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Editorial
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Editor's column

Food resources are an important factor in geopolitics and global economy. The Russian agro-industrial complex raises its production and introduces new technologies. It provides the population with a wide range of all basic food products. Despite the natural disasters, sanctions, high interest rates, and personnel shortage, the year of 2024 witnessed remarkable results in the domestic staple food security and self-sufficiency. Russia remains a global agricultural leader in many key areas and strengthens its role as an agent of global food security.

According to the Presidential Decree of May 2024, the Russian agricultural sector is to increase its production volumes and conquer foreign markets. By 2030, Russia's agro-industrial complex will have become technologically independent, food production is to quadruple, and exports are to increase by half.

The new Food Security Doctrine of the Russian Federation adopted in March 10, 2025, sets up the main strategic goal as maintaining the stability of world food markets on a multilateral and bilateral basis by increasing the export potential of domestic agricultural products, raw materials, foods, and fertilizers. The Doctrine also provides for the international scientific and industrial exchange of advanced agricultural technologies and experience to boost global food production and reproduction of agricultural breeds and cultivars.

According to the Russian Agricultural Bank, the year of 2024 saw twelve records in production and consumption of some livestock products. The Russian meat industry produced 16.74 million tons of meat cattle and poultry. According to Federal State Statistics Service, the meat production reached 3.76 million tons, which was by 1.8% as much as in 2023. Russians consumed 83 kg of meat per person, with pork and poultry responsible for 32 and 33 kg, respectively. In addition, Russia reached a 435-thousand-ton record in turkey meat production.

The meat industry is a key sector of the Russian agro-industrial complex that ensures the national food security. Today, it demonstrates significant export potential, having come a long way from import dependence to self-sufficiency. It keeps growing as a result of government support, private investments, and advanced technologies. The success of cattle farming has been brought about by the positive changes in forage production.

The positive trends in the meat industry are likely to continue, especially in the aspects of quality and safety provided by stricter production standards and modern technologies.

Professor Andrey B. Lisitsyn, Doctor of Engineering Sciences, Academician of the Russian Academy of Sciences, Laureate of the State Prize of the Russian Federation

The flourishing meat farming has boosted the production of meat foods, which keeps expanding its product range, meeting the domestic needs and creating a strong export potential.

Russian meat producers have demonstrated a reliable ability to adapt and develop in the face of global changes and challenges, which is the key to the stability and prosperity of the meat industry in the future. A stronger meat industry remains a priority area of the Russian agro-industrial complex.

Russia's meat industry possesses a significant export potential. It is increasing its exports of poultry, pork, and beef to Asia, the Middle East, and Africa. Further development of export potential requires high standards of quality, safety, and logistics, not to mention new partnerships with foreign companies and organizations from friendly countries.

At present stage, the meat industry can increase its competitiveness by interacting with science. R&D organizations that design and test new meat production and processing methods provide the meat industry with new production power, better product quality, stricter safety measures, and more sustainable technologies.

Professor Andrey B. Lisitsyn, Doctor of Engineering Sciences, Academician of the Russian Academy of Sciences, Laureate of the State Prize of the Russian Federation, has made an enormous contribution to the cooperation between science and the meat industry.

Professor Lisitsyn devoted many years of his professional career to the All-Russian Scientific Research Institute of the Meat Industry (VNIIMP). He became its rector in 1992, during the time of turbulent changes. Not only did he save the Institute, but turned it into an R&D leader of the entire Russian agro-industrial complex.

Professor Lisitsyn and his team design novel high-tech technologies to promote the meat processing industry, increase its competitiveness, enhance the range of domestic meat products, strengthen the national food security, intensify quality control, improve production safety, and create new functional products that will make Russians a healthier and stronger nation.

Professor Lisitsyn has a wide and diverse range of scientific interests. He forecasts agricultural development by analyzing the nutritional and technological standards of meat raw materials as an object of a single exotrophic chain of industrial processing, consumption, and absorption of nutrients. He develops methods of complex physicochemical assessment to reveal the effect of meat quality groups DFD, NOR, and PSE on its functional and technological profile. Professor Lisitsyn and his students study the formation, predicting, and management of meat quality and safety during production and storage; they design new functional meat foods and identify optimal technological modes that provide high-quality meat products for general, special, therapeutic, and prophylactic purposes. Their scientific achievements are published in top Russian and foreign journals.

Professor Lisitsyn has made a great contribution to the fundamentals of genomics, proteomics, and nutrigenomics as applied to the meat industry. He developed a complex quantitative assessment of the nutritional and technological profile of meat raw materials for industrial processing. This methodology is implemented worldwide in advanced meat processing equipment. He also developed a field-to-fork system of quality and safety that covers environment, raw materials, food, and human health.

Professor Lisitsyn's publications possess undeniable scientific relevance and practical significance. His theoretical and experimental studies of the effect of anatomical, morphological, physical, and biochemical profile of meat raw materials on the functional and technological properties of the final products made it possible to develop high technological, quality, and nutritional standards, as well as to design effective meat production technologies. This approach solves the problem of meat quality in meat products meant for different social and age population strata.

Professor Lisitsyn heralds the cooperation between progressive scientific achievements and industrial production. For instance, targeted biological value of meat products takes into account the digestibility of protein in muscle and connective tissue, as well as the amino acid composition of individual formulation ingredients and morphological differences in various muscles.

The novel technological processes and methods developed by Professor Lisitsyn and his team rely on decades of fundamental research. They have been introduced at more than 600 enterprises in Russia and the CIS.

Professor Lisitsyn also deserves credit for improving the system of continuous education for food engineers and technical workers in primary cattle processing, sausage production, canning, baby food, preschool / school canteens, special nutrition, veterinary, economy, quality control, safety management, power engineering, metrology, marketing, and numerous vocations, e.g., trimming, mincing, butchering, etc.

Today, Professor Lisitsyn continues his active teaching activities. Under his direct supervision, scientific research is conducted with both domestic and foreign organizations.

His active life position, responsible attitude, R&D achievements, organizational talents, and vast professional experience have been acknowledged at the state level. Professor Lisitsyn was awarded the State Prize of the Russian Federation in Science and Technology (1999), the Gold Medal for Agro-Industrial Development (2005), and the Order of Honor (2006), as well as the titles of Veteran of Labor (2000), Honored Worker of Moscow Industry (2007), Honored Worker of the Russian Agro-Industrial Complex (2007), and Honored Scientist of the Russian Federation (2013).

His achievements are remarkable not only in the aspect of their countrywide application, but also in their timeliness and usefulness.

We sincerely congratulate, Professor Andrey B. Lisitsyn, Doctor of Engineering Sciences, Academician of the Russian Academy of Sciences and Laureate of the State Prize of the Russian Federation, on his 80th birthday, which this outstanding scientist, wise leader, wonderful organizer, and talented mentor celebrates on April 16, 2025. We wish him good health, inexhaustible energy, and inspiration for new ideas and scientific achievements, as well as happiness and peace shared with family, colleagues, and students!

Editor-in-Chief,
Corresponding Member of the Russian Academy of Sciences,
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Acid curd (Karish) cheese supplemented with ashwagandha and/or probiotics: Modulatory efficiency on induced behavioral and neurochemical changes in rats

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Abstract:
Neurodegenerative disorder leads to a progressive memory loss that has only limited known medications. The use of ashwagandha, probiotics, or their combination may improve cholinergic activity, consequently providing therapeutic potency against amnesia and neuroplasticity disorders. We aimed to explore the modulatory benefits of ashwagandha extract and probiotics against induced behavioral and neurochemical retardations.

Acid curd (Karish) cheese samples were supplemented with ashwagandha extract and/or probiotics and subjected to chemical, microbiological, rheological, sensorial, and biological investigations by standard techniques.

The supplementation of Karish cheese with ashwagandha never deteriorated its chemical composition or rheological parameters. On the contrary, it exerted high antioxidant and phenolic potentials. Also, ashwagandha extract performed antimicrobial action against the tested pathogenic bacteria and showed better prebiotic effects with *Lactobacillus plantarum*. The biological study revealed that treating dementia-modeled rats with Karish cheese supplemented with ashwagandha and/or probiotics resulted in a detectable improvement in the behavioral and neurochemical measurements. However, the cheese supplemented with a formula of ashwagandha and probiotics had the greatest regenerating effect.

The supplementation of Karish cheese with ashwagandha and/or probiotics exhibited a modulatory efficiency against experimentally induced behavioral and neurochemical disorders.

Keywords: Ashwagandha, Karish cheese, *Lactobacillus plantarum*, probiotic, therapeutic effect, dementia, rodents

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INTRODUCTION

White soft cheese is a rudimentary product at the breakfast table and a meal, or a basic sandwich, for school children at the beginning of the day [1]. Acid curd, or Karish, cheese is an excellent choice of white soft cheeses mixed with many natural additives such as herbs, spices, or vegetables. Made from defatted milk, acid curd cheese can also be added to edible oils, with olive oil being the most famous. So, this cheese can be used with many additives that increase its health and nutritional value [2]. Several studies have shown that fermented dairy products improve memory functions. In addition, individuals who consume low-fat dairy products, including acid curd (Karish) cheese and yogurt (at least once a week), have a higher level of awareness than others. A close link has also been found between the consumption of fermented dairy products and a lower risk of dementia [3–6].

The physiological effects of fermented dairy products are due to the presence of fatty acids and bioactive peptides. They are naturally released during the fermentation process that occurs due to the presence of lactic acid bacteria in milk and its products [7].

Alzheimer's disease has serious effects on the patient, their family, and society. It is linked with severe cognitive damage and some metabolic defects. Prevention is the best way to fight this disease since its

treatment can neither delay nor stop its progress [8]. Studies indicate links between lower pathogens, lifestyle changes, lower injury rates, and better prevention [9]. Accordingly, following a healthy diet and exercising are some of the most successful ways to avoid many diseases to date [10]. Using herbs and medicinal plants in food for health benefits has been a trend over the past few years. Since milk and dairy products are commonly preferred by different segments of society, they are among the most important carriers of phytochemicals present in herbs (mainly polyphenols) for health benefits [1, 11].

Ashwagandha (*Withania somnifera* L.), Indian ginseng or winter cherry, has many health and medical benefits, both therapeutic and protective [12]. It can efficiently prevent thyroid dysfunction, reduce its complications for the nervous system, and treat hypertension [13, 14]. Ashwagandha is also considered an adaptogen, a memory enhancer, and a cardiovascular protector. It is known for its antioxidant, anxiolytic, antiparkinsonian, antivenom, anti-inflammatory, antitumorous, immunomodulatory, hypolipidemic, and antibacterial properties [15]. Ashwagandha extract was reported to possess antioxidant and anti-inflammatory effects against aluminum neurotoxicity, reduce cholinergic activity by maintaining normal acetylcholine esterase activity, and enhance memory [16]. It is added to many formulations to increase energy, improve overall health and longevity, as well as prevent various diseases. The dried roots of the plant are used to treat nervous and sexual disorders [17].

Probiotics have a positive effect on psychological well-being through enhancing human mood and sleep quality, etc. [18–20]. Different studies on experimental animals indicated a close relation between probiotic consumption and cognitive function. For example, a 12-week probiotic consumption had a positive effect on the cognitive function and some metabolic conditions in Alzheimer's patients [21]. Many previous studies confirmed anti-Alzheimer's properties of *Lactobacillus plantarum* [22]. In particular, this probiotic enhances the production of acetylcholine neurotransmitter, prevents memory deficit, and improves learning ability [23]. Combining probiotics, mainly *L. plantarum*, with ashwagandha extract as a supplement has proven to have a positive effect against aluminum chloride-induced neurotoxicity. Recently, Mustafa *et al.* confirmed the amelioration effect of ashwagandha extract combined with a probiotic strain in bio-yogurt against $AlCl_3$ neurotoxicity in rats [24]. In this regard, we aimed to evaluate the effect of ashwagandha ethanolic extract (AEE) on the properties of acid curd (Karish) cheese as a milk model system. For this, AEE and/or a probiotic were added to UF-retentate to deliver health benefits. The supplemented cheese was subjected to chemical, physical, textural, and microbiological determination, as well as a biological evaluation for cognitive or learning difficulties in rats.

STUDY OBJECTS AND METHODS

Our materials were obtained from the following sources:

– skimmed UF-retentate: from the Animal Production Research Institute, the Agriculture Research Center (Giza, Egypt);
– ashwagandha (*Withania somnifera* L., NCBI: txid126910) roots: from Imtenan Co. (Giza, Egypt);
– aluminum chloride ($AlCl_3$, 99%, BN AC196): from Alpha-Chemika (India);
– *Bacillus cereus* (ATCC133018), *Salmonella typhimuum* 9027, and *Staphaurius* (ATCC 25175): from the stock cultures of the Agricultural Research Centre (Giza, Egypt);
– *Escherichia coli* O157:H7 (ATCC 6933), *Listeria monocytogenes* V7, and *Yersenia enterocolitica* (ATCC9610TM): from Liofilchem S.r.l. (Rosetodegli Abruzzi, TE, Italy);
– *Streptococcus thermophiles*, *Lactobacillus delbrueckii* spp. *bulgaricus*, and *Lactobacillus casei*: from the stock cultures of the Dairy Microbiology Lab., the National Research Centre (Giza, Egypt);
– *Lactobacillus rhamnosus* (Tistr 541), *Lactobacillus plantarum* (Dsaz 0174), and *Lactobacillus reuteri* (B-14171): from the Cairo Microbiology Resources Center, the Faculty of Agriculture at Ain Shams University;
– *Bifidobacterium bifidum* (Bb-12) and *Lactobacillus acidophilus* (N4495): from Chr. Hansen's Lab. A/S (Copenhagen, Denmark).

Based on the microbiological results, *L. plantarum* was selected for this study.

Ashwagandha ethanolic extract (AEE) was prepared as previously described by Mustafa *et al.* [24]. In brief, ashwagandha roots were milled into fine powder, soaked in ethanol (70%), and stirred overnight. The mixture was then filtered (Whatman No. 1) and the solvent was evaporated using a rotary evaporator (under reduced pressure at 40°C), whereas the water residuals were freeze-dried. Finally, the dry extract was stored at –20°C until further *in vitro* and *in vivo* assessments.

The **total phenolic content** of ashwagandha ethanolic extract was analyzed spectrophotometrically by the modified Folin-Ciocalteu colorimetry and expressed as catechin equivalents using the catechin standard curve [24].

Radical scavenging activity. The capacity of ashwagandha antioxidants to quench the DPPH radical was determined as described by Salama *et al.* [25]. The radical scavenging activity (RSA, %) of the extract was calculated according to the following Eq. (1):

$$RSA = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Preparation of acid curd (Karish) cheese. Karish cheese was made by using skimmed UF-retentate heat-treated at 90°C/5 min and cooled to 42°C. For the experiments, 200 mg of ashwagandha ethanolic extract (AEE) was added to one litter of UF-retentate, then inoculated with a 2% active starter-mixture and/or a 2% probiotic (*L. plantarum*). The cheese treatments were

divided as follows: 1) the control (C) contained the active starter-mixture (without AEE); 2) the first treatment (T1) contained the active starter-mixture and the probiotic (*L. plantarum*); 3) the second treatment (T2) contained the active starter-mixture and AEE; 4) the third treatment (T3) contained the active starter-mixture, the probiotic (*L. plantarum*), and AEE. The different treatments were packed into plastic cubs (100 mL) and left at 42°C until complete coagulation. After coagulation, the treated cheeses were cooled and stored at 5°C until sensory, chemical, and microbiological analyses before and during storage [2].

Chemical analysis. The cheese samples were analyzed for the content of moisture, fat, total solids, total nitrogen, soluble nitrogen/total nitrogen ratio, and ash according to the Association of Official Analytical Chemists [26]. The pH value was measured using a digital laboratory pH-meter. Diacetyl and acetaldehyde levels were determined spectrophotometrically.

Antioxidant activity and total phenolic compounds. The antioxidant activity and total phenolic content were determined in both the pure ashwagandha ethanolic extract and the supplemented cheese samples according to Salama *et al.* [25].

The texture profile analysis of all the cheese samples was carried out according to the standards of the International Dairy Federation [27].

Screening for anti-microbial activity. The anti-bacterial activity of ashwagandha ethanolic extract (AEE) against pathogenic strains was evaluated according to Tepe *et al.* [28]. For this, the crude dry extract was considered as level 100%; AEE was diluted with distilled water to obtain levels 50 and 25%.

Evaluation of prebiotic activity. The prebiotic activity of ashwagandha ethanolic extract was based on the growth of the given probiotic strains according to the methods of Lourence & Viljoen [29].

Microbiological analysis. The total aerobic colony count, as well as mold, yeast, and coliform counts of the cheese samples were microbiologically examined as previously described by El-Shenawy *et al.* during different storage periods [30].

Sensory evaluation. The sensory properties of the cheese samples during different storage periods were evaluated according to Salama [1]. Ten (4 women (35–55 years) and 6 men (25–60 years)) expert panelists from the staff of the Dairy Science Department at the National Research Center (Egypt) were previously trained with commercial samples of cheese according to Cost-95. They evaluated appearance (10), body & texture (40), and flavor (50) of the cheese samples. The cheese samples were cut into cubes (1.5×1.5×1.5 cm), covered with plastic wrap to prevent dehydration, coded with three-digit random numbers, and held for at least 1 h at 20°C to equilibrate. Each panelist was given three cheese cubes to score each sample on the hedonic scale. The evaluation took place in a tasting room equipped as specified in Standard IS0 8589 (1988).

Animals and experimental study. Thirty adult female Wistar albino rats (150–200 g) were obtained from the Animal Colony of the Research Institute of Ophthalmology, Egypt. They were housed in suitable plastic cages and maintained under controlled conditions (temperature 25 ± 2°C, humidity 50 ± 5%, and 12 h light-dark cycles). The rats had free access to a commercial ration used as a basal diet and water ad libitum. After seven days (acclimatization period), they were randomly assigned into five groups (6 animals each) as follows. Group I included rats that orally received 2 mL/kg/day of starter-emulsified cheese for one month and served as the control. Group II included rats orally intoxicated with $AlCl_3$ (300 mg/kg/day) for one month to induce dementia [31]. Group III included rats that orally received 2 mL/kg/day (equivalent to 10^9 CFU/kg/day) of probiotic/starter-emulsified cheese in addition to intoxication with $AlCl_3$ for one month [32]. Group IV included rats that orally received 200 mg/kg/day of ashwagandha ethanolic extract-emulsified cheese alongside their intoxication with $AlCl_3$ for a similar period [24]. Group V included rats that orally received 2 mL/kg/day of starter/probiotic/AEE-emulsified cheese, as well as being $AlCl_3$-intoxicated for a month. All the animals were gavaged once a day, with the doses adjusted on a weekly basis according to their body weights. At the end of the experiment, behavioral observations and biochemical measurements were conducted.

Behavioral profile. ***Open field test.*** In this test, the rats were gently placed into a corner of a cleaned and sterilized planed arena and observed for 3 min. Both exploratory behaviors (ambulation and crossing of squares as well as rearing) and non-exploratory measures (grooming) were scored as absolute counts [33].

Modified elevated plus maze test. Depending on the aversion of rats to the open space, their spatial long-term memory was measured as described by Hliňák & Krejčí [34].

The ***novel object recognition test*** (hippocampus-dependent memory impairment) was performed via an automated tracking of rats with a video tracking system (Anymaze 4.20, Stoelting, USA). All exploratory actions were measured automatically and manually as explained by Gümüş [35].

Y-maze test. The short-term memory and locomotor activity were measured according to Wright [36]. The rats were video-tracked for 5 min using a video tracking system (Anymaze 4.20, Stoelting, USA) that recorded the number of arm entries and the distance travelled by each animal. The alternation percent was calculated according to the Eq. (2):

$$\text{Alternation persent} = \frac{\text{Total alternations}}{\text{Entries number} - 2} \times 100 \qquad (2)$$

Brain tissue sampling. After the last administration and behavioral tests, the animals were fasted overnight and euthanized by sudden decapitation. The brains of some animals (in each group) were dissected out and anatomized into two similar halves. The first half was

homogenized in phosphate buffer (0.1 M, pH 7.4) to determine oxidative stress markers. The second half was homogenized in 0.1 M perchloric acid containing 3, 4-dihydroxybenzylamine at a final concentration of 25 ng/mL to assess acetylcholinesterase activity and the level of biogenic amines. The brains of the rest of the animals (in each group) were dissected out and immersed in formaldehyde-saline (10 %, v/v) buffer for histopathological examination.

Acetylcholinesterase activity, dopamine, and serotonin. Acetylcholinesterase activity was determined according to the modified method of Ellman [37]. Dopamine and serotonin levels were measured by high-performance liquid chromatography (Waters, Milford, USA) following the method of Kim [38].

Oxidative stress status. Oxidative stress markers (glutathione, nitric oxide, malondialdehyde, and superoxide dismutase) were determined in brain phosphate buffer homogenates using reagent kits obtained from Biodiagnostic (Giza, Egypt).

Histopathological examination. Formaldehyde-saline (10 %, v/v) buffer-immersed brains were hydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Then, 5-µm-thick sections were cut and stained with hematoxylin and eosin for pathohistological examination using light microscopy.

Statistical analysis. Comparisons between means were carried out using one-way ANOVA, followed by a post-hock (Duncan) multiple comparisons test at $p \leq 0.05$, using the statistical analysis system (SAS) program software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The total antioxidant capacity and total phenolic content of ashwagandha ethanolic extract (AEE) are presented in Fig. 1. As can be seen, AEE can be considered an excellent source of antioxidants and phenolic compounds, as previously reported by Munir *et al.* [39]. In addition, the probiotic (*Lactobacillus plantarum*) and starter used in our study performed a detectable antioxidant activity resulting from metabolic and milk protein hydrolyses via the spontaneous action of the starter and beneficial bacterial strains, as previously reported by Ali *et al.* and Wang *et al.* [40, 41].

Figure 2 illustrates the main chemical analysis of different acid curd (Karish) cheese samples supplemented with AEE and/or probiotic bacteria. As we can see, neither AEE nor the probiotic bacteria made any changes in the chemical composition of manufactured Karish cheese, as previously reported [1, 22].

Antimicrobial activity. Table 1 illustrates the antimicrobial activity of ashwagandha ethanolic extract (AEE) at different concentrations (25, 50, and 100%) evaluated against the selected foodborne microorganisms. The AEE showed a varying ability to inhibit the growth of the pathogenic strains, depending on its concentration. Particularly, the highest concentration (100%) exhibited high antimicrobial activity against all the selected micro-

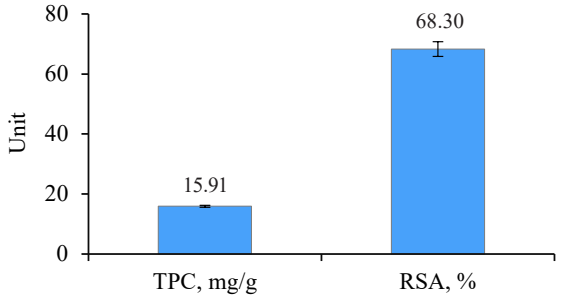


Figure 1 Total pnolic content (TPC) and radical scavenging activity (RSA) of ashwagandha ethanolic extract

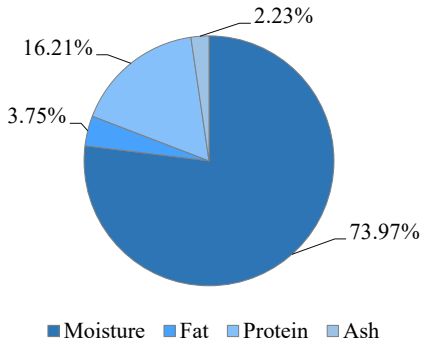


Figure 2 Chemical analyses of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract, probiotic bacteria, or their combination

Table 1 Antimicrobial activity of ashwagandha ethanolic extract (AEE)

Bacterial strains	Inhibition zone, mm		
	Pure AEE	Diluted AEE 50%	Diluted AEE 25%
Escherichia coli O157:H7	$7.0^{Af} \pm 0.3$	$2.0^{Bf} \pm 0.4$	$2.0^{Bd} \pm 0.4$
Yersenia enterocolitica ATCC 9610	$15.0^{Ad} \pm 0.2$	$12.0^{Bc} \pm 0.6$	$6.0^{Cb} \pm 0.4$
Salmonella typhimurium ATCC 9027	$12.0^{Ae} \pm 0.8$	$6.0^{Be} \pm 0.3$	$3.0^{Cc} \pm 0.2$
*Listeria monocytogenes*V7	$20.0^{Ac} \pm 0.1$	$8.0^{Bd} \pm 0.4$	$3.0^{Cc} \pm 0.7$
Staphylococcus aureus ATCC 25175	$22.0^{Ab} \pm 0.7$	$15.0^{Bb} \pm 0.1$	$3.0^{Cc} \pm 0.1$
Bacillus cereus ATCC 33018	$25.0^{Aa} \pm 0.3$	$20.0^{Ba} \pm 0.7$	$12.0^{Ca} \pm 0.3$

The data are represented as mean ± SD. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within a row, the means with different capital (A, B, C) superscript letters are significantly different. Within a column, the means with different small (a, b, c) superscript letters are significantly different

organisms, especially *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Gram-positive), with inhibition zones of 25, 22, and 20 mm, respectively. The minimum inhibition zones of 7 and 12 mm, however, were noted with *Escherichia coli* and *Salmonella* (Gram-negative), respectively. These findings confirmed the previous results demonstrated in [42, 43]. According to Khanchandani *et al*., AEE is more effective against gram-positive than Gram-negative bacteria [43]. This might be due to bioactive compounds in AEE that confer resistance against microbial pathogens. Alternatively, AEE contains phenolic compounds associated with antimicrobial efficiency through their hyper-acidification effect at the plasma membrane interface of the pathogen, consequently disrupting the cell wall synthesis [42]. More than 80 chemical compounds have been found in AEE, mainly alkaloids, flavone glycosides, steroidal lactones, and polyphenols [44–46]. These compounds play an active role in antibiotic, anti-inflammatory, and cytotoxic activities [14]. A recent study has demonstrated that AEE showed antagonistic potential against pathogenic bacteria due to the presence of endophytes, which have biocontrol potential against pathogens [44].

Antioxidant activity and total phenolic content. Interestingly, our study evidenced that adding ashwagandha ethanolic extract (AEE) and probiotic bacteria to Karish cheese improves its antioxidant activity and total phenolic content (Table 2). Among the fresh cheeses, the highest antioxidant activity and total phenolic content (6.49% and 6.24 mg/100 g, respectively) were found in the AEE/probiotic-supplemented sample (T3), followed by the AEE-treated cheese without probiotic bacteria (T2) and the probiotic-supplemented sample without AEE (T1). The lowest values were found in the control. However, 15 days of cold storage significantly decreased the antioxidant activity and total phenolic content of all the cheese treatments. The values were still quite high though, with the lowest found in the control (1.94% and 4.89 mg/100 g, respectively). The decrease in antioxidants and total phenols during storage agrees with the previous studies [24, 25].

Physicochemical properties of acid curd (Karish) cheese. Table 3 shows the physicochemical analysis of different treatments of Karish cheeses before and after 15 days of cold storage. In the fresh samples, the soluble nitrogen/total nitrogen ratio significantly increased in the cheese treated with ashwagandha ethanolic extract (AEE) and probiotic bacteria, while being equal in the control and the probiotic-supplemented cheese (T1). During cold storage (7 and 15 days), the soluble nitrogen/total nitrogen ratio also significantly increased. This is attributed to the addition of AEE, which markedly enhanced the effect of starter and probiotic bacteria as a prebiotic improving the growth and fermentation. This finding clarifies the upsurge of the soluble nitrogen/total nitrogen ratio in the fresh samples or during storage [47, 48].

The pH values decreased slightly between the fresh samples as AEE was added alone or in combination with probiotic bacteria. However, they decreased significantly throughout storage up to 15 days. As mentioned before, the decrease in pH spontaneously resulted from the action of the starter and probiotic bacteria, which was markedly enhanced by the presence of AEE.

Total volatile fatty acids and diacetyl values increased significantly among the treatments, as well as during storage, with the highest values noted in the cheeses treated with AEE and/or probiotics.

Acetaldehyde values significantly elevated in the fresh treatments and markedly decreased during storage, with the lowest values recorded at the end of storage. The samples supplemented with AEE and/or probiotics had higher acetaldehyde levels compared to the control.

These physicochemical changes were consistent with several previous studies [25, 49, 50].

Texture analysis is an important test that reflects the product's acceptability, varying with the type of cheese. Figure 3 presents the texture profile of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract (AEE) and/or probiotic bacteria. While no significant differences were observed in the examined texture profile parameters among the treatments, there was a slight difference when comparing them with the control. This difference might be due to the addition of AEE, probiotics, and/or starter culture. Alternatively, it could result from the changes in pH and soluble nitrogen/total nitrogen ratios that affected the texture profile during storage. It was previously reported that the texture profile correlated with the physicochemical changes and the chemical composition of Karish cheese [51]. Generally, the addition of AEE and probiotic bacteria does not have a significant effect on the texture properties compared with the control.

Sensory evaluation is one of the most important tests that determines the extent of acceptance of the

Table 2 Antioxidant activity and total phenolic content of acid curd (Karish) cheese supplemented with ashwagandha ethanolic extract and/or probiotic bacteria before and after 15 days of cold storage

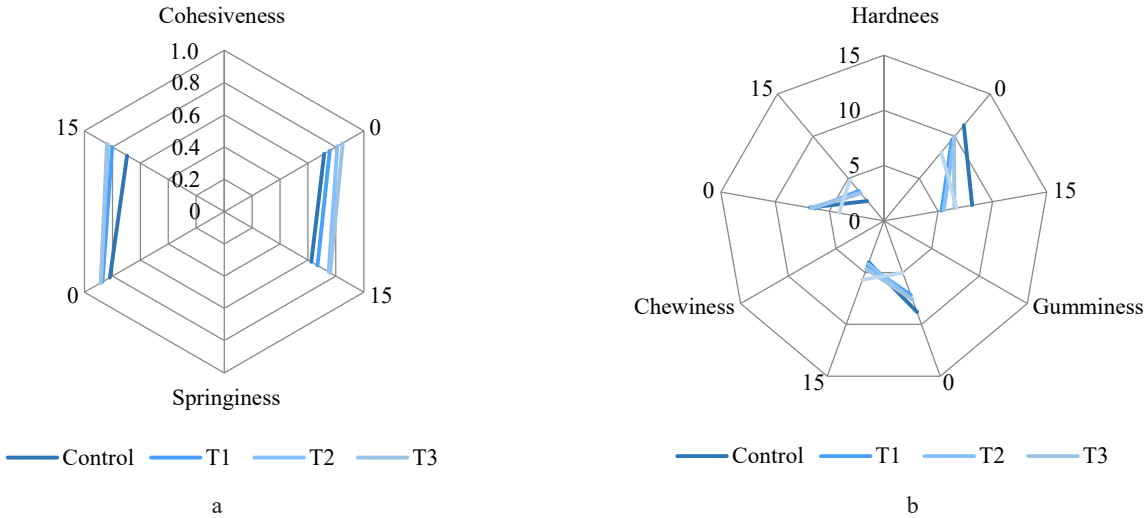
Property	Treatment	Storage	
		Fresh	15 days
Antioxidant activity, %	Control	4.97 ± 0.13^{B}	1.94 ± 0.03^{D}#
	T1	6.34 ± 0.09^{A}	2.25 ± 0.10^{C}#
	T2	6.39 ± 0.07^{A}	2.57 ± 0.13^{B}#
	T3	6.49 ± 0.06^{A}	3.51 ± 0.12^{A}#
Total phenolic content, mg/100 g	Control	5.80 ± 0.13^{B}	4.89 ± 0.04^{C}#
	T1	5.94 ± 0.13^{B}	5.28 ± 0.03^{B}#
	T2	5.96 ± 0.04^{B}	5.87 ± 0.09^{A}
	T3	6.24 ± 0.05^{A}	5.89 ± 0.10^{A}#

The data are expressed as mean ± SD of three replicates. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with different superscript letters are significantly different; Control (starter only); T1 (starter and probiotics); T2 (starter and AEE); T3 (starter, AEE, and probiotics). Within the same row, # is significantly different from the fresh sample

Table 3 Physicochemical properties of acid curd (Karish) cheese supplemented with ashwagandha ethanolic extract, probiotic bacteria, or their combination before and during cold storage

Property	Treatment	Cold storage		
		Fresh	7 days	15 days
Soluble nitrogen/total nitrogen ratio	Control	7.47 ± 0.02^{C}	8.07 ± 0.05^{D}#	9.41 ± 0.08^{C}#
	T1	7.47 ± 0.02^{C}	8.29 ± 0.08^{C}#	9.58 ± 0.13^{C}#
	T2	7.83 ± 0.01^{B}	9.19 ± 0.01^{B} #	13.29 ± 0.01^{B} #
	T3	7.91 ± 0.06^{A}	9.30 ± 0.02^{A}#	14.44 ± 0.01^{A}#
pH	Control	5.27 ± 0.01^{A}	$5.13 \pm 0.03^{A\#}$	4.76 ± 0.04^{A} #
	T1	5.25 ± 0.05^{A}	$5.03 \pm 0.02^{B\#}$	4.69 ± 0.01^{B} #
	T2	5.15 ± 0.04^{B}	4.95 ± 0.02^{C}#	4.58 ± 0.00^{C}#
	T3	5.18 ± 0.03^{B}	4.88 ± 0.01^{D}#	4.49 ± 0.01^{D}#
Total volatile fatty acids, mL/100 g	Control	5.00 ± 0.20^{D}	8.00 ± 0.10^{D}#	10.00 ± 0.20^{D}#
	T1	8.00 ± 0.10^{C}	11.00 ± 0.02^{C}#	14.00 ± 0.40^{C}#
	T2	10.00 ± 0.40^{B}	15.00 ± 0.30^{B}#	17.50 ± 0.85^{B}#
	T3	13.00 ± 0.20^{A}	18.00 ± 0.20^{A}#	21.00 ± 0.20^{A}#
Diacetyl, µm/100g	Control	25.20 ± 0.20^{D}	34.18 ± 0.32^{C}#	39.20 ± 0.30^{D}#
	T1	28.00 ± 0.50^{C}	35.67 ± 0.22^{B}#	42.40 ± 0.20^{C}#
	T2	29.60 ± 0.20^{B}	36.97 ± 0.08^{A}#	46.40 ± 0.40^{B}#
	T3	31.60 ± 0.10^{A}	37.33 ± 0.21^{A}#	48.40 ± 0.20^{A}#
Acetaldehyde, µm/100 g	Control	20.84 ± 0.11^{D}	16.56 ± 0.24^{D}#	5.06 ± 0.03^{D}#
	T1	23.80 ± 0.16^{C}	19.75 ± 0.10^{C}#	7.70 ± 0.01^{C}#
	T2	25.60 ± 0.60^{B}	21.48 ± 0.16^{B}#	9.06 ± 0.04^{B}#
	T3	28.62 ± 0.02^{A}	24.23 ± 0.28^{A}#	12.82 ± 0.28^{A}#

The data are expressed as mean ± SD of three replicates. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with different superscript letters are significantly different; within the same row, # is significantly different from the fresh samples. C (starter only), T1 (starter and probiotics), T2 (starter and AEE), T3 (starter, AEE, and probiotics)



Control – starter only; T1: starter and probiotics; T2: starter and AEE; T3: starter, AEE, and probiotics

Figure 3 Texture profile of Karish cheeses supplemented with ashwagandha ethanolic extract (AEE) and/or probiotic bacteria: (a) cohesiveness & springiness; (b) hardness, chewiness, & gumminess

product by the consumer. Table 4 shows different sensory properties of the Karish cheeses fortified with ashwagandha ethanolic extract (AEE) and/or probiotics before and during refrigerated storage up to 15 days. As can be seen, the addition of AEE and/or probiotic bacteria had no effect on the appearance of the fresh or cold-stored samples compared to the control. Neither did we find any significant changes in the body, texture, or taste of the treated samples. The total acceptance of the cheeses showed significant differences, with T3 given the highest acceptance, followed by T2, T1, and the control. Generally, all the samples were acceptable. This may be because we added a minimal amount of AEE that did not affect the taste, appearance, or any of the

Table 4 Sensory properties of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract and/or probiotic bacteria before and during cold storage

Sensory properties	Storage period	Cheese treatments			
		Control	T1	T2	T3
Appearance (10)	Fresh	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
	7 days	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
	15 days	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
Body and texture (40)	Fresh	$34.00^{A} \pm 3.00$	$35.00^{A} \pm 5.00$	$36.00^{A} \pm 2.00$	$36.50^{A} \pm 2.50$
	7 days	$34.80^{A} \pm 2.20$	$36.50^{A} \pm 1.50$	$36.80^{A} \pm 2.20$	$37.00^{A} \pm 2.00$
	15 days	$35.40^{A} \pm 3.40$	$37.60^{A} \pm 1.40$	$37.45^{A} \pm 1.55$	$37.80^{A} \pm 1.20$
Flavor (50)	Fresh	$34.00^{A} \pm 1.00$	$34.37^{A} \pm 2.63$	$35.00^{A} \pm 3.00$	$36.50^{A} \pm 1.50$
	7 days	$34.00^{A} \pm 2.00$	$34.50^{A} \pm 2.50$	$36.43^{A} \pm 1.57$	$37.00^{A} \pm 2.00$
	15 days	$34.53^{C} \pm 1.47$	$35.23^{BC} \pm 0.77$	$36.73^{AB} \pm 1.27$	$37.65^{A} \pm 0.75$
Total (100)	Fresh	$76.50^{C} \pm 1.00$	$77.87^{BC} \pm 2.13$	$79.50^{AB} \pm 1.00$	$81.50^{A} \pm 1.50$
	7 days	$77.30^{B} \pm 1.30$	$79.50^{AB} \pm 0.50$	$81.73^{A} \pm 0.73$	$82.50^{A} \pm 3.50$
	15 days	$78.43^{B} \pm 0.57$	$81.33^{A} \pm 2.33$	$82.68^{A} \pm 0.95$	$83.95^{A} \pm 1.45$

The data are presented as mean ± SD. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same row, the means with dissimilar superscript letters are significantly different. Control (starter only), T1 (starter and probiotics), T2 (starter and AEE), T3 (starter, AEE, and probiotics)

Table 5 Prebiotic activity of ashwagandha ethanolic extract on bacterial counts at different incubation times (log CFU/mL)

Strains	12 h		24 h		48 h	
	Control	Treatment	Control	Treatment	Control	Treatment
Lactobacillus reuteri	8.07 ± 0.06^{Ba}	8.63 ± 1.00^{A}	8.13 ± 0.11^{Ba}	$8.74 \pm 0.05^{A\#}$	8.19 ± 0.00^{Ba}	$8.79 \pm 0.07^{A\#}$
Lactobacillus plantarum	8.29 ± 0.07^{Aa}	8.54 ± 1.00^{A}	8.38 ± 0.09^{Ba}	$9.35 \pm 0.03^{A\#}$	8.39 ± 0.19^{Ba}	$9.40 \pm 1.00^{A\#}$
Lactobacillus acidophilus	7.89 ± 0.10^{Bb}	8.39 ± 0.09^{A}	8.10 ± 0.10^{Bab}	$9.80 \pm 0.03^{A\#}$	8.60 ± 0.53^{Ba}	9.41 ± 0.11^{A}
Lactobacillus rhamnosus	8.88 ± 0.12^{Ac}	8.92 ± 0.12^{A}	9.762 ± 0.200^{Aa}	$9.47 \pm 0.03^{A\#}$	9.200 ± 0.006^{Ab}	$9.23 \pm 0.20^{A\#}$
Bifidobacterium bifidum	8.13 ± 0.12^{Bb}	8.80 ± 0.10^{A}	8.99 ± 0.17^{Ba}	9.40 ± 0.62^{A}	9.26 ± 0.30^{Ab}	$9.55 \pm 0.23^{A\#}$
Enterococcus facium	8.30 ± 0.11^{Ab}	8.20 ± 0.10^{A}	8.90 ± 0.02^{Aa}	$8.80 \pm 0.11^{A\#}$	8.30 ± 0.06^{Ab}	$8.11 \pm 0.01^{B\#}$
Lactobacillus casei	7.95 ± 0.05^{Ac}	7.54 ± 0.05^{B}	8.18 ± 0.01^{Bb}	$8.20 \pm 0.01^{A\#}$	8.28 ± 0.01^{Aa}	$8.27 \pm 0.25^{A\#}$

The data are presented as mean ± standard deviation. The data were subjected to one-way ANOVA followed by a post-hoc (Duncan) test at $p \leq 0.05$. Within the row of the same incubation period, the means with different superscript capital letters (A, B) are significantly different. Within the same row, the means with different superscript small letters (a, b, c) are significantly different from the control at different incubation times.

studied sensory parameters. Therefore, Karish cheese supplemented with AEE and/or probiotic bacteria can be consumed by different age groups, especially children, to gain benefit from its nutritional and health properties. The same observation was made by Mustafa *et al.* who used ashwagandha extract with probiotic bacteria to manufacture a healthy functional yogurt [24]. Ashwagandha has been reported to improve the chemical quality of fermented dairy products containing probiotic bacteria, which in turn is reflected in its sensory quality [52]. Many researchers recommend the use of ashwagandha in dairy products, including yogurt, ice cream, and fermented milk, as well as in other food products such as probiotic juice [24, 47, 53, 54].

Prebiotic activity. Ashwagandha ethanolic extract (AEE) exhibits excellent prebiotic properties due to its high contents of phytochemicals, polyphenols, and other bioactive compounds. Therefore, we evaluated the prebiotic activity of various concentrations of AEE against foodborne microorganisms to choose the best strain with high prebiotic activity (best growth) for the manufacture of acid curd (Karish) cheese. Table 5 presents the selected probiotic strains in culture media containing AEE in comparison with the control. We found no significant changes in bacterial counts during the first 12 h. After that period, particularly after 24 h, the growth significantly increased. When the bacterial population reached its maximal growth, it remained constant during 48 h. *L. plantarum* showed significant growth after 24 h to reach a count of 9.35 CFU/mL, 1.0 log higher than that of the control group (8.38 log CFU/mL). As a result, we selected *L. plantarum* as a good carrier of AEE and probiotic bacteria to use in manufacturing Karish cheese. We also found increasing growth rates of the other probiotic strains at all the interval times. This is because AEE is a rich source of carbohydrates with high prebiotic potential [55]. Generally, herbs contain a good amount of carbohydrates, protein, minerals, and some vitamins. They serve as prebiotics and are a source of carbon and nitrogen enhancing the growth of different probiotic strains [56]. On the other hand, ashwagandha contains steroidal lactones and flavonoids, while its roots have suitable amounts of sominie, somniferin, somniferinine, withanine, and withanonine that supply the pH and titratable acidity [57].

Viability of *L. plantarum* and starter culture in Karish cheese. The viability of probiotics, or the number of viable counts, in the final product until the end of its storage is an important quality indicator. A functional product should have a viable count of at least 10^{-9}–10^{-10} CFU/mL. Therefore, all fermented products are tested for viable counts until consumption. Table 6 shows the viability of *L. plantarum* and starter culture in Karish cheeses supplemented with ashwagandha ethanolic extract (AEE). As can be seen, the presence of AEE enhanced the growth of *L. plantarum* in fresh Karish cheese and during storage at 4°C, compared to the control, increasing the production of lactic acid. The viable counts of *L. plantarum* reached 7.51 log CFU/mL after 15 days of cold storage.

According to the Codex Alimentarius, a commercial probiotic beverage should possess a minimum viable count of 10^6 CFU/mL at the time of consumption [58]. The growth rates recorded in our study showed that ashwagandha is a good medium for probiotic growth and it can be considered a prebiotic. Also, the starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) showed greater growth in the AEE-treated cheese (T2) compared to the sample free from the extract, although the growth of *S. thermophiles* was higher in the control. However, the starter cultures were still above the minimum viable counts recommended by the Codex Alimentarius. These results are in contrast with those reported by Momin and Prajapati, who indicated that the increase in the log viable count was lower in the fermented milk supplemented with ashwagandha [47]. Nevertheless, our results are consistent with those of Khatoon and Gupta, who reported a suitable growth of starter culture in various combinations with AEE [54]. At the end of the storage period (15 days), the total bacterial count in all the Karish cheese treatments ranged from 7.8 (Control) to 8.37 (T3) log CFU/g. Molds, yeasts, or coliform bacteria were not detected in any of the samples throughout cold storage. This indicates the microbial quality of the final product until the end of storage, as well as good hygiene during preparations, manufacture, and storage.

Biological study. Regarding behavioral measurements (Figs. 4–7), we found that the co-administration of rats with cheese supplemented with ashwagandha ethanolic extract (AEE), probiotics, or their combination significantly restored the behavioral deteriorations resulting from $AlCl_3$ intoxication. This was evidenced by the marked improvement in locomotion deficits (vertical and horizontal activities) in the open field test.In particular, AEE significantly increased the number of arms in the maze, while the formula (AEE plus probiotics) increased the spontaneous alternation percentage, compared to the $AlCl_3$-intoxicated rats' group. In the modified elevated plus maze, the probiotics, AEE, and their formula significantly decreased the transfer latency. Further, the AEE-treated rats spent significantly more time exploring in the novel object recognition test, compared to the probiotic-treated rats, whereas the formula-treated rats showed a high discrimination ratio, as well a high recognition index, compared to the other groups.

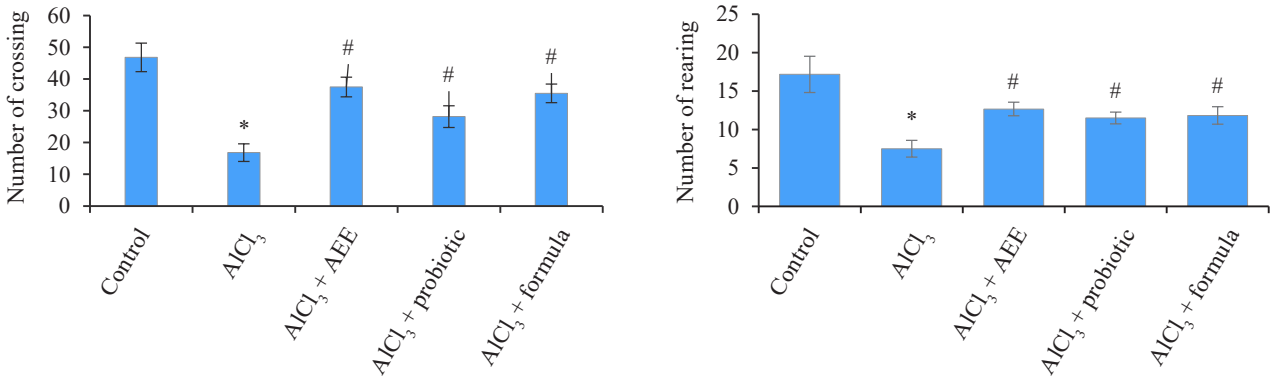
The open field test was used to assess the rats' locomotion and emotionality [59]. We found that AEE was able to alleviate some motor deficits and anxiety caused by $AlCl_3$, which might be due to its chemical constituents with antioxidant properties. Thus, AEE can reverse the $AlCl_3$-induced cognitive defects. These findings are consistent with some previous experiments [60]. The effect of AEE on the rats' motor activity was confirmed by the number of arms entered in the Y-maze, which also increased significantly in the AEE-treated group when compared with the other groups. In the Y-maze test, the formula was found better able to treat short-term memory deficits in the rats, compared to AEE or probiotics.

The modified elevated plus maze test is used to measure the long-term spatial memory of rats [61]. In our study, both AEE and probiotics, as well as their formula, markedly decreased the time to transfer to both

Table 6 Viability of starter culture and *Lactobacillus plantarum* in Karish cheeses supplemented with ashwagandha ethanolic extract during storage at 4°C**,** log CFU/g

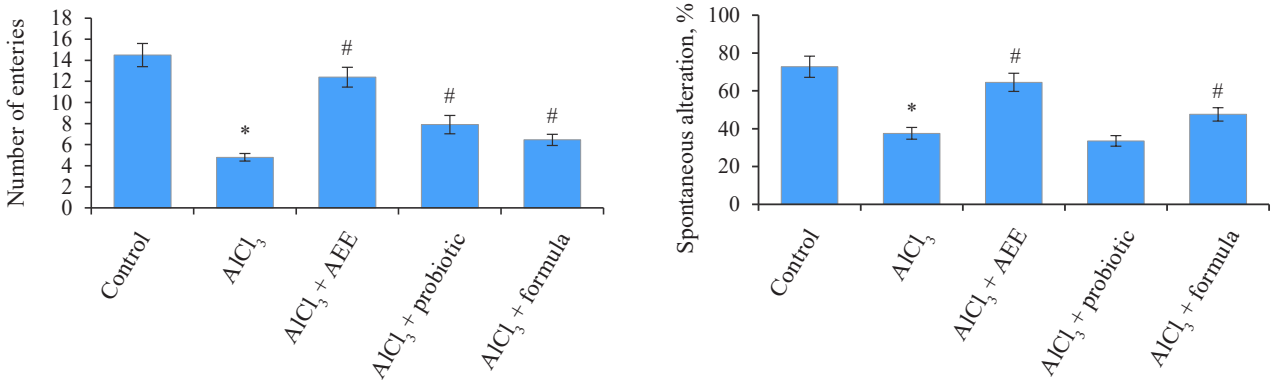
	Storage time	Control	T1	T2	T3
Total bacterial count	Fresh	$7.80^{A} \pm 0.11$	$8.10^{A} \pm 0.09$	$7.20^{A} \pm 0.08$	$8.20^{A} \pm 0.10$
	7 days	$7.90^{B} \pm 0.18$	$8.20^{A} \pm 1.01$	$7.18^{C} \pm 0.23$	$8.21^{A} \pm 0.21$
	15 days	$7.90^{B} \pm 0.10$	$8.32^{A} \pm 0.02$	$7.30^{C} \pm 0.03$	$8.30^{A} \pm 0.02$
Streptococcus thermophilus	Fresh	$7.90^{A} \pm 0.10$	$7.53^{B} \pm 0.41$	$7.65^{B} \pm 0.05$	$7.69^{B} \pm 0.51$
	7 days	$8.00^{A} \pm 0.10$	$7.14^{C} \pm 0.02$	$7.70^{B} \pm 0.01$	$7.73^{B} \pm 0.08$
	15 days	$8.14^{B} \pm 0.11$	$7.25^{D} \pm 0.22$	$7.73^{C} \pm 0.21$	$7.82^{A} \pm 0.07$
Lactobacillus delbrueckii spp. *bulgaricus*	Fresh	$7.10^{B} \pm 0.10$	$7.50^{A} \pm 0.05$	$7.50^{A} \pm 0.10$	$7.60^{A} \pm 0.02$
	7 days	$7.11^{C} \pm 0.01$	$7.67^{B} \pm 0.13$	$8.10^{A} \pm 0.10$	$8.16^{A} \pm 0.04$
	15 days	$7.30^{B} \pm 0.10$	$8.12^{A} \pm 0.01$	$8.13^{A} \pm 0.03$	$8.14^{A} \pm 0.12$
Lactobacillus plantarum	Fresh	–	$7.30^{B} \pm 0.06$	–	$7.80^{A} \pm 0.10$
	7 days	–	$7.51^{B} \pm 0.01$	–	$7.69^{A} \pm 0.01$
	15 days	–	$7.495^{A} \pm 0.100$	–	$7.51^{A} \pm 0.05$

The data are expressed as mean ± SD of three replicates. Within the same row, the means with different capital letters are significantly different at $p \leq 0.05$. Control (starter only); T1 (starter and probiotics); T2 (starter and AEE); T3 (starter, AEE, and probiotics)



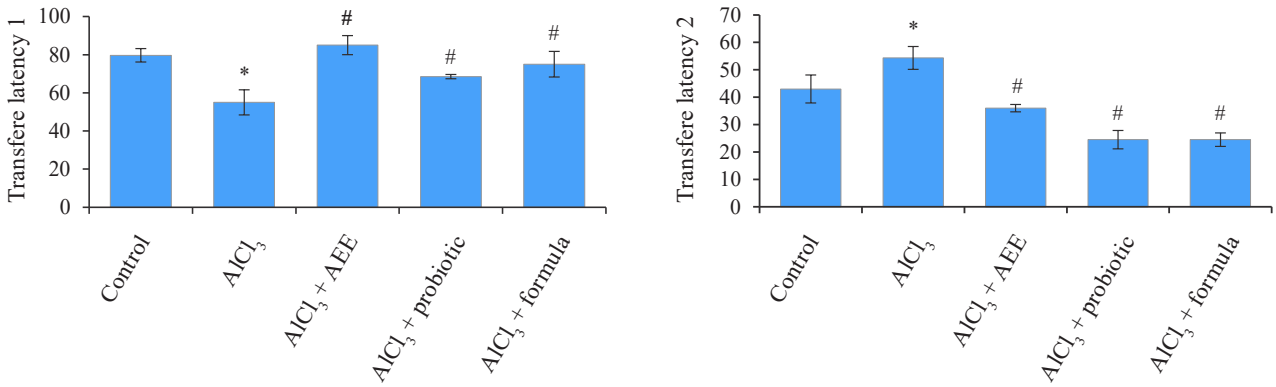
The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AEE – ashwagandha ethanolic extract

Figure 4 Effect of AEE- and/or probiotic-supplemented Karish cheese on the number of crossings and rearings in $AlCl_3$-intoxicated rats in the open field test



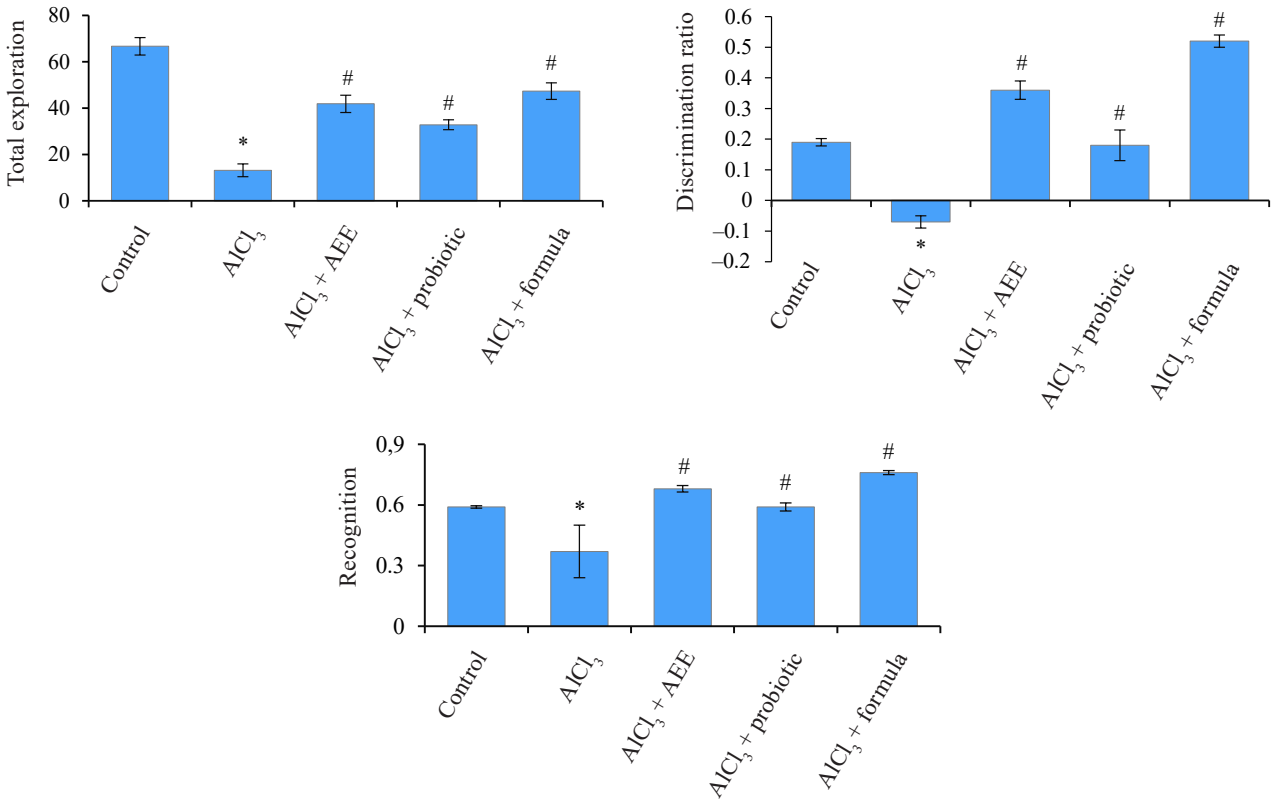
The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AEE – ashwagandha ethanolic extract

Figure 5 Effect of AEE- and/or probiotic-supplemented Karish cheese on the number of arm entries and spontaneous alternation percentage in $AlCl_3$-intoxicated rats in the Y-maze test



The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AEE – ashwagandha ethanolic extract

Figure 6 Effect of AEE- and/or probiotic-supplemented Karish cheese on transfer latency 1 and 2 in $AlCl_3$-intoxicated rats in the modified elevated plus maze test



The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a post-hoc (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AEE – ashwagandha ethanolic extract

Figure 7 Effect of AEE- and/or probiotic-supplemented Karish cheese on total exploration time, discrimination ratio, and recognition index in $AlCl_3$-intoxicated rats in the novel object recognition test

closed arms. This indicated their ability to restore the long-term memory in the treated rats compared to the untreated-$AlCl_3$ rats.

The novel object recognition test is used to assess the ability of rats to recognize an object or stimulus seen in the previous 24 h [62]. The test requires intact dorsal hippocampus and cortex [63]. Our data revealed that the administration of AEE- or formula-supplemented cheese remarkably restored the cognitive deficits and recognitive memory disruptions that accompanied $AlCl_3$-intoxication. This was indicated by the time spent exploring a novel object, a recognition index, and a discrimination ratio. In animal model investigations, strong associations have been reported between gastrointestinal microbiota and stress behavior. Particularly, the disruption or absence of gut bacteria was shown to increase the neuroendocrine stress response and behaviors associated with anxiety and stress-induced memory dysfunction [64, 65]. It was reported that probiotics reduced anxiety behavior in rodents, effectively reversing the effects of stress and improving memory and learning performance in terms of object recognition [66–68].

$AlCl_3$ is known to induce behavioral, biochemical, and histopathological effects that are linked with cognitive impairments [69–72]. In our study, the supplementation of Karish cheese with AEE and/or probiotics appreciably ameliorated the neuronal, biochemical, and behavioral aberrations in the $AlCl_3$-challenged demented rats. This indicated their curative and neuroprotective actions against dementia complications.

With respect to the neuro-and-biochemical investigations, we found that $AlCl_3$ significantly decreased brain monoamines (dopamine and serotonin) and raised acetylcholinesterase activity, as previously observed [72–74]. The levels of monoamines were similar to those in patients with Alzheimer's disease, suggesting a prominent role of $AlCl_3$ in aging and development of neurodegenerative diseases [75]. Interestingly, the supplementation with AEE and/or probiotics was found to significantly alleviate the effect of $AlCl_3$ on the biogenic monoamines, producing a therapeutic effect against neurodegenerative disorders. In addition, AEE and probiotics possessed significant antioxidant properties, which may be related to their contents of polyphenols, flavonoids, and other compounds. Compared with the control group, $AlCl_3$-intoxication resulted in a significant decrease in dopamine and serotonin levels (–37.9 and –49.7%, respectively). However, a significant elevation (63.1%) was shown in acetylcholinesterase activity. Interestingly, the treatment of rats with Karish cheese supplemented with AEE, probiotics, or their combination (formula), in

line with $AlCl_3$-intoxication, markedly restored (at different degrees) the $AlCl_3$-deteriorated neurochemical measurements. This was evidenced by a significant reduction in acetylcholinesterase activity (–28.4, –18.8, and –24.1%, respectively), as well as a notable increase in dopamine (45.3, 17.2, and 26.6%, respectively) and serotonin (69.6, 39.1, and 57.4%, respectively), compared with the $AlCl_3$-intoxicated group. The highest improvement was performed by the AEE-supplemented cheese (Table 7).

Our data showed a significant increase in the levels of malondialdehyde (59.6%) and nitric oxide (183.6%) in the brains of $AlCl_3$-intoxicated rats. We also found a significant drop in the anti-oxidative battery resulting from a marked decrease in glutathione (–49.4%) and superoxide dismutase activity (–36.6%), compared to the control group. Noteworthily, the treatment of $AlCl_3$-intoxicated rats with the Karish cheese supplemented with AEE, probiotics, or their combination (formula) alleviated some $AlCl_3$-induced oxidative deteriorations. This was evidenced by a marked decrease in the levels of malondialdehyde (–28.6, –12.8, and –16.3%, respectively) and nitric oxide (–42.1, –21.2, and –30.2%, respectively), as well as a remarkable rise in glutathione (39.8, 13.9, and 23.2%, respectively) and superoxide dismutase activity (39.8, 14.4, and 28.5%, respectively), compared to the $AlCl_3$-intoxicated rats (Table 8).

Regarding the histopathological examination (Fig. 8), the brains of the control animals had a normal histological structure in different brain regions, with only the cerebral cortex exhibiting some congested blood vessels (Fig. 8, plate 1). In particular, the striatum was histologically normal (Fig. 8, plate 2), and both the hippocampus and the cerebellum were also normal (Fig. 8, plates 3 & 4). However, the intoxication of rats with $AlCl_3$ resulted in serious histological alterations in brain tissue, with many dark degenerated neurons observed in the cerebral cortex (Fig. 8, plate 5) with neuronophagia. The hippocampus showed changes in blood vessels accompanied by mild glial infiltrations (Fig. 8, plate 6), as well as marked neuronal damage resulting in an obvious loss of cell density. Pink fibrillar material was seen between the degenerating neurons of the hippocampus with glial cells infiltration (Fig. 8, plate 7). Purkinje cell necrosis was commonly seen in almost all the examined cerebellar sections (Fig. 8, plate 8).

According to our results, the treatment of $AlCl_3$-intoxicated rats with AEE-supplemented Karish cheese resulted in a moderate protection against brain injury. In particular, the cerebral cortex appeared normal (Fig. 8, plate 9) and free from angiopathies; the hippocampus also appeared histologically normal (Fig. 8, plate 10). Similarly, the treatment of the $AlCl_3$-intoxicated animals with probiotic-supplemented Karish cheese resulted in a mild alleviative action against $AlCl_3$-induced brain damage. However, the cerebral cortex appeared normal, except for a few degenerating neurons with focal glial infiltrations (Fig. 8, plate 11). Cerebral angiopathy was also noticed with mild perivascular lymphocytic cuffing (Fig. 8, plate 12). A few degenerating neurons were observed in the hippocampus and the cerebellum (Fig. 8,

Table 7 Effect of Karish cheese supplemented with ashwagandha ethanolic extract and probiotics on $AlCl_3$-induced neurochemical changes

Parameter	Dopamine		Serotonin		Acetylcholinesterase	
	pg/g	%	pg/g	%	µmol/min/g	%
Control	2431 ± 54^{A}		863.0 ± 17.2^{A}		6814 ± 65^{E}	
$AlCl_3$	1509 ± 38^{E}	–37.9*	434.0 ± 15.9^{E}	–49.7*	$11\ 112 \pm 57^{A}$	63.1*
$AlCl_3$ + AEE	2192 ± 42^{B}	45.3#	736.0 ± 13.8^{B}	69.6#	7054 ± 64^{D}	–36.5#
$AlCl_3$ + probiotic	1769 ± 34^{D}	17.2#	604.0 ± 15.4^{D}	39.1#	9019 ± 55^{B}	–18.8#
$AlCl_3$ + formula	1911 ± 33^{C}	26.6#	683.0 ± 13.5^{C}	57.4#	8437 ± 49^{C}	–24.1#

The data are presented as mean ± standard error, subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. The means with dissimilar superscript letters are statistically significant. AEE (ashwagandha ethanolic extract); * is percentage of change calculated for the control group; # is percentage of change calculated for the $AlCl_3$ group. Formula is cheese supplemented with starter and AEE

Table 8 Effect of Karish cheese supplemented with ashwagandha ethanolic extract and/or probiotics on $AlCl_3$-induced oxidative stress status of the animals' brain

Parameter	Malondialdehyde		Nitric oxide		Glutathione		Superoxide dismutase	
	µmol/g	%	µmol/g	%	µmol/g	%	U/g	%
Control	43.26 ± 3.65^{C}		4.03 ± 0.52^{D}		318.4 ± 12.5^{A}		299.0 ± 32.5^{A}	
$AlCl_3$	69.15 ± 4.57^{A}	59.8*	11.43 ± 0.75^{A}	183.6*	160.9 ± 9.3^{D}	–49.4*	189.30 ± 28.7^{D}	–36.7*
$AlCl_3$ + AEE	49.34 ± 3.98^{C}	–28.6#	6.62 ± 0.25^{C}	–42.1#	224.9 ± 11.7^{B}	39.8#	264.9 ± 38.7^{B}	39.8#
$AlCl_3$ + probiotic	60.29 ± 2.78^{B}	–12.8#	9.01 ± 1.08^{B}	–21.2#	183.3 ± 8.9^{C}	13.9#	216.5 ± 26.8^{C}	14.4#
$AlCl_3$ + formula	57.82 ± 4.57^{B}	–16.3#	7.98 ± 0.98^{C}	–30.2#	198.2 ± 7.7^{C}	23.2#	243.2 ± 21.7^{C}	28.5#

The data are presented as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with dissimilar superscript letters are statistically significant. AEE (ashwagandha ethanolic extract); Formula (AEE + probiotic); * is percentage of change calculated for the control group; # is percentage of change calculated for the $AlCl_3$ group

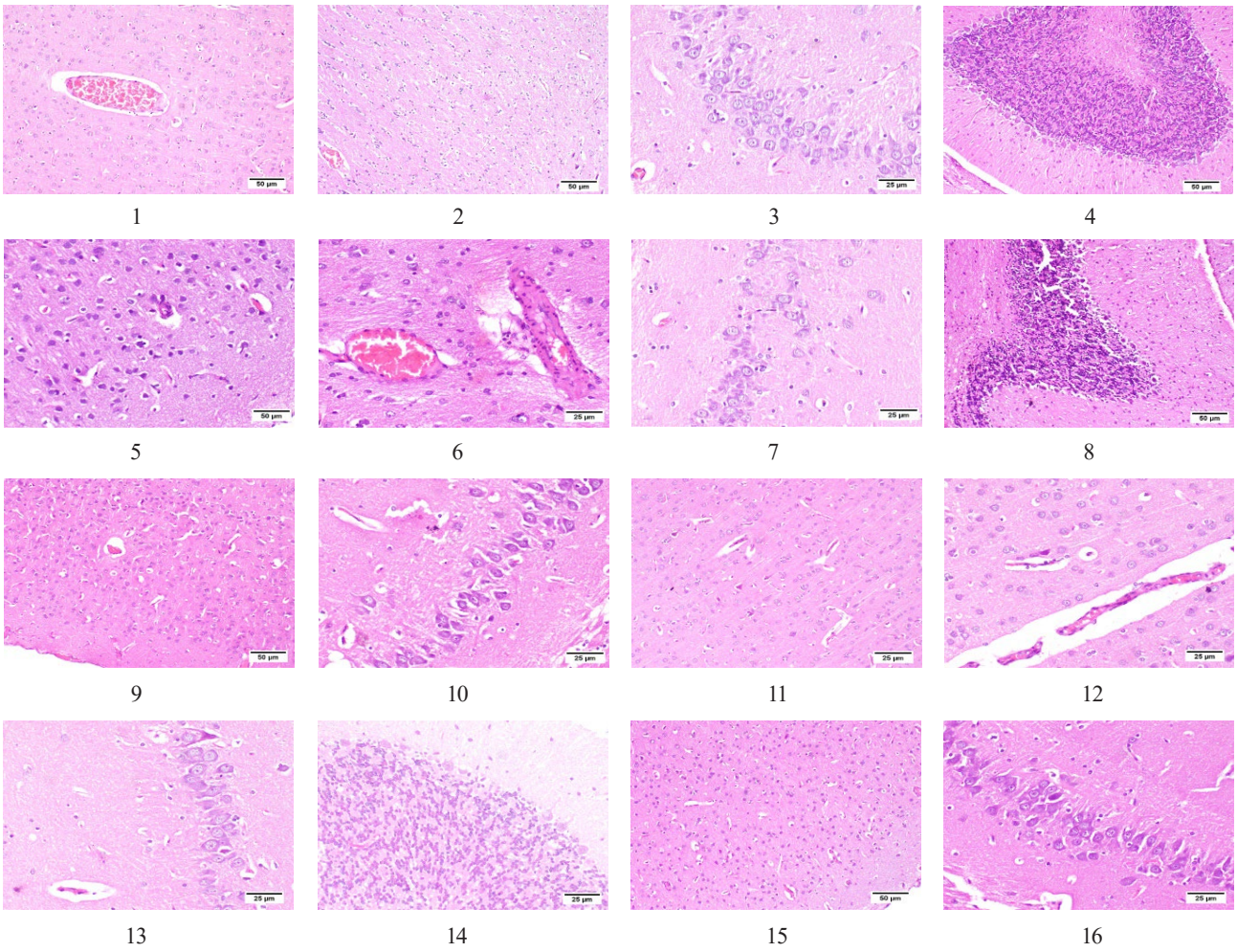


Figure 8 Photomicrograph of rat brain, H&E-stained: 1 – control group, showing normal cerebral cortex with congested cerebral blood vessels; 2 – control group showing normal striatum; 3 – control group, with normal architecture of hippocampus; 4 – control group, showing cerebellar cortex, normally appearing molecular layer, Purkinje cell layer, and granule cell layer; 5 – $AlCl_3$ group, showing degenerating (dark) neurons with neuronophagia, noticeable angiopathy; 6 – $AlCl_3$ group, showing a congested blood vessel with thickening in its wall, as well as focal perivascular lymphocytic aggregation; 7 – $AlCl_3$ group, showing loss in hippocampus cells with presence of pink fibrillar material and glial cells infiltrations; 8 – $AlCl_3$ group, showing necrosis of Purkinje cells; 9 – ashwagandha group, showing apparently normal cerebral cortex; 10 – ashwagandha group, showing apparently normal hippocampus; 11 – probiotic group, showing a few degenerating neurons in cerebral cortex with focal gliosis; 12 – probiotic group, showing cerebral vessels angiopathy with perivascular lymphocytic infiltration; 13 – probiotic group, showing a few degenerating neurons in the hippocampus; 14 – probiotic group, showing a few degenerating Purkinje cells; 15 – formula group, showing apparently normal cerebral cortex; 16 – formula group, showing apparently normal hippocampus

plates 13 & 14). The treatment of the animals with the formula (AEE and probiotics)-supplemented Karish cheese exerted the best neuroprotective action. The cerebral cortex appeared normal (Fig. 8, plate 15), although both the hippocampus and the cerebellum showed a few necrotic cells (Fig. 8, plate 16).

The $AlCl_3$-induced significant elevation in malondialdehyde and reduction in enzymatic (superoxide dismutase and CAT) and non-enzymatic (glutathione) antioxidant efficiency, as well as the extensive neuronal damage in the hippocampus were not consistent with the reports of Igbokwe *et al.* and Makhdoomi *et al.* [76, 77]. Since Al^{3+} and Fe^{3+} have similar ionic radii, Al^{3+} can bind to the Fe^{3+}-binding protein transferrin and pass Al^{3+} by transferrin receptors to deliver Al^{3+} to the brain and initiate oxidative damage [78]. Moreover, Al^{3+} alters Ca^{2+} flux and causes abnormal augmentation of intracellular Ca^{2+}, which can increase the production of reactive oxygen species via mitochondrial dysfunction [79]. This can result in oxidative damage, neuronal degeneration, neurochemical changes, and cognitive impairments. Since both inflammation and oxidative stress are interrelated, the latter induces inflammatory cytokine genes [80]. Thus, exposure to metals such as aluminum can increase the levels of proinflammatory cytokines in the brain [81]. Moreover, elevated nitric oxide inhibits microglia proliferation and stimulates glutamate release from astrocytes, leading to excitotoxicity of neurons and glia. Our study revealed that AEE- and/or probiotic-fortified cheese efficiently alleviated the $AlCl_3$-

associated pathophysiological deteriorations. It has been reported that AEE exhibited neuroprotection against oxidative stress by activating the Nrf2 pathway and up-regulating cytoprotective genes, as well as Keap-Nrf2-ARE signaling [82, 83]. Ashwagandha appears to increase the expression and translocation of Nrf2 that binds on ARE and causes the induction of several phase I and phase II metabolizing enzymes, phase III detoxifying proteins, and antioxidant proteins. The up-regulation of detoxification enzymes enhances cell survival and protection due to an improved redox state which prevents glutathionylated protein accumulation. Also, AEE-mediated neuroprotection was reported showing that Withanolide-A (the main constituent of AEE) increased glutathione synthesis in neuronal cells [84].

CONCLUSION

Acid curd (Karish) cheese as a dairy product model can consider an excellent delivery system for ashwagandha and probiotics, mainly *Lactobacillus plantarum*, to provide maximum health benefits. The addition of ashwagandha extract and/or *L. plantarum* enhances the nutri-pharmaceutical value of Karish cheese without affecting its properties. The combination of ashwagandha extract and *L. plantarum* performed a modulatory effectiveness against induced neurotoxicity, especially related Alzheimer's disease and learning difficulties. This effect was achieved through a remarkable improvement in the behavioral, neurochemical, and oxidative status.

CONTRIBUTION

L.K. Hassan and H.H. Salama were involved in the original research and manuscript writing. K.G. Abdel-Wahhab and H.M.A. Khalil conducted the biological study. S.M. Abdelhamid performed the microbiological assessment. All the authors were involved in editing, reviewing, and proofreading the manuscript. All the authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest which could hinder the publication of this article.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATION

The sensory test was carried out in compliance with the standard institutional criteria established by the Ethical Committee of the National Research Centre. After the participants had been given a full explanation of the study, they had to provide their signed written informed consent to participate in the research. The experimental study was approved by the Veterinary Institutional Animal Care and Use Committee (Approval No. Vet CU23012020113) in accordance with the American Guidelines for Animal Care and Use.

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Encapsulated polyphenols in functional food production

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Abstract:
Polyphenols are present as nutrient components in many functional food formulations. However, their bioavailability is quite low, and they tend to degrade under extreme technological conditions, e.g., heating, pH, etc. Moreover, polyphenols are known for their specific bitter taste. As a result, a large amount of polyphenols spoils the sensory properties of the finished product. Encapsulation seems a prospective solution to this problem. This article provides a comprehensive review of scientific publications on various methods of polyphenol encapsulation.

The review covered publications registered in PubMed, Google Scholar, ResearchGate, Elsevier, eLIBRARY.RU, and Cyberleninka in 2002–2023 with a focus on original research articles published after 2012. The search involved such keywords as *polyphenols*, *encapsulation*, *flavonoids*, *delivery systems*, and *functional products*.

Encapsulating materials are made of organic or inorganic substances, as well as of their combinations. Mineral salts delay the contact between polyphenols and taste buds. However, they are not resistant enough to gastric juice. In this respect, organic matrices are more effective. Carbohydrates protect active molecules from degradation in the stomach. Liposomes increase the bioavailability of polyphenols. Milk or whey proteins also proved quite effective for a number of reasons. First, they mask the astringent taste, which makes it possible to include more polyphenols in functional food formulations. Second, the resulting product is fortified with valuable proteins and essential amino acids. Third, high concentrations of polyphenols possess enough antioxidant properties to increase the shelf-life.

Polyphenol encapsulation is an effective method of functional product design, especially in the sphere of foods made for dietary nutrition, sports, preventive diets, etc.

Keywords: Polyphenols, biological activity, encapsulation, functional ingredients

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INTRODUCTION

Recent decades have seen a surge of scientific interest in phenolic compounds of plant origin in the fields of physiology, medicine, and functional foods. Polyphenols possess antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory, and organoprotective properties [1–7]. Foods fortified with primary elements, micronutrients, and biologically active substances contribute to treatment and prevention of major cardiovascular, endocrine, and oncological diseases.

Polyphenols with their beneficial properties are an important part of any healthy diet. The Federal Service for Surveillance on Consumer Rights Protection and Human Welfare was the first to specify the content of polyphenols and flavonoids in its Dietary Recommendations [8].

In nature, polyphenols occur in very small quantities. Moreover, they usually remain out of human consumption, being discarded as production waste, e.g., grape peels and seeds in wine production, citrus peels and membranes in juice production, olive pomace in

olive oil production, etc. [9]. Given their low bioavailability, mere dietary adjustments cannot provide human body with polyphenols, which makes food fortification the only option available. Unfortunately, their bitter and astringent taste gives polyphenols a rather unfavorable sensory profile. Free polyphenols interact with salivary mucin and weaken its coating effect on taste buds, resulting in a specific taste. Encapsulation can camouflage the unpleasant taste of polyphenols while improving their resistance to some external factors, preserving biological activity, and even increasing bioavailability [9–14]. For instance, the pharmaceutical industry uses encapsulation to create new polyphenol-based drugs [15–17]. In the food industry, phenolic compounds with their antioxidant effect can extend the shelf-life of finished products [18, 19]. Therefore, the success of new polyphenol-containing functional foods depends on the correct choice of encapsulating material and technological conditions.

In our study, we focused on scientific publications on various methods of polyphenol encapsulation.

STUDY OBJECTS AND METHODS

This review covered publications registered in PubMed, Google Scholar, ResearchGate, Elsevier, eLIBRARY.RU, and Cyberleninka databases in 2002–2023, with the focus on original studies published within the last 10 years in peer-reviewed sources. The search involved such keywords as *polyphenols*, *encapsulation*, *flavonoids*, *delivery systems*, and *functional products*.

RESULTS AND DISCUSSION

Encapsulation technologies. During encapsulation, a certain carrier substance develops particles, or capsules, that capture some other substance or a group of similar substances. Encapsulation isolates the biologically active substance from the environment. Various physicochemical interactions allow the carrier substance to include biologically active substances in the capsule wall or envelop them. Capsule particles can be of several types. Microspheres contain the active substance inside or as part of the wall. In microcapsules, the active substance is coated with the wall of some natural or synthetic carrier (Fig. 1) [17]. Particles vary in size from several micrometers to several millimeters.

Encapsulation is a well-tested reliable technology that makes it possible to modify the necessary properties and deliver all kinds of polyphenolic compounds. This method improves the bioavailability of polyphenols, masks the astringency, increases the shelf-life, and delivers molecules to specific parts of the intestine for metabolism or absorption.

Modern research publications feature such encapsulation technologies as spray (freeze) drying, spray cooling, extrusion coating, fluidized bed encapsulation, liposome capture, simple/complex coacervation, gelation, etc.

All encapsulation methods can be divided according to the strategy employed as top-down or bottom-up. The top-down strategy occurs in extrusion of emulsification. It breaks large solid or liquid structures into smaller fragments by external force, e.g., cutting, impact, or pressure. This method requires special equipment, e.g., homogenizers, which increases its operating and maintenance costs.

The bottom-up strategy uses self-organization potential to form larger molecules. Microcapsules appear when the components to be encapsulated and the carrier are mixed together in a solution. The process is sensitive to pH, temperature, ionic strength, etc., but it is energy efficient and provides microcapsules of preprogrammed shape and size. Coacervation, spray drying, electrospinning, bonding, and antisolvent precipitation are the most popular bottom-up strategy methods [20].

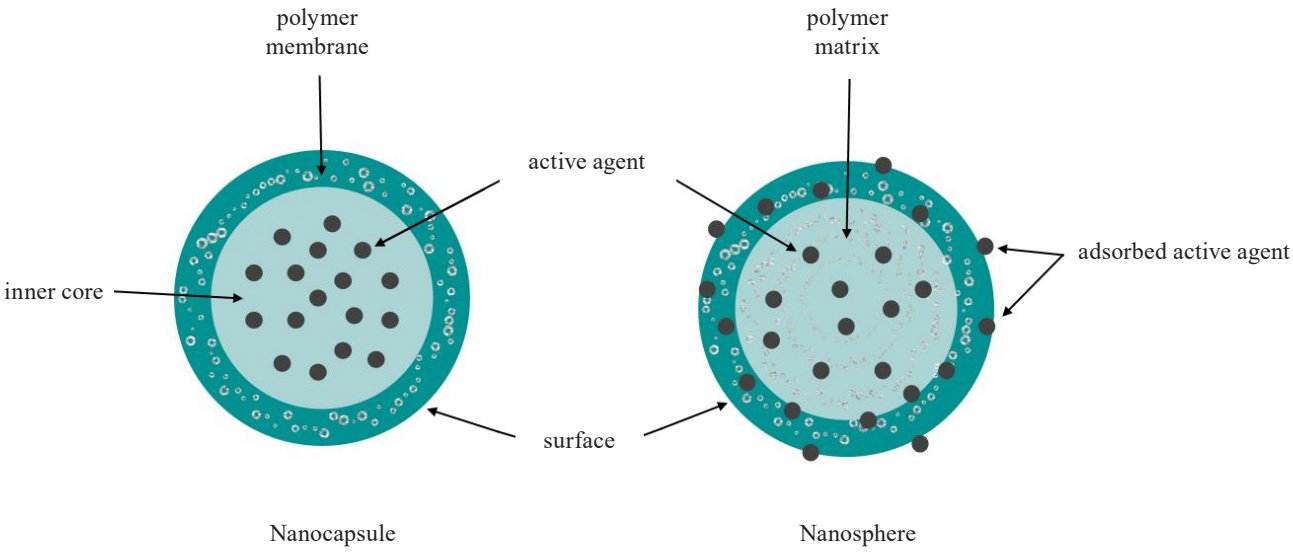

Figure 1 Nanospheres with the active substance absorbed and dispersed on the polymer surface vs. nanocapsules with the active substance in its core surrounded by the polymer membrane

Table 1 illustrates the advantages and disadvantages of each method [20].

In each specific case, the choice of encapsulation method depends on the chemical nature and properties of the molecules and the equipment available. Properly selected conditions make it possible to obtain capsules of the required structure, shape, and size.

The choice of optimal carrier medium is also of great importance because it delivers active molecules to certain parts of digestive tract, protects them from aggressive enzymes, ensures their bioavailability and thermal stability, improves sensory properties, etc. [30–34].

A wide range of mineral and organic substances can serve as carrier media during encapsulation.

Inorganic carriers. The bitterness of some polyphenols, e.g., green tea catechins, can be avoided by binding with inorganic salts, e.g., carbonates, phosphates, calcium and sodium chlorides, etc. Elabbadi *et al.* reported that this method masked the bitter taste without affecting the bioavailability of catechins, which did not degrade in the gastrointestinal tract [35]. They used the co-precipitation method by mixing a green tea extract with sodium carbonate and/or phosphate and adding calcium chloride. The resulting microcapsules proved resistant to washing, the maximal binding efficiency being as high as 65%. Such variables as flow rate, cations-to-anions ratio, and total ions also affected the degree of binding, particle sizes, and their tendency to agglomerate. The carbonate content was especially important. The optimal carbonate content was 75% since catechins appeared to be unstable in alkaline environment. When the proportion of carbonate was low, catechins were leached from the capsules during washing. Green tea extracts usually contain a mix of different catechins. Some catechins, e.g., catechin gallate, gallocatechin gallate, and gallocatechin methyl gallate, could bind more efficiently than the corresponding epicatechins. Gallates were better binders than similar molecules with no gallate radical.

More complex microcapsule compositions may include calcium chloride. For example, the bonds between calcium cations and carbonyl groups of pectinamide resulted in a network that stabilized the structure of the capsule [36]. When Wang *et al.* tried to encapsulate ferulic acid with zein, calcium chloride affected the structure of the resulting complexes [37]. The proportion of α-helices and β-sheets dropped whereas the proportion of disordered turns increased, resulting in smooth spherical particles. Calcium chloride was also reported as a hardening agent in combination with organic carriers [38].

Mineral carriers mask the bitter taste of polyphenols because capsules pass through the oral cavity very quickly to dissolve elsewhere in the digestive tract. The biggest disadvantage of mineral salts is that they cannot modify the breakdown and subsequent adsorption of polyphenols in the gastrointestinal tract. In this regard, bioorganic carriers seem to have better prospects. Carbohydrates, lipids, proteins, and their combinations are valuable nutrients that are able to protect polyphenols from aggressive hydrochloric acid and enzymes. They deliver polyphenols to certain parts of the gastrointestinal tract and/or increase their bioavailability.

Carbohydrates. The food industry knows a wide variety of carbohydrates, e.g., maltodextrin, β-cyclodextrin, pullulan, gelled glucan, curdlan, sodium alginate, pectin, etc. Their polysaccharides are stable at low pH; as a result, active components manage to reach the intestine intact to be absorbed there.

Dey *et al.* constructed a model of artificial digestion to encapsulate quercetin with gellan gum and registered the maximal release of quercetin at pH 7.4 [39]. Vallejo-Castillo *et al.* used a pectin-alginate composite to obtain enteric capsules with a papaya exocarp extract, which is rich in rutin and transferulic acid [40]. Guzmán-Díaz *et al.* included green tea extracts in double emulsions of chia seed mucilage [41]. After adding thixogam and carrageenan, they ensured the maximal release of catechins in the intestinal phase while preserving their antioxidant activity. Another study used chitosan, maltodextrin, and gum arabic to mask the astringent taste, increase the antioxidant activity, and prolong the shelf-life [42].

Although the encapsulation mechanisms are similar, each carrier has some specific properties. For example, alginate is stable in acidic environment while alkaline

Table 1 Advantages and disadvantages of the most common encapsulation methods

Methods	Approach	Advantages	Disadvantages
Emulsification	Top-down	Applicable to both hydrophilic and hydrophobic compounds [21]	Sophisticated equipment [22]
Spray drying	Bottom-up	Simple, fast, cheap [23]	Low product yield; not suitable for heat-sensitive materials [22]
Extrusion	Top-down	Soft process; suitable for both hydrophilic and hydrophobic compounds; long shelf-life [24]	Large particle size; few types of matrix materials; complex and expensive scaling [24]
Electrospinning	Bottom-up	Simple; cheap; applicable to heat-sensitive interactions; yields fibers with a very high surface-area-to-volume ratio and of all shapes and sizes [25, 26]	Porous fibers [27]
Coacervation	Bottom-up	Hihg encapsulating efficiency [28]	Unstable in aqueous solution; high production cost; sensitive to many factors, e.g., pH [21, 22, 29]

environment makes its capsules swell and disintegrate. Chitosan has favorable antibacterial and antioxidant properties, as well as effective mucoadhesion [43, 44]. Chitosan binds cholesterol and controls the release of active substances but dissolves only under acidic conditions. Guar gum is highly resistant to digestive enzymes and very stable in the upper gastrointestinal tract [45]. Between corn starch and quinoa-derived starch, quinoa starch appeared to form smaller quercetin capsules, which were more effective in protecting the active substance during storage [46]. Similar data were reported by Remanan and Zhu for rutin [47].

Polyphenols are known for their low hydrophilicity, which restricts the technological process and complicates absorption in the gastrointestinal tract. Cyclodextrin glucanotransferase triggers the development of cycloamylose. As it encapsulates resveratrol, its solubility in water increased by 6000 times, but the chemical stability, antioxidant effect, and anti-inflammatory properties remained the same [48].

The matrix material also affects the stability of the encapsulated active substance. Capsules based on a combination of alginate and guar gum were able to preserve anthocyanins during two weeks of storage [45]. Other research teams reported similar results [49]. In addition, alginate capsules proved very effective in preserving phenolic acids [45].

Polyphenol may both increase and decrease in content during storage as condensed molecules depolymerize and monomers bind to the capsule matrix. Composite matrices maintain higher antioxidant values of polyphenols, both in fresh capsules and after two or three weeks of storage, especially with guar gum.

Anthocyanins are the most common plant pigments, which makes them popular food colorants. The color intensity of encapsulated concentrates was reported to grow during storage, especially when a composite matrix was involved. Chitosan increased the intensity of the yellow color while alginate or its combination with guar gum intensified the red color [45]. Vergara *et al.* encapsulated extracts of purple potatoes, which owe their color to anthocyanins [50]. Purple potatoes have good prospects as a natural and safe food colorant. The scientists achieved 86% encapsulation efficiency by using maltodextrin. The resulting capsules provided higher stability of anthocyanins during storage. As much as 45% of the encapsulated anthocyanins remained after 138 days at 60°C while the control sample managed to maintain only 10% of the original amount after two days. A similar protective effect was reported for a gastrointestinal digestion model.

Costanzo & Angelico used encapsulation to preserve the active components of *Silybum marianum* extracts, improve their antioxidant and anti-inflammatory activity, and increase their bioavailability, thus raising the effectiveness of the final medicinal product [51]. Such extracts exhibit various important physiological effects and include a lot of biologically active substances, e.g., flavolignans, flavonoids, silibin A, silybin B, isosilybin A, isosilybin B, silicristin, isosilicristin, silydianin, taxifolin, etc. [52–55].

Sansone *et al.* used carboxymethylcellulose as coating and sodium lauryl sulfate as surfactant [56]. The liquid phase consisted of water, ethyl alcohol, and acetone in a ratio of 50:15:35. Spray drying made it possible to obtain the desired particle size and increase the bioavailability of plant components. If modified, this method can be used to encapsulate other natural biologically active substances.

Lachowicz *et al.* used maltodextrin and inulin to produce juneberry extracts, which they dried by vacuum or freeze drying to obtain powder [57]. Maltodextrin rendered the powders a higher content of polyphenols from berries and juices while inulin was more effective with berry peel samples. Freeze drying provided more active components and better antioxidant indicators than vacuum drying. Such powders can become an effective fortification means for functional foods with biologically active components.

Ćorković *et al.* performed a comparative analysis of pectin and alginate as encapsulating carriers for polyphenols and volatile substances in chokeberry juice [38]. Spectroscopy and high-performance liquid chromatography showed that alginate capsules had a higher concentration of polyphenols. Also, alginate capsules had better antioxidant results according to some other tests, e.g., FRAP (Ferric Reducing/Antioxidant Power), CUPRAC (CUPric Reducing Antioxidant Capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl), and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

Haładyn *et al.* used a combination of polysaccharides to encapsulate chokeberry concentrates and powder [45]. The researchers obtained the maximal content of active substances using a three-component carrier while the minimal one belonged to alginate capsules. Three-component matrices also demonstrated the greatest agreement between the polyphenolic profile of the capsules and the original concentrate and powder. Apparently, different polysaccharides differed in their abilities to bind fractions of phenolic compounds. Therefore, multicomponent carriers proved more effective in encapsulating natural extracts with complex chemical compositions.

To sum it up, carbohydrates solve a wide range of problems, from stabilizing the active substances, maintaining favorable physiological properties, and increasing bioavailability, to improving the sensory profile of the final product. By choosing the optimal carrier and technological conditions, food producers can obtain particles of a particular size with a lot of active substance.

Lipids. Emulsions, liposomes, nanoliposomes, and solid lipid particles are popular means of encapsulation. They deliver biologically active molecules in the food industry, pharmacy, and cosmetics [58–60]. Hydrophilic components are included in the aqueous fraction in the center of the liposome while fat-soluble components are distributed in the lipid layer. Amphiphilic phospholipids are natural components of cell membranes and

reliable liposome agents. They are found in soy, sunflower, and egg lecithin. Phospholipids from rice bran were reported to produce nanoliposomes with quercetin [61]. Jahanfar *et al.* prepared encapsulated rosemary extract using phosphatidylcholine by freeze-drying at 70°C [62].

Lipid microparticles can be a mix of hydrogenated and interesterified vegetable oils. Cutrim *et al.* heated oils and tea extracts to 80°C to dry them in a cooled medium in a mini spray dryer [63]. As a result, they achieved an encapsulation efficiency of 83.5%.

Pan *et al.* applied the pH-driven encapsulation method to low-soluble polyphenols [64]. The method relied on the deprotonation of hydroxyl groups in a highly alkaline environment. As a result, negatively-charged molecules increased the solubility of the capsule. However, this method is quite limited because some polyphenols become unstable in alkaline environment. A greater number of hydroxyl groups increases the chance of oxidation. Therefore, quercetin is less stable than resveratrol and curcumin, which reduces its encapsulation efficiency.

Peng *et al.* reported that encapsulation depends on the concentration [65]. For example, curcumin exhibited its maximal solubility at a concentration of 0.36 mg/mL. By increasing the concentration of curcumin in the solution, the scientists achieved no increase in its uptake by liposomes. Resveratrol was reported to demonstrate a similar pattern. However, quercetin increased its encapsulating abilities at larger concentrations because it went unstable in alkaline environment. Therefore, this type of encapsulation depends on two factors, i.e., solubility in water and stability at high pH values.

Moghaddasi *et al.* combined black pepper oil with polysorbate 80 to obtain an emulsion [66]. They managed to optimize the solubility of curcumin and obtain microcapsules of 16 nm in size with a better antioxidant profile than in an aqueous curcumin suspension. Kumar *et al.* encapsulated resveratrol using the ultrasound method in a nanoemulsion with lecithin in combination with polysorbate 80 [67]. The particle size reached 20 nm, and the binding efficiency was as high as 99%.

Resveratrol emulsion turned out to be more resistant to ultraviolet irradiation than its aqueous or alcoholic solutions. Both liquid (medium chain fatty acids + polysorbate 80) and semi-solid resveratrol nanoemulsions (laurylpolyoxyl-32 glyceride + caprylcaproylpolyoxyl-8 glyceride) were more bioavailable as they were more efficient in penetrating the wall of the small intestinal [68]. Emulsion stability and encapsulation efficiency could be increased by adding magnesium salts and poloxamers, i.e., molecules containing central hydrophobic and lateral hydrophilic regions [69].

Liposomes make it possible to encapsulate not only isolated substances, but also their combinations. For instance, liposomes with curcumin and resveratrol were more stable during storage than liposomes containing only one type of polyphenol. Spectroscopic and fluorescence analyzes made it possible to evaluate the distribution of different molecules in a liposome. Thus, curcumin predominated in the hydrophobic region while resveratrol predominated in the hydrophilic region. The resulting synergistic effect of polyphenols on liposome stability provided an encapsulation efficiency of 80.42% and a better preservation of antioxidant properties [70].

Chen *et al.* succeeded in increasing antioxidant activity by encapsulating epigallocatechin gallate and quercetin in liposomes [71]. Conversely, polyphenols were reported to reduce lipid oxidation [72]. This property can be used to fortify products with valuable oils and essential fatty acids.

Exosomes are phospholipid-based membrane vesicles. In humans and animals, exosomes are produced by many tissues and are responsible for intercellular transport. Some technologies make it possible to extract vesicles from biological fluids, e.g., cow's milk, and incorporate various biologically active or medicinal substances [73–76]. Vashisht *et al.* described the technology for producing exosomes from milk. In their study, exosomal curcumin was more stable and bioavailable, compared to the free control [77]. Other authors reported similar data [78, 79]. Extracellular vesicles of plant origin are known to contain biologically active components, including polyphenols [80, 81]. Such vesicles have good prospects as a delivery system for nutrients and pharmacological substances meant for oral administration.

Thus, lipid encapsulation increases the stability and bioavailability of polyphenols while maintaining their physiological activity.

Proteins. Encapsulation of polyphenols in protein-based nanoparticles is a popular research topic. Proteins seem an appropriate solution to the sensory characteristics' problem that producers of fortified foods often have to face. However, proteins affect the color of the final product and may make the solution opaque [82].

Protein-polyphenol interaction is based on both covalent and non-covalent bonds, e.g., hydrogen, hydrophobic, or Van der Waals bonds [83]. Larger and more hydrophobic molecules attach to protein more effectively. Hydrophobic bonds develop the interaction between the aromatic rings of polyphenols and the pyrrolidine rings of proline residues. Hydrogen bonds are formed by hydrogen acceptors in the protein and hydroxyl groups of polyphenols. Catechins bind to β-lactoglobulin (β-Lg) as the number of hydroxyl groups increases in the series of epigallocatechin gallate > epigallocatechin > epicatechin > catechin [84].

Flavonols follow the same pattern when interacting with collagen. An increase in the number of hydroxyl groups in the kaempferol – quercetin – myricetin chain raises the melting point of the complex, which indicates its greater stability [85]. The amount of gallate radicals is another important factor. Spectroscopy and fluorescence tests showed that the bond between epigallocatechin gallate and β-Lg is stronger than that of epigallocatechin [86].

The mature of protein also affects the encapsulation process. Hydrophobic proteins attach better to polyphenols if the former are rich in proline and basic residues,

as well as possess an open and mobile structure. In a study of whey proteins, the more hydrophobic β-lactoglobulin (β-Lg) had a greater affinity for polyphenols than α-lactalbumin (α-La) [87]. If preheated, proteins facilitate the formation of bonds with polyphenols. At ≥ 60°C, covalent bonds between epigallocatechin and β-Lg occurred fast, with lysine residues acting as a binding site [88]. Open-structured native globular proteins often demonstrate intramolecular interactions that promote protein aggregation [89].

These interactions depend on the molecular structure. For instance, a comparative analysis of galangin and genistein showed that galangin had a greater affinity for whey proteins and caseinate due to its flatter stereochemical structure and random B-ring rotation. Genistein, on the other hand, demonstrated localized isomerism and a twisted stereochemical structure [87, 90].

Jia *et al.* observed hydrogen and Van der Waals bonds during the formation of complexes of β-Lg with phenolic chlorogenic and ferrulic acids whereas hydrophobic interactions predominated in complexes with epigallocatechin gallate [91]. Temperature also proved an important variable. Under thermal treatment, chlorogenic and ferullic acids developed weaker bonds with β-Lg while epigallocatechin gallate bonds grew stronger [91].

The ability of proteins to bind to phenolic compounds are limited. For example, flexible gelatin molecules were reported to be able to bind to punicalagins (pomegranate polyphenols from the tannin group), whereas bovine serum albumin failed [92]. To select a protein carrier, functional food developers are to find out how a specific protein binds to a specific phenolic compound.

Scientific articles describe numerous methods for encapsulating polyphenols with proteins.

For example, Rodríguez-Félix *et al.* produced quercetin nanoparticles from zein by electrospraying [18]. They used Fourier transform infrared spectroscopy (FTIR) to establish the hydrogen bonds between quercetin and protein molecules. Such nanoparticles can increase the bioavailability of quercetin in medicine. Their antioxidant properties can prolong the shelf-life of functional foods. Bruni *et al.* also used zein to encapsulate leaf extracts of Paraguayan holly (yerba mate tea) by the method of electrospinning [93]. The resulting fibers retained antioxidant activity and increased the thermal stability of phenolic extracts, compared to the non-encapsulated samples.

When encapsulated by gelation of β-Lg matrix, epigallocatechin gallate could protect catechins in the stomach, thus preserving them intact to be absorbed in the intestine [94]. Another study involved thermally denatured β-Lg [95]. In the abovementioned studies, particle size and encapsulation efficiency depended on such variables as pH, temperature, molar ratio of β-Lg vs. epigallocatechin gallate, and β-Lg concentration.

Lestringant *et al.* encapsulated epigallocatechin gallate into a native β-Lg carrier, which they heated to 85°C to remove the solvent from the solution [96]. They tested the resulting native complex of β-Lg and epigallocatechin gallate for stability, physical properties, and bioactivity. The complexes showed comparable stability and binding efficiency to heated β-Lg nanoparticles at a molar ratio of 1:1 epigallocatechin gallate/β-Lg. The heated and desolvated β-Lg nanoparticles were of similar size; however, the desolvated samples possessed the highest binding affinity for epigallocatechin.

Cheng *et al.* reported that resveratrol, folic acid, and α-tocopherol bound with the central cavity of the outer surface of β-Lg [97]. The binding occurred near the amino acids of tryptophan (position 19) and arginine (position 124). Another binding area was the hydrophobic pocket in the space between α-helix and β-sheet structure of the protein. Therefore, β-Lg can simultaneously bind three biologically active substances to form protein-polyligand complexes. Xiang *et al.* described β-Lg as a transport molecule capable of binding low molecular weight lipophilic ligands, including polyphenols [98].

A pH-dependent encapsulation with casein can be applied to substances with low hydrophilicity, e.g., curcumin. The same method was once applied to nanoliposomes [99]. At pH 12, sodium caseinate dissociated and curcumin deprotonated. The subsequent neutralization triggered encapsulation of curcumin into self-assembling casein capsules, which were then spray dried. Ultraviolet and nuclear magnetic resonance spectroscopies revealed that sodium caseinate prevented curcumin from degradation, as a rule, in alkaline environment. This cheap technology enhances the biological properties of curcumin, in particular, its antiproliferative effect against colon and pancreatic cancer cells.

Xue *et al.* used a nanocomplex of glycosylated casein to encapsulate epigallocatechin gallate [100]. The resulting capsules did not aggregate during storage. An intestinal digestion model *in vitro* showed that the nanocomplex had a slow and prolonged release. Li *et al.* encapsulated naringenin with micellar casein and detected an increase in its solubility, as evidenced by its concentration in the aqueous phase [101]. Such capsules demonstrated a good potential as carriers of hydrophobic nutrients, e.g., flavonoids.

Polyphenols themselves can modify the biological and technological properties of proteins, increased whey proteins. Phenolic compounds are able to change the secondary structure of proteins, which is a scientifically proven fact.

Kanakis *et al.* used spectroscopic methods, i.e., circular dichroism and IR spectroscopy with Fourier transform [84]. Hydrophobic and hydrophilic bonds formed during the interaction between β-Lg and epigallocatechin gallate could increase the proportion of β-sheets and α-helices in the protein. The resulting protein structure proved reliably stable. In another study, the transformation of β-sheets into α-helices induced fewer twists and turns [91, 102]. The intensity depended on the specific features of individual molecules [103]. Flavonoids reduced the hydrophobicity of the protein surface and the number of sulfhydryl groups, which may denote increased protein aggregation [91, 103].

Thus, proteins can be used in functional food products. If combined with polyphenols, they can act as antioxidants, emulsifiers, foaming agents, and gelling agents [104–106]. However, the effect may be quite opposite. For example, baicalein, alone or with chrysin, was reported to enhance the foaming ability of *β*-Lg, while chrysin, on the contrary, weakened it [103].

Polyphenols were able to reduce the allergenic effect of milk protein complexes by weakening their binding to immunoglobulin epitopes in specialized foods for patients with food allergies to milk proteins and, in particular, *β*-Lg [102, 107].

Multicomponent capsules are getting more and more scientific attention [108–110]. By combining different materials in the matrix, researchers obtain capsules with predesigned properties, e.g., particle size, binding efficiency, pH-related solubility, stability, release rate, absorption by enterocytes, nutraceutical value, etc.

Luo *et al.* used dextran-zein fibers obtained by electrospinning to encapsulate curcumin [111]. By adding zein to dextran, they achieved a nonlinear increase in the hydrophobicity of the resulting fibers: 30% of zein increased the flexibility, elasticity, and tensile fiber strength. However, low concentrations had quite the opposite effect. The ratio of the protein and polysaccharide components also affected the release rate of active molecules. The maximal rate of curcumin release was registered at the 15% zein content.

Gómez-Mascaraques *et al.* encapsulated a grape juice extract using a mix of gelatin and carrageenan [30]. The optimal ratio protein to carbohydrate was 85 to 15. This ratio provided the maximal absorption rate of biologically active substances. The release of the extract from the matrix turned out to be pH-dependent and increased in alkaline environment. Calcium chloride boosted the encapsulation efficiency but eliminated the pH-dependence of the release. As a result, the extract release did not run well in alkaline environment. The researchers, however, managed to solve the problem by changing the sequence of encapsulation: they extruded carrageenan and extract through gelatin in the presence of calcium chloride. In this way, they obtained capsules for intestinal release that were resistant to acidic gastric juice.

Wang *et al.* used nanoparticles made from chitosan in combination with bovine serum albumin to encapsulate polyphenols obtained from *Pinus koraiensis* pine cones [112]. The capsules showed high stability over a long storage period at room temperature. An artificial digestion model proved that the biologically active substances could reach the intestine.

Caballero *et al.* combined pea protein and high methoxyl pectin (1:1) at pH 4.0 to encapsulate hesperidin [113]. The resulting capsules were 10 times more soluble in water while exhibiting higher antioxidant activities and a better bioavailability *in vitro* than unencapsulated hesperidin. The researchers mentioned their good commercial prospects in food and drink fortification.

Viljanen *et al.* observed anthocyanins in a whey-based emulsion [114]. Anthocyanins obtained from black currant, raspberry, and lingonberry exhibited dose-dependent antioxidant properties in relation to both proteins and lipids. Such effect was brought about by the optimal ratio of delphinidin and cyanidin. Most anthocyanins were distributed in the aqueous fraction, exhibiting antioxidant activity against proteins. The presence of lipids reduced the amount of anthocyanin in the aqueous phase. About 20% of anthocyanins manifested their antioxidant properties right where they bound to proteins, i.e., on the border between lipid and water phases [115].

Aceituno-Medina *et al.* used the method of electrospinning to encapsulate quercetin in amaranth protein isolates in combination with pullulan ultrafine fibers [116]. Quercetin molecules distributed quite evenly throughout the smooth fibers. A digestion test *in vitro* showed that the encapsulated compounds had a better antioxidant capacity than the non-encapsulated ones.

Yadav *et al.* combined whey protein concentrate, maltodextrin, and gum arabic to encapsulate grape juice extract by the ultrasound method [117]. They achieved an encapsulation efficiency of 87.9–91.13% with the smallest particle size and maximal preservation of antioxidant properties. These results belonged to microcapsules coated with whey protein concentrate and maltodextrin or gum arabic at ratios of 4:1 and 3:2, with a core-to-coating ratio of 1:5.

Emulsion thermal gelation is another common encapsulation method. Betz & Kulozik encapsulated blueberry extracts in whey protein [118]. The average diameter of the resulting microcapsules was 0.5–2.5 mm. However, the particles were large (≥ 100 μm), which spoiled the sensory properties of the finished products. The particle size decreased as the stirring speed reached 1350 rpm. Further acceleration did not reduce the particle size. Emulsifiers reduced the surface tension, thus preventing the particles from aggregation and sticking. This measure made it possible to reduce the particle size to 70 μm. However, the emulsifier promoted the transition of anthocyanins from the protein solution to the oil phase. The pH value was another important factor that affected the particle size. When blueberry extract was added at ≥ 10% of total volume at pH 3.0, the resulting aggregates were irregular in shape an did not form spherical capsules. The scientists explained it by the effect of pH on the electrostatic interactions between protein and polyphenol molecules.

Ha *et al.* proposed an interesting technological solution by combining all three types of organic encapsulating agents, i.e., proteins, carbohydrates, and lipids [119]. They used chitosan bound to linoleic acid and combined with *β*-lactoglobulin (chitosan – linoleic acid/*β*-Lg) as a carrier to encapsulate quercetin. The nanoparticles were prepared by modified ionic gelation. They treated a mix of quercetin with chitosan-bound linoleic acid and *β*-Lg at 5, 10, 15, and 20°C, followed by tripolyphosphate. As the amount of linoleic acid increased and the temperature went down, the association efficiency of chitosan and *β*-Lg increased, as did the quercetin encapsulation

efficiency. The researchers explained the effect by an increase in the hydrophobicity of *β*-Lg, which, in its turn, triggered an increase in hydrophobic interactions between molecules. Lower temperatures also reduced the particle size.

Guo *et al.* used a three-component matrix to encapsulate curcumin [120]. Pea protein isolate, high-methoxyl pectin, and rhamnolipid served as surfactants. When the ratio of protein to curcumin was 40 to 1, the encapsulation efficiency reached 93%. According to the Fourier transform infrared spectroscopy, the complexes appeared as a result of hydrogen bonds and electrostatic interaction. Curcumin was not crystalline. The capsules showed greater stability with respect to ultraviolet irradiation, thermal effects, and a gastrointestinal model.

Polysaccharides, e.g., gum arabic, can act as emulsion stabilizers as evidenced by the size distribution of the resulting particles. For example, Zhang *et al.* registered two size groups of particles in an emulsion of whey proteins and sunflower oil [72]. The smaller particles (130 nm) corresponded to aggregated proteins, and the larger ones (550 nm) were formed by the emulsion. By adding gum arabic, the researchers reduced the proportion of the smaller particles in a dose-dependent manner. At 1% gum arabic and 1.6 mM calcium chloride, only one peak was observed, which meant that all particles were ≤ 2580 nm. The *ζ*-potential also became more negative as the concentration of gum arabic and calcium chloride continued to grow. This effect occurred because the electrostatic repulsion of the particles grew and the steric stabilization of the emulsion prevented its aggregation. The encapsulation of resveratrol occurred at the hydrophobic part, i.e., at the water and oil interface, so the presence of gum arabic and calcium chloride affected the efficiency of the process. Consequently, the resveratrol fluorescence increased together with the concentration of these matrix components.

Shao *et al.* used an emulsion of whey protein and medium chain triglycerides stabilized with gum arabic to reach a 50% resveratrol encapsulation efficiency [121].

Not only the capsule, but also its filling can be multicomponent, i.e., several biologically active substances can be encapsulated at once. For example, a combination of curcumin and *β*-carotene was reported to increase the bioavailability and stability of *β*-carotene [32].

Another study demonstrated a higher photostability of caffeic and folic acids when encapsulated together, compared to single encapsulation [122]. A combination of various polyphenols, e.g., low molecular weight genistein and high molecular weight icariin, increased the total flavonoid content in capsules, compared to isolated encapsulation [123].

Biological properties of encapsulated polyphenols. Polyphenol capsules must be safe to be used in the food and pharmaceutical industries. Scientist developed several methods to measure the release of active molecules and the preservation of the beneficial effects during technological processing.

Several *in vitro* studies on various models demonstrated the absence of cytotoxicity in polyphenol capsules [62, 124]. The increased bioavailability of polyphenols is also well documented. For example, consumption of resveratrol capsules made from casein or casein with *β*-cyclodextrin increased its concentration in the blood by 10 times, compared to free resveratrol tests [125, 126].

Kardum & Glibetic suggested that protein binding could stabilize or even increase antioxidant properties and reduce autoxidation brought about by the changes in pH or temperature during food processing [82].

Polyphenols encapsulated with chitosan, maltodextrin, gum arabic, gelatin, and whey proteins also resulted in good antioxidant properties, long shelf life, and appropriate sensory profile in regard with taste [42]. For example, Pedrozo *et al.*, who used bovine serum albumin, recorded a twofold increase in antioxidant activity [127]. Quercetin nanoliposomes from rice bran phospholipids showed a thousandfold increase in antioxidant activity, compared to free quercetin [61]. By preserving the antioxidant activity of quercetin, capsules from chitosan and alginate were able to protect cells from oxidative stress [128]. However, encapsulation of blueberry extract using whey proteins had no significant effect on its antioxidant profile [129]. Some research teams even reported a decrease in antioxidant properties of quercetin, myricetin, and morin when they were bound to albumin, milk, and soy proteins [130].

This contradiction may be explained by different experimental conditions. In [88], the antioxidant properties of epigallocatechin gallate in combination with *β*-Lg did not change after heat treatment at 25–60°C. However, this indicator dropped when the temperature reached 85°C. The slower release of the active substance from microcapsules could be another explanation, but a slow release usually means a prolonged effect [131].

Capsules with curcumin made of zein and propylene glycol alginate preserved anticarcinogenic properties *in vitro* and demonstrated a 7.2-time increase in bioavailability *in vivo*, compared to crystalline curcumin [99].

Liposomes with rosemary extract retained their antiproliferative effect on cell lines and antimicrobial properties against Gram-positive bacteria [62]. Beconcini *et al.* studied the anti-inflammatory properties of encapsulated cherry extract, i.e., suppression of IL-6 and TNF-*α* and inflammatory cytokines *in vitro* [132]. They used chitosan derivatives and polylactic-glycolic acid. The abovementioned beneficial properties proved more pronounced in the capsuled cherry extract than in the pure one.

The anti-inflammatory effects of nanocurcumin were studied in COVID-19 patients. The mRNA expression and secretion of interleukins IL-1*β*, IL-6, TNF-*α*, and IL-18 were significantly lower in patients treated with nano-curcumin [133]. Piceatannol is a hydroxylated stilbene derivative with excellent anticarcinogenic properties. Encapsulated with bovine serum albumin, it demonstrated better anticarcinogenic and anti-inflammatory activity in laboratory mice [134].

In a human retinal cell line, epigallocatechin gallate liposomes had a more pronounced protective effect against oxidative stress compared to pure epigallocatechin gallate [69]. Haładyn *et al.* studied antidiabetic effects based on the inhibition of the glycolytic enzymes *α*-amylase and *α*-glucosidase. In their research, the encapsulated chokeberry concentrates and powders were more active than the non-encapsulated ones [45]. Capsules made of nanoporous silicone and *β*-cyclodextrin were able to preserve the antioxidant and antiangiogenic properties of polyphenols, in particular, of caffeic acid [135]. The organoprotective properties of curcumin in experimental gentamicin-induced nephropathy were stronger in liposomes than in crystalline forms [136].

Resveratrol liposomes also demonstrated greater stability than resveratrol nanoparticles. As a result, resveratrol liposomes had a better physiological effect on the 3T3-L1 fibroblast cell line. Nanoparticles, however, provided a longer release. Both encapsulation methods increased the solubility and bioavailability of resveratrol [137].

The longer storage stability is an important advantage of encapsulation. Quercetin nanoliposomes made from phospholipids retained their properties at 4°C for 6 months and at 27°C for 5 months [61]. Liposomes with chitosan or epigallocatechin gallate conjugates retained activity against Gram-positive and Gram-negative bacteria during storage for 28 days at 30°C [138]. *β*-Lactoglobulin-encapsulated quercetin, as well as ferulic and vanillic acids, maintained good photo- and thermal stability and anti-glycation properties [139]. Co-encapsulation of various polyphenols, such as epigallocatechin gallate and quercetin, made it possible to preserve their antioxidant properties [71].

Thus, encapsulation preserves the biological activity of polyphenols and even optimizes it because it protects active molecules from aggressive environment, increases solubility, and improves bioavailability.

CONCLUSION

The review revealed a great scientific interest to various methods of encapsulating various biologically active substances, such as polyphenols. Some of them use isolated carriers while others develop complexes. The efficiency of polyphenol encapsulation vary from 50 to 90% or more, depending on the method, encapsulation conditions, and the carrier. In most cases, encapsulated polyphenols retain their biological activity, e.g., anticancer, antioxidant, or anti-inflammatory properties.

To sum it up, micro- and nanocapsules are able to:
– mask the astringency typical of pure polyphenols and their extracts in functional foods;
– protect molecules from digestive juices, control their release, and deliver them to certain parts of the gastrointestinal tract;
– increase the bioavailability of polyphenols by improving their absorption by the walls of the digestive tract; as well as
– extend the shelf-life of the finished products.

Food producers may obtain polyphenols not only as pure substances or plant extracts: they can be harvested from by-products, e.g., pomace, cake, peels, etc. Since these discarded materials are rich in polyphenols, their recycling can open way to sustainable technologies in food production [140–145].

Polyphenol encapsulation technologies make it possible to create a wide range of fortified functional products to be used in healthy diets, preventive treatment, sports food, etc.

CONTRIBUTION

I.A. Evdokimov, G.S. Anisimov, and R.O. Budkevich developed the research concept. T.N. Bobrysheva, M.S. Zolotoreva, and A.K. Muravyev performed the research. T.N. Bobrysheva wrote the draft. G.S. Anisimov, M.S. Zolotoreva, and R.O. Budkevich edited and proofread the manuscript. G.S. Anisimov supervised the research.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Antihyperlipidemic and antioxidant potential of *Olea europaea* L. leaves: An experimental study *in vivo*, *in vitro* and *in silico*

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Abstract:
Hyperlipidemia is an enduring metabolic ailment that affects glucose and lipid processing.
The research objective was to measure the total phenolic, flavonoid, and tannin contents in *Olea europaea* L. leaves and to to identify their antioxidant and antihyperlipidemic potential. The study included an *in silico* model of interaction for hydroxytyrosol, oleuropein, and xanthine dehydrogenase. The *in vivo* experiment involved rabbits that received olive leaves (150 mg/kg) and 10 mL of egg yolk as a high-fat diet. At the end of the experimental period, blood samples were tested for lipid profile, and tissue specimens were used for liver histology.
The total phenolic content was 119.84 ± 3.86 mg GAE/g, the total flavonoid content was 2.22 ± 0.07 mg CE/g, and the total tannin content was 21.25 ± 1.24 mg REQ/g dry weight. According to DPPH and FRAP analyses, the antioxidant capacities were 0.34 ± 0.06 µg/mL and 6.35 ± 0.52 µmol Fe(II)/g dry weight, respectively. In the experimental animals, *O. europaea* leaves reduced such parameters as total cholesterol, low-density lipoprotein, total triglycerides, total cholesterol vs. high-density lipoprotein, and low-density lipoprotein vs. high-density lipoprotein. The histopathological liver assay showed no signs of tissue damage while the samples obtained from the control group demonstrated steatosis deposits and cellular necrosis. Based on the energy and RMSD results, hydroxytyrosol proved an effective xanthine dehydrogenase inhibition.
These findings constitute a good scientific basis for the complementary future research on the potential of *O. europaea* leaves as ingredients of functional foods or medical drugs.

Keywords: *Olea europaea* L., hyperlipidemia, phenolic compounds, antioxidant activity, antihyperlipidemic activity, xanthine dehydrogenase

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INTRODUCTION

Hyperlipidemia refers to increased presence of one or more lipid types in the bloodstream. This condition is evident through high cholesterol, sometimes accompanied by triglycerides [1–3]. Hyperlipidemia can be classified into two groups as either primary (familial) or secondary (acquired) hyperlipidemia. Primary hyperlipidemia originates from various genetic disorders while secondary hyperlipidemia typically arises as a result of inadequate diet, certain medications (amiodarone, glucocorticoids, etc.), hypothyroidism, uncontrolled diabetes, and unhealthy lifestyle [4]. The global incidence of hyperlipidemia has been progressively rising, which is commonly attributed to lifestyle and dietary factors [5].

As a multifaceted and enduring metabolic ailment, hyperlipidemia encompasses disruptions in glucose and lipid processing, along with broader systemic imbalances within the body. These imbalances include thickening of blood vessel walls, obesity, and heigh blood sugar [6]. Hyperlipidemia can directly trigger metabolic disorders, thus causing significant disruptions in metabolism and exerting adverse effects on intestinal well-being [7, 8]. Hyperlipidemia could be linked to an increased cumulative risk of myocardial infarction, ischemic stroke, and

premature mortality [9]. High cholesterol is a significant factor that contributes to a third of global ischemic (coronary) heart disease cases. In total, high cholesterol is accountable for 2.6 million deaths, making up 4.5% of the total mortality [10, 11].

Managing hyperlipidemia includes such strategies as dietary regulation, physical activity, and medication. According to Gold *et al.*, only a quarter of adults with significantly elevated low-density lipoprotein cholesterol (LDL-C) managed to attain the target reduction suggested by LDL-C guidelines [12]. The Spanish COHORT Familial Hypercholesterolemia Study provided individuals with familial hypercholesterolemia with lipid-lowering therapy; however, only a minority of 11.2% patients achieved the treatment objective of reducing low-density lipoprotein cholesterol below 100 mg/dL [13]. Several studies showed that hypercholesterolaemia correlates with the generation of reactive oxygen species that are known to cause oxidative damage to human cells. This process can be inhibited by antioxidant agents, e.g., polyphenols. They inhibit the propagation of free radicals either directly or indirectly, i.e., by reacting with enzymes involved in the production of reactive oxygen species [14].

The past few decades have seen a significant upsurge in alternative anti-hypercholesterolaemia remedies, particularly herbal medicines and their supplements [15]. Plants tend to yield compounds with fewer toxic side effects than synthetic medications, which makes them prospective raw materials for novel therapeutic agents [16]. Aumeeruddy *et al.* conducted a major review of popular herbal remedies utilized worldwide to manage high cholesterol [17]. The review covered a total of 174 surveys from 2001–2020 and revealed records of 390 plant species, encompassing 109 families and 294 genera. The studies were published by research teams from 37 countries and involved leaves (29%) and fruit (15%) as anti-hypercholesterolaemia remedies. Many of them featured *Olea europaea* L., commonly known as the olive tree. This species was reported across six countries: Algeria, Argentina, Greece, Palestine, Portugal, and Turkey. Olive (*O. europaea*) leaves contain a plethora of potentially bioactive compounds with antioxidant, antihypertensive, antiatherogenic, hypoglycemic, anti-cancer, and anti-inflammatory properties [18, 19].

Presumably, olive leaf extracts owe their biological properties to such potentially bioactive compounds as oleuropein and hydroxytyrosol [20, 21].

This article introduces the anti-hypercholesterolaemia potential of *O. europaea* leaves. We measured the total phenolic, flavonoid, and tannin contents to define their antioxidant and antihyperlipidemic potential. In literature sources, we did not find research on the antihyperlipidemic activity of *O. europaea* leaves using a high-fat diet and induced hypercholesterolaemia in rabbits as model animals. This research also featured an *in silico* interaction model of such phenolic compounds as hydroxytyrosol and oleuropein with xanthine dehydrogenase, which is a key enzyme in generating reactive oxygen species.

STUDY OBJECTS AND METHODS

Collecting the plant material. The olive leaves (*Olea europaea* L.) of a local Hamri variety were harvested in the Skikda region, North-East of Algeria, in early 2022. After a thorough cleaning, the leaves were dried at 40°C for 10 days to be ground to fine powder. The resulting powder was kept in a hermetically sealed bottle at room temperature in a dry and dark place until further use.

Preparing the extract. For extraction, we used a method described by Bhatia *et al.* [22]. After soaking 20 g of the olive leaf powder in 100 mL of 70% methanol at room temperature for 24 h, we filtered the mix and performed a triplicate maceration with solvent renewal in order to extract the maximum of the bioactive product. Then, we removed methanol in a rotary vacuum evaporator at 40°C and stored the resulting extract in airtight bottles at 4°C until use.

Total phenolics assay. To assess the total phenolic content, we applied the spectrophotometric method as described by Al-Farsi *et al.* [23]. In line with the procedure, we added 200 µL of the extract to 1.5 mL of the Folin-Ciocalteu reagent. After mixing and incubating the solutions in the dark for 5 min, we added 1.5 mL of sodium bicarbonate (60 g/L) to the reaction medium. Following 90 min of incubation at room temperature, we measured the absorbance with an ultraviolet-visible spectrophotometer at 725 nm against the blank without extract. The phenolic content was expressed as mg GAE/g dry weight, i.e., milligrams of gallic acid equivalent per 1 g dry weight.

Total flavonoids assay. Here, we followed the protocol described by Biglari *et al.* [24]. To assess the total amount of flavonoids in the extract, we added 4.0 mL of distilled water and 1 mL of the extract to 0.3 mL of 5.0% sodium nitrite ($NaNO_2$) and 0.3 mL of 2.0% aluminum chloride ($AlCl_3$) dissolved in methanol. After 5-min incubation at room temperature, we added 2 mL of 1.0% sodium hydroxide (NaOH) dissolved in methanol. The mix was then diluted to 10 mL with distilled water, and the absorbance was measured at 510 nm against the blank. The flavonoid content was expressed as mg CE/g dry weight, i.e., milligrams of catechin equivalent per 1 g dry weight.

Total tannins assay. To determine the total amount of tannins, we used the acidic vanillin method as described by Qaisar *et al.* [25]. We prepared the vanillin reagent by mixing the same volumes of 8% (v/v) HCl, 37% (v/v) methanol, and 4% vanillin in methanol (w/v). After storing the mix at 30°C, we added 200 µL of the extract to 1000 µL of the vanillin reagent. The mix was agitated and incubated in the dark at 30°C for 20 min. To measure the absorbance at 500 nm, we used a blank that consisted of a mix of methanol (37%) and HCl (8%) in equal volumes. The results were expressed as mg QE/g dry weight, i.e., milligrams of quercetin equivalent per 1 g dry weight.

Antioxidant activity. *DPPH assay.* As recommended by Blois, we measured the antioxidant potential of the olive leaf extract to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [26]. We mixed 60 µL of different concentrations of the extract with 1500 mL of the DPPH solution (6×10^{-5} M). After 30 min of incuba-

tion at room temperature, we monitored the absorbance at 517 nm. The percentage inhibition (I, %) of the DPPH radical was calculated according to the Equation below:

$$I = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control; A_{sample} is the absorbance of the test extract with the DPPH solution.

The same procedure was repeated for butylated hydroxytoluene solutions as positive control. The antioxidant activity of the extract was expressed as IC_{50}, which represents the concentration (μg/mL) of the extract required to scavenge 50% of the DPPH free radical.

FRAP assay. The ferric-reducing antioxidant power (FRAP) of the methanolic extract of *O. europaea* leaves was measured according the method described by Benzie & Strain [27]. In line with the protocol, we added 20 μL of the extract to 1.5 mL of the FRAP reagent. The obtained blue solutions remained at room temperature at 37°C for 20 min for the absorbance to be measured at 593 nm. We used ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) concentrations (100–2000 mmol/L) to calibrate the standard curve. The ferric-reducing antioxidant power was expressed as mmol Fe(II) per 1 g dry weight.

Experimental animals. *Ethic.* All the experiments on animals were confirmed and approved by the Department of Natural and Life Sciences, University of Skikda, Algeria. The experiments were conducted in line with the Guide for the Care and Use of Laboratory Animals.

Animals. The research involved healthy male rabbits (*Oryctolagus cuniculus* L.). The animals were eight weeks old and weighed 1.6–2.5 kg. They were obtained from a local supplier (Hama Bouziane, Constantine, Algeria). All the animals were kept under standard environmental conditions: 12 h light/12 h dark cycle, 20 ± 2°C. They had free access to tap water and food. The animals were quarantined for ≥ 10 days before the experiment.

The **antihyperlipidemic activity of *O. europaea* leaves** was measured based on the method proposed by Djerrou [28]. We divided a total of 25 rabbits randomly into five experimental groups with five animals in each (n = 5):

– group 1 represented normal control and received a standard diet;
– group 2 were subjected to oral feeding (gavage) with *O. europaea* leaves (150 mg/kg);
– group 3 received standard diet and 10 mL of egg yolk, i.e., a high-fat diet;
– group 4 received atorvastatin (2.5 mg/kg) followed by 10 mL of egg yolk after 30 min; and
– group 5 animals were fed with olive leaf powder (150 mg/kg) followed by 10 mL of egg yolk after 30 min.

Assay of plasma lipid and hepatic parameters. At the end of the experimental period (45 days), the rabbits were sacrificed to collect blood samples. Within 1 h, the samples were sent to a diagnostic laboratory for a lipid profile test using a BS-240 Mindray autoanalyzer. The test involved the following parameters: total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total triglycerides, total cholesterol vs. high-density lipoprotein, and low-density lipoprotein vs. high-density lipoprotein.

Histopathological study. The livers were washed with ice normal saline and fixed in a 10% formalin solution for histological assessment. The sections were assessed on hematoxylin and eosin to be examined with optic microscopy (Carl Seiss Microlmaging GmbH, Germany).

***In silico* study of the interaction of xanthine dehydrogenase and phenolic compounds.** We used a method described by Patel & Kukol to study the interaction between xanthine dehydrogenase and phenolic molecules [29]. Xanthine dehydrogenase is a key enzyme in generating reactive oxygen species while hydroxytyrosol and oleuropein are the major phenolic compounds in *O. europaea* leaves.

Preparing protein. The structure of xanthine dehydrogenase was downloaded from the Protein Data Bank Archive (www.pdb.org) with PDB ID: 1N5X at 2.80 Ångström resolutions. Then, we used the AutodockTools software to add Gasteiger-type atomic charges and hydrogen atoms. The structures were saved in the PDBQT format.

Preparing ligand. This part of the research involved two phenolic compounds, i.e., hydroxytyrosol and oleuropein. We downloaded the ligands from the PubChem site (https://pubchem.ncbi.nlm.nih.gov) in the SDF format to be reformatted into PDB using the PyMOL software. To increase the energy evaluation of the system, the receptor was immersed in a three-dimensional grid, which largely encompassed the active site of the protein and allowed the ligand to rotate freely in this site. The center of the grid box was determined by the coordinates X, Y, and Z with the dimensions of 3 Ångström. The spacing of the grid was fixed at 1 Ångström. The grid box was then centered on the ligand, its dimensions being proportional to the size of all the ligands in the study.

Calculating root mean square deviation. The reliability of a docking program is evaluated in terms its ability to reproduce protein-ligand complexes. Using the docking program, we determined the root mean square deviation (RMSD) between the conformation and the orientation of the ligand. The place it occupied in the experimental complex had to be as small as possible. The allowed ratio was a maximal difference of 2 Ångström. We used Discovery Studio 4.0 Client to calculate the root mean square deviation while AutoDock Vina served to determine the root mean square deviation and perform the visual analysis.

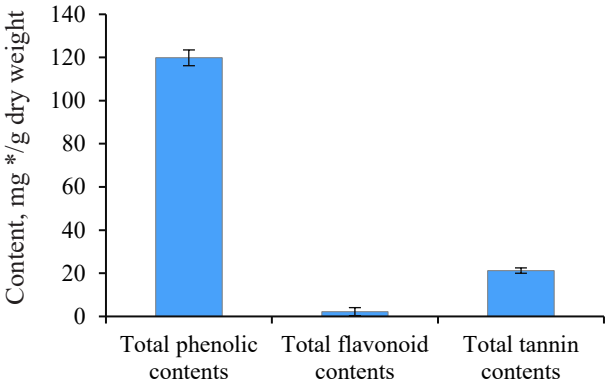
Statistical analysis. The results were given as mean ± standard deviation. The statistical analysis involved SPSS Statistics 16.0 (Chicago, USA). The analysis of variance (ANOVA) made it possible to identify the differences in the mean values. We applied the Tukey's test to register statistically significant difference. The significance level was $p < 0.05$.

RESULTS AND DISCUSSION

Bioactive content. Figure 1 shows that the methanolic extract of *Olea europaea* L. leaves was rich in phenolic compounds. The total phenolic, flavonoid, and tannin contents were 119.84 ± 3.65 mg GAE/g dry weight, 2.22 ± 1.86 mg CE/g dry weight, and 21.25 ± 1.24 mg REQ/g dry weight, respectively.

Our results were in agreement with those obtained by Ines *et al.*, who reported the phenolic contents of olive leaf extract (China) as 151.74 mg GAE/g [30]. Bixia *et al.*, who worked with Tunisian *O. europaea* leaves, reported a higher total phenolic content of 905.96 mg GAE/g [31]. Results of Turkish and Saudi Arabian researchers for the total phenols were lower than ours, namely 37.8 and 45.48 mg GAE/g, respectively [32, 33]. Sirajudheen *et al.*, who also worked with *O. europaea* leaves from Saudi Arabia, reported the total flavonoid content between 3.11 ± 0.67 and 6.44 ± 0.12 mg CE/g [33]. Our findings more or less coincided with these results. However, Nashwa & Abdel-Aziz reported the total flavonoid content as 21.45 mg EQ/g for the methanolic extract of olive leaves from Egypt [34]. These results exceeded those obtained in our study. To our best knowledge, no data regarding the total tannin contents in *O. europaea* leaf extracts have been published so far.

Chromatographic separation of aqueous extracts from Egyptian genotypes of the *O. europaea* plant revealed the presence of gallic acid, hydroxytyrosol, catechol, *p*-hydroxy benzoic acid, caffeine, vanillic acid, caffeic acid, syringic acid, oleuropein, vanillin, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, benzoic acid, o-coumaric acid, salicylic acid, and cinnamic acid [35, 36]. Several studies identified oleuropein as a primary compound in *O. europaea*. These phytochemical components are known for their potent biological activities [37, 38]. Sirajudheen *et al.* mentioned 6-C-glucopyranosyl-8-cxylopyranosylchrysoeriol, quercetin 3-galactoside-7-rhamnoside, isovitexin, 6-hydroxyluteolin 5-rhamnoside, melanoxetin, calomelanol D-1, monotropein, tephrodin, robinetin 3-rutinoside, isovitexin 7-O-rhamnoside, isovitexin, and kaempferol 3-(2''-(Z)-p-coumaroylglucoside) as the dominant flavonoids in the methanolic extract of *O. europaea* leaves [33]. The phytochemical composition of *O. europaea* leaf extracts depends on the geographic origin, climate, variety, growing conditions, maturity, season, soil, cultural specifics, processing methods, and solvent [39].



*Contents are represented as follows: total phenolics – milligram gallic acid equivalent per 1 g dry weight (mg GAE/g dry weight); flavonoids – milligram catechin equivalent per g dry weight (mg CE/g dry weight), and tannins – milligram quercetin equivalent per g dry weight (mg REQ/g dry weight)

Figure 1 Bioactive contents of the extract of *Olea europaea* L. leaves

Table 1 Antioxidant activities of *Olea europaea* L. leaf extract

	DPPH IC_{50}, µg/mL	FRAP, moles Fe(II)/g DW
Olea europaea L. leaves	0.34 ± 0.06[b]	6.35 ± 0.52
Butylated hydroxytoluene	0.18 ± 1.50[a]	–

a–b: If values (mean ± standard deviation, n = 3) in the same column have different letters, they are significantly different ($p < 0.05$). DPPH – 2,2-diphenyl-1-picrylhydrazyl; FRAP – ferric-reducing antioxidant power; IC_{50} – the extract concentration that scavenges 50% DPPH

Antioxidant activity. Table 1 summarizes the values of the antioxidant activities of *O. europaea* leaves based on DPPH and FRAP assays. The butylated hydroxytoluene, which was used as standard, had a stronger capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (0.18 ± 1.5 µg/mL) than the extract of *O. europaea* leaves (0.34 ± 0.06 µg/mL). As for the ferric-reducing antioxidant power, the olive extract demonstrated a rather high antioxidant potential of 6.35 ± 0.52 moles Fe(II)/g dry weight, as well as an ability to reduce the ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}).

Mansour *et al.* studied three *O. europaea* cultivars from Egypt, namely, *picual*, *tofahi*, and *shemlali* [36]. Their IC_{50} ranged from 48.14 ± 0.15 to 56.00 ± 0.13 µg/mL. As for the Turkish variety, its IC_{50} was 3.80 mg/mL [40].

Our findings agree with those reported by Cheurfa *et al.*, whose team studied the antioxidant activity of the ethanolic and aqueous extracts of *O. europaea* leaves cultivated in Chlef, Algeria [41]. Their FRAP assay rendered the values of 07.53 ± 0.06 and 4.01 ± 0.01 moles Fe(II)/g dry weight, respectively. Presumably, *O. europaea* leaves owe their antioxidant properties to their high oleuropein [42]. This natural phytochemical protects human cells against free radicals that react with cellular molecules of fats, proteins, and DNA, thus producing oxidative damage [43]. The antioxidant potencies of oleuropein may be due to its capacities to chelate copper and iron. Ions of these metals generate reactive oxygen species, which are known to trigger cancer, hypertension, cardiovascular diseases, and inflammatory disturbances [37].

Antihyperlipidemic activity of *Olea europaea* L. leaves. The concentration of total cholesterol significantly increased ($p < 0.05$) in the group of rabbits fed with egg yolk (1.07 ± 0.306 g/dL) compared to the untreated control rabbits (0.49 ± 0.72 g/dL). The concentration of total cholesterol also increased compared to the group of rabbits treated with olive leaf powder (0.67 ± 0.08 g/dL). However, both groups fed with egg yolk and treated with either atorvastatin (0.71 ± 0.12 g/dL) or olive leaf

powder (0.76 ± 0.08 g/dL) showed a significant decrease ($p < 0.05$) compared to Group 3, which received a standard diet and 10 mL of egg yolk. We detected a significant increase ($p < 0.05$) in high-density lipoprotein in the rabbits that received a high-fat diet (0.482 ± 0.070 g/dL) compared to the control (0.27 ± 0.06 g/dL) and Group 2 (0.30 ± 0.03 g/dL), which received olive leaves. Furthermore, we observed a significant decrease in Group 4 (0.35 ± 0.71 g/dL), treated with atorvastatin and egg yolk, compared to Group 3, which received olive leaves and egg yolk. However, the group of rabbits fed with olive leaves showed some decrease (0.44 ± 0.05 g/dL), although it was not significant, compared to Group 3.

The concentration of low-density lipoprotein-cholesterol dropped ($p < 0.05$) in Group 3 (0.44 ± 0.21 g/dL) compared to Group 2 (0.09 ± 0.05 g/dL) and increased insignificantly compared to Group 1 (0.26 ± 0.25 g/dL), which received a standard diet. In contrast, Groups 4 and 5, which both received egg yolk and atorvastatin or olive leaf powder, showed an insignificant decrease ($p > 0.05$) compared to Group 3, which received egg yolk and a standard diet, with respective levels of 0.22 ± 0.05 and 0.24 ± 0.08 g/dL.

The concentration of total triglycerides increased insignificantly in Group 3 (0.76 ± 0.27 g/dL) compared to Group 2 (0.61 ± 0.09 g/dL) and Group 1 (0.70 ± 0.18 g/dL). However, the triglyceride level decreased insignificantly in Group 4 (0.66 ± 0.14 g/dL) and the group fed with olive leaf powder (0.55 ± 0.22 g/dL) compared to Group 3, which received egg yolk and a standard diet.

The results showed no significant increase ($p > 0.05$) in the ratio of total triglycerides and high-density lipoprotein in Group 3 (2.10 ± 0.45 g/dL), compared to control (1.91 ± 0.60 g/dL). However, we observed no significant decrease in the group of rabbits fed with olive leaves (1.75 ± 0.35 g/dL) and atorvastatin (2.01 ± 0.25 g/dL), compared to Group 3. We also detected no significant increase in the low-density lipoprotein vs. high-density lipoprotein ratio in Group 3 (0.89 ± 0.32 g/dL), compared to Group 2 (0.33 ± 0.21 g/dL). No significant decrease ($p > 0.05$) was registered in Group 3 compared to Group 1 (1.03 ± 1.10 g/dL). Similarly, we found no significant decrease ($p > 0.05$) in the low-density lipoprotein vs. high-density lipoprotein ratio in Group 4 (0.46 ± 0.16 g/dL) and Group 5 (0.57 ± 0.23 g/dL), which received atorvastatin or olive leaves with egg yolk, compared to Group 3, which received a standard diet and 10 mL of egg yolk.

Epidemiological data correlate a high consumption of plant extracts with a lower risk of several cardiovascular and degenerative diseases, hence the increasing scientific interest in their therapeutic properties as sources of health-promoting phytochemical molecules. Several experiments on people, mice, and rats reported that the extract of *O. europaea* leaves improved the plasma lipids profile (Table 2). However, to our best knowledge, no publications have so far reported the antihyperlipidemic activity of *O. europaea* leaves using a high-fat diet and induced hypercholesterolaemia in rabbits as model animals.

Our findings were in line with several previous studies. For instance, Cheurfa *et al.* studied administered aqueous extract of *O. europaea* leaf cultivated in Chlef, Algeria, on serum total cholesterol, triglycerides, high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins in hypercholesterolemic mice [41]. The mice treated with the extracts showed lower levels of total cholesterol, low-density lipoproteins, and triglycerides. In addition, they reported rutin and luteolin to be naturally present in the leaves of *O. europaea*. These substances were anchored against HMG-CoA reductase, the enzyme that limits cholesterol metabolism.

Atorvastatin is a synthetic lipid-lowering agent; it is a competitive inhibitor of HMG-CoA-reductase. It reduces total cholesterol, triglyceride levels, and low-density lipoprotein cholesterol [44].

Jang *et al.* reported that such phenolic compounds as gallic acid and linoleic acid, as well as their mixes, improved the serum lipid profile in hypolipidemic C57BL/6 mice by decreasing serum triglyceride and low-density lipoprotein cholesterol [45].

Hadrich *et al.* studied the effect of orally-administered oleuropein (50 mg/kg) on adiponectin secretion [46]. Oleuropein exerts hypocholesterolemic effect by inhibiting peroxisome proliferator-activated receptor γ, sterol regulatory element-binding protein-1c, and fatty-acid synthase expression. The team reported a protective effect of oleuropein and hydroxytyrosol derived from olive leaves on high-fat diet-induced lipid metabolism disorders. These phenolic compounds exerted their hypolipidemic and hepatoprotective effects by improving

Table 2 Effect of *Olea europaea* L. on plasma profile lipids

Variables, mg/dL	Group 1 (standard diet)	Group 2 (olive leaves)	Group 3 (standard diet + yolk)	Group 4 (atorvastatin + yolk)	Group 5 (olive leaves + yolk)
Total cholesterol	0.49 ± 0.72[a]	0.67 ± 0.08[a]	1.07 ± 0.30[b]	0.71 ± 0.12[a, c]	0.76 ± 0.08[a, c]
High-density lipoprotein	0.27 ± 0.06[a]	0.30 ± 0.03[a]	0.48 ± 0.07[b]	0.35 ± 0.71[a, b, c]	0.44 ± 0.05[b]
Low-density lipoprotein	0.26 ± 0.25[a]	0.09 ± 0.05[a]	0.44 ± 0.21[a, b]	0.22 ± 0.05[a, b]	0.24 ± 0.08[a, b]
Total triglycerides	0.70 ± 0.18[a]	0.610 ± 0.096[a]	0.76 ± 0.27[a]	0.66 ± 0.14[a]	0.55 ± 0.22[a]
Total cholesterol vs. high-density lipoprotein cholesterol	1.91 ± 0.60[a]	2.23 ± 0.16[a]	2.10 ± 0.45[a]	2.01 ± 0.25[a]	1.75 ± 0.35[a]
Low-density vs. high-density lipoprotein	1.03 ± 1.10[a]	0.33 ± 0.21[a]	0.89 ± 0.32[a]	0.46 ± 0.16[a]	0.57 ± 0.23[a]

antioxidative defense system and blocking the expression of proteins involved in inflammation and liver damage [30].

Hepatoprotective activity of *Olea europaea* L. leaves. Figure 2 illustrates the results of the histopathological tests. The morphological features of the liver in the positive control group showed a normal architecture of hepatocytes (Fig. 2a). The histology of the rabbits that received a standard diet together with olive leaves also demonstrated a normal architecture of hepatocytes (Fig. 2b). The microscopic observation of the liver obtained from the rabbits that received a hyper-lipid diet (control group) revealed some changes in the tissue architecture, namely, steatosis deposits, bloating, and cellular necrosis (Fig. 2c). However, the histopathological architecture of the liver sections obtained from the rabbits treated with the olive leaves (test group, Fig. 2d) and atorvastatin (reference group, Fig. 2e) demonstrated no histological changes in comparison with Group 3, which received a standard diet with egg yolk.

Increasing incidences of some chronic diseases, including hyperlipidemia, have raised awareness regarding the importance of diet. Numerous investigations determined that animals fed with high-fat and high-cholesterol feeds developed nonalcoholic fatty liver diseases. This pathology is characterized by steatosis, necroinflammation, ballooning, and fibrosis. Gaube *et al.* reported that the oral administration of ethanolic olive leaf extract induced no changes in histopathology, biochemical profile, and hematological parameters after single or repeated doses in a rat model [47]. According to Taamalli *et al.*, the attenuation of hepatic tissues correlated quite well with the biochemical contents [48]. They explained the hepatoprotective potentials of *O. europaea* leaves by the high phenolic contents that were able to reduce the inflammatory and oxidant disorders in hepatic cells.

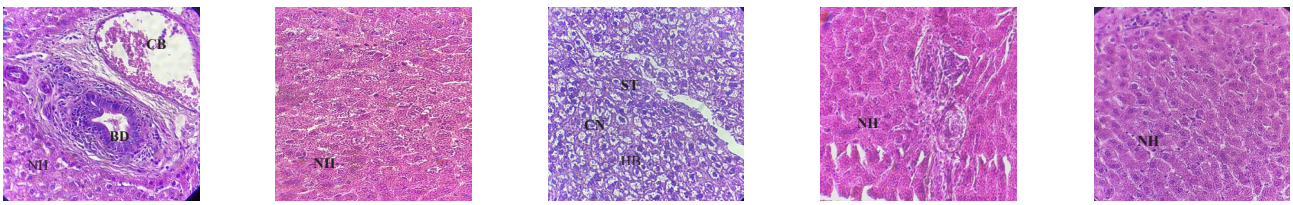
The hepatoprotective activity of *O. europaea* leaves has been elucidated against hepatic damages induced by cadmium, carbon tetrachloride (CCl_4), and diazinon. Jemai *et al.* evaluated the hepatoprotective potential of oleuropein at 16 mg/kg body weight against cadmium-induced hepatotoxicity in mice [49]. Orally-administered oleuropein restored significantly such biomarkers of liver injury as alanine transaminase, aspartate transaminase, lactate dehydrogenase, and phosphatase alkaline, compared to the animals that received cadmium alone. The histological and immunohistochemical tests showed a significant suppression of the inflammation scores, as well as the oxidative damage induced by cadmium in hepatic tissues. Rats pretreated with olive leaf extracts showed less oxidative damage in ischemic and non-ischemic parts of liver, as well as a significant improvement in physiological and histopathological disorders. The predominant mechanism of hepatoprotective action is due to the high antioxidant potential and the capacity to scavenge free radicals [50]. Vidičević *et al.* studied the protective mechanisms of dry olive leaf extract in hepatotoxic rats treated with carbon tetrachloride (CCl_4) [51]. The simultaneous treatment with CCl_4 and the extract of *O. europaea* leaves significantly reduced the expression of protein kinase activated by adenosine monophosphate (AMPK) and inhibited the expression of autophagy-related protein LC3II compared with the control group. Omagari e*t al.* confirmed the protective effect of oleuropein in reducing numerous hepatic genes involved in the production of reactive oxygen species and the regulation of cholesterol or lipid metabolism [52]. These effects are due to the properties of antioxidants that act as reducing agents by donating hydrogen and quenching singlet oxygen or by acting as chelators and trapping free radicals.

Interaction *in silico* between xanthine dehydrogenase and phytochemicals. *Binding free energy.* In this test, oleuropein formed the most stable complex with xanthine dehydrogenase with binding free energy (ΔG) of –7.5 kcal/mol, followed by hydroxytyrosol with an energy of –6.4 kcal/mol.

Interaction between hydroxytyrosol and xanthine dehydrogenase. A three-dimensional visualization of the interactions between the active site of xanthine dehydrogenase and hydroxytyrosol showed that the amino acids involved in the interaction were represented by leucine 788 and serine 1064 (Fig. 3).

According to the Discovery Studio model, hydroxytyrosol penetrated well into the active site of xanthine dehydrogenase by forming different pi-cation, pi-alkyl, hydrogen, pi-anion, and hydrogen carbon interactions (Table 3).

Interaction between oleuropein and xanthine dehydrogenase. A three-dimensional visualization of the interactions between the active site of xanthine



a b c d e

NH – normal hepatocytes, BD – bile duct, CB – capillary blood, HB – hepatocyte ballonisation, ST – steatosis deposit, CN – cellular necrosis

Figure 2 Light microscopy of rabbit liver tissue stained with hematoxylin and eosin (×400): a – Group 1, standard diet; b – Group 2, olive leaves; c – Group 3, standard diet and yolk; d – Group 4 atorvastatin and yolk; e – Group 5, olive leaves and yolk

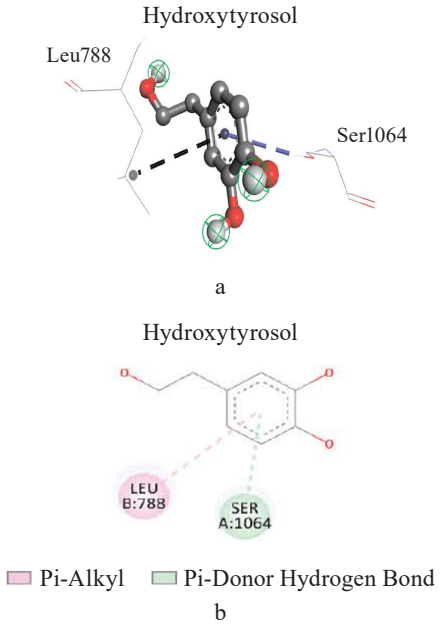


Figure 3 Three-dimensional (a) and two-dimensional (b) visualizations of interactions between the active site of xanthine dehydrogenase and hydroxytyrosol

Table 3 Interactions between hydroxytyrosol and xanthine dehydrogenase

Residues involved in the interaction	Type of interaction	Distances (Ångstrom)
Leucine 788	Pi-Alkyl	2.88938
Serine 1064	Pi-donor-hydrogen-bond	4.00545

Table 4 Interactions between oleuropein and xanthine dehydrogenase

Residues involved in the interaction	Type of interaction	Distances (Å)
Asparagine 595	Unfavorable acceptor-acceptor	2.24364
Proline 597	Carbon hydrogen bond	3.19379
Proline 753	Conventional hydrogen bond	2.41202
Lysine 754	Conventional hydrogen bond	2.43452
Arginine 37	Conventional hydrogen bond	3.06085
Histidine 821	Conventional hydrogen bond	2.31595
Glycine 588	Conventional hydrogen bond	2.34949

dehydrogenase and oleuropein showed that the amino acids involved in the interaction were represented by glycine 38, glycine 588, glycine 35, lysine 95, and proline 753 (Fig. 4).

According to the Discovery Studio model, oleuropein penetrated well into the active site of xanthine dehydrogenase by forming hydrogen and carbon hydrogen bonds (Table 4).

Reliability testing of the molecular docking program. ***Root mean square deviation.*** Interaction mode prediction consists of determining the correct positioning of the ligand in relation to its receptor. The ability of a program to perform this work is usually judged

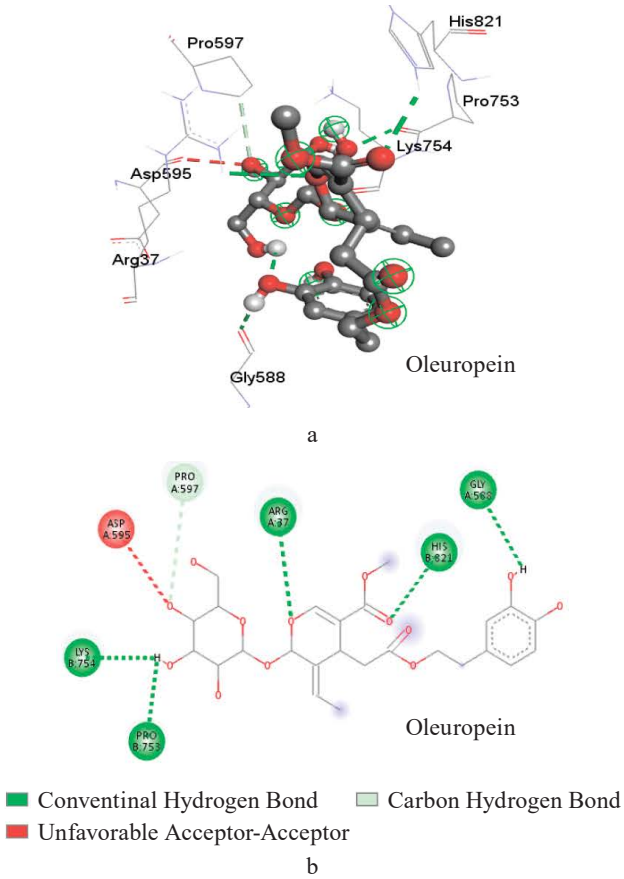


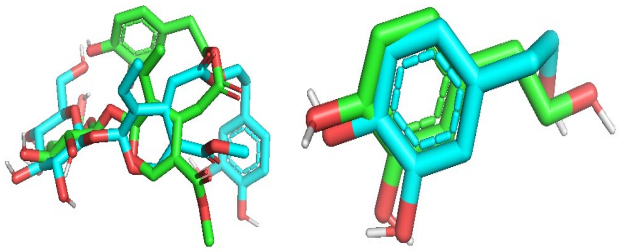
Figure 4 Three-dimensional (a) and two-dimensional (b) visualization of interactions between the active site of xanthine dehydrogenase and oleuropein

Table 5 Root mean square deviation values of ligand interaction with xanthine dehydrogenase

Ligands	Root mean square deviation (Ångström)
Hydroxytyrosol	0.994
Oleuropein	2.126

by means of the root mean square deviation (RMSD) of the model designed by the Vis-à-vis software regarding the structure of the reference (co-crystallized) ligand. The permissible limit is 2A°, beyond which the prediction is considered inadequate [53]. The enzyme complex used in this study is xanthine dehydrogenase (pdb: 1n5x). This procedure is followed by extracting the co-crystallized ligand from the given target and repositioning it by docking in the active site. The root mean square deviation of the best exposure of the reference ligand (after docking) is calculated with its crystallographic binding mode (before docking). The RMSD value of hydroxytyrosol of the software-designed model does not exceed 2 Å (Table 5).

Visual analysis is an essential step in judging the results described by the numerical value of the root mean square deviation. Visualization of the complexes selected, namely, xanthine dehydrogenase (pdb: 1n5x) and ligands made in the PyMOL molecular visualization system showed that the ligands predicted by



a b

Figure 5 Superposition of inhibitors (PyMOL) to calculate the root mean square deviation: a – hydroxytyrosol; b – oleuropein

AutoDock-Vina and the reference ligand were well positioned. Indeed, these results confirmed the root mean square deviation values.

The design of new plant-based computer-aided products (*in silico* study) is a relatively recent method for high-throughput screening of an extensive chemical database. It produces results for major compounds in less time and at lower costs. The *in silico* virtual screening method facilitates the development of innovative medicines. Based on the root mean square deviation results, the selected ligands, i.e., hydroxytyrosol and oleuropein, proved quite effective in inhibiting xanthine dehydrogenase (Fig. 5).

Xanthine oxidase results from the oxidation and/or proteolytic conversion of xanthine dehydrogenase. Xanthine oxidase is expressed in vascular cells and can circulate in plasma and bind to the extracellular matrix of endothelial cells. This enzyme catalyzes the metabolism of NADH, i.e., nicotinamide adenine dinucleotide (NAD) + hydrogen (H), molecular oxygen, hypoxanthine, and xanthine to produce O and HO. It is an important source of reactive oxygen species [54]. Most evidence about the involvement of xanthine oxidase in endothelial dysfunction and in the development of vascular diseases stems from the studies where the use of inhibitors of this enzyme (oxypurinol, allopurinol, febuxostat, topiroxostat) indicated improved endothelial function and vascular reactivity [29].

Recent studies showed that the inhibition of this enzyme protects against diabetic kidney disease and endometrial hyperplasia through the amelioration of oxidative stress by improving uterine-reduced glutathione and superoxide dismutase, as well as inhibiting the expressions of phosphatidylinositol-3-kinase (PI3K), Akt, and VEGF [54, 55]. Phenolic acids and flavonoids are antioxidants that can inhibit xanthine oxidase activity. The method was validated by attaching the inhibitory ligand to xanthine oxidase. Tran Minh *et al.* reported the root mean square deviation values ranging from 1.019 to 2.35 [56]. Cinnamon phenolic acids showed a remarkable activity of xanthine dehydrogenase inhibition [57]. Similarly, Serrano *et al.* inhibited xanthine oxidase with cinnamic acid [58]. Mehmood *et al.* studied the xanthine oxidase inhibition mechanism of eight structurally diverse phenolic compounds commonly present in fruit plant (quercetin, quercetin-3-rhamnoside, 4,5-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, 3,4-O-di-caffeoylquinic acid, 4-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, and caffeic acid) [59]. They used proton nuclear magnetic resonance (^{1}H NMR), atomic force microscopy, and various computational techniques. The study suggested that these phytochemicals had a potent inhibition and interaction with this enzyme. The inhibition of xanthine oxidase by phenolic compounds can be used to prevent several pathologies.

CONCLUSION

In the present study, *Olea europaea* L. leaves were rich in phenolic compounds, which possess potent antioxidant and antihyperlipidemic activities. *O. europaea* leaves improved the lipid profile in test animals by reducing such parameters as total cholesterol, low-density lipoprotein cholesterol, total triglycerides, total cholesterol vs. high-density lipoprotein cholesterol, and low vs. high density lipoprotein cholesterol. According to the histopathological tests, the liver of rabbits that received a hyper-lipid diet revealed changes in tissue architecture represented by steatosis deposits, bloating, and cellular necrosis. The histopathological architecture of liver sections obtained from rabbits treated with olive leaf powder demonstrated no damage in comparison with the group which received a high-fat diet only. Based on the energy and root mean square deviation, hydroxytyrosol was effective in inhibiting xanthine dehydrogenase. Thus, *O. europaea* leaves proved to contain a wide array of phytochemicals that could be used in the therapeutic context as an effective antihyperlipidemic agent and an inhibitor of xanthine dehydrogenase responsible for the production of free radicals. This potential can be used to prevent a number of diseases.

CONTRIBUTION

A. Amraoui and Z. Djerrou conceived and designed the analysis. A. Amraoui and S. Ali Haimoud collected the data. A. Amraoui, Z. Djerrou, K. Zerouki, and S. Elmokli worked with the data and analysis tools. A. Amraoui and Z. Djerrou performed the analysis. A. Amraoui and S. Ali Haimoud wrote the manuscript.

CONFLICT OF INTEREST

The authors declared no conflict of interests related to the publication of this article.

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Immunomodulatory effect of ashwagandha (*Withania somnifera* L. Dunal) and its impact on COVID-19

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Abstract:
Ashwagandha (*Withania somnifera* L. Dunal) is an Ayurvedic medicinal herb that has been known for its therapeutic properties for millennia. Ashwagandha contains several bioactive compounds, including withanolides, alkaloids, and saponins. They make ashwagandha a potent adaptogen and a versatile herb that can maintain optimal health and overall well-being. Ashwagandha reduces stress and anxiety, as well as boosts the immune system. Its anti-inflammatory properties treat arthritis, asthma, diabetes, and inflammatory bowel disease.
Ashwagandha produces an immunomodulatory effect on natural killer cells, lymphocytes, and leukemia cells. It enhances the activity of natural killer cells, increases lymphocyte function, and induces apoptosis in leukemia cells. However, its mechanism of action still remains understudied.
Ashwagandha has an impact on COVID-19: phytochemical withanone blocks or weakens the interaction between S-protein and Angiotensin-converting enzyme 2. Withanoside V and somniferine inhibit viral transcription and replication caused by SARS-CoV-2 M^{pro}.
This review explores the potential utilization of ashwagandha in the food industry, i.e., its safety and toxicity, as well as the mechanism behind its immunomodulatory effect.

Keywords: Ashwagandha, immunity, lymphocytes, natural killer cells, cancer, COVID-19

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INTRODUCTION

Ashwagandha (*Withania somnifera* L. Dunal) is also known as Indian ginseng or Indian winter cherry. It is a shrub with the following taxonomy: kingdom *Plantae*, phylum *Angiosperms*, class *Eudicots*, subclass *Asterids*, order *Solanales*, family *Solanaceae*, genus *Withania*, species *somnifera*. It is one of the most important herbs in Ayurveda, i.e., an ancient Indian medical system. Due to its versatile medicinal properties, ashwagandha has been used for millennia, usually as *Rasayana*. *Rasayana* is an herbal or metallic concoction that promotes physical and mental health, as well as overall well-being [1]. Other medicinal benefits include anti-tumor, anti-inflammatory, hypoglycemic, and anti-oxidant effects. As a result, many researchers have taken interest in the chemical composition of ashwagandha and identified flavonoids, tannins, alkaloids, glycosides, and steroid lactones in its leaves, stems, and roots. Steroid lactones are represented by withanolides, which are the main secondary metabolites responsible for the beneficial properties of this plant [2].

Ashwagandha affects the nervous, immune, energy production, endocrine system, a well as reproductive system [3]. Its root promotes vigor, endurance, strength, and health in general, as well as enhances the production of essential fluids, muscle fat, blood, lymph, semen, and cells [4]. In India, ashwagandha is used as a traditional immunomodulator. From 1956 onwards, numerous studies have reported the immunomodulatory, anti-inflammatory, anti-cancer, cardio-protective, anti-stress, anti-diabetic, anti-oxidant, neuro-protective, anti-microbial,

anti-arthritic, anti-Parkinson, anti-Alzheimer, and rejuvenating properties of ashwagandha [5, 6].

The earliest notes on its therapeutic purposes date back to 4000–5000 BC, with the Chinese being the pioneers in utilizing ashwagandha herbal blends as a medicine. The Rigveda, which is believed to have been composed between 3500 and 1600 BC, contains the oldest allusions to the medicinal use of ashwagandha in India. The ancient physicians investigated and documented the medicinal properties of ashwagandha in Ayurveda [7]. The word *ashwagandha* has two possible etymologies: either its roots smell of horse, or its extract grants the power and vigor of a horse [8].

Ayurvedic treatment has a multi-target approach in addressing health concerns and associated chronic stress. Ayurveda's comprehensive yet tailored approach is intended to restore and maintain the body's hemodynamics while enhancing immunity [9]. Ashwagandha has a long list of pharmacological properties: it is an aphrodisiac, apoptogenic, diuretic, anti-helminthic, astringent, tonic, narcotic, etc. Ashwagandha is an immuno-stimulator, anti-inflammatory, health promoter, and rejuvenator that reduces stress and combats rheumatism, goiter, boils, pimples, piles, flatulent colic, oligospermia, leukoderma, constipation, insomnia, nervous breakdown, etc. It even relieves the effect of snake and scorpion venom [10]. The herb is classified as *Rasayana*, i.e., a decoction or extract with immunomodulatory properties based on non-specific activation of macrophages, granulocytes, complement systems, natural killer cells, and lymphocytes. It also boosts the production of various effecter molecules generated by activated cells (para-immunity), thus giving protection against different pathogens, i.e., bacteria, fungi, viruses, etc. In this respect, it constitutes an alternative to conventional chemotherapy [11].

Nowadays, herbal products become more and more popular because they have numerous health benefits and almost no side effects [12, 13]. However, ashwagandha-based functional foods require more scientific research to prove their potential benefits. This review featured publications on ashwagandha and its immunomodulatory effect aimed at understanding how ashwagandha affects the immune response and whether it can enhance the body's ability to fight off infections. The results contributes to the development of new treatments against immune-related disorders, as well as identifies a number of potential uses of ashwagandha in complementary or alternative medicine. The study also focused on ashwagandha as a complementary treatment of COVID-19 patients, namely its impact on the immune response potential ability to alleviate the severity of COVID-19 symptoms. The findings from this study may provide insights into the potential use of ashwagandha as an alternative or complementary treatment for COVID-19.

RESUITS AND DISCUSSION

Ashwagandha: morphology and distribution. Ashwagandha (*Withania somnifera* L. Dunal) is a small shrub that can grow as tall as 1–2 m. Almost the entire plant is coated with silver-grey, extremely short, fine, and branching hairs called tomentum. Ashwagandha has tall brownish-dark stems that can have few or no leaves on the lowest section [14]. Ashwagandha leaves are simple, petiolate, whole, exstipulate, pointed, hairless, and up to 10 cm in length. Vegetative shoots have alternating big leaves that are laterally paired: one large and one small leaf. Floral leaves are laterally arranged opposite each other, in pairs of one large and one small leaf; they have a cymose cluster of 5–25 subtle pale green flowers in their axil [15]. Ashwagandha fruit is a spherical hairless berry of 5–8 mm in diameter. It is orange-red to scarlet when ruptured and covered by an expanded calyx. An average seed is 2.5 mm in diameter, occasionally kidney-shaped, and compressed; it is very light brown, rough, and netted [14].

Ashwagandha grows at an altitude of 1500 m above sea level. A semi-tropical location with 500–800 mm of yearly rainfall is optimal. During the growing season, the plant needs a dry environment, with the ideal temperature for cultivation of 20–38°C. Other favorable conditions include sandy loam or light red soil, as well as partial shade. The cultivation of ashwagandha primarily takes place in north-western and central India, the major producers being the states of Madhya Pradesh, Gujarat, Haryana, Maharashtra, Punjab, Rajasthan, and Uttar Pradesh. It also grows in Nepal, China, and Yemen [5]. Figure 1 shows the edible parts of ashwagandha.

Bioactive compounds in ashwagandha. The biologically active constituents of ashwagandha include alkaloids, e.g., isopellertierine or anferine; steroidal lactones, e.g., withanolides and withaferin; saponins that contain acyls; withanolides with glucose attached to carbon 27 (Table 1). Ashwagandha is also rich in iron. Withanolides are the major constituents found in ashwagandha roots: they are believed to account for its exceptional therapeutic properties [7]. Ashwagandha's major bioactive

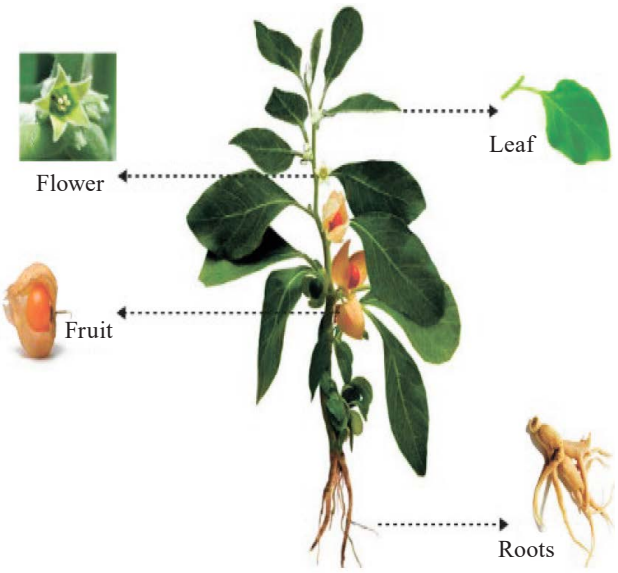
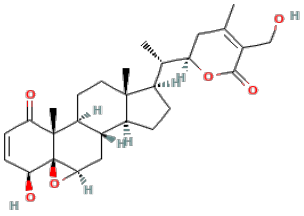
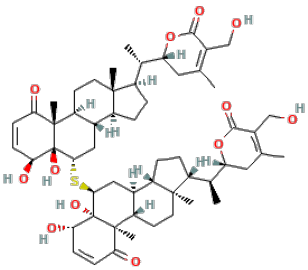
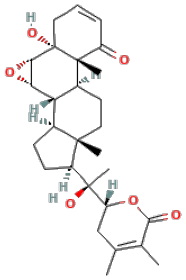
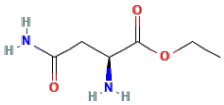
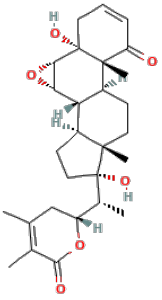
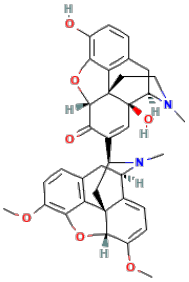
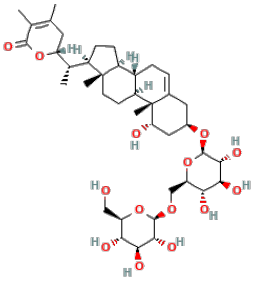


Figure 1 Different parts of ashwagandha plant

Table 1 Bioactive immunomodulatory molecules in Ashwagandha

Bioactive compound	Plant part	Structure	Immunomodulatory effect	Reference
Withaferin A	Roots		It increases activation and proliferation of macrophages, controls cytokine production, enhances the function of natural killer cells, and stimulates cytokine production	[18]
Ashwagandhanolide	Roots		It prevents NF-κB activation induced by tumor necrosis factor	[7]
Withanolide A	Roots		It amplifies the levels of T-helper 1 cytokines, as well as CD4 and CD8 counts, and promotes the activity of natural killer cells	[7]
L-Asparaginase	Fruits		It inhibits lymphoblastic leukemia	[19]
Withanone	Leaves & roots		The ACE2-RBD complex reduces the electrostatic part of the binding free energy, thus preventing coronavirus from infiltrating the human body or weakening the process of infiltration	[20]
Somniferine	Roots		It reduces the process of viral transcription and replication	[21]
Withanoside V	Roots		It reduces viral transcription and replication, as well as weakens the infiltration of COVID-19	[21]

components are withanone, withaferin A, withanolide A, and withanolide D. They have been tested for potential therapeutic properties, including anticancer, immunomodulatory, and neuroregenerative activities [16]. Withaferin A and withanone exhibit anticancer effects by activating tumor suppressor proteins, inducing oxidative stress, and reducing cellular metabolism and structure. Withanolides can enhance cell-mediated immunity in different models, including drug-induced myelosuppression in mice. Withaferin A is known as a natural anti-inflammatory agent [17].

Immunomodulatory effect of ashwagandha. The immune system protects the body against such pathogens as viruses, bacteria, and toxins [22]. Most ashwagandha studies feature withanolides, particularly withaferin A, which improves the immune system and exhibits anti-inflammatory properties. Ashwagandha roots and leaves are good for immune cells, particularly macrophages. These cells are equipped with lysosomal enzymes that facilitate the elimination of their own harmful waste. Alcoholic extracts of the entire ashwagandha plant enhance the ability of macrophages to engulf alien particles and decrease excessive immune responses, while stimulating the production of lymphocyte immune T-cells [23].

Esmaealzadeh *et al.* reported that ashwagandha boosts the production of antibodies [24]. Antibodies are known to engulf toxins, which are later extracted with sweat, mucus, feces, or urine. In addition, ashwagandha facilitates the production of nitric oxide, which activates macrophage actions of the immune system, thus improving their ability to ingest invader cells. An infection usually inhibits the production of nitric acid. Finally, ashwagandha has anti-inflammatory properties because it reduces the amount of C-reactive protein in the body [25].

As for the immunomodulatory effect, ashwagandha increases the number of $CD4^+$ helper T-cells and, to a lesser degree, $CD8^+$ helper T and B cells. For instance, Singh *et al.* reported that ashwagandha increased the total activated white blood cells [26]. $CD56^+$ cells, also called natural killers, are especially effective cell activators. These cells define the organism response to influenza, tumors, or mumps.

Saleem *et al.* developed an ashwagandha-based innovative herb mineral test formulation that may function as an efficient immunomodulatory and anti-inflammatory product [27]. This product can serve as a supplemental and alternative treatment against many inflammatory and auto-immune diseases. In their research, ashwagandha reduced the viral load in infected lymphocyte cells. Therefore, the immunomodulatory effect of ashwagandha makes it possible to use it as an antiviral herb.

Effect of ashwagandha on B- and T-lymphocytes. Ashwagandha causes polarization of T-helper cells by boosting T-lymphocyte development and proliferation, as well as their capacity to secrete IL-2 and IFN-γ, while only mildly downregulating IL-4 and causing B-cells to convert over to secrete IgG2a. Maurya *et al.* used flow cytometry to measure the lymphocyte proliferation [18]. They found that T ($CD3^+$) and B ($CD19^+$) cell count increased in the experimental animals treated with ashwagandha. The diversification of $CD4^+$ and $CD8^+$ T cells into their subtypes also increased.

Ashwagandha proved effective against hepatitis C: lymphocyte normal cells proliferated after 25 mg/mL ashwagandha water extract therapy. Mofed *et al.* studied the impact of ashwagandha water extract on tumor necrosis factor-α (TNF-α) in lymphocyte-non-malignant cells infected with hepatitis C [28]. The research showed a considerable reduction in TNF-α activity in infected lymphocytes treated with ashwagandha water extract. Pathogenic infections triggered the release of pro-inflammatory cytokine known as the tumor necrosis factor (TNF). Grunz-Borgmann *et al.* also studied an aqueous ashwagandha extract, and it showed anti-inflammatory properties by lowering CCL_2 and CCL_5 gene expression in response to TNF-α stimulation [29]. The impact of ashwagandha water extract on lymphocyte proliferation suggests that ashwagandha may have an immune-boosting effect [28].

Tharakan *et al.* chemically standardized an ashwagandha leaf extract, which proved able to stimulate the immune system [23]. The extract increased the *in vitro* production of Th1 cytokine IFN^- in Con A primed splenocytes. The extract was administered orally to BALB/c mice vaccinated with OVA-FCA. It enhanced the production of IL-2 and IFN^- by T-cells, facilitated a dose-dependent proliferation of T-cells, and slightly reduced the expression of Th2 cytokine IL-4. The flow cytometric examination of T-cells, B-cells, $CD4^+$, and $CD8^+$ revealed a significant increase in lymphocyte proliferation and differentiation.

In immunized mice, the ashwagandha leaf extract elicited upregulation of β-integrins LFA (CD11a) and Mac-1 (CD11b) in splenocytes. The spleen-derived macrophages isolated from the experimental mice demonstrated co-stimulatory molecules CD80 and CD86. These molecules are considered to be important secondary markers of immune activation. According to the chemical standardization of the extract, it was withanolide 2,3-dihydro-3-sulphonile withanone that was responsible for skewing the immune polarization of T-helper cells. It stimulated the expression of IFN^- while B-cells started to secrete IgG2a, thus increasing the expression of co-stimulatory molecules and integrins. The ashwagandha leaf extract has a potential of a Th1 immune adjuvant for chronic infections that suppress Th1 immunity [6].

Pal *et al.* studied the impact of ashwagandha root extract on DNA damage in rat lymphocytes [30]. The extract significantly reduced the DNA damage brought on by H_2O_2 in rat cells by about 88%. It reduced the oxidative stress in lymphocytes because it had a scavenging effect on H_2O_2, which may have helped to avoid DNA damage in rat lymphocytes.

Withaferin A and withanolide E exhibited specific immunosuppressive effects on human T- and B-lymphocytes and on rat thymocytes. Withanolide E had a specific

effect on T-lymphocytes whereas withaferin A affected both T- and B-lymphocytes [10].

Effect of ashwagandha on T-cell leukemia. T-cell acute lymphoblastic leukemia (T-ALL) is a severe form of blood cancer distinguished by abnormal proliferation of immature thymocytes. A genetic lesion accumulates in the thymus during T-cell development. This process leads to differentiation arrest and abnormal proliferation of immature progenitors and, eventually, to T-cell acute lymphoblastic leukemia [31]. Turrini *et al.* reported cytostatic and cytotoxic effects of ashwagandha on a human T-lymphoblastoid cell line [32]. It also induced immunogenic cell death and caused genotoxicity, as evidenced by multiple flow cytometric assays. An ashwagandha extract demonstrated a significant cytotoxic and cytostatic potential, which also caused immunogenic cell death. As a component of the proapoptotic pathway, $Ca2^{+}$ accumulated within cells, leading to the production of reactive oxygens.

Yang *et al.* treated leukemic cells with withaferin A and reported phosphorylation of c-Jun N-terminal kinases (JNKs) [33]. JNK-signaling pathway facilitates apoptosis. The induction of mitochondrial-mediated apoptosis in leukemia cell lines treated with withaferin A may be attributed to the activated N-terminal kinases. They increased the activation of pro-apoptotic proteins Bad, Bim, and Bax while inhibiting anti-apoptotic proteins Bcl-2 (B-cell lymphoma 2) and Bcl-XL (B-cell lymphoma-extra-large). An activated JNK-signaling pathway is crucial in catalyzing the apoptotic death of t(4;11) acute lymphocytic leukemia lines.

Effect of ashwagandha on natural killer cells. The immune system relies on natural killer cells to combat viral infections and cancer. Natural killer cells achieve this by both directly killing infected or cancerous cells and producing cytokines. These cells can also recognize specific signals on target cells and respond to inflammation. They inhibit virus replication either by producing IFN-γ or by destroying infected cells directly [34]. The recognition of dangerous molecular patterns is a key factor of innate immune defense against infection. Upon recognizing these pathogen signals, the organism reacts by stimulating different kinds of immune cells [35]. Natural killer cells cause cytotoxicity by granule exocytosis. It occurs when immunological synapse forms perforin and granzymes appear in premade cytoplasmic granules in natural killer cells that mimic secretory lysosomes [36].

Ashwagandha is known to have a cytotoxic effect on several human tumor cell lines. Khan *et al.* wrote that ashwagandha could enhance the availability of natural killer cells in a tumor [37]. The tumor cells secrete MICA to prevent the destroying tumor cells by natural killer cells. MICA expression is linked to cellular stresses, such as those induced by a growing tumor. When secreted, MICA interacts with circulating natural killer cells that are NKG2D-expressing, thus decreasing the ability of natural killer cells to destroy tumor cells. The increase in MICA expression by the tumor decreases the natural killer cell count. Ashwagandha could reduce the stress caused by tumor cells by enhancing anti-oxidant activity. Antioxidants are synthetic or organic substances that might stop or slow down various forms of cell damage [38]. By reducing stress, Ashwagandha was able to reduce the secretion of MICA from the surface of tumor cells. A low level of MICA in the blood may increase the number of natural killer cells with unbound NKG2D receptor ability to permeate the tumor area [38].

Tharakan *et al.* used an ashwagandha extract for 30 days to increase the population of $CD3^{+}$, $CD4^{+}$, $CD8^{+}$, $CD19^{+}$, and natural killer cells [23]. Numerous *in vivo* studies also suggested its ability to support both natural and adaptive immune response.

Therefore, ashwagandha decreases the oxidative stress caused by abnormal cells, thus increasing the availability of natural killer cells. By reducing MICA on tumor surface, ashwagandha also enhances the destruction of tumor cells (Fig. 2).

Ashwagandha as an immunity booster against COVID-19. In January 2020, amid the pneumonia outbreak, the identification of the seventh human coronavirus, referred to as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was confirmed in Wuhan, Hubei Province, China [39]. SARS CoV type II is a species of the coronavirus genus that generates COVID-19. By January 2020, it had resulted in ≥ 98 million documented cases and caused 2.2 million mortalities [40]. The WHO classified the outbreak as a pandemicin March 2020. SARS-CoV-2 is a positive-sense single-stranded RNA virus with a diameter of 80–120 nm and a mortality rate of 3–4%. Coronaviruses (CoV) are the beta strain from the *Coronaviridae* family. They cause diseases of the nervous, respiratory, gastrointestinal, and hepatic systems and may damage other vital organs if untreated [21].

For millennia, knowledge about medicinal herbs has been passed down from one generation to the next. All conventional medical systems have a substantial share of herbal remedies. The history of medicinal plants is as old as humanity itself: they are fast-acting, available, and affordable [41]. Flora is a wonderful collection of potential medications, and the significance of medicinal plants is gradually gaining more and more scientific attention. Tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids are some examples of bioactive components found in medicinal plants. These bioactive compounds produce various physiological effects on the human body. These compounds are synthesized by primary or secondary metabolism [42]. For more than 3000 years, Ayurvedic and traditional medicine have used ashwagandha (*W. somnifera* L.) as a medicine with numerous health advantages. Ayurveda sees ashwagandha as a powerful stimulant with sedative and anti-stress properties that helps stay young and extends life expectancy [7].

Humans become infected with SARS-CoV-2 when SPIKE Protein (S-protein) of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2). Coronaviruses employ angiotensin-converting enzyme 2 (ACE2) as

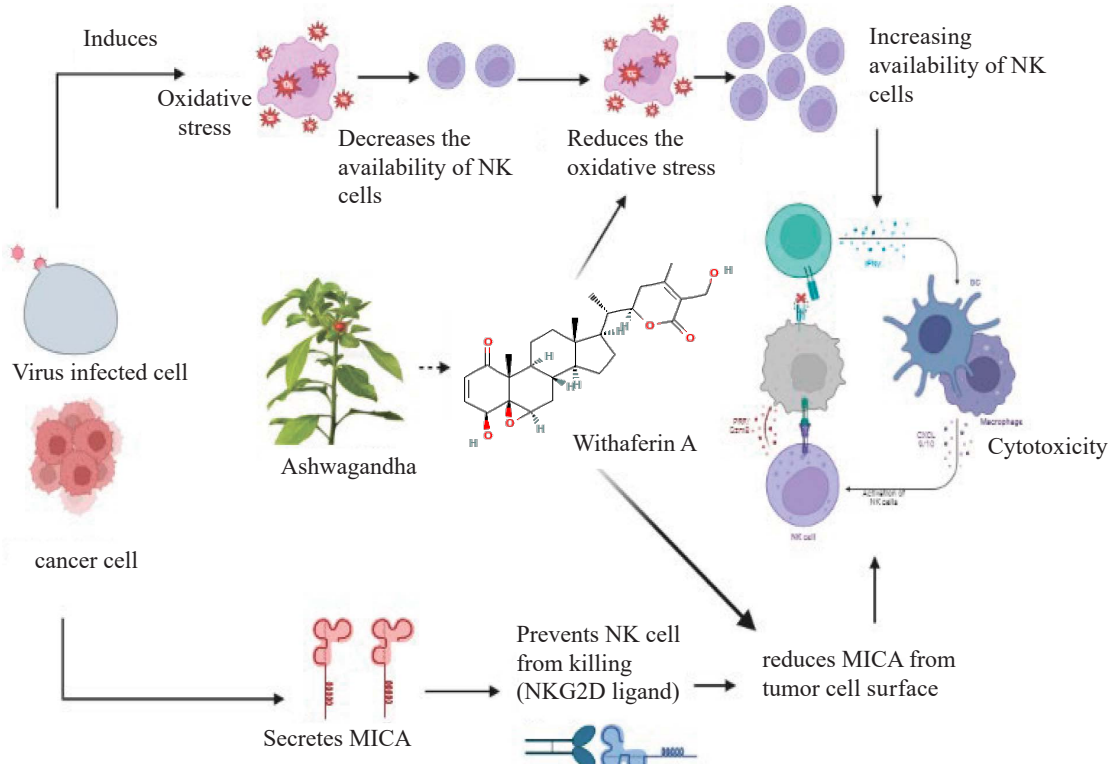


Figure 2 Effect of ashwagandha on natural killer cells

its entry receptor. Through S-proteins on their surface, coronaviruses detect matching receptors on the target cells. The attachment of the virus to the target cell surface is facilitated by the interaction between surface unit S1 of the S-protein and a cellular receptor (Fig. 3) [43]. The phytochemicals present in ashwagandha can prevent COVID-19 by blocking or weakening the interaction between S-protein and angiotensin-converting enzyme 2. Withanone interacts with the interface of angiotensin-converting enzyme 2 (ACE2) and S-protein receptor binding domain (RBD). Using two hydrogen bonds, withanone binds the interface of ACE2-RBD complex. As a result, withanone decreases the electrostatic component of binding free energies of ACE2-RBD complex, thus blocking or weakening the entry of coronavirus into the human body [20, 44]. The maturation of viral RNA into functional proteins, e.g., RNA polymerase, endoribonuclease, and exoribonuclease, impairs the host's normal protective mechanisms. SARS-CoV-2 M^{pro} facilitates this process, in which the crucial role belongs to M^{pro}. SARS-CoV-2 M^{pro} interacts with native ligand N3 and forms 16 NSPs, which leads to viral transcription and replication. As compared to ligand N3, withanoside V and somniferine present in ashwagandha have a substantial amount of binding energy. By slowing down the cleavage of polyproteins that releases NSPs, these bioactive phytochemicals bind with M^{pro}, thus reducing viral transcription and replication [21].

In India, the Ministry of AYUSH is responsible for education, research, and propagation of traditional medicine systems. The AYUSH suggests that ashwagandha powder (3–5 gm) combined with Harindra milk gargling is a safe and effective immuno-booster for COVID-19 prophylaxis and treatment. According to Panda & Kar, 30 mg/kg body weight of ashwagandha increased IgM and IgG titers, indicating a boost in cell-mediated immunity [45]. Surface markers T-cells $CD3^+$, $CD4^+$, and $CD8^+$, as well as B-cell $CD19^+$, showed increased proliferation and differentiation of lymphocytes. The extract was found to stimulate type 1 immunity by boosting the production of Th1 cytokines, namely interferon (IFN)-gamma and interleukin (IL)-2. The research also reported a slight reduction in the expression of Th2 cytokine IL-4.

Withanone decreases the interaction between angiotensin-converting enzyme 2 (ACE2) and spike protein, as well as blocks SARS-CoV-2 M^{pro} by inhibiting replication.

Bioactive natural substances usually include a wide range of phytoconstituents, such as phenols, steroids, and flavonoids. As a result, they have a strong biocompatibility and bioavailability, as well as low toxicity. Several bioactive molecules found in ashwagandha show significant therapeutic effects against SARS-CoV-2.

As a rule, 3–5% of the entire ashwagandha plant repsents an acceptable raw material for functional value-added products that demonstrate great physiochemical properties.

Application and utilization of ashwagandha. Traditionally, ashwagandha has been available as a supplement in capsules and powder. However, it can now be found in various food products, such as honey, ghee, and kombucha. Recently, ashwagandha was introduced into baked

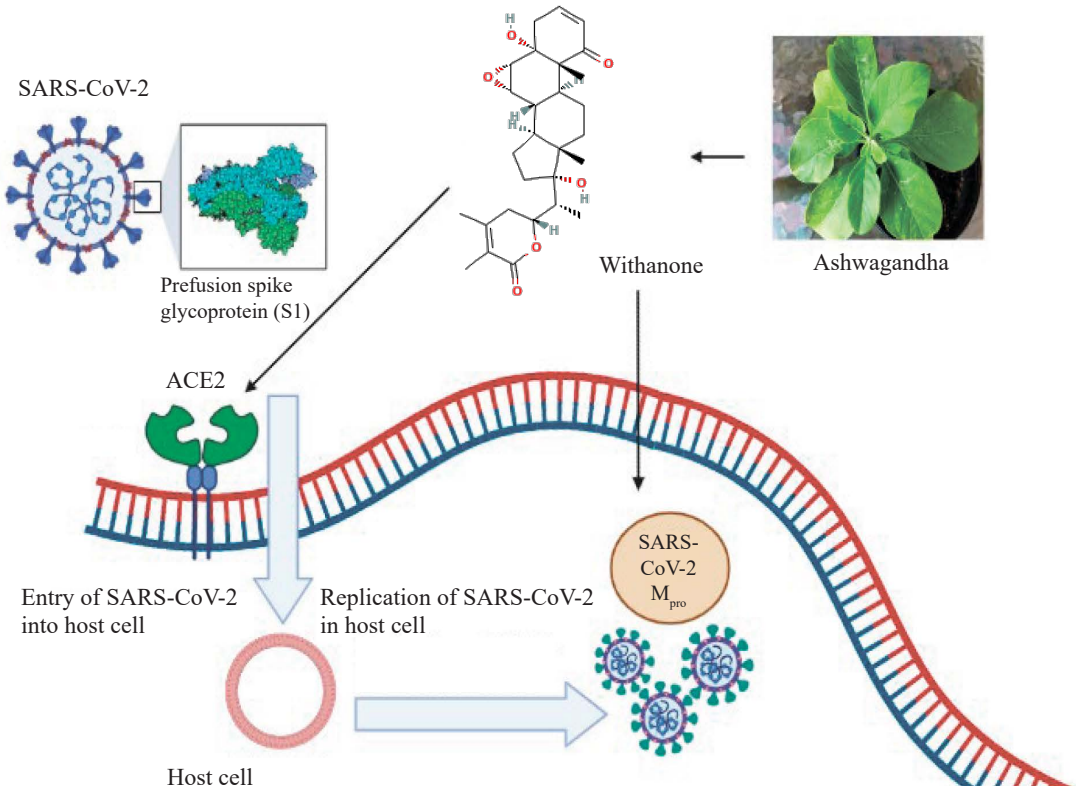


Figure 3 Effect of ashwagandha on SARS-CoV-2

goods, juices, sweets, and dairy products marketed as functional foods. The share of ashwagandha varies from 1 to 10%, depending on the type of product (Table 2).

According to Sharifi-Rad *et al.*, ashwagandha remains sensory acceptable if its share stays below 5% [46]. Baghel *et al.* incorporated ashwagandha root powder into biscuits to increase their nutritional value and add potential health benefits [47]. These biscuits may offer a natural remedy for a variety of ailments, in addition to promoting overall health and well-being.

The growing health-risk awareness increases the demand for functional low-calorie beverages [48]. Ashwagandha milk treats hypertension without affecting body mass index or weight. Therefore, ashwagandha can be considered a natural alternative or adjunct to conventional agents with fewer side effects [49]. Guava cheese fortified with ashwagandha had a good sensory profile [50]. Adding ashwagandha to confectionary products can enhance their nutritional value, fiber, and micronutrient content. Laddu sweets fortified with ashwagandha had a greater nutritional value, specifically in terms of crude fat, crude fiber, total dietary fiber, and mineral content [51].

Although ashwagandha has a great potential for boosting longevity and immunity without causing overstimulation, further research is needed, especially in the areas of bioavailability and bioactive compounds. No evidence is available on possible synergistic effects with other substances. Besides, new standardization methods have to be developed to define the percentage of active compounds in commercial products [46].

Toxicity, safety, and dietary recommendations. Ashwagandha is considered safe when taken in recommended doses. To date, toxicity tests have revealed no adverse effects, making ashwagandha safe for human use in treating both acute and chronic medical conditions [10]. A study on male and female Wistar rats proved that the hydroalcoholic ashwagandha root extract was safe and not toxic. The extract caused neither mortality nor any remarkable alterations in blood counts, biochemistry, physical appearance, or tissue structure. The highest oral dose of the extract used in the sub-acute study was 2000 mg/kg/day and triggered no adverse consequences [52]. An aqueous extract of ashwagandha roots without withanolides had no harmful effect even when administered in a dose of 3000 mg/kg body weight to Foster rats and Swiss albino mice [6].

Mandlik & Namdeo gave Swiss albino mice daily intraperitoneal injections of ashwagandha extract at the amount of 1100 mg/kg body weight [10]. No mortality occurred within 24 h. However, a greater dose led to death. The LD_{50}, which is the dose that kills 50% of test subjects, was 1260 mg/kg. The peripheral blood showed no changes, but the weight of spleen, thymus, and adrenal gland decreased significantly.

Paul *et al.* studied the acute toxicity effect of alkaloids extracted from ashwagandha roots on the central nervous system [6]. The LD_{50} was 465 mg/kg in rats and 432 mg/kg in mice. The researchers evaluated the toxicity of the alcoholic ashwagandha seed extract dissolved in normal saline. The LD_{50} reached 1750 ± 41 mg in albino mice. On the other hand, the aqueous extract of the

Table 2 Application and utilisation of ashwagandha

Product	Formulation	Result and findings	References
Herbal biscuit with ashwagandha (*Withania somnifera* L. Dunal) and ragi (*Eleusine coracana* L. Gaerth)	30% wheat flour, 28% Ragi flour, 20% sugar, 15% white butter, 5% other compounds, 2% ashwagandha	Sensory evaluation: overall acceptability – 6 points. The biscuits are expected to improve the nutritional status of individuals and potentially assist in the management of various disorders	[46]
Green tea with Brahmi (*Bacopa monnieri* L. Pennell), ashwagandha (*Withania somnifera* L. Dunal), stevia (*Stevia rebaudiana* Bert.), and Ceylon Cinnamon (*Cinnamomum zeylanicum* Blume)	0.2% green tea and ashwagandha, 0.05% of Brahmi and cinnamon, 0.14% stevia in soluble solid (g/100 mL)	Total plate count: 6.6×10^2 CFU/mL; yeast and mould count: 5.2×10^1 CFU/mL. Total polyphenol content: 59.68 ± 0.05 GAE/100 mL; DPPH scavenging activity (IC_{50}): 126.23 ± 0.53 μg/mL; pH 6.36 ± 0.01; titratable acidity: 0.488 ± 0.210%; total soluble solids: 0.700 ± 0.021	[47]
Milk fortified with ashwagandha (*Withania somnifera* L. Dunal) root powder	Milk and ashwagandha root powder in proportion 99.8:0.2, 99.6:0.4, and 99.4:0.6	Sensory assessment: the most acceptable formulation involved 4% ashwagandha	[48]
Guava cheese with ashwagandha	Sugar, 700 g/kg guava pulp, 90 g/kg butter, 2 g/kg citric acid, 0.5 ginger powder, 1.0 g lemon grass extract, 1.5 g/kg ashwagandha powder	Guava cheese treated with ashwagandha powder was the best value-added product in terms of physiochemical propertie	[49]
Legume based ladoo with ashwagandha	3–5% ashwagandha, wheat flour, soy flour and barley flour in the ratio of 40:30:30	Ashwagandha increased fat, fibre, and micronutrient content of sweets while also adding value and imparting advantageous medicinal characteristics	[50]

plant, administered as a 100 mg/kg/day dose in drinking water, was found to be non-toxic in rats after 8 months of exposure.

Taking ashwagandha with milk proved more beneficial in treating hypertension. In addition, it had no impact on body mass index or weight [53]. A recent clinical study showed that ashwagandha extract administered in doses of either 225 or 400 mg for 30 days could enhance cognitive flexibility, visual memory, reaction time, psychomotor speed, executive functioning, and stress response [54]. The daily ingestion of 240 mg ashwagandha extract over 60 days caused no significant adverse effects but was able to decrease cortisol [55]. Safety studies revealed no negative effects. However, they still lack a comprehensive understanding of the potential synergistic effects that may arise when ashwagandha is combined with other foods. Also, more research is required to investigate the effects of high doses and its potential impact during pregnancy [46].

Research perspective. Numerous studies indicate that ashwagandha has a considerable effect on the immune system. As a result, ashwagandha has all the chance to become a popular natural medicine against various immune-related conditions, particularly those linked to aging and chronic inflammation. Nevertheless, further research is required to understand the mechanisms underlying these effects, as well as to determine the most effective doses and formulations for clinical use. Natural remedies keep gaining more and more scientific attention as alternative approaches to managing various health conditions. As the popularity of natural remedies continues to grow, ashwagandha is anticipated to become increasingly important in integrative medicine, supplementing conventional pharmaceutical treatments. The rising public awareness of chronic inflammation and immunosenescence in age-related diseases demands new effective immunomodulatory agents. Ashwagandha's ability to regulate the immune system suggests that it has the potential to become a primary natural remedy for many conditions, particularly as the world's population ages and becomes more prone to such age-related diseases as Alzheimer's, cardiovascular disease, and cancer.

Ashwagandha treatment is impossible without medical consultations since it may interact with certain medications and lead to side effects. Nonetheless, the future for ashwagandha as an immunomodulatory agent looks promising despite some limitations. Ashwagandha is expected to continue to gain recognition and popularity as a versatile natural remedy. As the research interest in its immunity-boosting properties continues to grow, ashwagandha might one day play a more significant role in integrative medicine and become an important natural remedy for numerous immunological conditions. The outlook for the immunomodulatory effect of ashwagandha is optimistic, and its importance in the future of medicine cannot be overestimated.

CONCLUSION

Ashwagandha has shown significant immunomodulatory effects on various immune cells, including natural killer cells, B- and T-lymphocytes, and T-leukemia cells.

Ashwagandha contains such active components as withanolides, which can enhance the functioning of the immune system by elevating the production of immune cells and cytokines. These effects have been observed in both animal and human studies. Ashwagandha boosts the number and activity of natural killer cells, which are crucial components of the innate immune response. It also increases the count of B- and T-lymphocytes, which are responsible for the adaptive immune response. Furthermore, ashwagandha inhibits the growth of T-leukemia cells and affects the development of COVID-19. Withanone interacts with the interface of angiotensin-converting enzyme 2 and S-protein receptor binding domain. This interaction can prevent COVID-19 by weakening or blocking the interaction between S-protein and angiotensin-converting enzyme 2. Withanoside V and somniferine provide more binding energy than natural ligand N3. By reducing the binding with M^{pro}, they inhibit viral transcription and replication.

Therefore, ashwagandha may one day become an alternative or complementary treatment for COVID-19. Due to its immunomodulatory properties, ashwagandha has found its usage as a functional ingredient in the food industry. Ashwagandha can boost the immune response in farm animals, resulting in enhanced health and performance. After its millennia-long use in traditional medicine systems, modern medicine has officially recognized the medicinal properties of ashwagandha. Safety studies show low toxicity and a shielding effect against environmental toxins and chemotherapeutic agents. However, caution is still advisable while taking high doses of ashwagandha or using it with other medications. Further research is needed to establish its long-term safety and effectiveness.

CONTRIBUTION

All the authors participated in developing the research concept and writing the original draft. All the authors approved of the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interests regarding the publication of this article.

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Fruit phytochemicals: Antioxidant activity and health-promoting properties

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Abstract:
Synthesized in plants, polyphenols are powerful antioxidants and protect against stressful conditions. We aimed to identify different kinds of phytochemicals in fruits and provide detailed information on the roles they play in promoting good health in the human body. We also discussed the biological activities of phytochemicals found in several fruits.
Google Scholar and PubMed databases were used to search for relevant information that could assist in answering our research questions. We selected and reviewed both research and review articles related to the purpose of our study.
Fruits contain numerous antioxidants which neutralize the negative impact of free radicals on the body. Free radicals are destructive species that can be produced during normal body metabolism or come from exogenous sources such as smoking or exposure to radiation. Due to their unstable nature, they can cause damage to cellular macromolecules, resulting in the development of degenerative diseases. Phytochemicals are diverse groups of bioactive compounds found in fruits that have potent antioxidant activity and exhibit several health-promoting properties in both *in vivo* and *in vitro* studies. There are two major groups of antioxidants: natural (or dietary) antioxidants and synthetic antioxidants. Natural antioxidants have gained much popularity in recent times because of the safety concerns surrounding the use of synthetic antioxidants.
The consumption of fruits plays a critical role in disease prevention, especially diseases resulting from oxidative damage to cells. The inclusion of fruits in one's daily diet helps improve their overall wellbeing.

Keywords: Fruits, antioxidants, bioavailability, polyphenols, carotenoids, biological activity

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INTRODUCTION

Fruits are important in the human diet because they provide the body with nutrients that promote growth and development, as well as maintain good health. Insufficient intake of fruits is one of the top ten risk factors responsible for death globally. Increasing fruit consumption can save the lives of about 2.7 million people each year [1]. Many individuals recognize the importance of diet in promoting good health and preventing disease. This is driving the search for new diet regimens with a positive impact on human health. Scientists are vigorously screening various food products, such as fruits, for potential disease-preventing properties. Fruits are referred to as "functional foods", and studies have established the need to include them in the human diet [2].

Reactive oxygen species are free radicals involved in oxidation reactions in the body. They are harmful and can cause the breakdown of cell membranes, damage to membrane proteins, and DNA mutations. This can result in aging and many diseases such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damage, coronary heart disease, or arthritis. Antioxidants can inhibit the oxidation of molecules by breaking the chains of free radical reactions by donating their own electrons to free radicals [3]. Fruits are endowed with many bioactive compounds such as vitamin C, vitamin E, carotenoids, flavonoids, tannins, and other phenolic constituents, which act as potent antioxidants [4].

Although mostly consumed fresh, fruits can be processed into different kinds of food products such as canned foods, juices, pastes, etc. due to their free radical scavenging and health-promoting properties [2]. The consumption of bioactive compounds from fresh fruits or their processed products is linked with the prevention and decreased risk of many degenerative diseases [2]. Fruit-based products such as juices are widely available

on the market, and they have been shown to contain a lot of compounds that exhibit antioxidant properties [5, 6]. Studies have found that polyphenols present in grape juice can protect individuals from heart disease, while juice from berries and apples can inhibit hypercholesterolemia [7]. Also, juice obtained from oranges, pineapples, and grapefruits contains good quantities of folic acid derivatives that are involved in the prevention of diseases of the nervous system and malformations such as spina bifida [8].

Studies are demonstrating that plant-based diets containing a lot of fruits and other nutrient-rich plant foods can lower the risk of diseases associated with oxidative stress [9]. Antioxidants from natural sources such as fruits are now gaining much popularity, especially in the food industry, due to their safety, unlike synthetic antioxidants, as well as their preventative and therapeutic effects on the body. Although synthetic antioxidants are used in food production to prevent lipid oxidation, butylated hydroxyanisole and hydroxytoluene are suspected to cause cancer and liver damage in animal studies [10, 11]. Diets containing adequate amounts of plant-based foods are highly important for maintaining good health. Food from plants such as fruits, vegetables, and whole grains contains enough antioxidants and minimizes the occurrence of chronic diseases [12]. Individuals who consume fruits in large quantities have a reduced risk of developing cancer and early death, especially from cancers involving epithelial cells, such as those in the cervix, lungs, stomach, esophagus, pancreas, and colon [12].

This review provides current information on phytochemicals present in fruits and shows them as potent antioxidants that promote good health by preventing diseases such as cancer, diabetes, etc. It also discusses some of the factors that can reduce fruit phytochemical bioavailability and thus decrease their health benefits for the human body.

STUDY OBJECTS AND METHODS

We reviewed relevant scientific literature found in Google Scholar and PubMed. Some of the search terms we used to retrieve articles included the effect of free radicals on the human body; phytochemicals and their fruit sources, biological activity, digestion, and bioavailability; and the importance of antioxidants for human health. The articles were selected based on the purpose of this review and our research questions.

RESULTS AND DISCUSSION

Generation of free radicals in the body and their role in cellular pathology. Free radicals are highly reactive molecules produced from either endogenous metabolic processes or external sources. They consist of reactive oxygen species and reactive nitrogen species. The endogenous sources include peroxisomal metabolism, mitochondrial respiration, phagocyte activity, inmation, arachidonate pathways, ischaemia, exercise, and reactions involving iron and other transition metals. The external sources involve exposure to radiation, ozone, cigarette smoke, air pollutants, and industrial chemicals [13, 14]. Nitric oxide radical ($NO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), perhydroxyl radical ($HOO^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), singletoxygen ($^1O_2$), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), hypochlorite radical (ClO^-), and lipid peroxides (LOPs) are examples of free radicals capable of causing damage to biomolecules such as DNA, proteins, and lipids [15]. Polyunsaturated fatty acids can undergo peroxidation to produce compounds such as malondialdehyde, isoprostanes, 4-hydroxy-2-nonenal, etc. These compounds are known to cause diabetes, neurodegenerative diseases, and heart disease. Lipoproteins can be destroyed by peroxynitrites, resulting in the lipid peroxidation of cell membranes. The production of free radicals can interfere with protein synthesis and protein functions [15].

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the body's antioxidant defense system [16]. Excessive production of free radicals may lead to a buildup of oxidative stress in cells and subsequent damage to proteins, DNA, lipids, and carbohydrate molecules. Many human diseases can be caused by oxidative stress, including brain dysfunction, heart disease, inflammatory diseases, diabetes, cardiovascular malfunctions, autoimmune diseases, and aging [2, 17]. Overproduction of reactive oxygen species may lead to overexpression of oncogenes, mutagen formation, and the initiation of atherogenic processes or inflammation. Maintaining redox homeostasis in the body is necessary for good health and disease prevention [11]. Since excessive production of free radicals is harmful, organisms possess natural antioxidant defense enzymes such as glutathione peroxidase, catalase, superoxide dismutase, and glutathione reductase to neutralize the effect of free radicals on the body [13]. However, certain conditions such as stress, illnesses, a high intake of processed foods, and environmental pollution can cause an imbalance in the body's natural antioxidant defense processes [8].

Free radicals become a problem for the body when its antioxidant defense system is inadequate to neutralize or scavenge them [18]. Oxygen plays a crucial role in supporting human metabolic processes. However, in the process of certain metabolic events, oxygen can be transformed into highly reactive species that are destructive to cells in the body. Most of these reactive species are free radicals, possessing unpaired electrons. Since free radicals are unstable, they can either donate or accept electrons from other cellular molecules [14]. Increasing the intake of dietary antioxidants from fruit sources can reduce the stress imposed on the body by reactive oxygen species by protecting biomolecules (proteins, nucleic acids, and lipids) from oxidative damage, suppressing inflammatory responses, and controlling vascular homeostasis [19, 20].

Major phytochemicals in fruits. Fruits are important sources of phytochemicals for human consumption. About 200 000 phytochemicals have been identified, of which 20 000 can be found in vegetables, fruits, and

cereals. Phytochemicals are bioactive compounds of plant origin that do not supply the body with energy but possess essential health benefits [21]. They are secondary metabolites present in plants. The main phytochemicals are phenolic compounds, carotenoids, and glucosinolates [12]. These chemicals are produced in plants to protect them from predators or diseases. They are referred to as "non-essential" nutrients because the human body can function without them [22]. Fruits contain different kinds of phytochemicals, such as phenolic acids, carotenoids, and flavonoids. These phytochemicals exert a wide range of biological activities and provide protection against chronic diseases. For instance, they may prevent the proliferation of cancer cells and regulate inflammatory and immune responses [23].

Carotenoids are lipid-soluble compounds synthesized in plants and microorganisms but not in animals. They are found in subcellular organelles such as chloroplasts and chromoplasts. Carotenoids are mainly conjugated with proteins in chloroplasts and serve as an additional pigment for photosynthesis. However, they exist in chromoplasts in crystalline form or as oil droplets. Carotenoids impart yellow, orange, and red colors to different kinds of plants and fruits. They are used in food processing as colorants and food supplements. During photosynthesis, they act as photosensitizers and protect plants from photodamage [12, 24]. Many carotenoids bind to chlorophylls, giving rise to xanthophyll-chlorophyll and carotene-chlorophyll complexes (giving fruits a variety of colors). When fruits mature, their chlorophyll content is reduced, retaining only colored pigments [25]. Fruit color can indicate the type of carotenoids present; for example, yellow-orange fruits are high in β-carotene and α-carotene [26].

Typically, carotenoids contain the C40 skeleton, also known as tetraterpenoids. Humans can obtain approximately 50 carotenoids through diet. There are two classes of carotenoids, namely carotenes and xanthophylls. Carotenes include α-carotene, β-carotene, β,ψ-carotenes, and lycopene, whereas xanthophylls include β-cryptoxanthin, zeaxanthin, lutein, astaxanthin, fucoxanthin, and peridinin. These carotenoids contain oxygen in the form of hydroxy, aldehyde, carbonyl, carboxylic, furanoxide, and epoxide groups. Chemical structure of major carotenoids in fruits is shown in Fig. 1. There is a growing interest in screening plants and underutilized fruits for carotenoids. This is because fruits are identified as rich sources of carotenoids in the human diet [27–29]. There are a lot of health benefits associated with the intake of foods containing carotenoids. Table 1 shows some major carotenoids, their fruit sources, and their potential biological activities.

Polyphenols are among the most abundant groups of substances present in fruits, with about 8000 phenolic structures identified so far [15, 42]. They are mainly classified into flavonoids, phenolic acids, lignans, and stilbenes. Flavonoids are the most abundant group of phytochemicals [21]. They possess a minimum of one hydroxyl group linked to an aromatic ring. Dietary polyphenols are grouped into flavonoids and nonflavonoids. Flavonoids include flavones (luteolin and apigenin), flavonols (quercetin, kaempferol, and myricetin), flavononones (narigenin), isoflavonoids (daidzein and genistein), anthocyanidins (malvidin and cyanidin), and flavanols (epicatechin, catechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate). Chemical structure of selected polyphenols in fruits is shown in Fig. 2. Nonflavonoids include stilbenes, phenolic acids, tannins, lignans, anthocyanidins, anthraquinones, and coumarins. They are characterized based on their carbon atom arrangements. Most polyphenols derived from plants, such as flavonoids and phenolic acids, are conjugated. They are bound to one or more sugar moieties or residues through their hydroxyl groups [37, 43]. They can also be conjugated to amines, organic acids, lipids, carboxylic acids, and other phenolic compounds [44].

Polyphenols are synthesized in plants as a form of protection against stressful conditions such as exposure to UV, temperature fluctuations, and infection by pathogens [45]. They are powerful antioxidants with metal chelating properties that can reduce lipid peroxidation and trap nitrates to prevent the formation of mutagenic nitroso compounds [13]. Phenolic acids, flavonoids, and tannins constitute the most common polyphenols present in the human diet [46]. The composition of phenolic compounds found in fruits is determined by fruit ripeness, cultivar, physiological conditions, weather, and soil conditions. Postharvest treatments (e.g., processing) and storage can also influence the content of polyphenols in fruits [47]. Higher concentrations of polyphenols can be found in the outer parts of fruits such as apples, watermelons, oranges, etc. The concentration of polyphenols present in some plant food can be as high as 500 mg per 100 g of food [48]. Table 2 lists various polyphenolic compounds and the fruits that contain them

Antioxidant and health-promoting properties of fruit phytochemicals. ***The role of phytochemicals in cancer prevention and management.*** Cancer is a disease characterized by the abnormal growth of cells able to invade and metastasize to other areas of the body. This disease develops due to the changes taking place within the genes that control normal body functions [71]. It is one of the major health problems affecting people globally, both in developed and developing countries. About 18.1 million new cases of cancer were recorded in 2018 worldwide, and this number is expected to increase to about 23.6 million by 2030 [72].

The current remedies for cancer treatment include surgical removal and treatment of cancer cells with radiation accompanied by chemotherapy [73]. Chemoprevention of cancer makes use of natural and/or artificial mediators to interrupt carcinogenesis by inhibiting specific molecular signaling pathways. The chemotherapeutic mediators involved in cancer treatment may be grouped as blocking and suppressing agents [74, 75]. Chemotherapy has certain disadvantages, such as drug resistance, cancer recurrence, and harmful effects on non-targeted tissues. These are some of the challenges arising from the

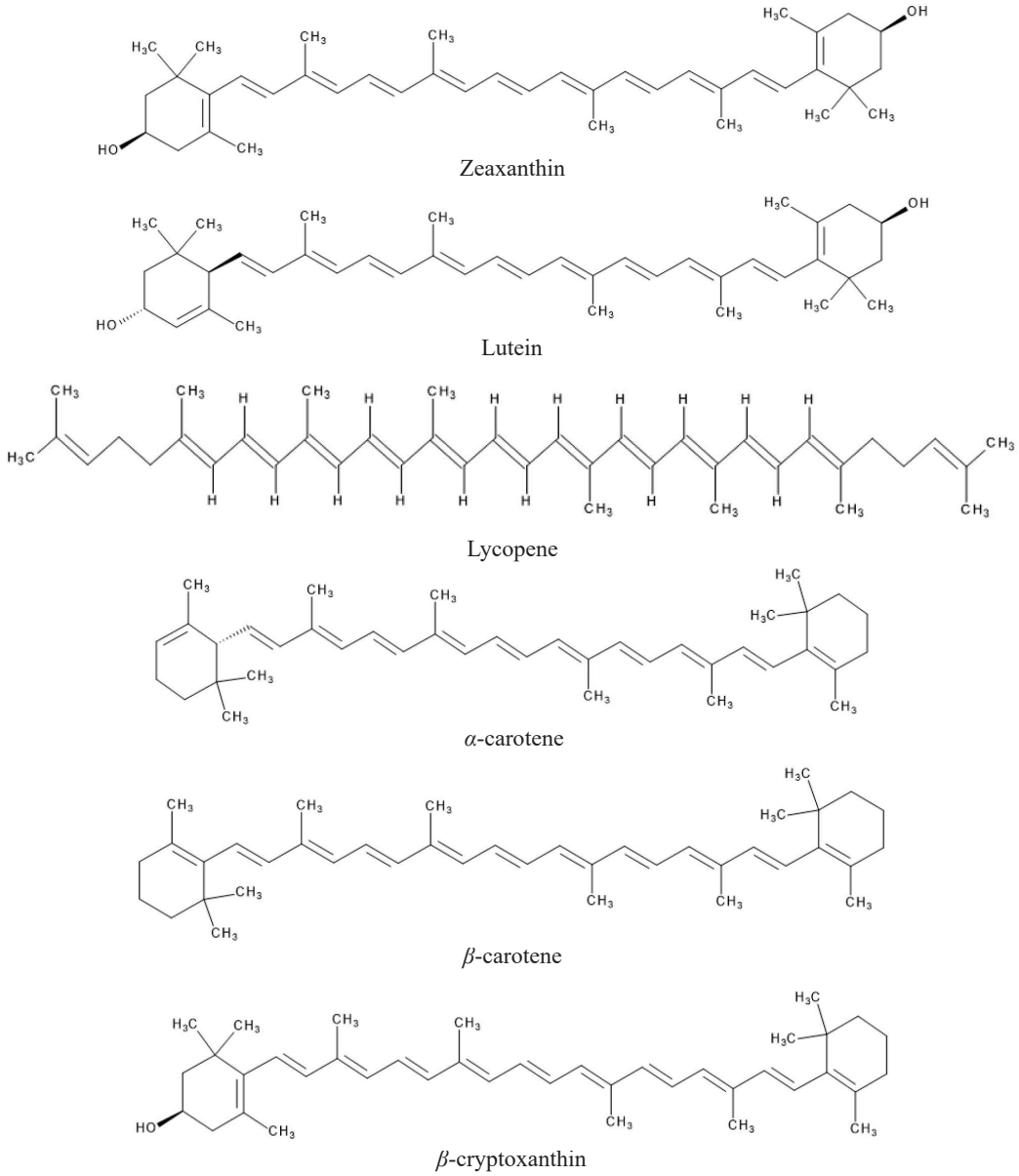

Figure 1 Chemical structure of major carotenoids in fruits

Table 1 Carotenoids and their fruit sources

Phytochemical	Fruit sources	Biological activity	References
Lutein	Avocado, melon, rosehips, black chokeberries, sea buckthorn, kiwi, and black currants	Treatment of osteoarthritis and optic nerve injury; antioxidant, anti-inflammatory, neuroprotection, and cardio-protection activities	[30–32]
Zeaxanthin	Avocado, melon, goji berries, Chinese wolfberry, orange, peaches, and mandarins	Prevention of acute and chronic coronary syndromes and age-related macular degeneration; maintenance of normal visual function and anticataract action	[30, 31, 33, 34]
β-cryptoxanthin	Peaches, tangerines, oranges, papaya, grape, mango, and watermelon	Provitamin A and antioxidant activities; promotes bone formation and inhibits bone resorption	[30, 35, 36]
Lycopene	Watermelon, pink grapefruit, red grapefruit, papaya, pink guava, and apricot.	Neuroprotective, antioxidant, cardiovascular, anti-hypertensive, and anti-platelet effects	[37, 34, 38, 39]
β-carotene	Mango, papaya, banana, grape, melon, orange, watermelon, pear, rosehips, black chokeberries, sea buckthorn, and black currants	Provitamin A, antioxidant, and anticancer activities; prevention of scleroderma and treatment of erythropoietic protoporphyria	[37, 36, 40, 41]
α-carotene	Pineapple, banana, grape, mango, melon, orange, and pear	Provitamin A activity	[36]

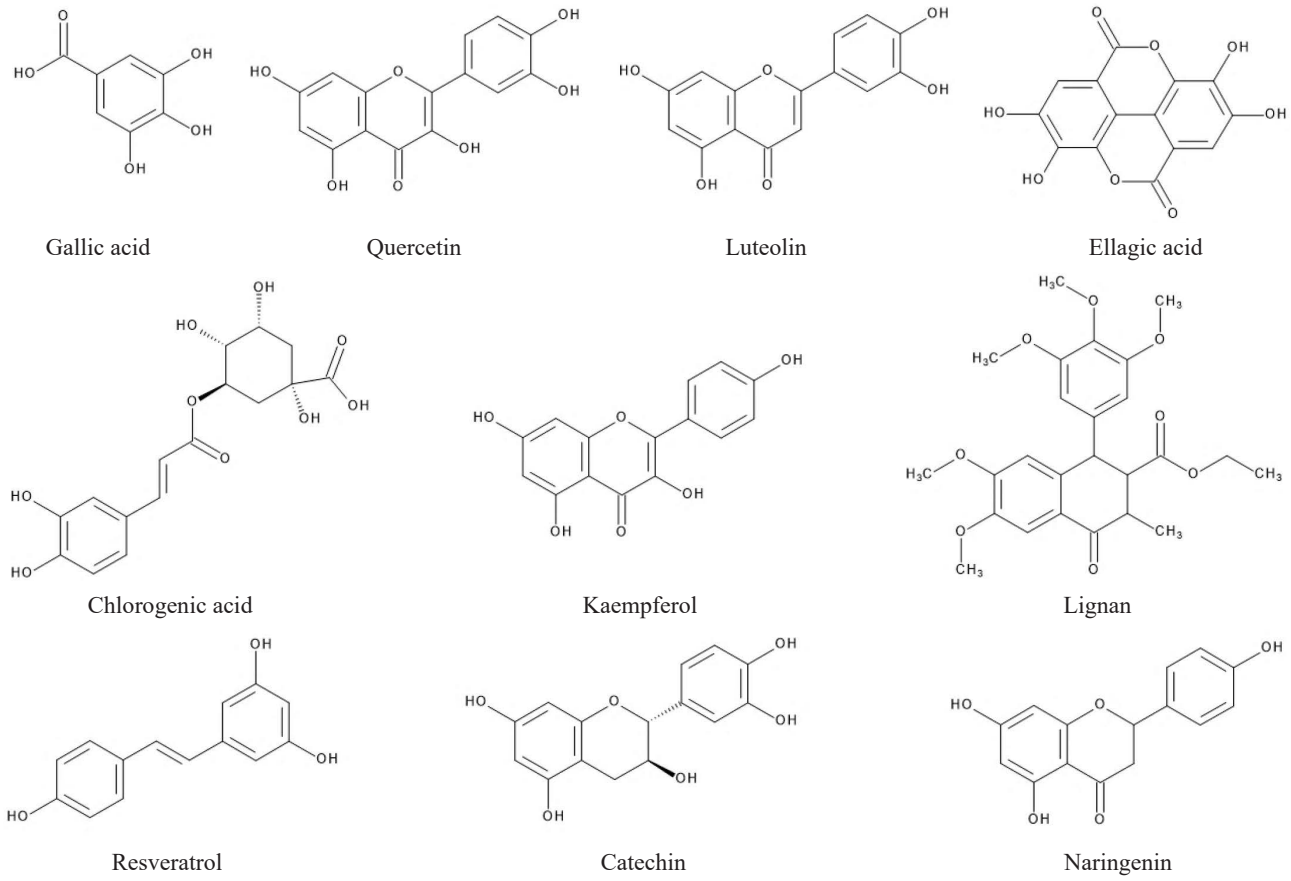

Figure 2 Chemical structure of selected polyphenols in fruits

use of anticancer drugs for cancer treatment. To avoid the side effects emerging from the use of chemotherapeutic agents, scientists are investigating new anticancer agents with better effectiveness and fewer or no side effects [76]. Several epidemiological studies have found that eating fruits on a regular basis can significantly reduce the risk of developing cancer. Polyphenols offer protection to healthy body cells, as well as kill, or are toxic to, premalignant and malignant cells [74].

Screening plants for potent phytochemicals is a better alternative for improving cancer treatment due to their lower side effects. Phytochemicals are biologically active compounds that possess potent anti-tumor properties [76]. They participate in slowing or preventing carcinogenesis by mobbing free radicals, suppressing the survival and spreading of malignant cells, and reducing the invasiveness and angiogenesis of tumors [77–79]. Phytochemicals prevent cancer progression by acting on several molecular targets and signal transduction pathways, such as membrane receptors, downstream tumor-activator or suppressor proteins, kinases, transcription factors, caspases, microRNAs, and cyclins [76].

Apigenin, a flavonoid found in fruits, possesses potent anticancer properties [80]. According to the research, apigenin in the amount of 5 mg/kg suppresses tumor growth while also reducing Ki67 expression and stimulating apoptosis in an athymic nude mouse xenograft with human chondrosarcoma Sw1353 cells. Apigenin can stimulate the expression of Bcl-2 family proteins and activate the caspase cascade to induce G2/M phase arrest and apoptosis [79]. The proliferation of tumor cells such as those in the lung, breast, colon, prostate, and liver can be suppressed by resveratrol (found in fruits such as grapes and berries) [81]. It has been shown to suppress the growth and metastasis of tumors in the lungs of mice with metastatic Lewis lung carcinoma tumors [82]. The antimetastatic and antitumor properties of resveratrol could be due to its ability to inhibit DNA synthesis, angiogenesis, and neovascularization [81]. Several signaling pathways can be altered by resveratrol to reduce the growth and proliferation of cancer cells, initiate programmed cell death, reduce inflammation and angiogenesis, as well as prevent tumor metastases [76]. Diets rich in lycopene content (e.g., fruits) are also shown to reduce the risk of prostate cancer diagnosis in men [83]. Polyphenolic compounds play a crucial role in cancer treatment and prevention by interrupting the initiation, promotion, and progression of cancer cells via the modification of several signal transduction pathways [84]. The role of phytochemicals in cancer prevention at various stages is demonstrated in Fig. 3 [74].

The role of phytochemicals in diabetes management. Diabetes mellitus is one of the main causes of death worldwide, with a prevalence of 8.8% in adults aged 20–79 years [52]. It is generally classified into three major groups, namely type 1 diabetes, type 2 diabetes,

Table 2 Polyphenols and their fruit sources

Phytochemical	Fruit sources	Biological activity	References
Gallotannins	Mangoes and pomegranate	Anti-allergic, anti-hyperglycemic, lipid-lowering, antioxidant, anti-inflammatory, and anticancer properties	[49, 50]
Ellagitannins	Strawberries, raspberries, blackberries, cloudberries, pomegranate, and grapes	Anti-angiogenic, anti-atherogenic, antithrombotic, anti-inflammatory, antioxidant, and antimicrobial properties	[49, 51]
Hydroxycinnamic acids	Apple, pear, plums, cherries, peaches, berries, and grapes	Neuroprotective, antioxidant, hepato-protective, antimicrobial, anti-hypertensive, anticancer, and cardioprotective properties	[40, 41]
Ellagic acids	Blackberry, raspberries, strawberries, mango, and pomegranate	Anti-atherogenic, anti-inflammatory, anti-ulcerative, antidepressant, antidiabetic, antioxidant, and antitumor activities	[40, 52, 53]
Gallic acids	Blackberry, grapes, banana, blueberry, cantaloup, guava, mango, persimmon, and pomegranate	Anti-inflammatory, anti-tumor, anti-obesity, anti-myocardial ischemia, and antioxidant properties	[40, 54, 55]
Anthocyanins	Grapes, blackberries, raspberries, blueberries, Malay apple, red currants, elderberry, and pomegranate	Anti-hypertensive, anti-diabetic, and anti-obesity effects; prevention of stroke, hypercholesterolemia, and hyperuricemia	[30, 40, 56, 57]
Quercetin	Apple, Indian gooseberry, bilberry, cranberries, blackcurrants, raspberry, strawberry, grapes, cherries, passion fruit, and pomegranate	Anti-ulcer, anti-allergic, and antioxidant activities; prevention of osteoporosis and degenerative diseases	[37, 40, 54, 56, 58]
Catechins	Apple, grapes, pear, persimmon, and pomegranate	Antioxidant, anti-allergenic, ant-inflammatory, and antimicrobial activities; protection of skin from UV radiation	[37, 40, 54, 59]
Chlorogenic acids	Cantaloup, apple, carambola, cherries, passion fruit, peach, and pineapple	Anti-obesity, antidiabetic, anti-hypertensive, antimicrobial, and antioxidant effects	[54, 60]
Hesperetin	Citrus fruits (orange, tangerine, lemon, and lime)	Induces apoptosis and cell cycle arrest; anti-angiogenesis, anti-metastases, antioxidant, and anti-inflammatory properties	[37, 40]
Naringenin	Citrus fruits	Anti-inflammatory, antioxidant, anti-atherogenic, anti-convulsant, antibacterial, and immunomodulatory activities	[37, 61]
Naringin	Banana and citrus fruits	Stimulates bone regeneration and repair; anti-inflammatory, anti-cancer, and antioxidant properties; ameliorates mitochondrial dysfunction	[40, 62, 63]
Resveratrol	Red grape, cranberry, blueberry, and plums	Induces apoptosis; cardioprotective, anti-tumor, and antioxidant effects; modulation of estrogen receptor activity	[64]
Lignans	Strawberry, cranberry, blackberry, cloudberry, red and green grapes, grapefruit, orange, tangerine, apricot, melon, kiwi, pineapple, and pear	Antitumor, anti-hypertensive, platelet-activating factor antagonistic, sedative, anti-estrogenic, and antioxidant activities	[64–66]
Kaempferol	Indian gooseberry, blackcurrants, raspberry, strawberry, grapes, banana, apple, guava, and lemon	Induces cell cycle arrest and apoptosis; anti-ulcerogenic, anti-inflammatory, anti-depressive, anticancer, and antioxidant effects; improves wound healing and reduced lung damage	[40, 54 56, 67–69]
Luteolin	Banana, apple, guava, and lemon	Antioxidant, anti-inflammatory, antimicrobial, and anticancer activities	[54, 70]

and gestational diabetes [85]. A lot of factors are responsible for the development of diabetes in individuals, with innate immunity regarded as the main factor in its pathophysiology [86]. Type 1 diabetes is caused by the immune system's inflammatory response to pancreatic islet cells, which causes β-cells to lose function. In type 2 diabetes, low-grade systemic inflammation is a common mediator for the initiation and development of micro- and macro-vascular problems [87]. It is distinguished by insulin resistance and a loss of β-cell function, resulting in an insufficient supply of insulin to meet the body's metabolic needs. People who suffer from type 2 diabetes are unable to control their blood sugar, which results in the excess accumulation of glucose in their blood and urine. Type 2 diabetes is an important public health concern because it accounts for 90% of all diabetes cases worldwide [88].

In recent times, phytochemicals have been under intense consideration for their use as drug candidates to prevent and treat several metabolic disorders such as hyperglycemia and dyslipidemia [52]. Phytochemicals induce important hypoglycemic effects and contribute

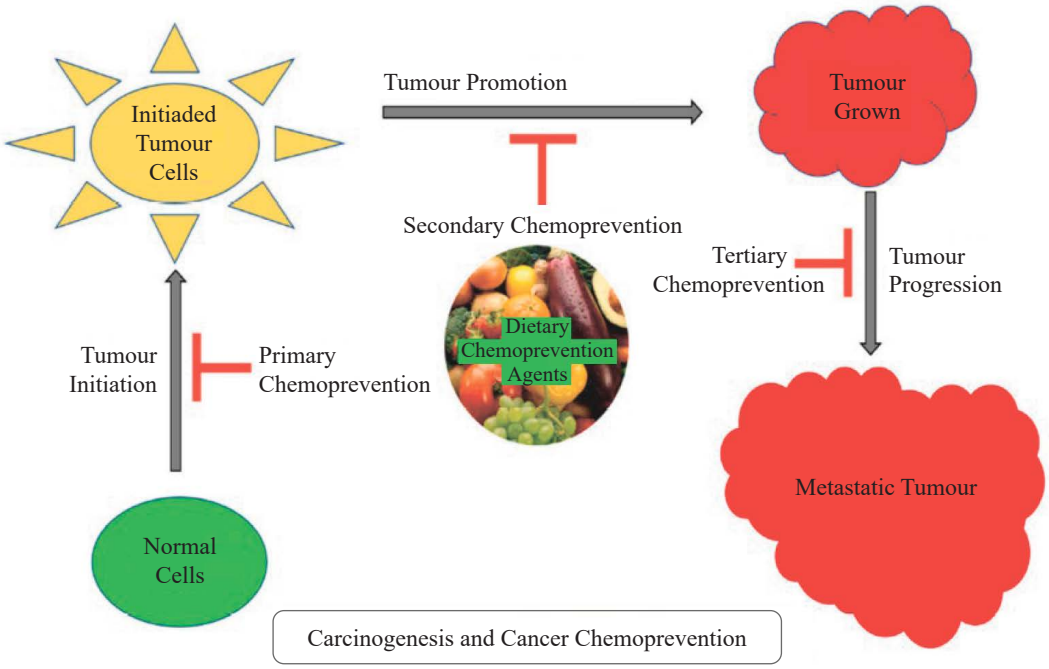

Figure 3 The role of phytochemicals in cancer prevention at various stages of cancer development [74]

to the prevention of diabetes-related vascular complications [89, 90]. Certain groups of flavonoids exhibit potent hypoglycemic properties by enhancing glucose and oxidative metabolism in diabetic conditions. The hypoglycemic property of phytochemicals has been established by studies in human and animal models of type 2 diabetes [91].

According to Keshari *et al.*, flavonoids such as naringenin, apigenin, and quercetin isolated from *Fiscus racemose* stems (these compounds are also present in fruits) demonstrated a hypoglycemic effect by reducing the levels of glucose from 300 to 185 mg/dL after one week of oral administration (100 mg/kg) *in vivo* [92]. Also, the administration of flavonoids to rats improves the glycogen content in the liver when compared with that of untreated diabetic rats. Flavonoids exert their antidiabetic activity by binding to glucose transporter 1 receptors and peroxisome proliferator-activated receptor gamma [92]. This promotes glucose uptake, lipid metabolism, improves insulin activity, and enhanced glucose tolerance in diabetic animals and humans [93]. Flavonoids act through different mechanisms, such as increasing the levels of superoxide dismutase, catalase, and glutathione in the pancreas, normalizing the levels of aspartate transaminase and alanine transaminase in plasma, and enhancing glucose uptake by the cells [94].

Anthocyanins, polyphenols present in fruits such as berries, can prevent type 2 diabetes and obesity. Anthocyanins influence glucose absorption, insulin secretion, level, and action, as well as lipid metabolism in both *in vitro* and *in vivo* studies. Several *in vitro* studies suggested that anthocyanins could decrease glucose absorption from the intestine by delaying the release of glucose during digestion [95]. Some phytochemicals with known anti-diabetic properties include ellagic acid (found in berries, dried fruits, and pomegranate), epigallocatechin gallate (cranberries, strawberries, cherries, pears, kiwi, peaches, black berries, apples, and avocados), naringenin (citrus fruits, cherries, and grapefruit), hesperetin (orange and lemon), chrysin (passion fruit), kaempferol (grapes, raspberry, strawberries, peach, cowberries, and apples), apigenin (grape fruit and orange), quercetin (apples and berries), and resveratrol (grapes) [52].

Anti-inflammatory properties of phytochemicals. Inflammation is a biological response to tissue damage which may be caused by harmful agents produced from biological, physical, or chemical sources. Inflammation can activate several signaling pathways, including phosphatidylinositol-3-kinase (PI3K), Janus-activated kinase (JAK), and mitogen-activated protein kinase (MAPK). Chronic inflammation activates chemokines, cytokines (IL-4, IL-5), inducible nitric oxide synthase (iNOS), signal transducer and activator of transcription 3 (STAT3), and cyclooxygenase enzyme (COX) [96]. Prolonged inflammation interrupts metabolism and induces stress in cells. As a result, it can cause inflammatory diseases such as asthma, autoimmune disorders, allergies, arthritis, and inflammatory bowel disease [96, 97]. Inflammatory diseases can be prevented or suppressed by blocking the pathways that produce inflammatory mediators, particularly proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins (IL-1β, IL-6) [98].

Phytochemicals mediate inflammation through kinases such as mitogen-activated protein kinase and protein kinase C. Phytochemicals inhibit the activity of these enzymes by changing the DNA-binding potential of transcription factors such as nuclear factor kappa-B (NF-κB), one of the main effector molecules that media-

tes inflammation [99, 100]. Flavonoids such as quercetin inhibit the activity of enzymes such as cyclooxygenase and lipoxygenase in arachidonic acid metabolism to reduce the production of prostaglandins and leukotrienes [101, 102]. Apigenin inhibits the production of prostaglandin E2 (PGE2) and the activity of cyclooxygenase (COX-2), as well as NF-κB-dependent pathways [64]. Hesperidin is another phytochemical that inhibits the synthesis of pro-inflammatory mediators such as arachidonic derivatives, thromboxane A2, and prostaglandins E2 and F2 [103]. Nitric oxide is one of the main mediators of inflammation, and phytochemicals that can prevent its production without damaging endothelial or neuronal nitric oxide synthase (NOS) may be considered potent compounds for treating inflammation [104].

Antioxidant activity of phytochemicals. Antioxidants refer to molecules with the potential to react with free radicals by neutralizing or terminating chain reactions initiated by free radicals to prevent them from destroying other molecules [14]. Antioxidants can protect cells by converting reactive oxygen species to non-radical species, breaking auto-oxidative chain reactions, or reducing the concentration of localized oxygen [105]. Evidence of oxidative stress is implicated in several diseases, such as cancer, cardiovascular, neurological, and pulmonary diseases, rheumatoid arthritis, nephropathy, ocular diseases, and the induction of pre-eclampsia during pregnancy. These conditions develop when free radicals begin to alter cell membranes, proteins, lipoproteins, and DNA; stimulate the overexpression of specific genes; activate various kinases and transcription factors such as AP-1 and NF-kappa B; and contribute to the production of toxic peptides (β-amyloid) [106].

Natural antioxidants are primarily obtained from plants, as well as fruits, in the form of vitamins, carotenoids, flavonoids, tannins, alkaloids, terpenoids, isothiocyanates, lectins, polypeptides, and other phenolic compounds [107–110]. Phenolic compounds exhibit potent antioxidant activity, which is shown through their ability to neutralize the deleterious effects of free radicals and serve as antitumor, cardioprotective, and antimutagenic agents [111]. Studies have shown that the intake of dietary antioxidants, especially from fruits with a high total phenolic content, increases serum antioxidant scavenging capacity and provides a boost for the body's antioxidant defense system [112, 113]. Flavonoids such as quercetin, combined with other bioactive compounds, exhibit strong antioxidant activities and are responsible for the disease-preventing properties of fruits. Quercetin is one of the most potent antioxidant compounds that protects the body against oxidative stress induced by amyloid deposits [64]. Rutin (quercetin 3-O-rhamnoglucoside), which is found in fruits, has a similar level of antioxidant activity. It exertsits antioxidant activity by donating electrons to free radicals to convert them into more stable and less reactive species. Enzymes that are involved in the production of reactive oxygen species can be inhibited by rutin to lower oxidative stress. This can prevent diseases caused by oxidative stress, e.g.,neurodegenerative diseases [64, 114].

Carotenoids such as zeaxanthin, astaxanthin, and lutein are powerful lipid-soluble antioxidants that are involved in mobbing free radicals mainly in lipid-soluble environments. High intakes of carotenoids can prevent lipid oxidation and oxidative stress caused by free radicals [30]. Some carotenoids serve as precursors for vitamin A, while others possess effective antioxidant properties that act to scavenge reactive oxygen species. Carotenoids such as α-carotene, β-carotene, and cryptoxanthin are shown to have provitamin A activity, while lutein and lycopene have strong antioxidant activity [115]. Photo-oxidative damage, which occurs because of UV irradiation of the skin, affects macromolecules such as proteins, lipids, and DNA. This may lead to conditions such as premature skin aging, erythema, photodermatoses, and skin cancer. Intake of carotenoids from diets has been shown to improve skin texture, color, strength, and elasticity. Because of their antioxidant activity, carotenoids offer protection to the skin from the sun and harmful ultraviolet radiation [116]. Several studies have found that diets rich in phytochemicals with high antioxidant activity can help prevent a variety of chronic degenerative diseases caused by oxidative stress. Regular consumption of fruits is important for maintaining good health and disease prevention [117].

Bioavailability of phytochemicals and factors that hinder their bioavailability in the body. ***Digestion and absorption of phytochemicals in fruits.*** The absorption of phytochemicals from the food matrix into the body depends on their solubility, structure, degree of glycosylation or acylation, molecular size, the individual's microbiome, and the presence of complementary compounds [118]. Most of phytochemicals exist as esters, glycosides, or polymers in food, while others covalently bind to cell wall components in food matrices, preventing their release for absorption in the gastrointestinal tract. Apart from isoflavonols, all flavonoids exist in their glycosylated form. However, aglycones and some glucosides, such as resveratrol and quercetin, are easily absorbed from the small intestine [22, 119, 120].

The absorption of phytochemicals from food (e.g., fruits) begins with chewing and digestion in the mouth. Chewing and the action of digestive enzymes, particularly α-amylase, aid in the reduction of food particle size and the release of bioactive compounds (polyphenols or carotenoids) from the food matrix [121]. The digest is delivered to the stomach, where digestive enzymes, an acidic pH, and further mixing disintegrate the food matrix to release more polyphenols or carotenoids. The acidic chyme from the stomach is released into an alkaline environment in the duodenum. Enzymes released by the liver and pancreas and the presence of bile salts at this stage also help release additional bioactive compounds and completely break down the food matrix into simple, absorbable macronutrient units. Polyphenols are then released into the bloodstream and delivered to target tissues. Those phenolic compounds which are not

digested are metabolized by the microbiota in the colon to produce metabolites, which are also absorbed into the bloodstream [122]. Carotenoids are lipophilic compounds that are insoluble in aqueous solutions. In the stomach, they are packaged into micelles (which help transport fat-soluble materials) to make them more accessible to the intestinal epithelium for absorption into the bloodstream [34, 36].

Factors that may hinder the bioavailability of phytochemicals in fruits. Bohn defines bioavailability as the amount or concentration of a nutrient or non-nutrient substance absorbed into the human body to stimulate physiological activities or for storage [48]. Bioaccessibility, on the other hand, is defined as the amount of nutrients released from a food matrix during digestion, making the nutrient available or easily accessible for absorption in the gastrointestinal tract [22]. The ability of phytochemicals to exert health-promoting effects on the body depends primarily on their bioaccessibility and bioavailability [123]. Phytochemicals such as polyphenols exist in foods as polymers or in glycosylated form, which may hinder their absorption. To improve the bioavailability of phytochemicals from fruits, they must undergo hydrolysis by colonic microbiota and intestinal enzymes for easy absorption [123, 124]. The involvement of colonic microflora reduces the efficiency of polyphenols absorbed due to the degradation of aglycones to produce several simple aromatic acids during the process [49].

Some phytochemicals, such as tannins, can interact with proteins to form insoluble complexes, and this can limit or prevent their absorption [125, 126]. β-casein can also bind to (+)-catechins and (–)-epicatechin through its proline residues [127]. Phytochemicals can undergo different kinds of changes during gastrointestinal digestion, and those that are able to pass through the intestinal walls can either be metabolized or excreted, which reduces the level (low bioavailability) delivered to target organs or tissues for therapeutic effect [128].

Phytochemicals (such as flavonoids) can bind to components in the digestive system secretions such as saliva, pancreatic, and gastric juices. This may also reduce the amount absorbed into systemic circulation for physiological activities. Research has shown that phenolic compounds have a good affinity for proline-rich proteins and histatins present in human saliva. They either form a covalent or non-covalent bond to increase the size of the phenolic compound to prevent passage through absorptive cells of the small intestine [129]. Enzymes, such as pancreatic α-amylase and trypsin, have been shown to lose activity in the presence of phenolic compounds [130]. In addition, polyphenols can also bind to enterocyte brush border enzymes, and this prevents their release for absorption into the body [129].

CONCLUSION

Fruits are rich sources of phytochemicals which perform several biological activities by acting as potent antioxidant, anticancer, antidiabetic, and anti-inflammatory agents. The modes of action of these bioactive compounds include scavenging free radicals or inhibiting the activity of enzymes and transcription factors that play a critical role in signaling pathways involved in disease initiation and progression. The excess accumulation of free radicals in the body and the inability of the body's antioxidant defenses to effectively destroy free radicals cause oxidative stress and may lead to diseases such as cancer, diabetes, etc. It is therefore imperative that fruits rich in phytochemicals be regularly consumed to reduce an individual's risk of suffering from several chronic disease conditions.

CONTRIBUTION

E.K. Danyo designed the research concept, wrote the original draft, and edited the manuscript. M.N. Ivantsova wrote, reviewed, and edited the manuscript. Both of the authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Microencapsulation by coacervation: Physicochemical and sensory properties of food flavorings

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Abstract:
Coacervation is a low-energy method that is ideal for encapsulating heat-sensitive materials, e.g., limonene, citral, linalool, and isoamyl acetate.
This research used a simple coacervation method to prepare flavoring beads with alginate and Tween 80. The methods of scanning electron microscopy (SEM) and fourier transform infrared (FTIR) spectroscopy made it possible to study the morphology and structure of the flavoring beads. After the extraction, the flavor retention and structure were described using the method of gas chromatography with mass spectrometry (GC-MS).
The microcapsules demonstrated a retention rate of 99.07–99.73% while the encapsulation efficiency remained as high as 96.40–97.07%. The microcapsules had a mononuclear structure and ranged from spherical to elongated ellipsoids; they were sealed without agglomeration. The particle size was below 1000 μm. The GC-MS chromatograms detected neither structural changes nor any new compounds. The FTIR spectra were similar to the control but demonstrated slight shifts, which suggested fundamental structural changes caused by the coacervation. We also fortified sponge cake and jelly with flavoring beads. The sensory analysis of the sponge cake samples revealed no significant differences compared to the control. All the fortified jelly samples had higher scores for smell, taste, texture, and overall preference than the control.
The coacervation method proved to be an excellent solution for the problem of heat-sensitive flavorings that often lose quality or sensory attributes in food products that undergo extensive thermal treatment.

Keywords: Sodium alginate, flavorings, coacervation, GC/MS, FTIR, sensory properties

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INTRODUCTION

The market for flavorings is expected to increase from 14.66 billion $ in 2021 to 20.12 billion $ by 2028, with a Compound Annual Growth Rate of 4.64% [1, 2]. Flavorings affect consumption, acceptance, and food palatability. However, they are sensitive to environmental conditions and industrial processing. In free form, the flavor loss may reach 90% because flavoring substances are extremely volatile and react easily with other components [3]. Microencapsulation is an important method of improving flavor stability. During microencapsulation, solids or liquids are enclosed in polymeric matrices, e.g., maltodextrin, starches, alginates, etc. In addition, microencapsulation extends storage life by preventing unfavorable chemical or sensory changes, as well as increases the solubility of hydrophobic flavorings. The method also makes it possible to control the release of active constituents and create new applications [4].

Microparticles can be obtained by a variety of processes and methods, including fluidized beds, extrusion, centrifugal extrusion, coating, spray-drying, spray-chilling, coacervation, liposomes, and inclusion complexation. Industrial microencapsulation must be simple, reproducible, and quick, with low dependency on the solubility properties of the core substance and the coating polymer. Microencapsulation by coacervation has a few

advantages over other methods. For instance, it provides effective control of the properties and quality of the final product. Microencapsulation by coacervation can be applied to heat-sensitive products and allows for continuous, cheap, and simple production. The obtained particles are highly soluble and stable while being uniform in size [5, 6].

The encapsulation of oils has received particular attention in scientific literature. Most publications feature citronella oil, sunflower oil, fish oil, linseed oil, orange oil, canola oil, olive oil, and thyme oil [7–14]. As for the application range, oil capsules are popular in medicine and pharmacy, where they provide controlled medication release and serve as antibacterial or nutraceutical agents. They can be used in cosmetics, molecular cuisine, food industry, animal feed, etc.

However, microencapsulation of flavorings by coacervation, especially with the help of alginate, has received very little scientific attention so far. The current study aimed at encapsulating limonene, citral, linalool, and isoamyl acetate using the coacervation method with alginate for coating. The flavorings belong to different chemical classes and are part of many commercial food formulations. In nature, they occur in oranges, bananas, lemons, etc.

This research focused on the physical and chemical properties of the microencapsulated flavorings. In addition, the fortified sponge cake and jelly samples underwent a sensory assessment to reveal the effect of processing conditions on the quality of the final product. The simple and reliable microencapsulation method could be applied to different flavorings in food products with extended shelf-life in order to control the flavor release and bring the flavor through severe processing conditions.

STUDY OBJECTS AND METHODS

Ingredients and chemicals. The wheat flour, butter, fresh eggs, sugar, fresh skimmed milk, salt, baking powder, and gelatin were obtained from a local market in Giza, Egypt. The linalool, citral, limonene, isoamyl acetate, and Tween 80 were purchased from Sigma-Aldrich, USA. The sodium alginate with a medium viscosity of ≥ 2000 cp was isolated from brown algae with a molecular weight of 80–120 kDa and a mannuronic to guluronic acid ratio of 1.56. It was purchased from Sigma-Aldrich, USA, while the calcium chloride was provided by Rankem, India.

Preparing beads by coacervation. The encapsulation method with the simple coacervation technique described by Müller *et al.* was used to prepare flavoring beads [11]. To prepare sodium alginate solution, we dissolved 2.5% (w/v) of sodium alginate in distilled water at 1000 rpm for 30 min using a magnetic stirrer. Then, we added 1% (w/v) of the concentrated liquid flavoring to the sodium alginate solution. To prepare the alginate emulsion gel, we added 1% (w/v) Tween 80 as an emulsifying agent and stirred the resulting mix for 1 h. The mix of aroma compounds and alginate solution underwent stirring at 500 rpm for 1 h. After being transferred to a separating funnel, it went into a wide-mouth beaker with a 3% (w/v) calcium chloride solution. The procedure involved an 18-gauge needle of 38 mm in length and 1.27 mm in diameter, as well as a 100-mL glass syringe. For additional hardening, the flavoring beads were stirred at 10 rpm for 1 h. After that, we filtered the beads through cheesecloth and washed them under distilled water. After rinsing, the beads were stored in airtight containers for analysis. The control sample contained neither sodium alginate nor calcium chloride.

Determining encapsulation efficiency. The coacervated beads with limonene, citral, and linalool were evaluated after 24 h of hydrodistillation. The method involved dissolving 5 g of beads in 150 mL of distilled water for 3 h using a Clevenger-type apparatus. We added 2 mL of ethyl ether to extract the volatiles from the aqueous phase. The extract was concentrated in a rotary evaporator, followed by nitrogen gas to vaporize any remaining solvent until constant total weight [15].

As for isoamyl acetate microcapsules, we dissolved 5 g of coacervated beads in 30 mL of distilled water in a screw-cap vial and then mixed it using a vortex mixer. Then, we added 40 mL of diethyl ether solution to extract the flavor from the water phase, as described by Moawad *et al.* [16]. After collecting the flavoring, the ethyl ether was allowed to evaporate by rotary evaporation at 40°C, followed by nitrogen gas to vaporize any remaining solvent until constant total weight. Finally, the retention percentage was calculated using the Eq. (1) below:

$$\text{Flavoring retention} = \left(\frac{\text{Total flavoring in the beads}}{\text{Initial flavoring load}} \right) \times 100 \quad (1)$$

Shahidi Noghabi & Molaveisi assessed the encapsulation efficiency by a modified method that allowed them to determine the surface oil or flavoring [17]. They added 30 mL of hexane to 5 g of beads, followed by stirring at 300 rpm for 10 min. After filtration and washing with hexane, the solvent was vaporized under vacuum at 50°C. All residual solvent was evaporated with nitrogen until constant weight. Finally, they calculated the encapsulation efficiency, %, by using the Eq. (2) developed by Rubén *et al*. [18]:

$$\text{Encapsulation efficiency} = \left(\frac{\text{Total flavoring on bead surfasce}}{\text{Initial flavoring load}} \right) \times 100 \quad (2)$$

Scanning electron microscopy analysis (SEM). The scanning electron microscopy revealed the surface morphology of the flavoring microcapsules. We used a Quanta FEG 250 field emission electron microscope (Czech Republic) to examine the samples at an accelerating voltage of 10 kV. The gold-sputtered samples were mounted on aluminum stubs with double-sided adhesive tape and coated with gold using an Edwards sputter coater S150 A (Crawley, England). The magnification range was 50–15 000×.

Determining flavoring retention and structure by gas chromatography with mass spectrometry (GC-MS).

The analysis involved a Hewlett-Packard 5890 gas chromatograph and a Hewlett-Packard 5970 mass spectrometer. The volatiles were separated in a J&W DB-5 MS column, USA (30 m, 0.25 mm, 0.25 μm). The oven temperature remained 50°C for 5 min before climbing to 250°C at a rate of 4°C per 1 min. Helium served as a carrier gas, with a flow rate of 1.1 mL per 1 min. The sample size was 2 μL; the split ratio was 1:10; the injector temperature was 220°C. The mass spectra were acquired in the electron impact mode at 70 eV and a scan m/z range of 29–400 amu. The retention indices of the isolated volatile compounds were computed using the retention time of a series of n-alkanes (C_6-C_{22}), which were analyzed under identical conditions. The isolated peaks were identified according to the repository of mass spectra established by the National Institute of Standards and Technology (NIST) [19].

Structural analysis by Fourier transform infrared (FTIR) Spectroscopy. This test involved a transmittance mode iS50 Thermo Nicolet Nexus 670 FT-IR (Thermo Scientific, USA) with a built-in diamond crystal to capture the FTIR spectra of alginate and beads [20]. With 32 scans at a 4 cm^{-1} resolution, the analysis covered a spectral range of 400–4000 cm^{-1}.

Preparing sponge cake and jelly. The sponge cake samples were prepared as recommended by Pasukamonset *et al.* [21]. The concentrations of flavoring beads followed the results of the encapsulation efficiency test and the allowable amounts reported for the creaming stage in the Fenaroli's Handbook of Flavor Ingredients [22]. We scaled the dough into two aluminum molds (10×20 cm) to be baked in an electric oven (Universal, Egypt) at 175°C for 25 min. After that, it stayed there to cool at ambient temperature for 30 min. The resulting products were stored in airtight polyethylene pouches at 4°C for further analysis. The jelly samples were prepared with modifications as proposed by Cano-Lamadrid *et al.*, who added 100 g of sugar to 200 mL of water and boiled it with 20 g of gelatin for 2 min [23]. The flavorings were added separately. Unflavored sponge cake and jelly served as control samples.

Sensory evaluation of sponge cake and jelly. The sensory evaluation involved 26 trained panelists from the Department for Food Technology and Nutrition, National Research Centre, Cairo, Egypt. They assessed the coacervated flavoring, sponge cake, and jelly samples using the nine-point hedonic scale, from 9 points for like extremely to 5 for neither like nor dislike and 1 for dislike extremely. Color, smell, taste, softness, and acceptability were the primary sensory attributes [24]. Each panelist was given a tray with core samples of cakes and jelly, a glass of water, and an evaluation sheet. The samples were randomly coded using a three-digit number. The panelists were instructed to rinse their palates between the samples. They had enough room to handle the samples and the questionnaire; the evaluation time was not limited.

Statistical analysis. The obtained data were evaluated using the analysis of variance (ANOVA), the Duncan's Multiple Range test (DMRT), and the SPSS 22 Statistical Package for Social Sciences. The results were presented as mean ± SD with significant differences at $p < 0.05$.

RESULTS AND DISCUSSION

Flavoring retention and encapsulation efficiency. The total retention percentage of the encapsulated flavoring ranged from 99.07 to 99.73% (Table 1). The high retention percentage indicated that the loss of the coacervation-encapsulated volatiles was extremely low. The percentages recorded for the different flavorings were very close despite the differences in volatility, vapor pressure, and molecular weight. The significant difference in the total retention between limonene and the rest of the samples could be correlated with the polarity between the core material and the environment. The method is known to be suitable for core materials. In addition, the procedure presupposes no higher-energy steps for homogenization or encapsulation, which prevents the active constituents from going loose.

We recorded no significant differences ($p \leq 0.05$) in encapsulation efficiency, which stayed between 96.40 and 97.07%. Table 1 demonstrates the excellent encapsulation of the core content despite the difference in some physicochemical properties. The non-significant differences were connected with the total retention content, where the highest efficiency percent (97.07%) belonged to isoamyl acetate, which also showed the highest retention percentage. The lowest efficiency percent (96.40%) was observed in the limonene sample, which also had the poorest retention properties.

Our findings agreed with those published by Müller *et al.*, who coacervated orange oil with alginate and found the encapsulation efficiency as 99.51% [11]. Benavides *et al.*, who studied coacervated thyme essential oil, established an inverse relationship between the content of encapsulated oil and the encapsulation efficiency percentage [14]. Similarly, Baranauskaite *et al.* reported that increasing oregano essential oil content decreased the encapsulation efficiency percentage of microspheres [25]. The authors explained it by the limited capacity of the capsule to contain oil. When they raised the oil content, a significant amount of it approached the microsphere surface. When the microsphere dried, this oil was lost by volatilization, thus reducing the encapsulation efficiency percentage [14].

Table 1 Mean values of total retention and encapsulation efficiency for flavorings microencapsulated by coacervation

Component	Encapsulation efficiency, %	Flavoring retention, %
Linalool	$96.87^{a} \pm 0.92$*	$99.53^{ab} \pm 0.23$
Citral	$96.93^{a} \pm 1.17$	$99.60^{a} \pm 0.20$
Limonene	$96.40^{a} \pm 0.72$	$99.07^{b} \pm 0.50$
Isoamyl acetate	$97.07^{a} \pm 1.10$	$99.73^{a} \pm 0.12$

*Means ± SD with the same superscripts in the same column are insignificant at $p < 0.05$

Encapsulation efficiency percentage depends on some other variables. For example, a faster stirring rate causes a higher degree of dispersion. As a result, small clusters of oil appear in the microsphere. They are finely distributed between the alginate chains. Emulsifying agent is another important variable. In our case, Tween 80 improved the oil retention and flavoring [26]. The wall vs. core ratio is an important factor in enhancing encapsulation efficiency percentage, which is known to go down when the amount of essential oil increases. A larger amount of wall material or crosslinking agent makes the wall more compact, which prevents the release of oil or flavoring from the microcapsules [27].

Morphological analysis of flavoring beads. Figure 1 demonstrates the results of the scanning electron microscopy, which we used to study the morphology of flavoring microcapsules. They showed spherical or elongated ellipsoids with few or no dents, cracks, or holes. These complete and sealed capsules protected the flavoring and the core material. Some irregular morphology was observed, but the structure was mononuclear, which means that the core material was sealed without agglomeration. The particle size was not homogeneous for different microcapsules. The average particle size was below 1000 µm.

Our findings confirmed those reported by Müller *et al.*, who obtained coacervated orange oil microparticles with a mean volume diameter of 908.63 µm and a particle size of 346.37–1867.31 µm [11]. Piornos *et al.* studied the microscopic outer and inner structures of optimized linseed oil beads of approximately 1.80 mm [10]. They reported various external morphologies, i.e., rough and smooth surfaces, as a consequence of drying and subsequent shrinking. Some beads had small depressions on the surface. However, the authors discarded the possibility of deeper pores by zooming on the problematic zones. The microencapsulation of limonene essential oil using simple coacervation by chitosan showed that microcapsules had a mean size of 10 µm. They were rough and not spherical in shape, which resembles our findings. The shrinking was caused by the loss of encapsulated oils, as seen by the pores in microcapsules [3]. Gawad & Fellner compared the encapsulation of glycerol using a simple coacervation with alginate and a complex coacervation with alginate and chitosan [20]. The size and appearance they obtained were comparable with our data. Therefore, other studies also revealed the ability of simple coacervation to present an efficient and excellent encapsulation.

Studying the effect of coacervation on flavoring structure by gas chromatography with mass spectrometry (GC-MS). Figure 2 illustrates the retention potential of coacervated flavorings based on their different physical and chemical properties. Coacervation is a low or no-energy method, compared to other intensive-energy protocols, e.g., spray-drying. Therefore, coacervated flavorings are expected to be more stable than spray-dried ones. The chromatograms revealed neither changes nor any new compounds.

These results correspond with those reported by Li *et al.* [28]. The authors encapsulated citral essential oil by simple coacervation and found that the process did not degrade the two main compounds of the essential oil, namely *α*-citral and *β*-citral. The microcapsules they obtained were highly efficient in preserving the qualities of peaches during storage. Similarly, encapsulation did not affect the antifungal or antibacterial activities of citral essential oil.

Besharati *et al.* found that encapsulating flaxseed oil with chitosan minimizes the ruminal biohydrogenation process of unsaturated fatty acids: the microcapsules had excellent flavor retention and stability [29]. D-limonene, the major component of orange oil, was well retained (60–85%) in microcapsules prepared by Baiocco & Zhang based on shear stress [30].

Coacervation is a good method for heat-sensitive materials, e.g., essential oils or flavorings, because it does not involve thermal treatment. Microparticles obtained by coacervation have higher thermal stability than those obtained by intensive-energy methods, e.g., spray-drying. As a result, coacervated microparticles may be used in foods that undergo intense thermal treatment. Consequently, the microencapsulation method may be selected based on the final product, equipment availability, and the relation between production cost and sales price of the microencapsulated flavorings [11].

Fourier transformed infrared spectroscopy (FTIR) analysis. Figure 3 shows the FTIR spectra of flavorings

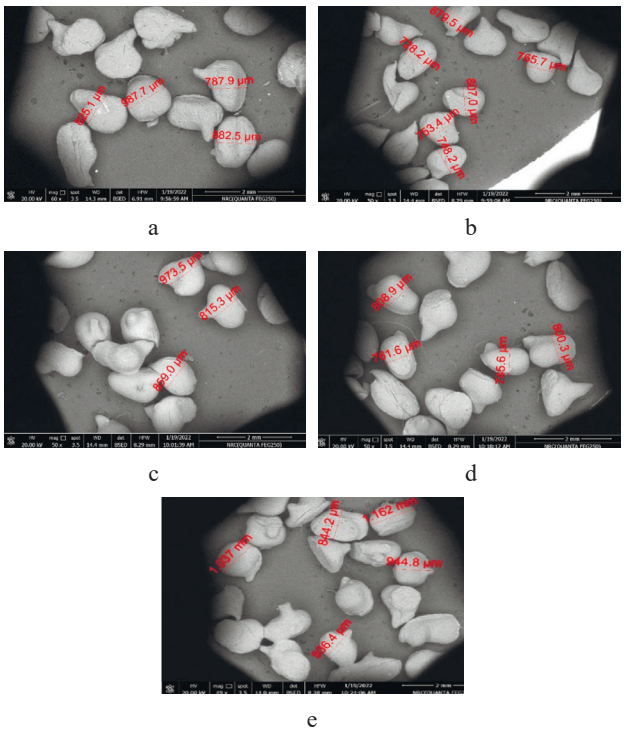

Figure 1 Morphology of beads for flavorings microencapsulated in alginate by coacervation (a – Sodium alginate (control), b – Linalool, c – Citral, d – Limonene, and e – Isoamyl acetate). Scanning electron microscopy

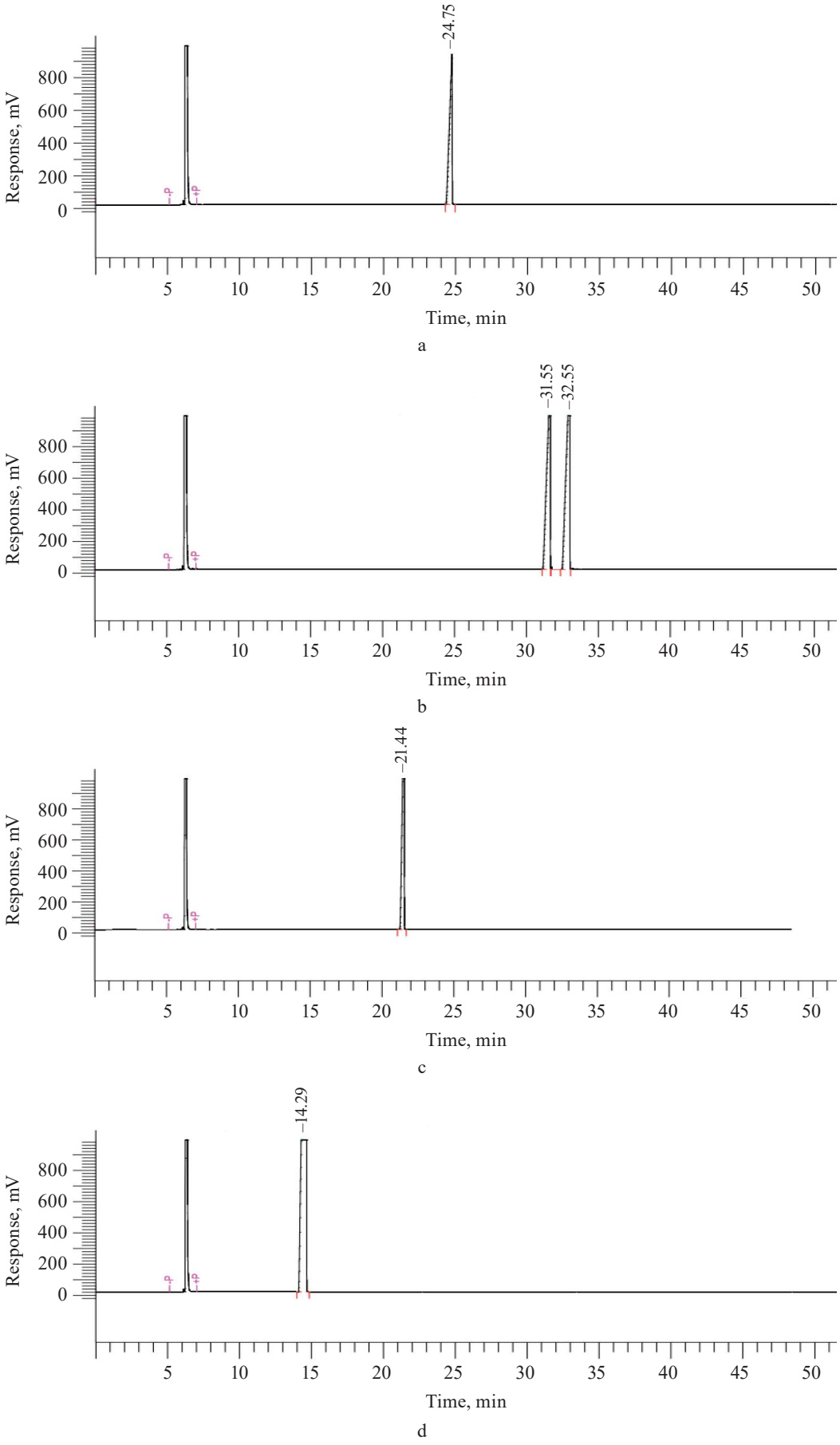

Figure 2 Flavorings microencapsulated in alginate by coacervation (a – Linalool, b – Citral, c – Limonene, and d – Isoamyl acetate). GC-MS chromatograms

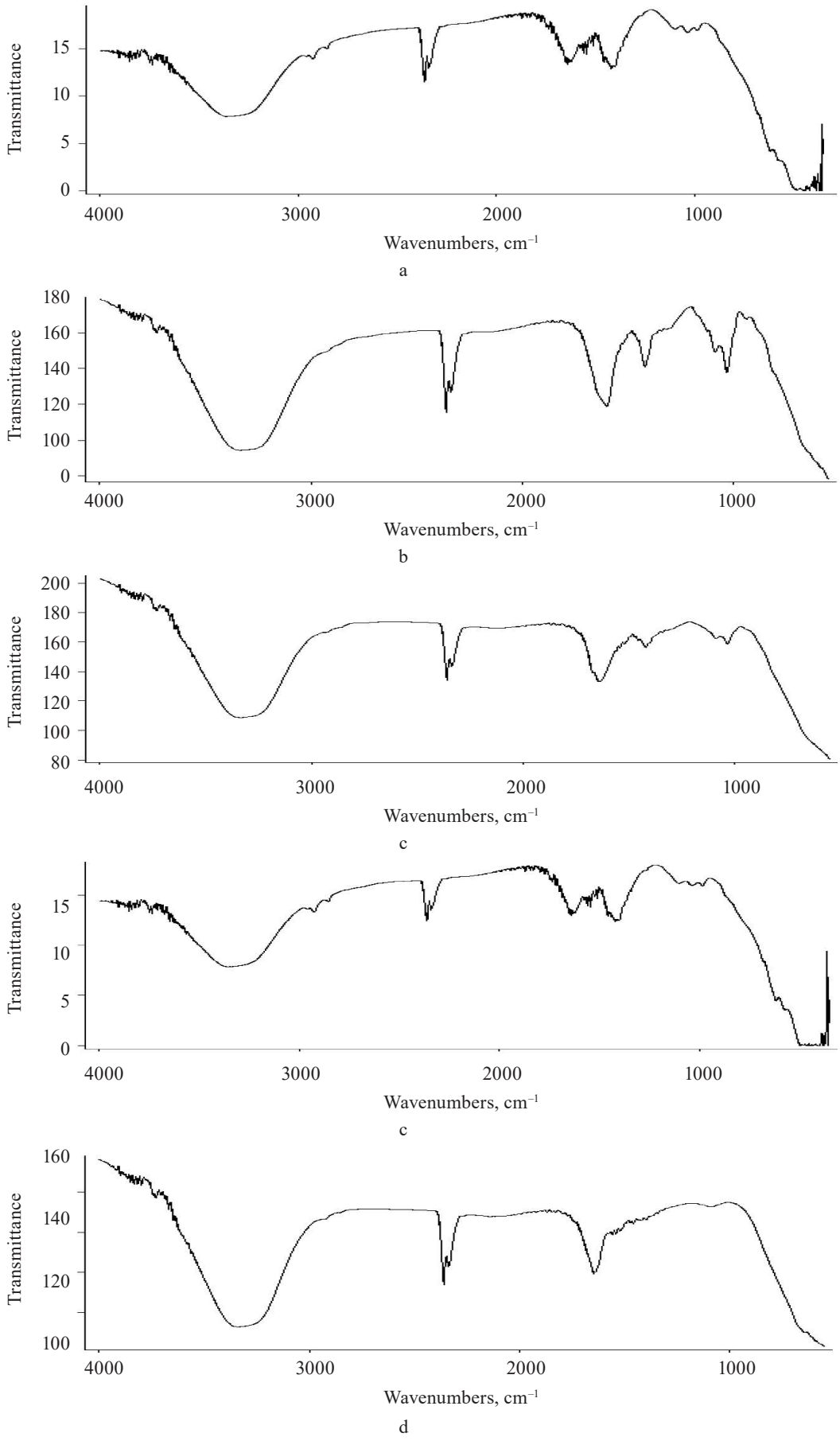

Figure 3 Flavorings microencapsulated in alginate by coacervation (a – control, b – Linalool, c – Citral, d – Limonene, and e – Isoamyl acetate). FTIR spectra

Table 2 Sensory profile of sponge cake samples fortified with coacervated beads (max 9)

Sample	Color	Smell	Taste	Softness	Overall preference
Control (unflavored)	$7.52^{a} \pm 1.25$*	$6.88^{a} \pm 1.56$	$7.08^{a} \pm 1.70$	$7.46^{a} \pm 1.53$	$7.38^{a} \pm 1.44$
Linalool	$7.40^{a} \pm 1.11$	$6.90^{a} \pm 1.04$	$6.98^{a} \pm 0.94$	$7.42^{a} \pm 1.16$	$7.27^{a} \pm 1.18$
Citral	$7.35^{a} \pm 1.35$	$7.25^{a} \pm 1.27$	$7.00^{a} \pm 1.26$	$7.65^{a} \pm 0.85$	$7.46^{a} \pm 0.97$
Limonene	$7.40^{a} \pm 1.28$	$7.02^{a} \pm 1.28$	$7.28^{a} \pm 1.31$	$7.60^{a} \pm 0.96$	$7.52^{a} \pm 1.00$
Isoamyl acetate	$7.48^{a} \pm 1.25$	$7.35^{a} \pm 0.98$	$7.60^{a} \pm 1.06$	$7.83^{a} \pm 0.90$	$7.73^{a} \pm 0.86$

* Means with the same superscripts in the same column are insignificant at $p < 0.05$

Table 3 Sensory profile of jelly samples fortified with coacervated beads (max 9)

Sample	Color	Smell	Taste	Texture	Overall preference
Control (unflavored)	$6.68^{b} \pm 1.49$*	$5.96^{b} \pm 1.62$	$6.48^{b} \pm 1.53$	$7.80^{a} \pm 1.44$	$6.38^{b} \pm 1.29$
Linalool	$8.32^{a} \pm 0.63$	$7.62^{a} \pm 0.73$	$7.60^{a} \pm 0.71$	$8.36^{a} \pm 0.70$	$7.96^{a} \pm 0.66$
Citral	$7.70^{a} \pm 1.00$	$7.45^{a} \pm 1.15$	$7.24^{a} \pm 1.45$	$8.12^{a} \pm 0.83$	$7.70^{a} \pm 0.87$
Limonene	$7.92^{a} \pm 1.04$	$7.32^{a} \pm 0.90$	$7.30^{a} \pm 0.79$	$7.98^{a} \pm 1.29$	$7.62^{a} \pm 0.88$
Isoamyl acetate	$7.86^{a} \pm 0.93$	$7.72^{a} \pm 1.02$	$7.56^{a} \pm 1.42$	$8.36^{a} \pm 0.86$	$7.90^{a} \pm 0.84$

*Means with the same superscripts in the same column are insignificant at $p < 0.05$

encapsulated by coacervation and alginate (control). The alginate spectrum (control) had an OH-related broadband at 3.200–3.400 cm^{-1} stretching with strong hydgen bonding. The peak at 2.924 cm^{-1} could be ascribed to the overlapping symmetrical and asymmetrical C-H stretching vibration of aliphatic chains ($-CH_2-$, $-CH_3$). The asymmetric and symmetric vibrational modes of carboxylate ions (O-C-O) were recorded at 1.633 and 1.414 cm^{-1}, respectively. The vibrational mode at 1.093 cm^{-1} was attributable to the C–O stretching vibration of a pyranose ring. Due to its polysaccharide structure, the stretching vibration of sodium alginate (C-O-C) manifested at 1.035 cm^{-1}.

A C-H stretching was also identified for uronic acid (982 cm^{-1}) and mannuronic acid (878 cm^{-1}). These findings confirmed those reported by Helmiyati & Aprilliza [31]. The spectra of microencapsulated flavorings were related strongly to the control but with slight shifts suggesting fundamental structure changes caused by coacervation. For example, linalool, citral, and isoamyl acetate had narrower and more intense bands at 3.200–3.400 cm^{-1} that reflected new hydrogen bonds between alginate and flavorings. However, nothing of similar kind was recorded for limonene due to the nature of this flavoring. The O-C-O signal was more intense in the alginate sample (control) than that in the microencapsulated samples. Again, an exception was the limonene beads, which revealed weaker ionic bonds between flavorings and carboxyl groups due to the absence of efficient functional groups in the flavoring. Generally, ionic bonds are essential evidence for the interaction during complex coacervation, e.g., between chitosan and alginate, as well as the broadening of some bands or the increase in the intensity of others [20].

Sensory evaluation of food products fortified with microencapsulated beads. Table 2 sums up the results of the sensory evaluation, which included color, smell, taste, texture, and overall preference of the control sponge cake and the cakes fortified with coacervated flavorings. No significant differences were obtained for color scores. The samples with coacervated citral, limonene, or isoamyl acetate got the best scores for smell, taste, softness, and overall preference. Again, no significant differences were observed between these experimental samples and the control. Choosing sponge cake as an application for the encapsulated flavorings was critical for assessing the success of the whole process and the limits of potential use for such formulated raw materials in different food products. Additionally, the application opens prospects for flavorings in many food products that need intensive cooking conditions without affecting the quality or sensory attributes.

The jelly samples fortified with coacervated flavorings received higher mean scores than the control, and this time the differences were statistically significant (Table 3). Texture was the only variable with no significant differences. In contrast, all the experimental samples showed significant differences in smell, taste, and overall preference. The lower molecular weight and vapor pressure of aroma compounds were responsible for the highest sensory attributes. Immersing in water solutions increased the aroma release in the gas phase because sucrose had the so-called *salting out* effect, which constituted more than 50% of the jelly formulation. Vatankhah Lotfabadi *et al.* used time-intensity analysis and HS-GC/MS spectrometry to study D-limonene flavor in rock candy crystal sticks and reported similar results [32]. Dubova *et al.*, who studied the natural flavor distillates obtained from melon and cucumber, also reported sensory characteristics that were closer to natural raw materials than the existing industrial analogs [33].

CONCLUSION

In this research, the method of coacervation proved quite efficient in encapsulating such popular industrial flavorings as limonene, citral, linalool, and isoamyl

acetate. The experimental samples demonstrated high retention percentage and excellent encapsulation efficiency. The capsules were complete and sealed, which means they provided good protection for the core material. The coacervated flavorings showed no physicochemical changes; no new compounds were detected. The experimental samples of sponge cake and jelly received better scores for all sensory attributes. Therefore, microencapsulation by coacervation could be an excellent solution for heat-sensitive food ingredients.

CONTRIBUTION

Amr Farouk, Mamdouh H. El-Kalyoubi, and Mohamed F. Khallaf developed the research concept, supervised the project, and performed the practical part of the study. Shimaa Moawad and Ramadan A. Gawad designed the methodology, as well as performed the statistical analysis, formal analysis, and data curation. Shimaa Moawad and Badr Saed prepared the samples and performed the extraction. Amr Farouk wrote the original draft, reviewed scientific articles, and edited the text. All the authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to the publication of this article.

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Phylogenetic identification of microbes from fermented botanicals used in gluten-free composite flour mixes

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Abstract:
Phylogenetic information on microbial communities involved in fermenting botanicals has important implications for the food industry since it can provide a valuable perspective on the diversity, composition, and techno-functional properties and characteristics of the final product. Microbial phylogenetic analysis illustrates the evolutionary history of microbes through visual representational graphs (phylogenetic trees) showing the beginning and advancement of their assemblage.
In this study, we used molecular methods to determine the phylogenetic identities of microbes occurring in spontaneously fermented sweet potato, maize, and pigeon pea samples after a 72-hourly evaluation every 12 h. The sequences obtained were edited using the bioinformatics algorithm against similar sequences downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN and aligned using ClustalX. The neighbor-joining technique was applied to extrapolate the chronicle of the isolates evolution.
Molecular identification from the BLASTN results showed the following bacterial isolates: *Lysinibacillus macrolides*, *Klebsiella pneumoniae*, *Lactococcus lactis*, *Providencia stuartii*, *Enterobacter cloacae*, *Limosilactobacillus fermentum*, *Lactobacillus fermentum*, *Staphylococcus edaphicus*, and *Bacillus flexus*, as well as the following fungal isolates: *Trichosporon asahii*, *Mucor irregularis*, *Cladosporium tenuissimum*, and *Aspergillus niger*. The sequences obtained from the isolates produced an exact match with the NCBI non-redundant nucleotide (nr/nt) database. *L. lactis* had the highest percentage occurrence for bacteria (38.46%), while *T. asahii* and *A. niger* showed the highest occurrence for fungi (37.50%).
Identifying and characterizing the microorganisms involved in the fermentation process would allow optimizing fermentation conditions to enhance the quality and nutritional value of the final products.

Keywords: Phylogenetic identification, fermented botanicals, gluten-free composite, flour mixes

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INTRODUCTION

Microbial fermentation is a bio-engineered process that has been employed for centuries in the production of various nutrient-enhancing fermented food products such as beverages, bread, and dairy foods [1–5]. During this procedure, microorganisms such as bacteria and fungi enzymatically break down complex organic compounds inherent in the food substrate to produce various metabolites, including organic acids, alcohols, and gases [6]. Microbial communities involved in fermentation are diverse and complex, and their compositions and activities are influenced by various factors such as temperature, pH, and the presence of nutrients [7–9].

In recent years, there has been a growing interest in the use of botanicals as a substrate for microbial fermentation, particularly in the production of traditional fermented foods and beverages. The main purpose is to develop agro-processed, highly-nutritive, gluten-free therapeutic foods from comparatively advantageous indigenous botanicals [10–12]. Such botanicals include various plant materials (grains, vegetables, fruits, roots, and tubers) that are rich in nutrients and bioactive compounds and

can influence the growth and metabolism of microorganisms [13]. Studies of microbial communities involved in the fermentation of botanicals have important implications for the food industry, as they can provide insights into the techno-functional properties and characteristics of the final product [12].

Customary methods for microbial characterization have been challenged overtime due to imprecise and ambiguous similarities they provide between different species [14]. This irregularity in morphological nomenclature for microorganisms necessitated a need for modern, more pragmatic and reliable taxonomic protocols. Phylogenetics is a molecular-based technique with an improved and more proficient method of characterizing and identifying microorganisms [15]. It entails studying progressive evolutionary relationships among organisms based on their genetic characteristics from a similar forebear [16]. This powerful tool for understanding the diversity and structure of microbial communities has become a ubiquitous part of biological analyses [17, 18].

Phylogenetic analysis of microbial isolates is one of the means by which one can learn about the evolutionary history of species by constructing comparative visual representational graphs of phylogenetic trees (illustrations showing the beginning and advancement of assemblage of organisms) using the organism's morphological features [15, 19]. Recently, sequences from deoxyribonucleic acid (DNA) and proteins from different organisms have been used to determine their evolutionary relationships from common forebears to different off-springs [16, 20, 21].

Studying phylogenetic properties of microbial isolates is crucial in understanding the evolutionary history and diversity of microbial populations. Their evolutionary relationships provide insights into the origins, diversification, and distribution of microbial species. Phylogenetic properties can provide a better understanding of the microbial world, its interactions with the environment, and its impact on human health and disease [22]. This knowledge can be applied in various fields, such as biotechnology, medicine, and agriculture, to develop innovative solutions to tackle the challenges we face today [23].

In this study, we aimed to phylogenetically identify microorganisms isolated from fermented botanicals used to formulate gluten-free composite flour mixes.

STUDY OBJECTS AND METHODS

Collection and confirmation of samples. Yellow-fleshed sweet potato tubers (*Ipomea batatas* L.), maize of the yellow-grain variety (*Zea mays* L.), and pigeon peas (*Cajanus cajan* L. Millsp.) were obtained from local food merchants in the Auchi metropolis, specifically in the Etsako-West Local Government Area of Edo State, Nigeria. The authenticity and quality of these samples were verified at the Herbarium Curation Division, Department of Basic Sciences, Edo University Uzairue, also located in Edo State, Nigeria.

Preparation and fermentation of samples. The samples of botanical materials were subjected to fermentation, which was carried out spontaneously for 72 h. The process took place at 28 ± 2°C, following the procedures described in [12].

Microbiological analysis. The fermented botanical samples underwent microbiological analysis every 12 h to determine the total microbial counts. The method used for this analysis followed the guidelines provided by the American Public Health Association [24, 25]. To initiate the analysis, 1 mL of the fermented samples was aseptically withdrawn and mixed with 9 mL of peptone water. Subsequently, we performed a sequential 10-fold dilution.

For the microbiological analysis, aliquots from the final dilutions were taken and introduced into specific agar media. Bacteria were cultured using Nutrient Agar (NA), MacConkey Agar (MCA), and De Mann-Rogosa-Sharpe Agar (MRS). Fungi, on the other hand, were cultured using Potato Dextrose Agar (PDA). The plates containing the samples and agar media were then incubated for 24–48 h at 37°C for bacteria and at room temperature (25 ± 2°C) for fungi.

All of these processes, including dilution, culturing, and incubation, were conducted in triplicate to ensure accuracy and reliability of the results.

Isolation and enumeration of bacteria and fungi. Distinct colonies with varying morphologies were counted and reported as colony-forming units per milliliter (CFU/mL) of the respective samples. To classify these colonies, they were separated as pure cultures and preserved in agar slants both at 4°C and at room temperature. Standard morphological, biochemical, and molecular techniques were employed to confirm the identification of the different bacterial and fungal species [26, 27].

Molecular identification. ***Bacterial genomic DNA extraction.*** Five milliliters of an overnight liquid culture of the bacterial isolate in Luria Bertani (LB) medium was centrifuged at 14 000 rpm for 3 min. The resulting pellet was then resuspended in 500 mL of normal saline and heated at 95°C for 20 min. After cooling on ice, the mixture was spun for another 3 min at 14 000 rpm. The resulting supernatant, which contained the DNA, was carefully removed and transferred to a 1.5-mL micro centrifuge tube. This DNA extract was then stored at –20°C for future downstream reactions.

DNA quantification. The genomic DNA obtained was measured for its quantity using a Nanodrop 1000 spectrophotometer. To initiate the process, the Nanodrop software was opened by double-clicking on the Nanodrop icon. The spectrophotometer was calibrated using 2 µL of sterile distilled water as the initial blank, which was replaced with normal saline. Next, 2 µL of the extracted DNA was carefully loaded onto the lower pedestal of the spectrophotometer, and the upper pedestal was lowered to allow contact with the DNA sample. When the "measure" button was clicked, the Nanodrop spectrophotometer provided the measurement of the DNA concentration.

16S rRNA amplification. The 16S ribosomal RNA (rRNA) region of the rRNA genes in the isolates was amplified using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3')

primers. The amplification process was carried out in a 50 µL final volume for 35 cycles, using an ABI 9700 Applied Biosystems thermal cycler. The PCR mix consisted of the X2 Dream Taq Master Mix provided by Inqaba, South Africa, which included taq polymerase, DNTPs, and MgCl, along with the primers at a concentration of 0.4 M. The extracted DNA served as a template for the PCR reaction. The PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s, which was repeated for 35 cycles, and final extension at 72°C for 5 min. The resulting PCR product was separated on 1% agarose gel, subjected to electrophoresis at 120 V for 15 min, and visualized using a UV transilluminator.

Fungal genomic DNA extraction. DNA extraction was performed using a ZR fungal DNA mini prep extraction kit provided by Inqaba, South Africa. Fungal isolates in pure culture were densely grown and suspended in 200 µL of isotonic buffer, to be then transferred to ZR Bashing Bead Lysis tubes. To this, 750 µL of lysis solution was added, and the tubes were placed securely in a bead beater equipped with a 2-mL tube holder assembly. The samples underwent processing at maximum speed for 5 min.

Subsequently, the ZR Bashing Bead Lysis tubes were centrifuged at 10 000 × g for 1 min. After centrifugation, 400 µL of the supernatant was carefully transferred to a Zymo-Spin IV spin Filter (orange top) positioned in a collection tube. The collection tube was then centrifuged at 7000 × g per 1 min. Then, 1200 µL of fungal/bacterial DNA binding buffer was added to the filtered liquid in the collection tube, resulting in a final volume of 1600 µL. Next, 800 µL of this mixture was moved to a Zymo-Spin IIC column placed in the collection tube, which was centrifuged at 10 000 × g for 1 min. The flow-through was discarded, and the remaining volume was retained within the Zymo-Spin IIC column.

Then, 200 µL of DNA pre-wash buffer was added to the Zymo-Spin IIC column in a fresh collection tube and centrifuged at 10 000 × g for 1 min. Following this, 500 µL of fungal/bacterial DNA wash buffer was added to the column, and centrifugation was performed at 10 000 × g for 1 min. The Zymo-Spin IIC column was then transferred to a clean 1.5-µL centrifuge tube. To elute the DNA, 100 µL of DNA elution buffer was added to the column matrix and centrifuged at 10 000 × g for 30 s. The resulting DNA, which was of high purity, was stored at –20°C for subsequent downstream reactions.

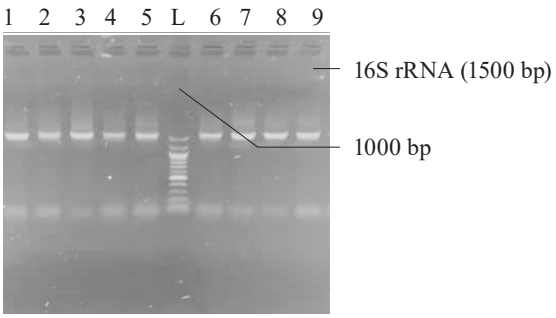
Internal Transcribed Spacer (ITS) amplification. The ITS region of the rRNA genes present in the fungal isolates was amplified using specific primers, namely ITS1-F: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4-R: 5'-TCCTCCGCTTATTGATATGC-3'. This amplification process was carried out on an ABI 9700 Applied Biosystems thermal cycler, with a final reaction volume of 50 µL for a total of 35 cycles. The PCR mixture consisted of the X2 Dream Taq Master Mix provided by Inqaba, South Africa, which contained taq polymerase, DNTPs, and MgCl, along with the primers at a concentration of 0.4 M. The extracted DNA served as a template for the PCR reaction. The PCR conditions involved initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. These steps were repeated for 35 cycles, and a final extension was performed at 72°C for 5 min. To visualize the PCR product, it was separated on a 1% agarose gel using an electric field of 120V for 15 min, and the resulting bands were observed under a UV transilluminator.

Sequencing was conducted at Inqaba Biotechnological in Pretoria, South Africa, using the BigDye Terminator Kit on a 3510 ABI sequencer. The sequencing reaction was prepared with a final volume of 10 µL, consisting of the following components: 0.25 µL of BigDye® Terminator v1.1/v3.1, 2.25 µL of 5× BigDye sequencing buffer, 10 micromolar Primer PCR primer, and 2–10 nanograms of PCR template per 100 base pairs. The sequencing process involved 32 cycles with the following temperature conditions: denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and extension at 60°C for 4 min.

Phylogenetic analysis of the isolates. The obtained sequences underwent editing using the bioinformatics algorithm Trace Edit. To identify similar sequences, we used the National Center for Biotechnology Information (NCBI) database. These sequences were then aligned using ClustalX. The evolutionary history of the isolates was inferred using the neighbor-joining technique [28]. The resulting phylogenetic tree displayed the most supported relationships, with a branch length summation of 0.73390024. To assess the reliability of the tree, a bootstrap test with 1000 replicates was conducted, showing the percentage of replicate trees in which the taxa were grouped together near the branches [29]. The tree was visualized with branch lengths reflecting the inferred evolutionary distances. The phylogenetic analysis involved the computation of the phylogenetic space using the Jukes-Cantor procedure, considering 17 nucleotide progressions [30]. Sites with less than 95% inclusion were removed, representing less than 5% of the sequence space, and positions allowing for cryptic bases were retained using a partial removal selection approach. The final dataset consisted of 324 positions. The evolutionary analysis was performed using MEGA X [31].

RESULTS AND DISCUSSION

Molecular identification from the BLASTN results of the DNA sequences shows that the 16S rRNA sequence obtained from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database (Fig. 1). The 16S rRNA of the isolates W1 showed the 99–100 % percentage similarity to other species. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Providencia*, *Enterobacter*, *Klebsiella*, *Lysinibacillus*, *Staphylococcus*, *Limosilactobacillus*, *Lactobacillus*, and

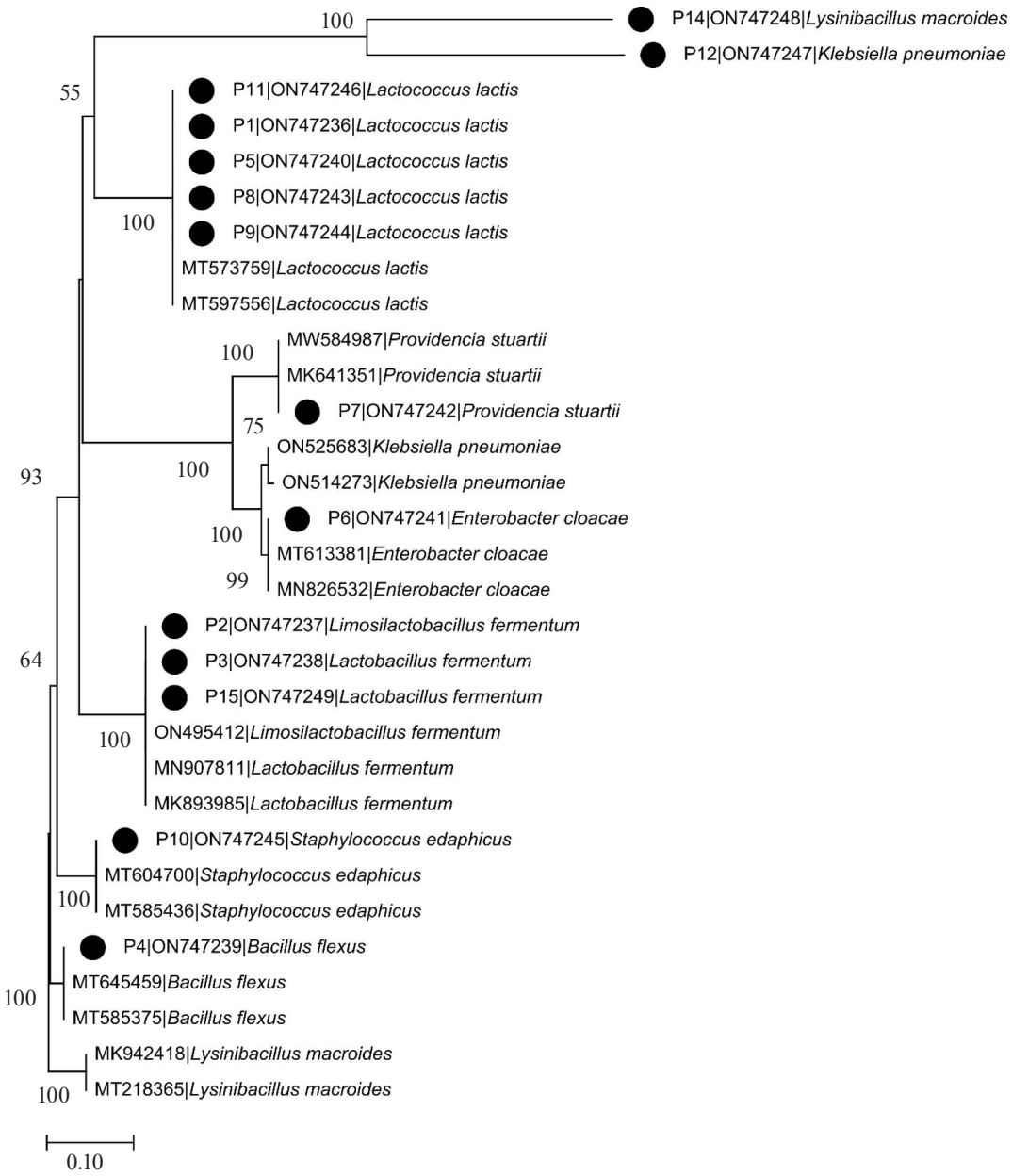


Note: lanes 1–9 represent the 16S rRNA bands at 1500 bp, while lane L represents the 100 bp molecular ladder

Figure 1 Agarose gel electrophoresis of the 16S rRNA of the bacterial isolates.

Lactococcus sp. They also revealed a close relatedness to *Providencia stuartii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Lysinibacillus macroides*, *Staphylococcus edaphicus*, *Limosilactobacillus fermentum*, *Lactobacillus fermentum*, and *Lactococcus lactis* (Fig. 2).

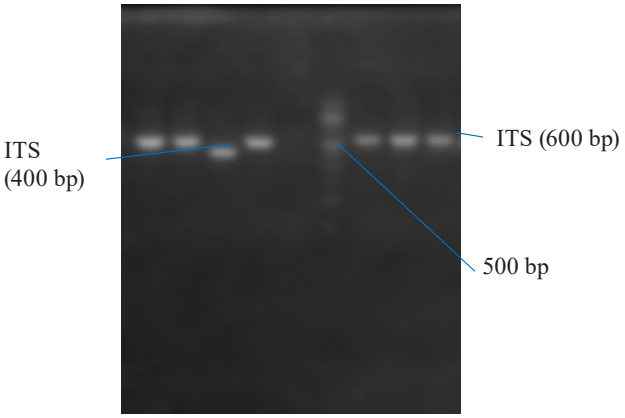
The ITS sequence obtained from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database (Fig. 3). The ITS of the isolates showed the 100% similarity to other species. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement within the *Aspergillus*, *Cladosporium*, *Mucor*, and *Trichosporon* sp., as well as revealed a close relatedness to *Aspergillus niger*, *Cladosporium tenuissimum*,



Note: The nodes without circular annotation represent highly similar species that were obtained as top hits from the NCBI (National Centre for Biotechnology Information)

Figure 2 A phylogenetic tree showing the classification and evolutionary relationship of the bacterial isolates

Mucor irregularis, and *Trichosporon asahii* (Fig. 4). The morphological characteristics of the identified microbial and fungal isolates, which help in authenticating these isolates, are presented in Tables 1 and 3, respectively, while their actual names and the summary of their closest BLASTN similarities are shown in Tables 2 and 4, respectively. The percent similarities between the isolated microorganisms and those from the GenBank database indicate that they all share a common ancestry [15].



Note: lanes 1–4 and 5–8 represent the ITS bands at 400 bp and 600 bp, while lane L represents the 100 bp molecular ladder

Figure 3 Agarose gel electrophoresis showing the amplified ITS fragment of the fungal isolates

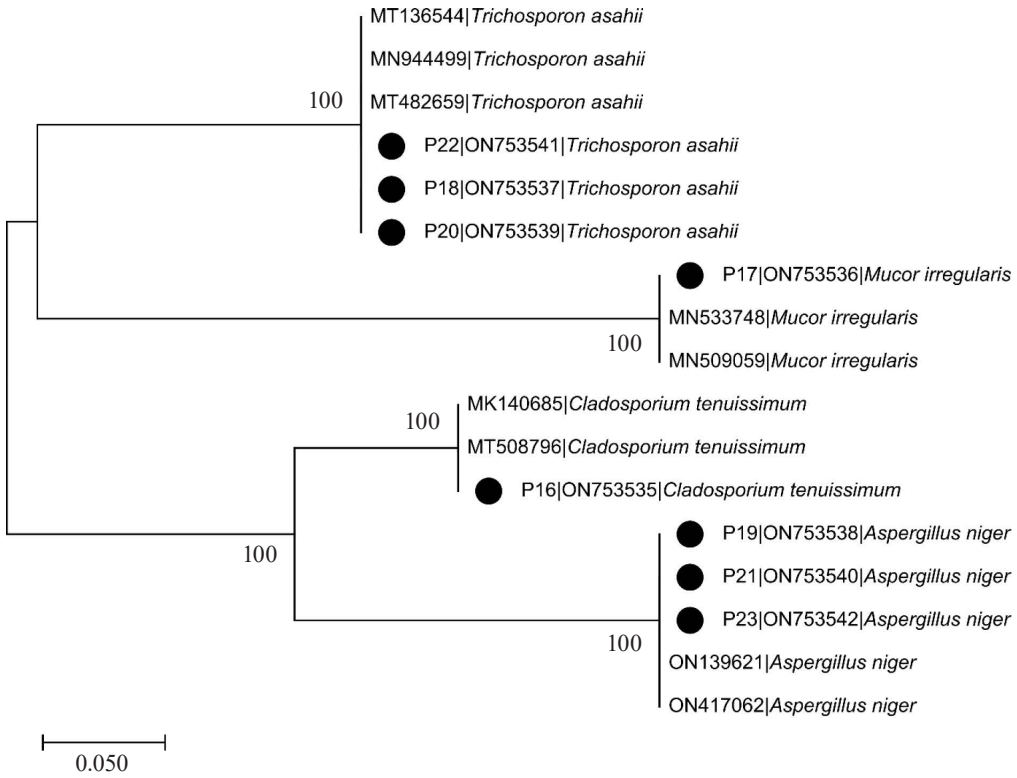
The DNA sequencing results using BLASTN identified the following fungi isolates (Fig. 4).

L. lactis and *L. fermentum* were the highest occurring bacteria isolates with percentage occurrences of 38.46 and 15.39%, respectively, while *L. fermentum* occurred least with a 7.69% occurrence (Fig. 5). Lactic acid bacteria are a phylogenetically heterogeneous group of Gram-positive bacteria that share metabolic and physiological characteristics [32]. These bacteria ferment carbohydrates into lactic acid (homofermentative), or into lactic acid, ethanol, and CO_2 (heterofermentative) [33, 34].

L. lactis is widely used in industry as a fast acidifier, starter culture, and flavor enhancer in milk fermentation [35–37]. In addition, *L. lactis* subsp. *lactis* is widely applied in cheese and butter production, as well as to inhibit pathogen development [38–41].

Food-associated *Lactobacillus* strains are "generally recognized as safe" (GRAS) microorganisms that have a major role in fermented milk production and in sourdough technology. In particular, some members of these strains called non-starter lactic cultures play a considerable role in developing cheese and dough aroma, texture, and flavor through ripening. They are currently being used in fortifying health-enhancing functional foods [42–44].

L. fermentum is a Gram-positive, non-spore-forming, rod-shaped probiotic bacterium belonging to the heterofermentative lactic acid bacterium. It is capable of fermenting carbohydrates and producing lactic and organic acids [45]. The bacterium has anti-diabetic probiotic



Note: The nodes without circular annotation represent highly similar species that were obtained as top hits from the NCBI (National Centre for Biotechnology Information)

Figure 4 A phylogenetic tree showing the classification and evolutionary relationship of the fungal isolates

Table 1 Morphological characteristics of the identified bacterial isolates

Bacteria	Shape	Color	Elevation	Surface	Margin	Transparency	Consistency	Diameter
Lysinibacillus macrolides	Gram-positive rod	Creamy	Raised	Smooth	Entire	Clear	Dry	Small
Klebsiella pneumoniae	Gram-negative rod	Greyish white to pink	Dome-shaped	Mucoid	Entire	Opaque	Dry	Medium
Lactococcus lactis	Gram-positive rod	Creamy	Raised	Smooth	Undulate	Clear	Dry	Small
Providencia stuartii	Gram-negative rod	Dull grey	Raised	Smooth	Entire	Clear	Dry	Large
Enterobacter cloacae	Gram-negative rod	Reddish	Flat	Smooth	Entire	Clear	Dry	Large
Limosilactobacillus fermentum	Gram-positive rod	Creamy	Raised	Smooth	Entire	Clear	Dry	Medium
Lactobacillus fermentum	Gram-positive rod	Creamy	Raised	Smooth	Entire	Clear	Dry	Medium
Staphylococcus edaphicus	Gram-positive cocci	Yellow	Raised	Smooth	Entire	Clear	Moist	Small
Bacillus flexus	Gram-variable rod	Creamy	Flat	Smooth	Entire	Clear	Dry	Small

Table 2 Actual names of the bacterial isolates and the summary of BLASTN similarities

Sample code	Accession number	BLASTN identity of sample	Identity, %	Actual name of organisms
P1	ON747236	*Lactococcus lactis* MT 597556 MT 573759	100	*Lactococcus lactis*
P2	ON747237	*Limosilactobacillus fermentum* ON 495412	100	*Limosilaclobacillus fermentum*
P3	ON747238	*Lactobacillus fermentum* MN 907811	100	*Lactobacillus fermentum*
P4	ON747239	*Bacillus flexus* MT 645459 MT 585375	100	*Bacillus flexus*
P5	ON747240	*Lactococcus lactis* MT 573759 MT 597556	100	*Lactococcus lactis*
P6	ON747241	*Enterobacter cloacae* MT 613381 MN 826532	99	*Enterobacter cloacae*
P7	ON747242	*Providencia stuartii* MU 584987	100	*Prodidencia stuartii*
P8	ON747243	*Lactococcus lactis* MT 573759 MT 597556	100	*Lactococcus lactis*
P9	ON747244	*Lactococcus lactis* MT 573759 MT 597556	100	*Lactococcus lactis*
P10	ON747245	*Staphylococcus edaphicus* MT 604700 MT 585436	100	*Staphylococcus edaphicus*
P11	ON747246	*Lactococcus lactis* MT 573759 MT 597556	100	*Lactococcus lactis*
P12	ON747247	*Klebsiella pneumonia* ON 514273	100	*Klebsiella pneumonia*
P14	ON747248	*Lysinibacillus marcrolides* MK 942418 MT 218365	100	*Lysinibacillus marcrolides*
P15	ON747249	*Lactobacillus fermentum* MK 893985	100	*Lactobacillus fermentum*

Table 3 Morphological characteristics of the identified fungal isolates

Fungi	Shape	Color	Mycelia	Surface	Margin	Transparency	Consistency	Diameter
Trichosporon asahii	Irregular	Light beige/white	Raised	Smooth	Wide	Waxy	Dry with irregular folds	Large
Mucor irregularis	Irregular/ ellipsoidal	Whitish to slightly yellowish	Aerial mycelia	Wool-like	Entire	Opaque	Cottony	Small
Cladosporium tenuissimum	Rough walled	Olive green to brown/ black	Mycellium with swellings	Smooth	Undulate	Clear	Verruculose	Small
Aspergillus niger	Plethora strains	White to yellow/black	Conidial heads are radiate	Cottony	Entire	Opaque	Dry	Large

Table 4 Actual names of the fungal isolates and the summary of BLASTN similarities

Sample code	Accession number	Identity of sample	Identity, %	Actual name of organisms
P16	ON753535	*Cladosporium tenuissimum* MK 140685 MT 508726	100	*Cladosporium tenuissimum*
P17	ON753536	*Mucor irregularis* MN 533748 MN 509059	100	*Mucor irregularis*
P18	ON753537	*Trichosporon asahii* MT 136544 MN 944499 MT 486259	100	*Trichosporon asahii*
P19	ON753538	*Aspergillus niger* ON 139621 ON 417062	100	*Aspergillus niger*
P20	ON753539	*Trichosporon asahii* MT 136544 MN 944499 MT 486259	100	*Trichosporon asahii*
P21	ON753540	*Aspergillus niger* ON 139621 ON 417062	100	*Aspergillus niger*
P22	ON753541	*Trichosporon asahii* MT 136544 MN 944499 MT 486259	100	*Trichosporon asahii*
P23	ON753542	*Aspergillus niger* ON 139621 ON 417062	100	*Aspergillus niger*

features which enable host cells to adjust their anti-inflammatory and antioxidant systems, resulting in improved glucose homeostasis capable of oxidative stress protection in diabetic conditions [46, 47]. It can be added to fermented foods like yoghurt and is found in some dietary supplements [48, 49]. In addition, *L. fermentum* was reported as the most predominant bacteria in Chinese cereal gruel, West African cereal dough, and Indian rice-based fermented beverage [50–52]. Furthermore, it is regarded as "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA) [53]. *L. fermentum* can also inhibit the growth of foodborne pathogens in food products [54]. In addition, foods obtained from fermentation by *L. fermentum* usually possess good palatability, high sensory quality, texture, stability, and nutritional properties [55, 56].

The spore-forming bacteria include *L. macroides* and *Bacillus flexus* with an occurrence of 7.69% (Fig. 5). They demonstrate an exceptional ability to adapt to their environment, and their presence in fermented flours could be due to their capacity to resist the acid produced by lactic acid bacteria [55–59]. Also, they are commonly found in beans and legumes [60]. *Bacillus* spp. present in fermented foods hydrolyze the substrate and produce enzymes, such as nattokinase, phytase, amylase, protease, cellulase, and lipase. These enzymes help break down complex compounds into simple biomolecules [61]. For example, amylase converts starch in legumes into sugar. Likewise, protease is used to convert proteins into amino acids [61, 62].

Bacillus species have also shown probiotic potential [63]. *B. flexus* biofilm has been used as a biologi-

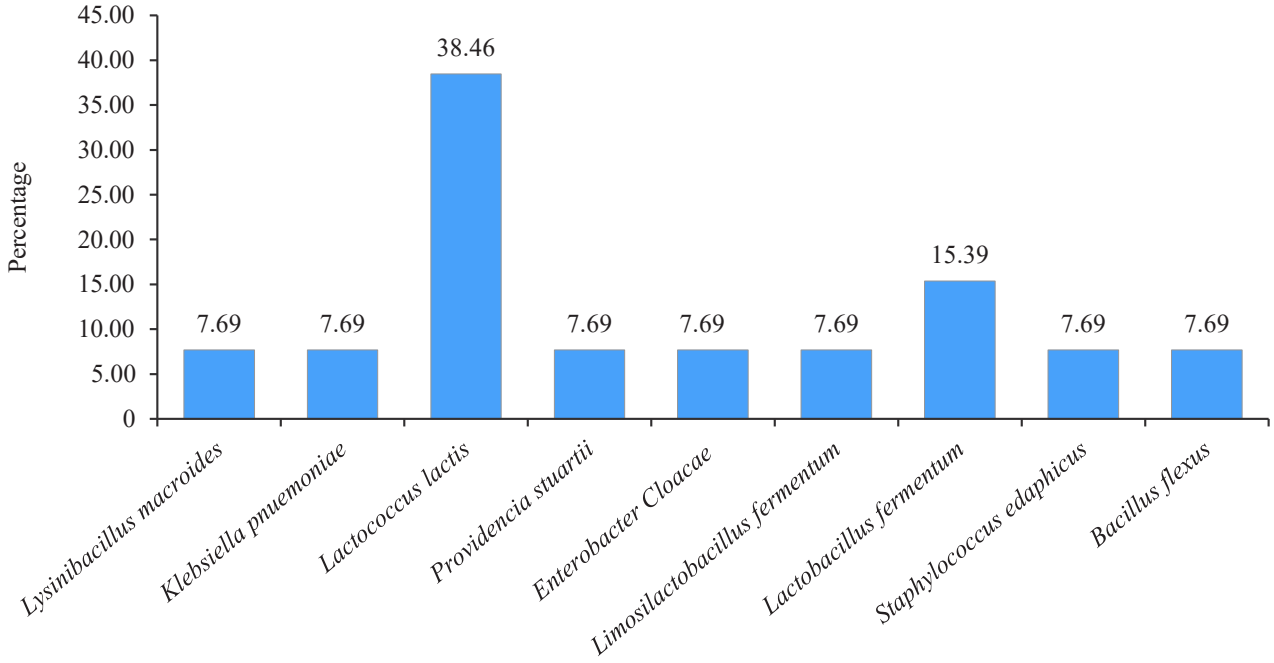


Figure 5 Percentage genera of the bacteria isolated from fermented botanicals

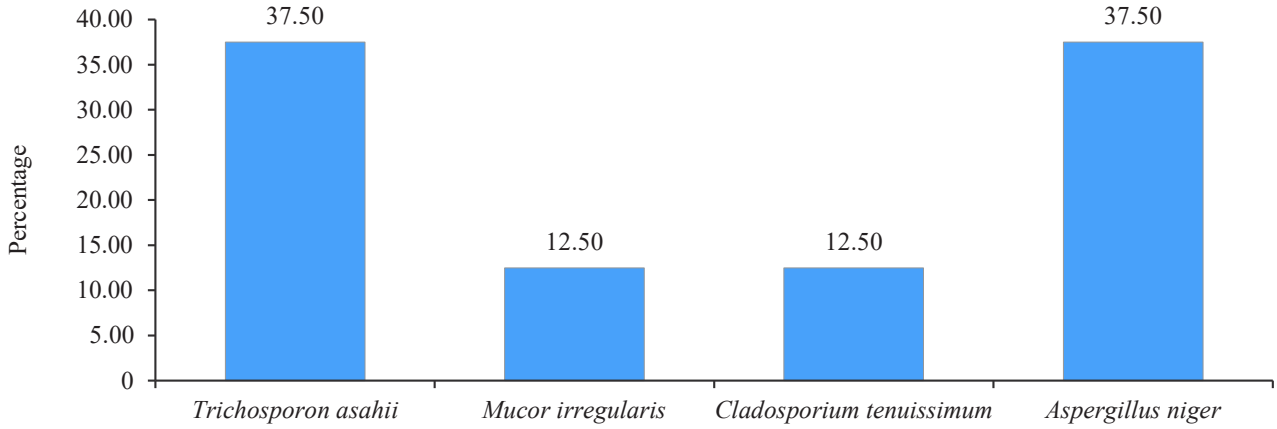


Figure 6 Percentage genera of the fungi isolated from fermented botanicals

cal control agent against the Cowpea pest *Callosobruchus maculates* [64].

The microbial profile of the fermented flour also showed the presence of *Enterobacteriaceae*. These include *Klebsiella pnuemoniae*, *P. stuarti*, and *E. cloacae* with a 7.69% occurrence each (Fig. 5). The *Enterobacteriaceae* family comprises a large group of Gram-negative non-spore-forming bacteria. These facultative anaerobic rods, which break down glucose-producing acid with/without gas, include some harmless commensal species, as well as important human and animal pathogens [65]. Their ubiquitous distribution means some members of the *Enterobacteriaceae* family will inevitably enter the food chain. While their low numbers are acceptable and do not directly lead to safety apprehension, their presence signifies inappropriate or poor processing and sanitary protocols around the food processing surroundings [66, 67]. Generally, *Enterobacteriaceae* are considered hygiene indicator organisms during food processing. Therefore, they are used to monitor the effectiveness of implemented preventive pre-requisite measures such as Good Manufacturing Practices and Good Hygiene Practices (GMP/GHP) [68].

According to percentage occurrence data for the fungi from fermented botanicals, both *T. asahii* and *A. niger* had a 37.50% occurrence, while *M. irregularis* and *Cladosporium temuissimum* had an occurrence of 12.50% (Fig. 6). These organisms are commonly present as contaminants in the human skin, cooking utensils, processing equipment, the environment, water, or in the seeds of cereals and legumes [69, 70]. They do not appear to play a significant role in the fermentation process, although they could be further exploited for their probiotic potentials [70–72].

CONCLUSION

The amalgamation of morphological attributes and molecular (DNA) markers have been accurately used for microbial nomenclature at the molecular level because the processes eliciting genetic changeability are the direct product of sequence changes from biochemical markers (genes and proteins). The phylogenetic properties of microbial isolates obtained from the fermentation of botanicals can provide valuable information on the diversity, composition, and functional properties of these microbial communities. Identification and characterization of the microorganisms involved in the fermentation process may optimize fermentation conditions to enhance the quality and nutritional value of the final products.

CONTRIBUTION

All the authors participated in developing the research concept and writing the original draft.

CONFLICT OF INTEREST

The authors have no conflict of interest concerning the conceptualization, research design, and publication of this work.

DATA AVAILABILITY

All the data associated with this study has been deposited in the NCBI GenBank database, with the accession numbers of ON747236-ON747249 (bacteria) and ON753535-ON753542 (fungi).

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Sustainable fish oil extraction from catfish visceral biomass: A comparative study between high-shear homogenization and high-frequency ultrasound on wet rendering process

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Abstract:
Traditional wet rendering leads to the degradation of polyunsaturated fatty acids in fish oil. Therefore, we combined this method with high-shear homogenization and high-frequency ultrasound to extract oil from *Clarias magur* visceral biomass. This way, we aimed to achieve higher oil yield, shorter extraction times, and a better preservation of polyunsaturated fatty acids.
High-shear homogenization and high-frequency ultrasound increased the oil yields by 9.17 and 10.55%, respectively, compared to traditional wet rendering. The oil quality was also improved, with lower acid and peroxide values. Scanning electron microscopy confirmed enhanced cell disruption for increasing the oil extraction efficiency. Fourier transfer infrared spectroscopy also proved the efficacy of homogenization and ultrasound pretreatment in enhancing the extraction of polyunsaturated fatty acids from *C. magur* visceral biomass. Their content showed a significant variation among different extraction methods. Specifically, the high-frequency ultrasound method resulted in a notable 15.1% increase, while the high-shear homogenization method demonstrated a significant 13.3% increase, compared to the wet rendering method (control). The oil extracted by the high-frequency ultrasound method demonstrated a 7.5% increase in eicosatetraenoic acid and a 11.7% increase in docosahexaenoic acid, as compared to the oil obtained from the control method. High-shear homogenization and high-frequency ultrasound shortened the extraction time and reduced the temperature requirements for oil extraction from wet biomass.
These techniques have potential for efficient fish oil extraction, valuable in the healthcare and food industries.

Keywords: Catfish waste management, wet rendering, high-shear homogenization, high-frequency ultrasound, polyunsaturated fatty acids, lipids

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INTRODUCTION

Fish oil, a key source of polyunsaturated fatty acids (PUFAs), is becoming more popular due to its health benefits. The fish oil market was worth USD 2.5 billion in 2020, and it is predicted to grow to USD 3.3 billion by 2025, with a 5.8% growth rate per year [1]. The demand for fish oil is increasing due to its wide applications in health supplements, medicines, and animal feed. PUFAs in fish oil can improve heart health and brain function, reduce inflammation, and even prevent cancer [2].

Catfish is one of the most widely farmed fish species globally, with a high production rate and low production costs, making it an affordable source of protein [3]. The viscera of catfish, including the liver and gut, are usually considered as waste and therefore discarded. However, catfish viscera contain significant amounts of lipids, proteins, and other bioactive compounds, making them an ideal source of PUFAs and other valuable components [4].

Traditional fish oil extraction methods, such as solvent extraction, cold pressing, enzymatic hydrolysis, and supercritical fluid extraction, have certain drawbacks, including safety concerns, low oil yield, high cost, and capital investment requirements [5]. As a result, researchers are now searching solvent-free, ecofriendly, and cost-effective alternatives. Wet rendering, a method that involves cooking fish in water or steam to release oil and separate it from other components, has emerged as a promising approach for fish oil extraction. This method offers potential advantages in terms of safety, oil yield, and affordability, making it a suitable option for recovering fish oil [6]. However, high temperature and prolonged cooking time lead to a thermal degradation of PUFAs and therefore affect the quality of fish oil.

By combining wet rendering with such methods as enzymatic treatment, ultrasonication, and high-shear homogenization, we can achieve higher oil extraction rates while minimizing thermal degradation [5]. Enzymatic treatment offers an advantage of breaking down cellular structures and releasing oils more effectively [7]. Ultrasonication, through application of high-frequency vibrations, disrupts cellular structures and enhances oil release, increasing the mass transfer rate and improving oil extraction efficiency with reduced thermal degradation [8]. Furthermore, incorporating high-shear homogenization into wet rendering significantly enhances overall oil extraction efficiency by mechanically disrupting cellular structures, improving mass transfer, and reducing the particle size [9]. This combination of methods contributes to higher oil yields with minimized thermal degradation. However, enzymatic treatment, despite its effectiveness, may have some limitations. For instance, it can exhibit limited specificity, impacting its efficiency on different types of fish tissues [10]. Moreover, enzymes require specific reaction conditions, such as pH and temperature, which can add complexity to the extraction process. Additionally, the cost associated with acquiring and using enzymes tends to be higher compared to high-frequency ultrasound and high-shear homogenization techniques [11].

In this study, we combined the wet rendering method with high-shear homogenization and high-frequency ultrasounds to extract fish oil from catfish visceral biomass. We investigated the effects of the extraction methods on the recovery/yield of oil, acid and free fatty acid values, oxidative stability, microstructure, compatibility, fat composition, and lipid profile. Our primary objectives were to enhance oil yield, reduce extraction time, and improve PUFAs preservation.

STUDY OBJECTS AND METHODS

Chemicals. Analytical-grade chemicals were utilized in this study. These include thiobarbituric acid, trichloroacetic acid, ammonium thiocyanate, ferrous chloride, and sodium hydroxide procured from Merck (Darmstadt, Germany). Solvents – chloroform, methanol, and hexane – were supplied by Lab-Scan (Bangkok, Thailand). Other chemicals like the Supelco® 37 component FAME mix, 1,1,3,3-tetramethoxypropane, and additional ammonium thiocyanate were sourced from Sigma-Aldrich (St. Louis, MO, USA). Cumene hydroperoxide and 2-thiobarbituric acid were obtained from Fluka Co. (Buchs, St. Gallen, Switzerland).

Raw material. Freshly available *Clarias magur* visceral biomass was procured from the Huatake fish market at Ladkrabang (Bangkok, Thailand). Approximately 2–3 kg of viscera was packed in polyethylene bags, placed into a polystyrene container with ice and transported to the School of Food Industry, King Mongkut's Institute of Technology Ladkrabang. Upon arrival, the visceral biomass was ground using a meat grinder (Model: HR271331, Philips, Netherlands), and the ground sample was used for further analysis.

Proximate composition. The standard AOAC method numbers such as 930.15, 923.03, 920.39, and 923.01 were used to determine the moisture, protein, fat, and ash contents in fresh *C. magur* visceral biomass [12].

Experimental design. We applied the Taguchi orthogonal array design to assess the effect of high-shear homogenization and ultrasonication on *C. magur* visceral oil extraction via wet rendering. A four-factor, three-level design was used to study the impact of each variable on the oil yield, recovery, quality (acid value, free fatty acids), and oxidative stability (peroxide value, thiobarbituric acid reactive substances) [13]. The variables for high-shear homogenization-assisted extraction included the homogenization speed and time (6500–26 000 rpm, 5–15 min), as well as the cooking temperature and time (40–80°C, 5–15 min). High-frequency ultrasound-assisted extraction included such variables as the amplitude and sonication time (40–80%, 5–15 min), as well as the aforementioned cooking conditions.

Oil extraction. ***High-shear homogenization-assisted extraction.*** Accurately weighed (100 g) ground visceral biomass was mixed with distilled water at a ratio of 1:0.5 (w/v). The mixture was subjected to a high-shear homogenization (T-25 Digital Ultra Turrax, IKA, Thailand) at different homogenization speeds and times (Table 1). The homogenized mixtures were exposed to wet rendering with various cooking temperatures and times (Table 1). The oil was separated as described by Pudtikajorn *et al.*, with slight modifications [14]. After each experimental run, the sample temperature was allowed to be brought down to room temperature and the extraction mixture was filtered through muslin cloth and then through Whatman No. 1 filter paper. The filtrate was further centrifuged at 2500 g for 15 min, the cell debris pellets were discarded, and the liquid sample was separated into the oil and aqueous phase using a separating funnel.

The recovered oil was used for analysis, the excess oil was transferred into an amber bottle, whereas headspace was flushed using nitrogen gas and stored at –20°C.

High-frequency ultrasound-assisted extraction. The minced visceral biomass (100 g) was mixed with distilled water at a ratio of 1:0.5 (w/v). The mixture was subjected to an ultrasound (Vibra-Cell processor, Sonics & Material, Inc., Newtown, CT, USA) at different amplitudes and times, followed by wet rendering with various cooking temperatures and times (Table 2). After each experimental run, the sample was filtered, and the oil was separated, as described earlier [14]. The recovered oil was used for further analysis. Additionally, the oil obtained from the simple wet rendering process was considered as a control. The process parameters were a viscera:water ratio (w/v) of 1:0.5, extraction temperature of 90°C, and extraction time of 20 min.

Extraction yield. The oil extraction yield, g/100 g (wet basis), was calculated using the following Eq. (1) [14]:

$$\text{Yield} = \frac{\text{Weight of obtained oil}}{\text{Weight of sample taken for extraction}} \times 100 \quad (1)$$

The oil recovery, %, was calculated using the following Eq. (2):

$$\text{Oil recover} = \frac{\text{Weight of obtained oil}}{\text{Total oil present in sample}} \times 100 \quad (2)$$

Oil quality. ***Acid value.*** The acid value was measured using a method suggested by Chaijan *et al.*, with slight modifications [15]. One gram of oil was dissolved in 10 volumes of hexane and titrated with 0.02 N KOH after adding two drops of phenolphthalein until the pink color was achieved. The acid value was expressed as milligrams of KOH neutralizing free acids in a gram of oil, and was calculated accordingly.

$$\text{Acid value} = \frac{56.1 \times N \times (Vs - Vb)}{W} \quad (3)$$

where N is the normality of KOH; Vs is the volume of KOH required for the sample; Vb is the volume of KOH required for the blank; W is the weight of the sample, g.

Free fatty acids (FFAs). The FFA, %, content in the oil sample was calculated as a percentage of oleic acid present, utilizing the following Eq. (4):

$$\text{FFA} = \frac{\text{Acid value} \times \text{MW}_{\text{oleic acid}} \times 10}{\text{MW}_{\text{KOH}} \times 1000} \quad (4)$$

where $\text{MW}_{\text{oleic acid}}$ is the molecular weight of oleic acid; MW_{KOH} is the molecular weight of KOH.

Oxidative stability of oil. ***Peroxide value.*** The peroxide value of oil was measured spectrophotometrically as described by Bruno *et al.*, using 20 mM ferric thiocyanate as a reducing agent [16]. The standard curve was plotted using cumene hydroperoxide at concentrations of 0 to 50 ppm and the peroxide value was expressed as mg cumene equivalents/kg of the oil sample.

Thiobarbituric acid reactive substances (TBARS). The TBARS analysis was also conducted as described by Bruno *et al.*, with minor modifications [16]. About 10 mg of oil was mixed with 2.5 mL of thiobarbituric acid, incubated at 95°C for 10 min, cooled, and then centrifuged at 3600 g for 20 min at 25°C. The absorbance of the upper layer was measured at 532 nm. The TBARS content was determined through a standard 1,1,3,3-tetraethoxypropane curve, expressed as mg MDA/kg oil.

Characterization of oil. Three oil samples were selected for characterization, namely: 1) the oil recovered by optimized high-shear homogenization-assisted extraction; 2) the oil recovered by optimized high-frequency ultrasound-assisted extraction; 3) the oil recovered by traditional wet rendering (viscera:water = 1:0.5, extraction temperature = 90°C, extraction time = 20 min), wich considered as the control.

Scanning electron microscopy. To evaluate the impact of each extraction method, the residues obtained from high-shear homogenization-assisted extraction, high-frequency ultrasound-assisted extraction, and wet rendering, were examined under a scanning electron microscope (XL30 FEI, Philips, France) at the magnification of 500×. In preparation for scanning electron microscopy, the residual material was dried at 50°C and then coated with gold using the sputter coating technique.

Fourier transfer infrared spectroscopy (FTIR). FTIR spectra were obtained using the ATR-FTIR model Equinox 55 (Bruker, Ettlingen, Germany) at a range of 400–4000 cm^{-1} with a resolution rate of 4 cm^{-1} [17].

Fatty acid composition. Fatty acid methyl esters (FAMEs) were prepared following Moula Ali *et al.* [18]. The derivatized FAMEs were analyzed via gas chromatography (GC, 7890B series, Agilent) using an HP 88 capillary column and a flame ionization detector. Gas chromatography conditions followed the manufacturer's guidelines. Peaks were identified using Supelco® standard retention time, integrated, and calibrated against the standard curve with Open LAB CDS software (Chem Station edition, Agilent Technologies, Santa Clara, CA, USA).

Lipids nutritional quality indexes. The data from the fatty acid composition analysis were used to calculate three indexes of nutritional quality: atherogenicity index (AI), thrombogenicity index (TI), and hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH).

AI represents the relationship between the cumulative amounts of primary saturated fatty acids and primary unsaturated fatty acids, with the former considered pro-atherogenic and the latter, anti-atherogenic [19]. The following Eq. (5) was used to calculate the AI:

$$\text{AI} = \frac{[\text{C12:0} + 4 \times \text{14:0} + \text{C16:0}]}{(\text{MUFA} + \text{n-6} + \text{n-3})} \quad (5)$$

TI indicates the relationship between pro-thrombogenetic (saturated) and anti-thrombogenetic fatty acids (MUFAs, n-6, and n-3 fatty acids) [19]. It was measured using the Eq. (6).

The HH ratio is associated with cholesterol metabolism. It was determined using the Eq. (7) [20].

$$TI = \frac{[C14:0 + C16:0 + C18:0]}{[(0.5 \times MUFA) + (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3 / n\text{-}6)} \quad (6)$$

$$HH = \frac{[C18:1(n9) + C18:2(n6) + C18:3(n3) + C20:5(n3) + C22:5(n3) + C22:6(n3)]}{[C14:0 + C16:0]} \quad (7)$$

Statistical analysis. The experiments were performed in triplicates and the data were presented as means ± SD. Duncan's multiple range test was used to indicate the significant difference between the mean values. Range analysis was carried out using SPSS (version 10.0.1.0, Stat-Ease) to measure the effect of individual variables and to determine their optimum level [13]. The average response (P_{ij}) for each variable (i = 1–3) was calculated at each level (j = 1–3). The difference between the highest and lowest values of P_{ij} was represented as R_j and calculated to determine which variable contributed most to the quality of the oil.

RESULTS AND DISCUSSION

The proximate composition of *Clarias magur* viscera contained 54.67 ± 0.13% of moisture, 12.21 ± 0.57% proteins, 24.35 ± 0.76% fat, and 1.86 ± 0.51% ash (w/w). Similar catfish species (i.e., *C. magur* from Kenya) were reported to contain 38.20% fat, which was relatively higher than the content of fat in the catfish from Thailand [21]. The difference in total fat could be due to the difference in geographical distribution and fish feed [22].

Effect of high-shear homogenization on extraction yield, oil quality, and oxidative stability. We found significant ($p < 0.05$) effects of various independent variables under study on the extraction yield (oil yield and recovery ranging from 4.62 to 12.31 g/100 g and 19.01 to 50.65%, respectively), oil quality (acid value and free fatty acids ranging from 10.52 to 29.31 mg KOH/g of oil and 0.53 to 1.47% oleic acid, respectively), and oxidative stability (peroxide value and thiobarbituric acid reactive substances ranging from 18.10 to 39.11 mg cumene hydroperoxide equivalent/kg of oil and 0.34 to 0.52 mg MDA eq/kg of oil, respectively) (Table 1).

Range analysis was carried out to optimize the extraction variables, consistent with ANOVA. The wet rendering time was the most significant parameter affecting the extraction yield (oil yield and recovery) while the homogenization speed was the most significant factor affecting the oil quality (acid value and free fatty acids) and oxidative stability (peroxide value and thiobarbituric acid reactive substances) (Table 1). The oil yield increased as the wet rendering time increased from 5 to 10 min. This could be correlated to a higher breakdown of adipose tissues with time and the release of more fat [23]. However, the 15 min wet rendering time resulted in lowering the oil yield. This could be caused by the prolonged wet rendering process leading to the emulsification of oil with water and proteinaceous material, making the subsequent separation steps more difficult [24].

The oil quality (acid value and free fatty acids) was significantly ($p < 0.05$) affected by the homogenization speed. The acid value and free fatty acids increased as the homogenization speed rose from 6500 to 26 000 rpm. Azab *et al.* observed that the acid value of mango kernel oil increased when extracted through high-shear homogenization at 8000 rpm [25]. Due to the strong mechanical action, the oil is more exposed to oxygen, which leads to increased oxidation [9]. The resulting free radicals and reactive oxygen species can react with fatty acids in the oil, leading to an increase in the acid value. Furthermore, the increased shear forces can cause more disruption of the oil droplets, leading to a release of more free fatty acids [9].

The breaking of the oil droplets during high-shear homogenization exposes a larger surface area of the oil to the aqueous phase, promoting hydrolysis and increasing the content of free fatty acids. Additionally, the intensified shear forces can accelerate the breakdown of lipid molecules, increasing the peroxide value [26]. The higher shear force generated at increased homogenization speeds facilitates greater oxidation, resulting in higher peroxide value levels. Moreover, the shearing action causes the breakdown of lipid molecules, leading to the production of secondary oxidation products, such as malondialdehyde (MDA), which reacts with thiobarbituric acid to form thiobarbituric acid reactive substances. Therefore, higher homogenization speeds can lead to higher thiobarbituric acid reactive substances, indicating an increase in lipid oxidation.

Effect of high-frequency ultrasound on extraction yield, oil quality, and oxidative stability. Table 2 shows that the extraction yield, oil quality, and oxidative stability of the extracted oil were significantly ($p < 0.05$) affected by various independent variables. The oil yield and oil recovery ranged between 3.18 to 12.28 g/100 g and 13.08 to 50.41%, respectively. The acid value and free fatty acids in the extracted oil ranged between 9.35 to 26.12 mg KOH/g of oil and 0.47 to 1.31% oleic acid, respectively. The peroxide value and thiobarbituric acid reactive substances (TBARS) ranged between 14.34 to 32.22 mg cumene hydroperoxide equivalent/kg of oil and 0.30 to 0.51 mg MDA equivalent/kg of oil, respectively.

Based on the range analysis, the ultrasound amplitude and sonication time significantly ($p < 0.05$) influenced the extraction yield, oil quality, and oxidative stability, while the wet rendering temperature and wet rendering time showed less significant effects. Raising the ultrasound amplitude from 40 to 60% increased the oil yield and recovery, which could be attributed to an increased cavitation effect (Table 2). This effect causes

Table 1 The yield, recovery, quality, and oxidative stability of the oil recovered from *Clarias magur* visceral biomass via high-shear homogenization-assisted wet rendering using Taguchi orthogonal array design

Run	A	B	C	D	Oil yield[a]	Oil recovery, %	Acid value[b]	Free fatty acids[c]	Peroxide value[d]	TBARS[e]
1	6500	5	40	5	4.62 ± 0.12[a]	19.01 ± 0.51[a]	10.52 ± 0.02[c]	0.53 ± 0.04[c]	18.10 ± 0.24[c]	0.34 ± 0.02[b]
2	6500	15	60	10	5.90 ± 0.09[b]	24.27 ± 0.36[b]	10.88 ± 0.03[d]	0.55 ± 0.03[d]	18.63 ± 0.29[d]	0.34 ± 0.01[b]
3	6500	25	80	15	7.16 ± 0.17[e]	29.46 ± 0.31[e]	11.09 ± 0.02[e]	0.56 ± 0.01[e]	19.25 ± 0.13[e]	0.36 ± 0.02[bc]
4	13 000	5	60	15	7.88 ± 0.08[f]	32.42 ± 0.56[f]	11.81 ± 0.05[f]	0.59 ± 0.04[f]	20.64 ± 0.28[f]	0.40 ± 0.02[c]
5	13 000	15	80	5	6.24 ± 0.23[d]	25.67 ± 0.42[d]	14.21 ± 0.04[g]	0.71 ± 0.06[g]	21.16 ± 0.23[g]	0.40 ± 0.03[c]
6	13 000	25	40	10	12.31 ± 0.11[i]	50.65 ± 0.54[i]	18.96 ± 0.03[h]	0.95 ± 0.05[h]	24.12 ± 0.31[h]	0.41 ± 0.01[d]
7	26 000	5	80	10	11.19 ± 0.16[h]	46.04 ± 0.29[h]	21.19 ± 0.05[i]	1.07 ± 0.06[i]	27.34 ± 0.25[i]	0.51 ± 0.02[e]
8	26 000	15	40	15	8.33 ± 0.13[g]	34.27 ± 0.31[g]	24.73 ± 0.10[k]	1.24 ± 0.03[k]	34.14 ± 0.16[k]	0.52 ± 0.03[e]
9	26 000	25	60	5	6.12 ± 0.21[c]	25.18 ± 0.25[c]	29.31 ± 0.07[l]	1.47 ± 0.07[l]	39.11 ± 0.22[l]	0.52 ± 0.01[e]
Optimal condition	13 000	5	60	10	11.89 ± 0.23	40.70 ± 0.18	12.94 ± 0.11	0.65 ± 0.03	19.32 ± 0.21	0.39 ± 0.02
Control	–	–	–	–	10.90 ± 0.29	37.31 ± 0.30	18.24 ± 0.23	0.91 ± 0.02	25.21 ± 0.24	0.46 ± 0.03
Factors	Levels	Values								
A	6500	P_{A1}			5.89	24.24	10.83	0.54	18.66	0.34
	13 000	P_{A2}			8.81	36.24	14.99	0.75	21.97	0.40
	26 000	P_{A3}			7.67	35.16	25.07	1.26	33.53	0.51
		R_A			2.92	12.00	14.24	0.72	14.87	0.17
B	5	P_{B1}			7.89	32.49	14.50	0.73	22.02	0.41
	15	P_{B2}			6.82	28.07	16.60	0.83	24.64	0.42
	25	P_{B3}			8.53	35.09	19.78	0.99	27.49	0.43
		R_B			1.71	7.02	5.28	0.26	5.47	0.02
C	40	P_{C1}			8.42	34.64	18.07	0.90	25.45	0.42
	60	P_{C2}			6.63	27.29	14.62	0.87	26.12	0.42
	80	P_{C3}			8.19	33.72	15.49	0.78	22.58	0.42
		R_C			1.79	7.35	3.45	0.12	3.54	0.00
D	5	P_{D1}			5.66	23.28	18.01	0.90	26.12	0.42
	10	P_{D2}			9.80	40.32	17.01	0.85	23.36	0.42
	15	P_{D3}			7.79	32.05	15.87	0.79	24.67	0.42
		R_D			4.14	17.04	2.14	0.11	2.76	0.00
Rank					D* > A* > C > B	D* > A* > C > B	A* > B* > C > D	A* > B* > C > D	A* > B* > C > D	A* > B*, C, D
Major contributing factor					D	D	A	A	A	A
Overall					Factor A					

A, B, C, and D represent variables such as homogenization speed (rpm), homogenization time (min), wet rendering temperature (°C), and wet rendering time (min), respectively

[a] Oil yield in terms of g/100 g, based on wet weight; [b] Acid value in terms of mg KOH/g of oil; [c] Free fatty acids in terms of % oleic acid; [d] PV in terms of mg cumene hydroperoxide equivalent/kg of oil; [e] Thiobarbituric acid reactive substances in terms of mg MDA eq/kg of oil

Control is the oil extracted by traditional wet rendering (viscera:water = 1:0.5, v/v; extraction temperature = 90°C; extraction time = 20 min)

The same lowercase letter above each measured parameter indicates significant difference ($p < 0.05$)

K_{ij} were the average values of each measured parameter from a nine-treatment set at level j (j = 1, 2, 3) of each factor i (i = A or B, or C, or D)

R_A and R_B were the differences between the highest and lowest values of K_{ij} within the same factor i

The rank was based on the largest to smallest order of R_A and R_B values, the factor with asterisk meaning significance

The two most contributing factors were selected based on the significance and rank order of R_i

Table 2 The yield, recovery, quality, and oxidative stability of the oil recovered from *Clarias magur* visceral biomass via high-frequency ultrasound-assisted wet rendering using Taguchi orthogonal array design

Run	A	B	C	D	Oil yield[a]	Oil recovery, %	Acid value[b]	Free fatty acids[c]	Peroxide value[d]	TBARS[e]
1	40	5	40	5	3.18 ± 0.16[a]	13.08 ± 0.21[a]	9.35 ± 0.04[c]	0.47 ± 0.02[c]	14.34 ± 0.24[c]	0.30 ± 0.02[b]
2	40	15	60	10	3.42 ± 0.09[b]	14.07 ± 0.14[b]	9.79 ± 0.02[d]	0.49 ± 0.10[c]	14.79 ± 0.29[d]	0.31 ± 0.01[b]
3	40	25	80	15	3.86 ± 0.21[c]	15.88 ± 0.51[c]	10.24 ± 0.05[e]	0.52 ± 0.05[cd]	15.90 ± 0.13[e]	0.34 ± 0.02[bc]
4	60	5	60	15	12.15 ± 0.13[g]	50.00 ± 0.34[g]	15.21 ± 0.02[h]	0.77 ± 0.02[f]	19.88 ± 0.31[h]	0.41 ± 0.01[d]
5	60	15	80	5	5.82 ± 0.23[f]	23.95 ± 0.12[f]	12.68 ± 0.04[g]	0.64 ± 0.04[e]	17.62 ± 0.23[g]	0.38 ± 0.03[c]
6	60	25	40	10	5.64 ± 0.19[e]	23.20 ± 0.36[e]	10.82 ± 0.02[f]	0.54 ± 0.02[d]	16.19 ± 0.28[f]	0.39 ± 0.02[c]
7	80	5	80	10	12.28 ± 0.07[h]	50.41 ± 0.21[h]	20.27 ± 0.07[j]	1.02 ± 0.10[h]	27.61 ± 0.16[j]	0.51 ± 0.03[e]
8	80	15	40	15	5.63 ± 0.12[e]	23.16 ± 0.12[e]	18.61 ± 0.06[i]	0.94 ± 0.12[g]	22.96 ± 0.25[i]	0.49 ± 0.02[e]
9	80	25	60	5	5.11 ± 0.20[d]	21.02 ± 0.15[d]	26.12 ± 0.05[l]	1.31 ± 0.07[i]	32.22 ± 0.22[l]	0.51 ± 0.01[e]
Optimal condition	60	5	60	10	12.05 ± 0.18	49.55 ± 0.21	14.15 ± 0.08	0.71 ± 0.04	19.83 ± 0.19	0.40 ± 0.01
Control	–	–	–	–	10.90 ± 0.29	37.31 ± 0.30	18.24 ± 0.23	0.91 ± 0.02	25.21 ± 0.24	0.46 ± 0.03
Factors	Levels	Values								
A	40	P_{A1}			3.48	14.34	9.79	0.49	15.01	0.32
	60	P_{A2}			7.87	32.38	12.90	0.65	17.90	0.39
	80	P_{A3}			7.67	31.53	21.66	1.09	27.60	0.50
		R_A			4.39	18.04	11.87	0.60	12.59	0.18
B	5	P_{B1}			9.20	37.83	14.94	0.75	20.61	0.40
	15	P_{B2}			4.95	20.39	13.69	0.69	18.45	0.39
	25	P_{B3}			4.87	20.03	15.72	0.79	21.43	0.41
		R_B			4.33	17.80	2.03	0.10	2.98	0.02
C	40	P_{C1}			4.81	19.81	12.92	0.65	17.83	0.39
	60	P_{C2}			6.89	28.36	17.04	0.85	22.29	0.41
	80	P_{C3}			7.32	30.08	14.39	0.72	20.37	0.41
		R_C			2.51	10.27	2.65	0.13	1.92	0.02
D	5	P_{D1}			4.70	19.35	16.05	0.80	21.39	0.39
	10	P_{D2}			7.11	29.22	13.62	0.68	19.53	0.40
	15	P_{D3}			7.21	29.68	14.68	0.74	19.58	0.41
		R_D			2.51	10.33	2.43	0.12	1.86	0.02
Rank					A* > B* > C, D	A* > B* > D > C	A* > C* > D > B	A* > C* > D > B	A* > B* > C > D	A* > B*, C, D
Major contributing factor					A	A	A	A	A	A
Overall					Factor A					

A, B, C, and D represent variables such as ultrasound amplitude (%), sonication time (min), wet rendering temperature (°C), and wet rendering time (min), respectively

[a] Oil yield in terms of g/100 g, based on wet weight; [b] Acid value in terms of mg KOH/g of oil; [c] Free fatty acids in terms of % oleic acid; [d] PV in terms of mg cumene hydroperoxide equivalent/kg of oil; [e] Thiobarbituric acid reactive substances in terms of mg MDA eq/kg of oil

Control is the oil extracted by traditional wet rendering (viscera:water = 1:0.5, v/v; extraction temperature = 90°C; extraction time = 20 min)

The same lowercase letter above each measured parameter indicates significant difference ($p < 0.05$)

K_{ij} were the average values of each measured parameter from a nine-treatment set at level j (j = 1, 2, 3) of each factor i (i = A or B, or C, or D)

R_A and R_B were the differences between the highest and lowest values of K_{ij} within the same factor i

The rank was based on the largest to smallest order of R_A and R_B values, the factor with asterisk meaning significance

The two most contributing factors were selected based on the significance and rank order of R_i

a breakdown of adipose tissues over time and leads to the release of more oil, consequently improving yield and recovery. However, when the amplitude exceeded 80%, it caused the emulsification of oil with water and protein, complicating the separation and reducing the oil yield [27]. Along with the amplitude, the sonication time significantly impacted the response variables. As the sonication time increased from 5 to 15 min, both the oil yield and recovery improved. This improvement could be due to the extended exposure to ultrasound, promoting further tissue breakdown and oil release [28]. Nevertheless, similarly to the ultrasound amplitude, excessive sonication time could lead to emulsification and a subsequent decrease in yield. Zheng *et al.* also observed

that the prolonged sonication time (30 min) resulted in the formation of an interfacial membrane over the lipids, thereby inhibiting the extraction of oil from *Schizochytrium* sp. [29].

Regarding the oil quality indicators (acid value and free fatty acids), both the ultrasound amplitude and sonication time significantly ($p < 0.05$) influenced the responses. As these factors increased, both the acid value and free fatty acids increased, too. This could be attributed to the intensification of mass transfer and mixing at the molecular level induced by cavitation, which stimulated the hydrolysis of triglycerides [30]. The resulting free radicals and reactive oxygen species can react with fatty acids present in the oil, thereby increasing the acid value. Zhang *et al.* observed similar trends during the ultrasound-assisted extraction of peanut oil [31]. The measures of oxidative stability, including peroxide value and thiobarbituric acid reactive substances (TBARS), also increased with higher ultrasound amplitude and sonication time.

The collapse of cavitation bubbles can result in the formation of hydroxyl radicals, which are highly reactive free radicals. These radicals can interact with fatty acids in the oil, inducing peroxidation and subsequently increasing the peroxide value. Hernández-Santos *et al.* observed an increase in peroxide value in pumpkin oil extracted at 80 to 100% of ultrasound amplitude [32]. Moreover, the increased cavitation resulting from higher amplitude and time facilitates the breakdown of lipid molecules, leading to the production of secondary oxidation products like malondialdehyde, which reacts with thiobarbituric acid to form TBARS [33].

In contrast, the wet rendering temperature and time exhibited less significant effects on the response variables, according to the range analysis. While these factors might influence the extraction process, their impacts were not as significant as those of the ultrasound amplitude and sonication time. This suggests that while the wet rendering conditions can be optimized, the primary focus for extraction optimization should be on ultrasound parameters.

Optimization and validation of extraction conditions. Utilizing the Taguchi orthogonal array design for optimization, the extraction conditions were aimed to maximize the oil yield and retain the quality. High-shear homogenization-assisted extraction (HAE) was optimized at 13 000 rpm homogenization speed, 5 min homogenization time, 60°C wet rendering temperature, and 10 min wet rendering time. High-frequency ultrasound-assisted extraction (UAE) was optimized with 60% amplitude, 5 min sonication time, 60°C wet rendering temperature, and 10 min wet rendering time.

The experimental validation of the optimized conditions significantly increased the oil yield while preserving the quality. Specifically, the oil yield from HAE was approximately 9.17% higher (11.89 g/100 g) than that from the control method (10.90 g/100 g). Meanwhile, the UAE method yielded a remarkable increase of about 10.55% (12.05 g/100 g), showing the efficiency of ultrasound-assisted extraction. In terms of the oil quality, both the HAE and UAE methods showed substantially lower acid values compared to the control method. In particular, the acid values of 12.94 mg KOH/g oil for HAE and 14.15 mg KOH/g oil for UAE were 29.06 and 22.46% lower, respectively, than the acid value for the control method (18.24 mg KOH/g oil). The achieved acid values were within the Codex standard for fish oil (20 mg KOH/g) [34].

The peroxide value was 19.32 mg cumene hydroperoxide equivalent/kg oil for HAE and 19.83 mg cumene hydroperoxide equivalent/kg oil for UAE. These values were 23.35 and 21.36% lower, respectively, as compared to the one for the control method (25.21 mg cumene hydroperoxide equivalent/kg oil). This indicates a reduction in oxidation levels in the oil extracted by these methods.

The scanning electron microscopy images provided additional validation of the optimized extraction methods, as they revealed a considerable degree of cell disruption in the residues after both the HAE and UAE procedures. This indicates a more rapid oil extraction at lower temperatures (Fig. 1). The effect was more evident in UAE, suggesting that ultrasound pre-treatment led to better oil extraction than homogenization. This may be attributed to the sonoporation effect, where the application of ultrasound generates cavitation bubbles, leading to the formation of tiny pores in cell membranes and enhancing the oil release [35]. In contrast, the residues from the traditional wet rendering (control) showed minimal cell disruption, highlighting the effectiveness of the optimized extraction methods.

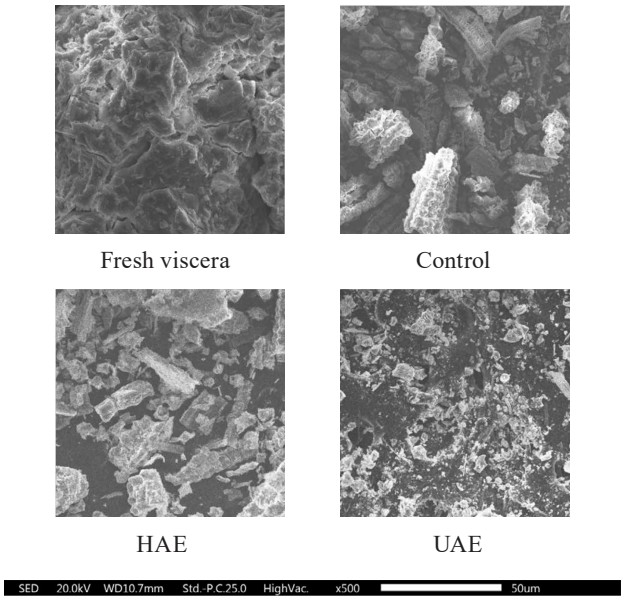

Figure 1 Scanning electron microscopy of fresh *Clarias magur* visceral biomass and visceral residues after oil extraction using different methods: HAE – optimized high-shear homogenization-assisted wet rendering; UAE – optimized high-frequency ultrasound-assisted wet rendering; control – traditional wet rendering (viscera:water = 1:0.5, w/v; extraction temperature = 90°C; extraction time = 20 min)

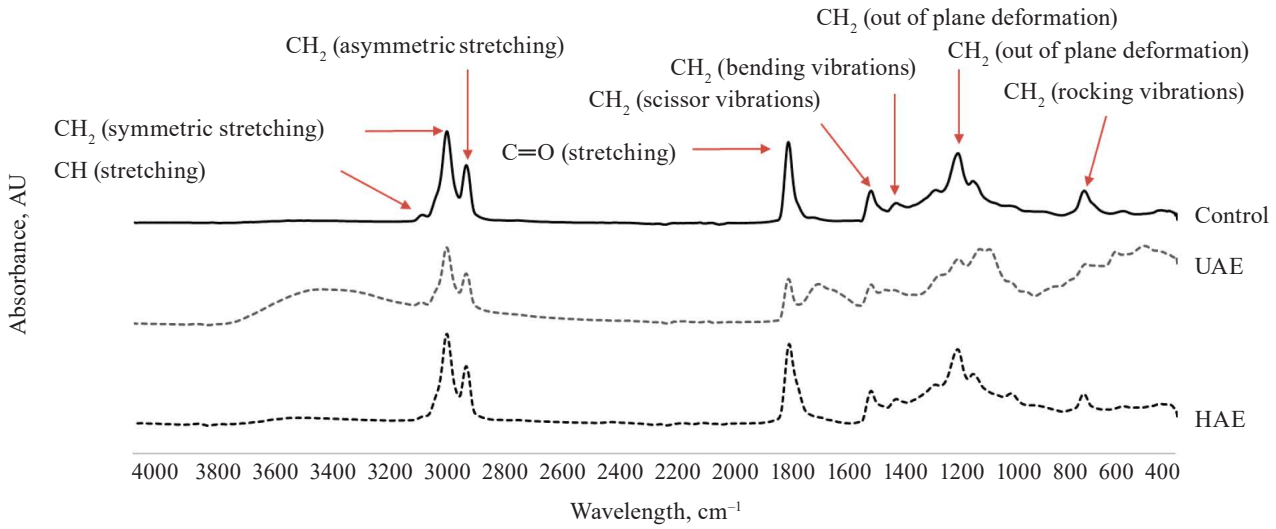

Figure 2 Fourier transfer infrared spectroscopy of *Clarias magur* viscera oils recovered using different methods: HAE – optimized high-shear homogenization-assisted wet rendering; UAE – optimized high-frequency ultrasound-assisted wet rendering; control – traditional wet rendering (viscera:water = 1:0.5, w/v; extraction temperature = 90°C; extraction time = 20 min)

Characterization. ***Fourier transfer infrared (FTIR) spectra*** of the oil samples obtained from high-shear homogenization-assisted extraction (HAE), high-frequency ultrasound-assisted extraction (UAE), and traditional wet rendering are shown in Fig. 2. The functional group region of the spectra contained five different peaks. The peak at 3010 cm^{-1}, which indicates the C─H stretching vibration of *cis*-double bonds in fatty acids, was observed at a minor intensity in the UAE and control samples (wet rendering) but was absent in the oil obtained from HAE. This difference might be due to the higher mechanical and thermal stress which may break down *cis*-double bonds or convert them into *trans*-double bonds or saturated fatty acids [36]. This process can also be increased due to higher air exposure during homogenization [9].

Additionally, two major peaks were observed at 2924 and 2852 cm^{-1}, which correspond to the symmetric and asymmetric stretching vibrations of CH_2, respectively. These peaks are indicative of the fatty acid chain length. Comparing these two peaks, we observed that the amplitude of the control oil sample (recovered without pretreatment) was lower than that of the oil samples obtained through HAE and UAE. The long-chain fatty acids are often tightly packed in adipose tissues, and without the mechanical disruption from homogenization or cavitation (ultrasound), these fatty acids might not be fully released, resulting in a lower intensity of the corresponding peaks in the control sample [24].

Furthermore, we identified a significant peak at 1742 cm^{-1}, which corresponded to the C═O stretching vibration of the ester group in triacylglycerols. The peak intensity of the oil obtained through HAE and UAE was higher, suggesting a higher presence of ester bonds in triacylglycerols. In contrast, the control oil sample had a very low peak intensity. This suggests that without the pretreatment steps (homogenization and ultrasound), the adipose cells might not have been effectively ruptured to release triacylglycerols [37]. The presence of unsaturated fatty acid chains in the oil samples was indicated by the peak at 1451 cm^{-1}, corresponding to CH_2 scissor vibration. Among all the oil samples, the oil obtained through UAE had the highest amplitude, followed by the oil obtained through HAE and the control method.

The fingerprint region contained three peaks: a) CH_2 bending vibration at 1362 cm^{-1}; b) out-of-plane deformation at 1153 cm^{-1}; and c) CH_2 rocking vibration at 721 cm^{-1}. Among these peaks, the one at 1153 cm^{-1} (out-of-plane deformation) corresponded to lipids and is generated due to the deformation of the C─H bonds in lipid molecules, indicating the degree of unsaturation. The oil samples recovered without pretreatment (control) had a lower amplitude compared to the oil samples recovered through UAE or HAE. This showed that the oil extracted without homogenization or ultrasound had a smaller amount of polyunsaturated fatty acids. These results were in agreement with the fatty acid composition of the oils.

Fatty acid composition. Table 3 displays the fatty acid profiles of the oils extracted using three methods: high-shear homogenization-assisted extraction (HAE), high-frequency ultrasound-assisted extraction (UAE), and traditional wet rendering process. Linoleic acid (C18:2 n-6) was the most dominant fatty acid across all three methods, with the highest concentration observed in the oil extracted by HAE (305.11 µg/g of oil). The second most abundant fatty acid was oleic acid (C18:1 n-9), with the highest level observed in the oil extracted by UAE (256.81 µg/g of oil). This finding was in agreement with Sathivel *et al.* who also reported an abundance of omega-6 and omega-9 fatty acids in catfish oils. Other fatty acids were found in lower or moderate amounts [38]. We also found that the total amount of polyunsaturated

fatty acids (PUFAs) was significantly higher than the total amount of saturated fatty acids (SFAs) ($p < 0.05$). However, eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), long-chain omega-3 fatty acids, were present in lower concentrations across all the samples, similarly to the observations made by Sathivel *et al.* [38].

A notable finding was a significant influence of the extraction method on the PUFA content. The UAE method yielded the highest PUFA content (417.06 μg/g of oil), followed by HAE (410.17 μg/g of oil) and the control method (362.93 μg/g of oil) ($p < 0.05$). This suggests that the thermal process involved in wet rendering could negatively affect the PUFA content, as suggested by Akoh, but using an optimized extraction condition, such as UAE or HAE, can retain a considerable amount of PUFAs [39]. The oil obtained from the UAE method exhibited significantly higher amounts of EPA and DHA (29.41 and 34.91 μg/g, respectively) compared to the oil extracted from the control method (27.40 μg/g EPA and 30.80 μg/g DHA). This difference could be attributed to a higher rate of oxidation in the oil extracted via the control method, leading to the production of secondary oxidation compounds.

Lipid nutritional quality indexes. Table 3 also presents the lipid nutritional quality indices of the oils extracted using three different methods: high-shear homogenization-assisted extraction (HAE), high-frequency ultrasound-assisted extraction (UAE), and traditional wet rendering. The Omega 6:Omega 3 ratios were 3.92:1, 3.68:1, and 4:1 for the HAE, UAE, and control (wet rendering) methods, respectively. These ratios are important because they can influence inflammatory responses in the body. A lower ratio is generally preferred, as it is considered to be healthier [40]. In this case, the oil extracted through UAE had the lowest Omega 6:Omega 3 ratio, implying a better balance between these two types of fatty acids and therefore potentially healthier inflammatory responses.

Lower atherogenicity index (AI) values are desirable as they indicate lower potential for atherogenesis, a process that can lead to atherosclerosis and cardiovascular disease [41]. The oils extracted by HAE and UAE had significantly lower AI values (0.22 and 0.20, respectively) compared to the control method (0.27), indicating their lower potential for atherogenesis. The thrombogenic index (TI) assesses the potential of the oil to contribute to the formation of thrombus or blood clots [19]. A lower TI is better for heart health. Again, the oils extracted via HAE and UAE showed lower TI values (0.31 and 0.29, respectively) compared to the control method (0.38). This suggests that the oils from HAE and UAE could have a lower propensity for clot formation.

The hypocholesterolemic/hypercholesterolemic ratio (HH) indicates the balance between fatty acids that de-

Table 3 Changes in fatty acid compositions of Clarias magur visceral oils recovered using HAE and UAE compared to traditional wet rendering method

Fatty acid	HAE	UAE	Control
		μg/g of oil	
C14	32.61 ± 0.02[a]	32.61 ± 0.01[a]	32.62 ± 0.01[b]
C14:1	3.32 ± 0.00[a]	3.32 ± 0.01[a]	3.51 ± 0.02[b]
C15	4.61 ± 0.01[a]	4.51 ± 0.01[b]	4.61 ± 0.02[a]
C16	99.81 ± 0.21[a]	110.71 ± 0.32[b]	145.92 ± 0.29[c]
C16:1 n-7	93.01 ± 0.31[b]	128.81 ± 0.23[c]	62.40 ± 0.24[a]
C17	4.12 ± 0.02[b]	4.22 ± 0.01[c]	4.01 ± 0.01[a]
C18	3.71 ± 0.02[a]	3.72 ± 0.00[a]	3.61 ± 0.01[a]
C18:1 n-9	249.80 ± 0.52[b]	256.81 ± 0.43[c]	229.90 ± 0.29[a]
C18:2 n-6	305.11 ± 0.19[c]	295.11 ± 0.20[b]	262.90 ± 0.22[a]
C18:3 n-6	0.72 ± 0.02[b]	12.21 ± 0.01[c]	0.61 ± 0.01[a]
C18:3 n-3	11.21 ± 0.01[b]	11.21 ± 0.02[b]	9.12 ± 0.02[a]
C18:4 n-3	6.21 ± 0.01[b]	6.21 ± 0.02[b]	6.01 ± 0.01[a]
C20	1.91 ± 0.03[b]	1.91 ± 0.02[b]	1.71 ± 0.02[a]
C20:1 n-9	12.92 ± 0.02[b]	13.71 ± 0.04[c]	10.11 ± 0.04[a]
C20:2 n-6	3.71 ± 0.01[a]	3.71 ± 0.00[a]	3.71 ± 0.01[a]
C20:3 n-6	2.41 ± 0.02[b]	2.42 ± 0.01[b]	2.31 ± 0.01[a]
C20:4 n-6	7.52 ± 0.02[b]	7.82 ± 0.02[c]	7.31 ± 0.03[a]
C20:3 n-3	0.22 ± 0.00[a]	0.22 ± 0.01[a]	0.20 ± 0.00[a]
C20:4 n-3	3.11 ± 0.03[b]	3.21 ± 0.02[c]	2.81 ± 0.02[a]
C20:5 n-3	29.11 ± 0.13[b]	29.41 ± 0.18[b]	27.40 ± 0.15[a]
C22:1 n-9	2.12 ± 0.03[b]	2.22 ± 0.03[c]	1.92 ± 0.02[a]
C22:2 n-6	6.82 ± 0.01[b]	7.11 ± 0.02[c]	6.02 ± 0.02[a]

Continuation of Table 3

Fatty acid	HAE	UAE	Control
		µg/g of oil	
C22:4 n-6	1.21 ± 0.00[a]	1.31 ± 0.01[b]	1.22 ± 0.02[a]
C22:5 n-6	2.41 ± 0.02[a]	2.41 ± 0.02[a]	2.51 ± 0.02[b]
C22:6 n-3	33.21 ± 0.12[b]	34.91 ± 0.16[c]	30.80 ± 0.10[a]
C24:1 n-9	0.91 ± 0.01[c]	0.82 ± 0.01[b]	0.71 ± 0.01[a]
Total SFA	146.77 ± 0.02[a]	157.68 ± 0.02[b]	192.48 ± 0.02[c]
Total MUFA	361.08 ± 0.01[b]	405.69 ± 0.02[c]	308.55 ± 0.01[a]
Total PUFA	410.17 ± 0.02[b]	417.06 ± 0.02[c]	362.93 ± 0.03[a]
		Lipids nutritional quality indexes	
Omega-6:Omega-3	3.92:1	3.68:1	4:1
AI	0.22[a]	0.20[a]	0.27[b]
TI	0.31[a]	0.29[a]	0.38[b]
HH	4.29[c]	4.17[b]	2.25[a]

HAE – oil extracted by optimized high-shear homogenization-assisted extraction; UAE – oil extracted by optimized high-frequency ultrasound-assisted extraction; and control – oil extracted by traditional wet rendering (viscera:water = 1:0.5, w/v; extraction temperature = 90°C; extraction time = 20 min)

AI – atherogenicity index, TI – thrombogenicity index, HH – hypocholesterolemic/hypercholesterolemic fatty acid ratio

Values are given as mean ± SD (n = 3)

Different lowercase superscripts in the same row denote significant differences ($p < 0.05$)

crease low-density lipoprotein cholesterol (hypocholesterolemic) and those that raise it (hypercholesterolemic) [42]. Higher ratios are healthier as they indicate more hypocholesterolemic activity. In this study, the oils extracted via HAE and UAE had significantly higher HH values (4.29 and 4.17, respectively) compared to the control method (2.25). This suggests that the oils from the HAE and UAE methods could be healthier in terms of cholesterol balance.

CONCLUSION

Our research showed that high-shear homogenization-assisted extraction and high-frequency ultrasound assisted extraction, which were optimized by using the Taguchi orthogonal array design, provide significantly improved *Clarias magur* oil extraction efficiency, quality, and oxidative stability compared to the traditional wet rendering method. The optimal conditions allo wed more efficient extraction of high-quality, PUFA-rich oil from *C. magur* viscera at lower temperatures. Analytical methods, including Fourier transfer infrared spectroscopy, fatty acid analysis, and nutritional quality indexes confirmed the superior quality of the obtained oil.

As indicated by scanning electron microscopy images, extensive cell disruption was observed in the high-shear homogenization and high-frequency ultrasound extract residues. These findings underscore the value of these methods as faster, more efficient, and low-temperature alternatives to the traditional extraction methods. Further research could expand on these findings by exploring other potential applications and scalability of these extraction methods. Our study provides promising pathways for the valuable use of catfish visceral biomass, contributing to waste minimization and enhancing the overall value chain in the fish industry.

CONTRIBUTION

Jaydeep Dave conducted research activities and investigations, as well as wrote some manuscript sections. Nishant Kumar, Ashutosh Upadhyay, and Sampatee Sanguanpuag provided critical inputs during the work. Daniel Tua Purba investigated some parts of the experiments. Pikunthong Nukthamna was involved in the sample collection and wrote some sections of the manuscript. Ali Muhammed Moula Ali devised the work plan and designed the experiments, corrected and reviewed the manuscript. Sri Charan Bindu Bavisetty conducted and documented some lab experiments and contributed to the manuscript.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Digital transformation metamodel in smart farming: Crop classification prediction based on recurrent neural network

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Abstract:
Agriculture 4.0 is an opportunity for farmers to meet the current challenges in food production. It has become necessary to adopt a set of agricultural practices based on advanced technologies. Agriculture 4.0 enables farms to create added value by combining innovative technologies, such as precision agriculture, information and communication technology, robotics, and Big Data.
As an enterprise, a connected farm is highly sensitive to strategic changes in organizational structures, objectives, modified variety, new business objects, processes, etc. To control the farm's information system strategically, we proposed a metamodel based on the ISO/IS 19440 standard, where we added some new constructs relating to advanced digital technologies for smart and connected agriculture.
We applied the proposed metamodel to the crop classification prediction process. This involved using machine learning methods such as recurrent neural networks to predict the type of crop being grown in a given agricultural area.
Our research bridges farming with modern technology through our metamodel for a connected farm, promoting sustainability and efficiency. Furthermore, our crop classification study demonstrates the power of advanced machine learning, guided by our metamodel, in accurately predicting crop conditions, emphasizing its potential for crop management and food security. In essence, our work advances the transformative role of digital agriculture in modern farming.

Keywords: Farm modeling, digital agriculture, agriculture 4.0, advanced technologies, connected farm, ISO 19440-2007

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INTRODUCTION

The agricultural sector is an important contributor to the Moroccan economy, employing over 40% of the population and accounting for nearly 15% of the country's Gross Domestic Product (GDP). The farming sector in Morocco comprises small-scale, family-owned farms that produce a variety of crops, including cereals, fruits, vegetables, and livestock. The most important crops in terms of production and revenue are cereals (wheat and barley) and fruits (citrus, olives, and dates). Over the past few decades, Morocco has invested greatly in the agricultural sector to develop irrigation systems and promote modern farming techniques. These efforts have helped increase productivity and improve the quality of agricultural products, which has led to greater exports and a stronger economy.

Despite these advancements, the farming sector in Morocco still faces challenges, such as water scarcity, soil degradation, and the need for greater infrastructure development. Nevertheless, the Moroccan government continues to prioritize the agricultural sector as a key driver of economic growth and development, and there is a growing interest in sustainable farming practices and organic farming methods.

Digital farming, also known as smart agriculture, is a modern farming approach that harnesses the power of data, technology, and automation to improve the efficiency and productivity of agricultural practices [1]. Digital farming involves the use of various tools and technologies, such as sensors, drones, GPS systems, and data analytics, to collect and analyze data about crops, soil conditions, weather patterns, and other factors that affect agricultural production [2].

One of the key benefits of digital farming is its ability to provide farmers with real-time, data-driven insights into their farming practices. For example, sensors

can be used to monitor soil moisture, plant growth, and nutrient levels, allowing farmers to optimize their use of water, fertilizers, and other resources. Drones equipped with high-resolution cameras can also be used to provide detailed images of crops, helping farmers identify potential problems, such as pests, diseases, and nutrient deficiencies. Digital farming can also help farmers optimize their planting schedules and improve crop yields by using data-driven decision-making tools. By analyzing data on weather patterns, soil conditions, and other factors, farmers can make more informed decisions about when and where to plant their crops, reducing waste and increasing yields [3].

Other benefits of digital farming include reduced environmental impact, improved crop quality, and increased efficiency and profitability. However, digital farming also requires significant investment in technology and infrastructure, as well as specialized knowledge and skills.

In this paper, we propose incorporating the ISO 19440 standard into the agricultural landscape. This can herald a new era of informed and efficient farming. By treating a farm as an organized enterprise and harnessing the power of standardized data modeling and information management, the agricultural sector can achieve unprecedented levels of productivity, sustainability, and collaboration. The principles embedded within ISO 19440 can be employed to streamline and elevate farm management by providing a structured approach to data modeling and information management.

Crop classification is an agricultural process that involves identifying and categorizing different types of crops based on their characteristics, such as appearance, growth habits, and agricultural practices. Crops can be classified in many different ways, including by their:

– use: some crops are grown for human consumption (wheat, rice, and corn), while others are grown for animal feed or industrial uses (cotton or tobacco);
– growing season: crops can be classified as annuals, which complete their life cycle within a single year, or perennials, which live for multiple years;
– growth habits: crops can be vines, shrubs, or trees;
– climate requirements: some crops require specific climatic conditions, such as a certain amount of rainfall or temperature range, while others are more adaptable to a range of conditions;
– soil requirements: crops have different soil requirements, such as pH levels, nutrient content, and drainage; and
– geographic origin: some crops are native to specific regions of the world, while others were introduced and adapted to new environments.

Crop classification is important for farmers and policymakers, as it helps them identify the best practices for growing and managing different types of crops, as well as understand their potential impact on the environment and local economies.

In this study, we aimed to outline a strategic plan for navigating the digital evolution of agricultural practices. For this, we introduced a model for transformation and examined its implications against ISO 19440:2007 "Enterprise Integration" by conceptualizing a farm as a structured enterprise. Then, we elucidated the empirical findings of our case study involving crop classification prediction and scrutinized our methodology. Lastly, we deliberated on the originality and practicality of our proposed metamodel, as well as proposed potential avenues for future research.

STUDY OBJECTS AND METHODS

From Agriculture 1.0 to Agriculture 4.0. The digital revolution is a societal shift that impacts both our personal and professional lives. The incorporation of novel technologies and the rise of innovative practices have fundamentally altered our society, influencing its culture, structure, and organization. Recent innovations, such as Cloud computing, Internet of Things (IoT), Big Data analytics, and Virtual Reality, have gradually become integral across various industries [4]. Beyond the mere utilization of these novel tools, the convergence between the digital realm and the professional sphere has prompted attention to several key aspects: digitalization, communication and collaboration, globalization, and competitiveness [5].

This transformation has had a profound effect on the agricultural sector, a pivotal player in the Moroccan economy. As a result, a new paradigm for farming has emerged – one that is interconnected, intelligent, adaptable, and community-oriented, with a special emphasis on customer involvement.

The evolution of agriculture can be categorized into different "Agriculture" eras, ranging from Agriculture 1.0 to Agriculture 4.0. These eras are characterized by different stages of technological advancement and significant changes in agricultural practices over time.

Agriculture 1.0 is often referred to as the "subsistence agriculture" era, where manual labor and traditional methods were used for farming. This era began around 10 000 years ago, during the Neolithic Revolution, and lasted until the 19th century.

Agriculture 2.0, also known as the "mechanized agriculture" era, began in the 19th century and was characterized by the introduction of machines, such as tractors, seed drills, and threshing machines. This era led to significant improvements in agricultural productivity and efficiency.

Agriculture 3.0, also known as the "Green Revolution" era, began in the mid-20th century and was characterized by the development of new technologies, such as high-yielding crop varieties, synthetic fertilizers, and pesticides. This era led to a significant increase in global agricultural productivity, enabling farmers to produce more food than ever before.

Agriculture 4.0, also known as the "digital agriculture" era, is the current stage of agricultural development, characterized by the integration of advanced digital technologies, such as the Internet of Things (IoT), artificial intelligence (AI), and robotics [6]. This era is focused on increasing agricultural efficiency, reducing waste, and improving sustainability through the use of

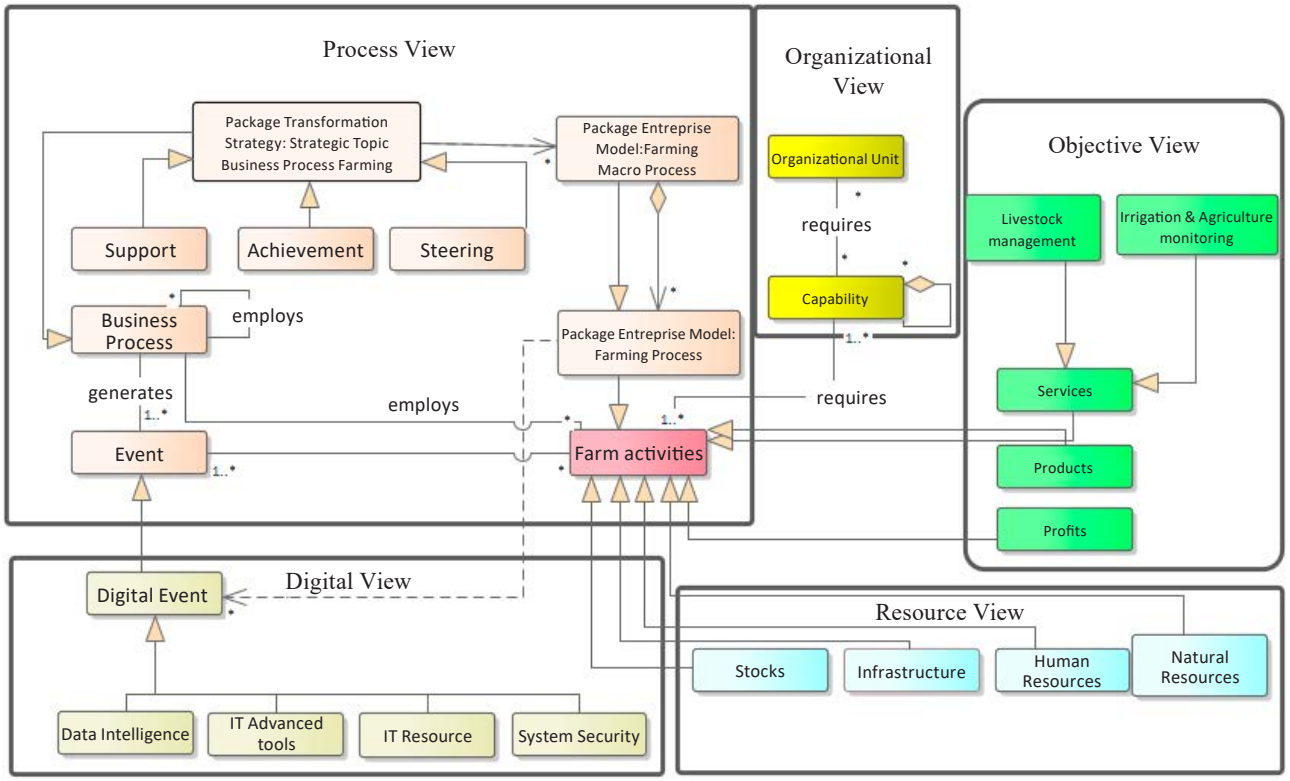

Figure 1 Metamodel of a connected farm

data-driven decision-making, precision farming, and other digital agricultural processes [7].

Overall, the transition from Agriculture 1.0 to Agriculture 4.0 has led to significant improvements in agricultural productivity, efficiency, and sustainability, enabling farmers to produce more food with fewer resources and reduce the environmental impact of agricultural practices.

Research methodology. Modeling a connected farm based on the ISO 19440 Enterprise Metamodel, which views a farm as an enterprise, involves a comprehensive approach to integrating technological advancements into agricultural operations [8]. This metamodel recognizes that a farm functions as a dynamic and interconnected entity, encompassing diverse processes such as crop cultivation, livestock management, resource allocation, and supply chain logistics. By applying the principles of ISO 19440, we can represent the farm as a network of interconnected systems, where data-driven decision-making becomes central to optimizing productivity and sustainability. Just as the enterprise model captures interactions between departments and stakeholders, this approach acknowledges intricate relationships between various aspects of agriculture, enabling the deployment of advanced technologies (IoT devices, sensors, and data analytics) to streamline operations, enhance resource efficiency, and ultimately achieve better outcomes across the agricultural value chain [9].

A metamodel of a connected farm encompasses several essential perspectives, namely the Process View, Organizational View, Objective View, Resource View, and Digital View (Fig. 1) [10].

The Process View focuses on various steps and activities involved in the operation of a connected farm. It includes processes such as crop management, livestock rearing, water and energy resource management, environmental monitoring, and harvesting. The Process View aims to identify workflows, dependencies, and interactions among these processes, ensuring efficient and streamlined operations throughout the farm.

The Organizational View delves into the structure and hierarchy of a connected farm. It defines the roles, responsibilities, and relationships among different stakeholders, including farm owners, managers, workers, and technicians. This perspective also considers communication channels and decision-making processes within the farm, ensuring clear lines of authority and effective collaboration.

The Objective View outlines the goals and objectives of a connected farm. It encompasses both short-term and long-term objectives such as maximizing crop yield, optimizing resource utilization, improving sustainability, ensuring animal welfare, and enhancing overall profitability. The Objective View provides a strategic framework for decision-making and guides the allocation of resources to achieve the desired outcomes.

The Resource View focuses on various resources required for the functioning of a connected farm. This includes physical resources (land, machinery, buildings, and infrastructure), as well as human resources (skilled labor, technicians, and farm workers). Additionally, it considers the availability and allocation of natural resources, such as water, energy, and nutrients. The Resource

View ensures efficient utilization of resources, whileminimizing waste and environmental impact.

The Digital View encompasses the technological aspects of a connected farm. It involves the integration of sensors, actuators, and other IoT (Internet of Things) devices to collect real-time data on various farm parameters such as soil moisture, temperature, humidity, livestock health, and equipment status. This data is then processed and analyzed using advanced analytics and machine learning algorithms to derive meaningful insights [11]. The Digital View enables data-driven decision-making, precision agriculture practices, and remote monitoring and control of farm operations.

With these perspectives, the metamodel of a connected farm provides a holistic framework for understanding and optimizing the complex interplay of processes, organizations, objectives, resources, and digital technologies. It facilitates the design, implementation, and management of an intelligent and efficient farm ecosystem that leverages technology to drive sustainable and profitable agricultural practices.

The Process View in a connected farm encompasses a detailed examination of various steps and activities involved in the farm's operation. It aims to optimize workflows, dependencies, and interactions among different processes to ensure efficient and streamlined operations throughout the farm ecosystem.

The Process View identifies and analyzes different processes, such as crop management, livestock rearing, water and energy resource management, environmental monitoring, harvesting, and others. Each process is broken down into its constituent activities, tasks, and subtasks, providing a granular understanding of the farm's operational procedures (Fig. 2).

For example, crop management involves such activities as soil preparation, seed sowing, irrigation, fertilization, pest and disease control, and crop monitoring [12]. The Process View examines the sequence of these activities, their interdependencies, as well as inputs and outputs associated with each step. This level of detail allows farm managers to identify potential bottlenecks, inefficiencies, or areas for improvement within the crop management process.

Similarly, in livestock rearing, the Process View encompasses activities such as animal feeding, health monitoring, breeding, housing management, and waste management. By analyzing these processes, farm operators can optimize feeding schedules, implement effective health monitoring systems, enhance breeding practices, improve waste management techniques, and ensure the overall well-being of livestock.

The Process View also considers cross-cutting processes such as water and energy resource management and environmental monitoring. It examines the utilization of water and energy resources throughout the farm, identifying opportunities for conservation, efficiency improvement, and sustainable practices. Additionally, it addresses environmental monitoring processes to ensure compliance with regulations and to proactively mitigate any potential environmental impact.

By understanding and optimizing the processes within a connected farm, farm owners and managers

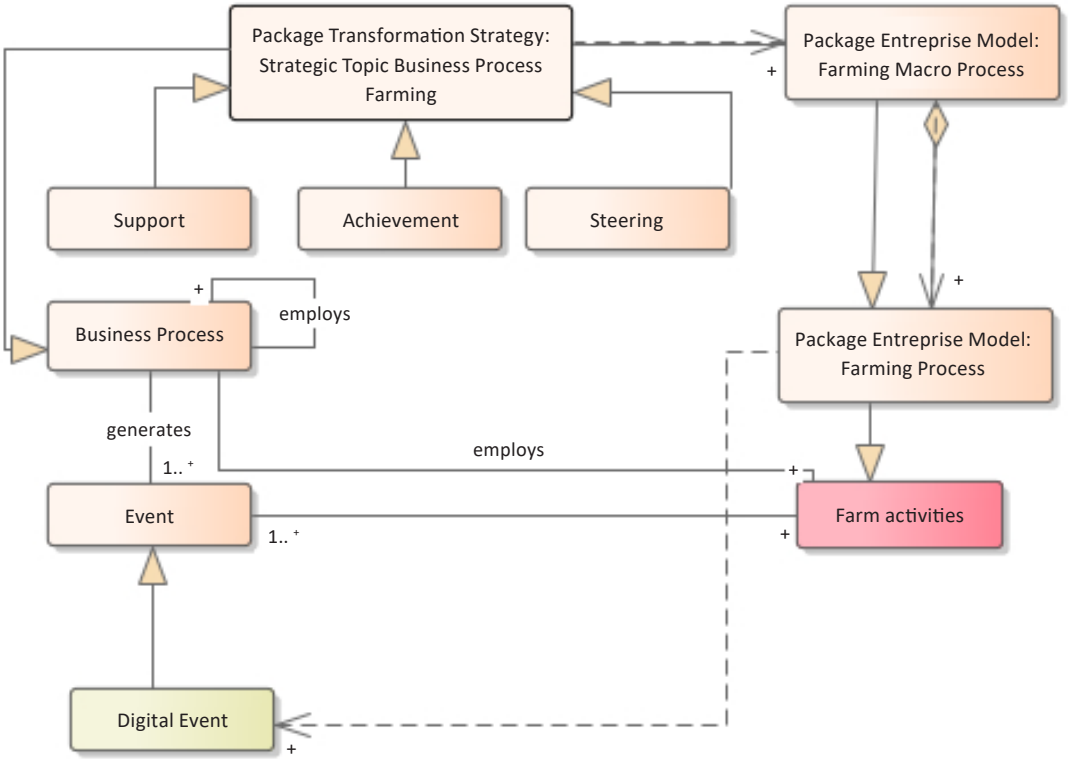

Figure 2 Process View

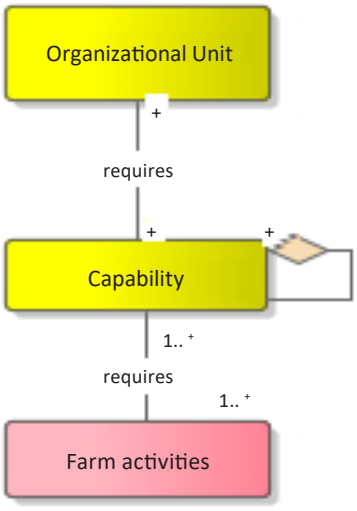

Figure 3 Organizational View

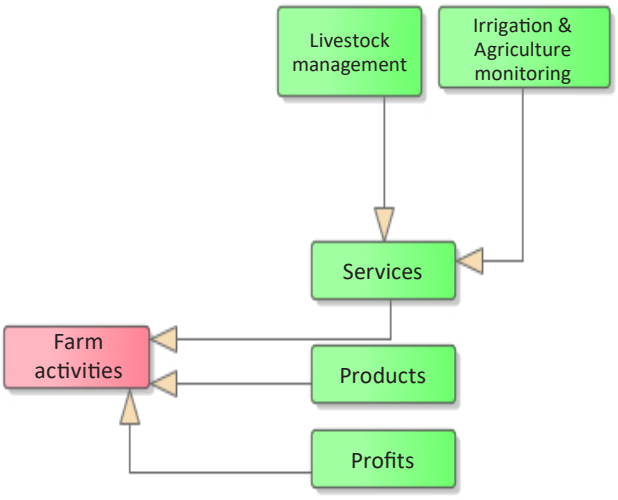

Figure 4 Objective View

can enhance operational efficiency, reduce waste, improve resource utilization, and ultimately achieve higher productivity and profitability. Thus, the Process View provides a foundation for continuous improvement and innovation, as well as a framework for implementing best practices and adopting emerging technologies in farm operations.

The Organizational View within a connected farm entails a comprehensive examination of the structure, hierarchy, and relationships among various stakeholders involved in the farm's operations. It aims to establish a clear understanding of roles, responsibilities, and communication channels to ensure effective collaboration and decision-making (Fig. 3).

Within the Organizational View, the farm's structure and hierarchy are defined, as well as different positions and levels of authority within the organization. This includes identifying farm owners, managers, supervisors, workers, technicians, and other key personnel. By clarifying these roles, the Organizational View establishes a framework for accountability and promotes efficient coordination within the farm.

Additionally, the Organizational View considers relationships among individuals and groups within the farm. It examines communication channels, reporting lines, and decision-making processes to facilitate effective information flow and decision-making. Clear lines of communication and well-defined reporting structures enable timely dissemination of information, quick problem resolution, and efficient coordination of activities.

The Organizational View also takes into account specific responsibilities and tasks assigned to each role within the farm. By clearly defining these responsibilities, it ensures that every stakeholder understands their role in contributing to the overall success of the farm. This clarity promotes efficiency, reduces duplication of efforts, and enhances productivity across the organization.

Furthermore, the Organizational View encourages a culture of collaboration and teamwork within the farm. It establishes mechanisms for effective collaboration, such as regular meetings, cross-functional teams, and knowledge-sharing platforms. By fostering a collaborative environment, the Organizational View enhances innovation, problem-solving, and continuous improvement within the farm.

Ultimately, the Organizational View aims to optimize the farm's human resources by aligning roles, responsibilities, and relationships to achieve the farm's objectives. It promotes a harmonious working environment, empowers individuals to contribute their expertise effectively, and facilitates efficient decision-making processes. By ensuring a well-structured and organized farm, the Organizational View sets a foundation for performing successful operations and achieving the desired outcomes.

The Objective View in a connected farm aims to define and align its goals and objectives to guide the farm's strategic direction and decision-making processes. It provides a framework for setting both short-term and long-term objectives that are in line with the farm's mission, vision, and values (Fig. 4).

Within the Objective View, the farm's goals and objectives are established based on various factors such as market conditions, customer demands, regulatory requirements, and the farm's unique circumstances. These objectives may include maximizing crop yield, optimizing resource utilization, improving sustainability practices, ensuring animal welfare, enhancing profitability, and adopting innovative technologies.

Short-term objectives focus on immediate targets that can be achieved within a relatively short timeframe, often in alignment with the current growing season or operational cycle. These objectives could include improving irrigation efficiency, reducing pesticide usage, or implementing precision agriculture techniques for specific crops [13].

Long-term objectives, on the other hand, outline broader aspirations and strategic aims for the farm. These objectives may encompass sustainable land management

practices, diversification of crop production, adoption of renewable energy sources, or implementation of advanced data analytics for the farm's decision-making.

The Objective View also involves prioritizing objectives based on their importance, feasibility, and potential impact. This prioritization ensures that resources, such as time, labor, and capital investments, are allocated effectively to achieve the desired outcomes. By setting clear objectives, the farm can focus its efforts and resources on areas that contribute most significantly to its success.

Furthermore, the Objective View plays a crucial role in measuring and evaluating the farm's performance against the defined objectives. Key performance indicators (KPIs) are established to track progress and assess the effectiveness of strategies and initiatives implemented to achieve the objectives. Regular monitoring and evaluation allow for adjustments, refinements, and identification of areas for improvement.

By integrating the Objective View into the farm's operations, owners and managers can effectively align their decision-making processes with the overall strategic goals. This alignment ensures that day-to-day activities, resource allocation, and investments are in line with the desired outcomes, driving the farm towards long-term success, sustainability, and profitability.

The Resource View within a connected farm encompasses a comprehensive assessment of various resources required for the farm's operations. It involves an examination of both physical and human resources, as well as efficient management and allocation of these resources to support sustainable and productive farming practices (Fig. 5).

In terms of physical resources, the Resource View considers assets such as land, machinery, buildings, infrastructure, and other agricultural inputs. It evaluates the availability, quality, and suitability of land for different crops and livestock rearing. Additionally, it examines the farm's infrastructure, including irrigation systems, storage facilities, and equipment, ensuring that they are properly maintained and utilized to support efficient farm operations.

The Resource View also addresses management of human resources within the farm. It involves assessing the skills, knowledge, and expertise of the farm's workforce, including skilled labor, technicians, farm workers, and management personnel. The Resource View aims to ensure that the right people are assigned to the right tasks, optimizing productivity and leveraging the strengths of individuals within the organization.

Furthermore, the Resource View encompasses the availability and allocation of natural resources such as water, energy, and nutrients. It considers sustainable practices for water management, including irrigation techniques that minimize water waste and maximize efficiency. It also examines energy consumption and explores opportunities for renewable energy sources to reduce the farm's environmental footprint. Additionally, nutrient management strategies are evaluated to ensure efficient and responsible use of fertilizers and other inputs, minimizing pollution and maximizing soil health.

Efficient resource management is a key aspect of the Resource View. It involves optimizing resources' utilization of while minimizing waste and environmental impact. This may include implementing precision agriculture technologies to precisely apply water, fertilizers, and pesticides only where and when they are needed, reducing resource waste. It may also involve adopting sustainable farming practices that promote soil health, biodiversity, and conservation of natural resources.

By analyzing and managing resources effectively, the Resource View supports the farm's overall sustainability and profitability. It ensures that resources are used efficiently, reducing unnecessary costs and environmental impact. The Resource View also enables farm owners and managers to make informed decisions regarding investments, resource allocation, and adoption of new

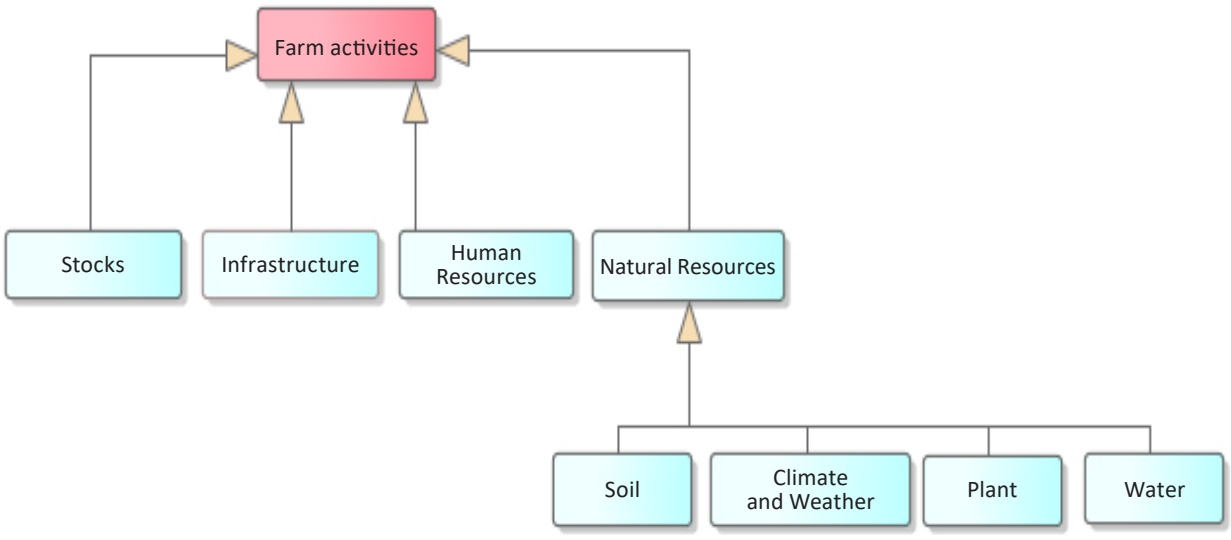

Figure 5 Resource View

technologies, optimizing the farm's performance and resilience in a rapidly changing agricultural landscape.

The Digital View within a connected farm focuses on the integration of digital technologies and data-driven systems to enhance the farm's operations and decision-making processes. It encompasses the use of sensors, actuators, and other Internet of Things (IoT) devices to collect real-time data on various aspects of the farm, enabling precision agriculture and intelligent farm management [14].

Within the Digital View, a network of sensors is deployed throughout the farm to gather data on parameters such as soil moisture, temperature, humidity, air quality, crop growth, livestock health, and equipment status. These sensors provide continuous monitoring and generate valuable data that can be analyzed and utilized for informed decision-making (Fig. 6) [15].

The collected data is transmitted to a centralized system or cloud platform where it is stored, processed, and analyzed using advanced analytics and machine learning algorithms [16]. This data analysis enables the extraction of valuable insights that can guide the farm's management strategies. For example, machine learning algorithms can detect anomalies in crop growth patterns or identify signs of disease in livestock, enabling early intervention and preventive measures [17].

The Digital View also includes the use of remote monitoring and control systems. Farm managers can access real-time data and insights through digital dashboards or mobile applications, allowing them to monitor the farm's operations, make data-driven decisions, and remotely control the farm's equipment and systems. This remote monitoring capability provides flexibility and enables timely interventions, even when farm managers are not physically present on-site [18].

Furthermore, the Digital View enables the implementation of precision agriculture techniques. With the help of GPS technology and mapping tools, farm managers can precisely determine the spatial variability of soil and crop conditions, enabling the targeted application of resources such as water, fertilizers, and pesticides. This targeted approach reduces resource wastage, enhances crop yield, and minimizes environmental impact.

The Digital View also encompasses the adoption of digital record-keeping systems, farm management software, and other digital tools that streamline administrative tasks, facilitate data organization, and improve overall efficiency. These technologies help in tracking the farm's inputs, managing inventories, planning schedules, and analyzing financial data, leading to better resource allocation and improved operational performance [19]. By leveraging the capabilities of the Digital View, connected farms can achieve enhanced productivity, optimized resource utilization, and improved sustainability. The integration of digital technologies enables real-time monitoring, data-driven decision-making, and automation of the farm's processes. Ultimately, the Digital View empowers farm owners and managers to harness the power of data and technology, driving innovation and transforming traditional farming practices into intelligent, efficient, and sustainable agricultural systems [20].

RESULTS AND DISCUSSION

This study offers an application of the proposed model to the agricultural system, employing a case study of crop classification that uses IT constructs.

There are several approaches to crop classification using machine learning. One of the most common methods is the use of satellite imagery or drones to capture high-resolution images of the area under study. These images are then processed using image processing algorithms to extract relevant crop features.

These features are then used as inputs for classification algorithms such as recurrent neural networks, which classify different crops based on the extracted image features. Once the training is completed, the trained model can be used to classify new types of crops with high accuracy.

Crop classification based on machine learning can be used for various applications, including crop management, yield prediction, disease detection, and crop growth monitoring. By combining this data with other sources of information such as weather and soil data, farmers can make more informed decisions to optimize their production and yield.

In our study, we aimed to determine the outcome of the harvest season, whether the crop would be healthy (alive), damaged by pesticides, or damaged due to other causes.

Below are the basic steps to perform crop classification prediction using recurrent neural networks (RNN) [21].

Data collection. We worked with an existing dataset that contains information about crops. We started by uploading the data file to the cloud storage location. Then, we created a cluster in Databricks by specifying the appropriate cluster configuration such as node type and Spark version. Next, we opened a notebook in Databricks by selecting "Create Notebook" in the navigation bar. In the notebook, we used commands to explore files in the cloud storage location. To load the data, we used the "spark.read" command to read the data from the cloud storage location and convert it into a Spark DataFrame. We also used the "display" command to show a preview of the data (Fig. 7).

Data cleaning. Data cleaning and preprocessing includes data normalization, feature scaling, and data formatting.

If there are unique values present in the columns, they will reduce dimensionality during subsequent processing (Fig. 8).

Using the dataset's isnull().sum() function, we verified that there were 233 missing values in the dataset in the "number_weeks_used" variable. Therefore, we could replace the missing values with the mode of the data. Similarly, upon checking for null values, we found that there were no null values in our dataset.

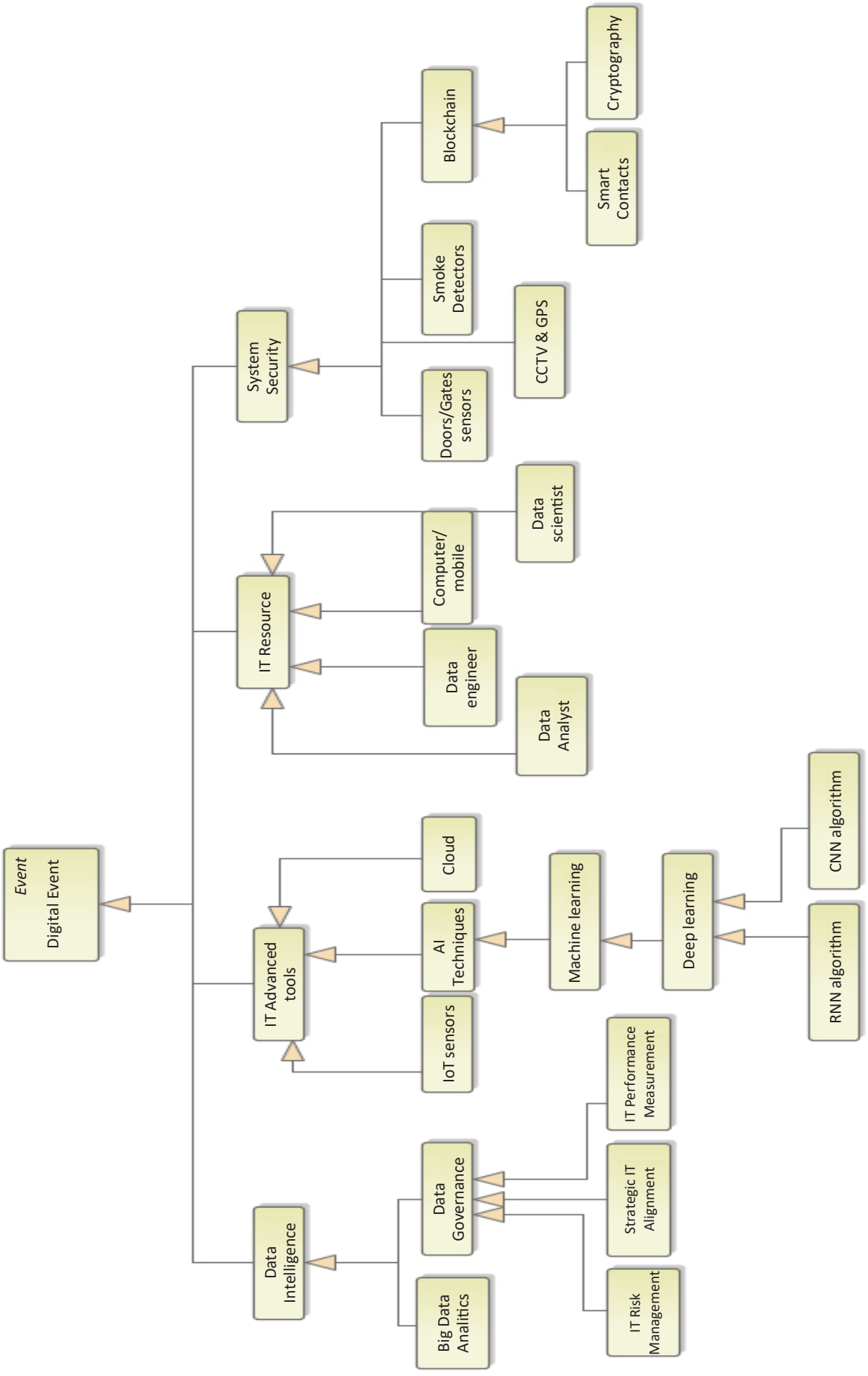

Figure 6 Digital View

	ID	Estimated_ Insects_ Count	Crop_ Type	Soil_ Type	Pesticide_ Use Category	Number_ Doses_ Week	Number_ Weeks_ Used	Number_ Weeks_ Quit	Season	Crop_ Damage
0	F00000001	188	1	0	1	0	0	0	1	0
1	F00000003	209	1	0	1	0	0	0	2	1
2	F00000004	257	1	0	1	0	0	0	2	1
3	F00155357	448	1	0	2	20	20	11	2	2
4	F00155265	3896	0	1	2	60	48	10	1	2

ID: Unique identifier; Estimated_Insects_Count: Estimated number of insects per square meter; Crop_Type: Crop category (0, 1); Soil_Type: Soil category (0, 1); Pesticide_Use_Category: Pesticide use category (1 – never, 2 – previously used, 3 – currently using); Number_Doses_Week: Number of doses per week; Number_Weeks_Used: Number of weeks used; Number_Weeks_Quit: Number of weeks since quitting; Season: Season category (1, 2, 3); Crop_Damage: Crop damage category (0 = alive, 1 = damage due to other causes, 2 = damage due to pesticides)

Figure 7 Crop classification dataset

```
How many NaN are there in the Crop Classification dataset? ID
Estimated_Insects_Count      0
Crop_Type                    0
Soil_Type                    0
Pesticide_Use_Category       0
Number_Doses_Week            0
Number_Weeks_Used          233
Number_Weeks_Quit            0
Season                       0
Crop_Damage                  0
source                       0
dtype: int64
```

Figure 8 Data cleaning

Data preprocessing. Data preprocessing is an essential step in the data analysis process. It involves preparing and transforming raw data to make it ready for analysis. Here are some common steps in data preprocessing:

Outlier analysis. Outliers were clearly present in the "number_doses_week" and "number_weeks_quit" columns. Consequently, the next step was to find the average value for each column to replace the outlier.

Skewness analysis. We checked the skewness of our data using the histogram and observed that all the data was normally distributed. Our dataset was now ready to be fed into the machine-learning model for classification analysis (Fig. 9).

Data splitting. The data can be split into training, validation, and test sets.

After preprocessing, we divided the data into training and testing subsets. We adhered to the 80% rule for data training and 20% for data testing.

Model training. The RNN model is trained using the training data. This involves optimizing the model parameters to minimize the prediction error.

The construction of an RNN model begins with the initialization of the model's weights and biases. The input, hidden, and output layers are then added, each with its own number of neurons and activation function. The model can be built using a deep learning library such as TensorFlow or PyTorch.

After the model is constructed, it needs to be compiled with a loss function and an optimizer. The loss function measures the difference between the model's predictions and the true values in the dataset. The optimizer adjusts the model's weights and biases to minimize the loss function.

The model is then trained by feeding the training data to the model and adjusting the weights and biases at each iteration. Training can be performed over multiple epochs to improve the model's accuracy.

After training, the model is evaluated using the test dataset. The model's accuracy is measured using metrics such as accuracy, precision, recall, and F1 score.

The Root Mean Square Error (RMSE) is a commonly used metric for evaluating the accuracy of a model's predictions. The RMSE measures the square root of the average of the squared differences between the predicted values and the true values (Fig. 10).

Model testing. The RNN model is tested using the test data. This involves evaluating the model's performance on the test set.

Prediction. Finally, the trained model can be used to make predictions on new data. Comparing the actual data to the forecasts of a model or statistical analysis is an important step in evaluating the accuracy and validity of the model or analysis (Fig. 11).

When a model or statistical analysis is created, it is often used to predict future outcomes or estimate trends

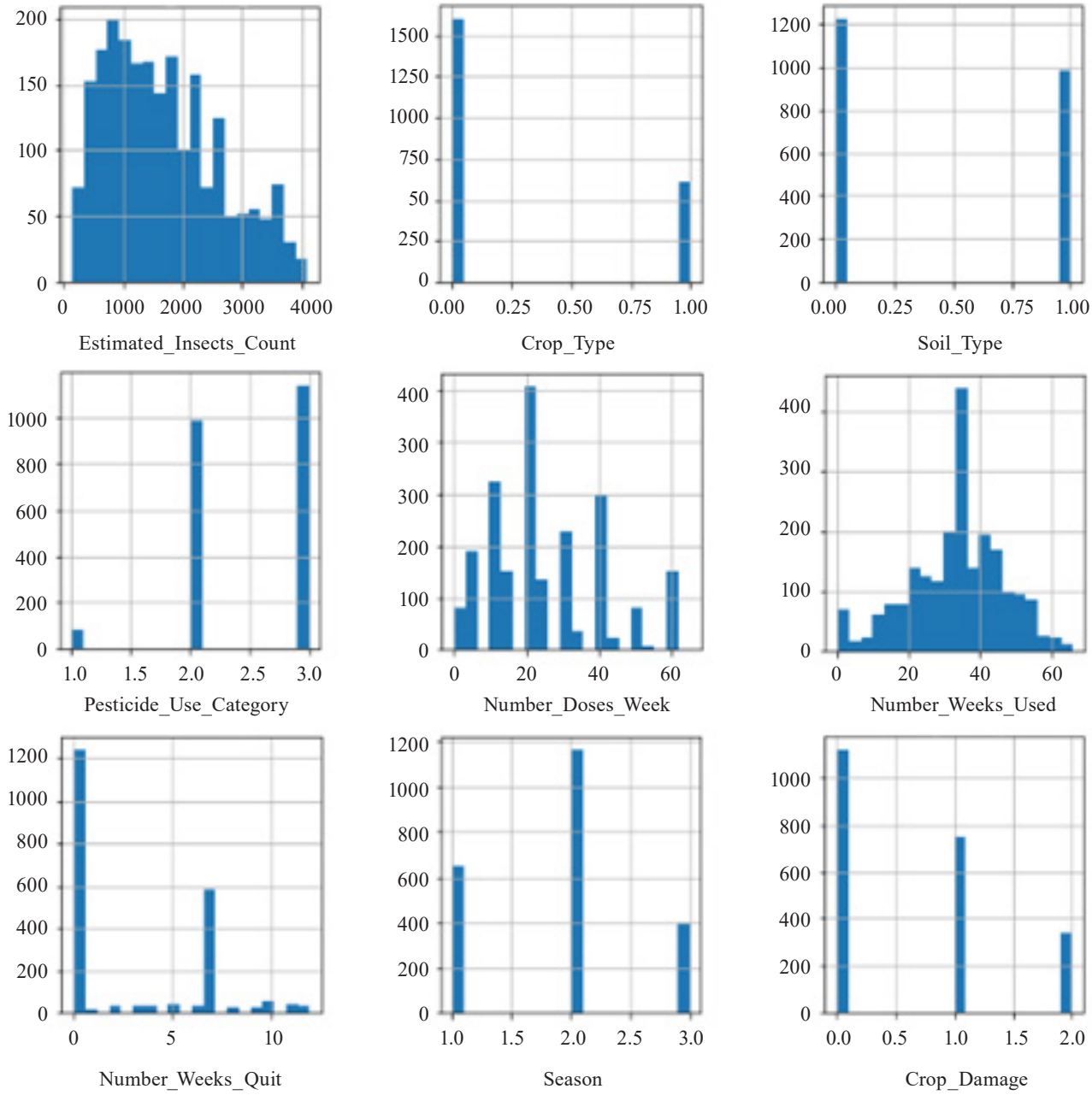

Figure 9 Skewness Analysis

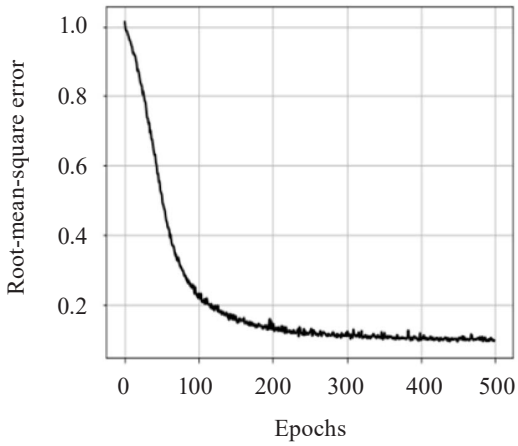

Figure 10 RMSE loss over epochs

based on historical data. Once the actual data becomes available, it is important to compare the model's or analysis predictions with the actual results to determine the accuracy of these forecasts.

If the predictions are close to the actual results, it indicates that the model or statistical analysis is reliable and can be used for future predictions. If the predictions deviate significantly from the actual results, it suggests that the model or statistical analysis needs to be revised to improve its accuracy.

It is important to note that even the most accurate models and statistical analyses are not perfect, and there may be unforeseen factors that can impact the actual results. Therefore, it is crucial to continue evaluating and adjusting the models and statistical analyses over time to ensure their ongoing accuracy.

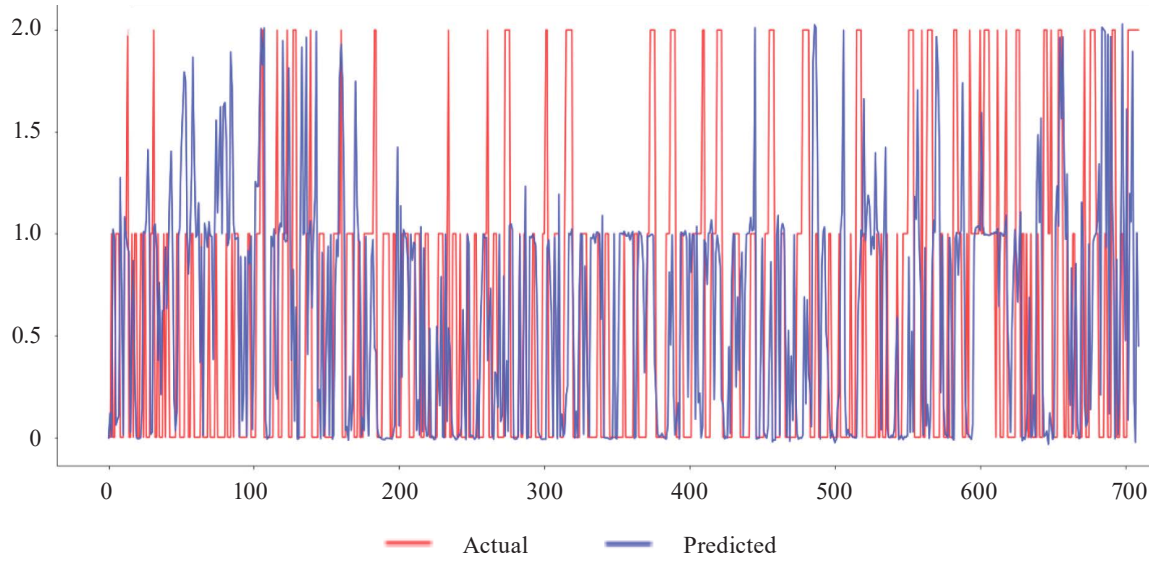

Figure 11 Prediction

CONCLUSION

Our research falls within the domain of digital agriculture. We introduced a metamodel for a connected farm, drawing inspiration from the ISO/IS 19440-2007 standard. This metamodel conceptualizes a farm as an enterprise encompassing four fundamental views (process, objective, organizational, and resource views) in addition to the digital view. In this framework, a farm is not merely a traditional agricultural operation but rather an integrated entity that seamlessly incorporates digital technologies. By considering these five interconnected views, we provided a comprehensive and holistic understanding of modern farming practices, where data-driven decision-making and digital tools play a pivotal role in optimizing agricultural operations, resource management, and sustainability.

Furthermore, our metamodel serves as a foundational blueprint for the development and implementation of smart farming systems. It enables farms to harness the power of data analytics, remote sensing, and automation to enhance crop yields, improve resource efficiency, and reduce environmental impacts. By embracing the digital view alongside the traditional business perspectives, farms can adapt to the evolving agricultural landscape, ensuring long-term viability and resilience in an increasingly technology-driven world.

Our case study delves into crop classification, offering a ground-breaking solution for predicting the condition of crops within a dataset. This predictive model differentiates between healthy crops, those damaged by pesticides, and those affected by other factors. Our metamodel, which harnesses recurrent neural networks, consistently delivered a Root Mean Square Error of less than 0.1, an impressive result with a dataset of over 2,210 rows. Our research underscores the potential of advanced machine learning techniques in revolutionizing crop management and ensuring food security.

CONTRIBUTION

The authors equally contributed to writing this manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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Oil emulsion stability in electrolyzed water solutions

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Abstract:
Metastable electrochemically activated water solutions possess unique properties that make it possible to modify food emulsions. This comparative analysis featured the stability of model oil-in-water emulsions with anolyte or catholyte as a dispersion medium, as well as the physical and morphometric profile of the emulsion system.
The research involved emulsions based on anolyte and catholyte. They consisted of refined sunflower oil, emulsifier (lecithin), and stabilizers, which were represented by sodium alginate, sodium carboxymethylcellulose, pectins, and agar. The study also covered such parameters as aggregative stability, viscosity, morphometry, oil particle size, and zeta potential.
Anolyte and catholyte affected the process of separation in the model emulsions. The samples stabilized with alginate and sodium carboxymethylcellulose proved to be the most stable emulsions while agar triggered gelation. The effect of substituting tap water with metastable electrolyzed water solutions depended on the oil proportion in the emulsion. Catholyte destabilized the samples with 20% of oil and liquified gel in the samples stabilized with agar. Anolyte was more aggressive in destabilizing emulsions with 30% of oil. The effective viscosity of these emulsions correlated with the stable phase fraction. The anolyte-based samples had low effective viscosity. The opposite results for emulsions with different oil fractions may have been caused by interface changes, i.e., surface tension, adsorption, coalescence, etc. In the emulsions with 46% of oil and animal origin emulsifier, neither anolyte nor catholyte had any significant effect on the aggregative stability of the system.
The revealed patterns can be used to control the properties of emulsion products with oil phase ≤ 30%, e.g., low-fat mayonnaises, sauces, emulsion drinks, etc. Metastable electrolyzed water solutions may provide a reagent-free control of properties and patterns of finished or semi-finished foods and biological raw materials.

Keywords: Electrolyzed water, anolyte, catholyte, oil-in-water emulsion, aggregative stability, scanning electron microscopy

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INTRODUCTION

The structure and properties of food products depend on such technological factors as temperature, pressure, mixing rate, etc., as well as on the interaction between ingredients, including water. Logically, modified water can change the properties of semi-finished or finished product. The method of electrolyzed water solution is quite promising in this respect. Its metastable fractions accumulate in the anode or cathode chamber of the electrolyzer [1, 2]. The resulting electrolysis produces anolyte and catholyte. These aqueous solutions possess high oxidizing or reducing abilities, respectively.

Water nanoclusters based on catholyte or anolyte were reported to show a correlation between the number of bound water molecules and the energy of hydrogen bonds between them. Experimental studies proved that water surface tension depends on the energy of hydrogen bonds [3]. Mathematical modeling demonstrated a decrease in the number of clusters in catholyte and an increase in the number of water molecules per cluster in anolyte [4]. Compared to water, catholyte has greater average energy of hydrogen bonds. For anolyte, however, the average energy is lower. This phenomenon indicates an increase or decrease in energy density, respectively.

It depends on the physiological effects produced by electrolyzed water solutions. Anolyte has acidic pH and an anomalous positive redox potential while catholyte has alkaline pH and a unique negative redox potential [5, 6].

Electrolyzed water solutions owe their useful properties to hydrogen and oxygen bubbles. Bubbles are stable due to the uncompensated electric charges on the interface between liquid and gas [7, 8]. During electrolysis, bubbles appear, grow, and detach themselves from the electrode surface [7]. Their growth rate depends on the current density and water flow rate. For instance, high current density increases gas bubble diameter while low flow rate reduces it.

Electrolyzed water solutions were reported to have a lower surface tension on the interface between liquid and air than the initial water solution. In catholyte, the surface of hydrogen bubbles started to adsorb atomic hydrogen and $H_3O_2^-$ ions when the surface tension on the liquid-air interface went down [9]. Anolyte accumulated hypochlorite ions and hypochlorous acid molecules on the surface of oxygen bubbles. During open storage, electrolyzed water solutions interacted with air, causing spontaneous restoration of the water solution. Catholyte demonstrated a correlation between the surface potential difference and the tension [8–10]. This effect was probably brought about by the neutralization of negatively charged hydroxyl ions on the solution surface. The fact that ions accumulate on the interface and reduce the surface tension could also explain the surface-active properties of catholyte and anolyte. As a result, electrolyzed water solution may obtain emulsifying properties.

Such dispersed systems as emulsions, suspensions, and foams are the main objects of directed modification in food production. Food producers control the water phase of dispersed systems by introducing food additives with certain functions and applying various physical and physicochemical methods, including the electrolyzed water solution technology [11, 12]. Ultrasound treatment was reported to facilitate the process in emulsions [12, 13]. Related studies usually feature such physicochemical properties as pH, redox potential, water activity, and color, as well as such technological parameters as yield, emulsion stability, and consistency. As a rule, the resulting emulsions prove stable and more abundant while the texture profile remain the same. For instance, ultrasound treatment combined with electrolyzed water was able to boost the extraction efficiency and solubility of krill proteins. Their emulsifying properties, foaming ability, and foam stability improved while the particle size decreased [14]. Other studies reported other factors that affected the conformation of molecules, their volume in the solution and, eventually, the rheological properties of the biomolecular solution. These factors include polyelectrolyte concentration, temperature, pressure, low molecular weight substances, pH, and electric field [15–20].

Emulsions with a surfactant, or emulsifier, are the most common dispersed systems in the food industry. By changing the physicochemical properties of the aqueous phase, food producers can adjust the emulsion, while giving it additional stability by applying protein or polysaccharide food thickeners. If a direct emulsion contains a high proportion of the hydrophilic phase and the emulsifier has an amphiphilic chemical structure, the system can become sensitive to such factors as pH, redox potential, electrical conductivity, ionic impurities, etc. [21]. As a result, dispersed systems based on electrolyzed water solutions have anomalous physicochemical parameters [22–25]. Their properties may differ from those of dispersed systems that were prepared using ordinary water.

Therefore, anolyte and catholyte require a deeper scientific insight into the way they affect emulsions. The current trends in fatty products dictate that food emulsions, e.g., mayonnaise and sauces, should have low energy value or that some animal ingredients should be replaced by their plant analogues [26–28]. This article introduces a comparative analysis of the aggregative stability of oil-in-water model emulsions with anolyte or catholyte as a hydrophilic dispersion medium.

STUDY OBJECTS AND METHODS

The experiment involved softened tap water (pH 7.2, redox potential +340 mV). We used a diaphragm modular electrochemical cell (Delfin Aqua, Russia) to obtain metastable fractions of electrolyzed water solution, an HI98120 device (Hanna, Germany) to measure pH, and a ST20R meter (Ohaus, China) to define redox potential. The anolyte had pH 4 and ORP +800 mV while the catholyte had pH 9 and ORP –400 mV.

Liquid fat-soluble soy lecithin (DENLEC, Louis Dreyfus Company, Brazil) served as emulsifier. To create the oil phase, we dissolved the lecithin in sunflower oil until 0.2% concentration (by weight). To obtain the aqueous phase, we dissolved plant polysaccharide in water/anolyte/catholyte until 0.5% concentration (by weight). This plant polysaccharide was a standard stabilizer for an oil-in-water emulsion. For comparison, we used several polysaccharides, namely agar (GELAGAR IT+, B&V S.R.L., Italy), sodium carboxymethylcellulose (Acucell AF2985, Akzo Nobel Chemicals A.G., Netherlands), sodium alginate (Shandong Jeling Group Corporation Inspection Repart, China), as well as low-methoxylated apple pectin and apple fruit pectin (APA105, Yantai Andre Pectin Co. Ltd, China). We produced direct emulsions by pouring the oil phase into the water phase while stirring the mix with a mechanical stirrer at 1200 rpm for 5–10 min. All the samples were stored at room temperature.

To study the separation process, we left the emulsion to settle at room temperature for 6, 7, and 13 days. The stable component (V_{st}) was determined as a percentage from total emulsion volume. Emulsion stability depended on how well the polysaccharides were dissolved in water. The effective viscosity was measured using an RVDV II+Pro rotational viscometer (Brookfield, Spain). The morphological studies involved a light microscope (Altami, Russia) and a JSM-6390A scanning electron microscope (JEOL, Japan). The droplet size distribution

(diameter, μm) was obtained by morphometric analysis of microphotographs of the emulsion monolayer using the ImagoJ software. We appealed to the dynamic light scattering method to study the hydrodynamic radius of droplets in a Zetasizer Nano ZS analyzer (Malvern, UK). The same device made it possible to define the zeta potential of emulsion droplets. The experimental data were processed using MS Excel and graphs.

RESULTS AND DISCUSSION

Aggregative stability of 20% oil emulsion. We used polysaccharides because polar groups could respond to changes in the redox potential of the water solution. The hypothesis was that by replacing water with anolyte or catholyte with a modified redox potential value we would be able to affect the consistency, stability, and gelation of the resulting emulsion. However, unique properties of electrolyzed water solutions do not depend on the chemical composition of the water solution. As a result, they gradually return to the initial values of softened tap water.

The water emulsion samples (redox potential +340 mV, pH 7.2) contained alginate, sodium carboxymethylcellulose, or pectins. They were initially homogeneous, but the liquid part eventually became separated. The emulsion stabilized with agar formed a stable gel that did not separate even after 6 days of storage. Table 1 illustrates the comparative analysis of model emulsions stabilized by several different polysaccharides.

Sodium carboxymethylcellulose yielded the most stable model emulsion. Pectins, however, were responsible for the least stable emulsions, especially apple pectin, so we excluded them from further studies. Figure 1 demonstrates a comparative analysis of the experimental data.

Anolyte and catholyte had different effects on the stability of the oil-in-water emulsion. However, the severity and direction of this effect depended on the polysaccharide.

The emulsion samples stabilized with sodium carboxymethylcellulose initially looked like homogeneous thick liquid. The samples with water or catholyte released the liquid component after 7 days of storage. Their aggregative stability dropped by 20% for the water sample and by 40% for the catholyte sample. The emulsion that contained anolyte maintained its initial state. These differences may be attributed to the chemical structure of sodium carboxymethylcellulose, which is an anionic polyelectrolyte with a functional acidic carboxymethyl group.

All the sodium alginate emulsions eventually lost their aggregation stability by approximately 20% (Fig. 1). The liquid volume was the same in all the samples.

The agar-stabilized emulsion exhibited two states. If the aqueous phase was water or anolyte, it was a stable gel. If the aqueous phase was represented by catholyte, the emulsion split into dense and liquid components. In the latter case, the aggregative stability decreased by 25% by the end of the experiment (Fig. 1). This effect took place because agar gel develops together with hydrogen bonds between agarose molecules, a process that depends entirely on electrostatic interaction, not chemical one [29]. Probably, the negative redox value of catholyte reduced the number of hydrogen bonds, which, in its turn, reduced the stability of agar gel. In terms of emulsion stability, the sample with sodium carboxymethylcellulose and anolyte demonstrated the best results, provided that the purpose was to maintain the liquid state of the emulsion.

Table 1 Stable component vs. total emulsion stabilized with different polysaccharides stored at room temperature, %

Polysaccharide / Storage time	Alginate	Sodium carboxymethylcellulose	Apple pectin	Fruit pectin	Agar
0 days	100	100	100	100	100
6 days	85	98	48	73	100

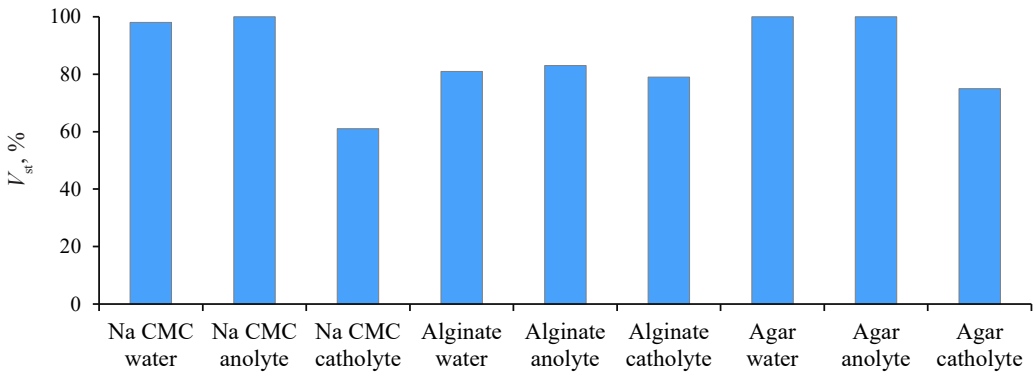

Figure 1 Stable component (V_{st}, %) vs. total emulsion (20% oil) stabilized with sodium carboxymethylcellulose, alginate, or agar after 7 days at room temperature: water – softened tap water (redox potential +340 mV, pH 7.2); Na-CMC – sodium carboxymethylcellulose; anolyte – oxidized fraction of electrolyzed water solution (redox potential +800 mV, pH 4); catholyte – reduced fraction of electrolyzed water solution (redox potential –400, pH 9)

Anolyte and catholyte failed to increase the stability of the sodium alginate emulsion.

Figure 2 demonstrates the state of the stable component in these two emulsions for 13 days, which gives an idea of how the composition stability may change during longer storage.

The curves in Fig. 2 clearly demonstrate that the stability trends for 7 days of storage persisted for 13 days. Catholyte triggered separation in both types of emulsions. This feature was most pronounced for the sample stabilized with sodium alginate. The samples with anolyte and softened water demonstrated the same changes. The emulsions stabilized with sodium carboxymethylcellulose had the best results.

In this experiment, we studied the effect of electrolyzed water solution on emulsions in a model oil-in-water system with 20% of oil and 0.2% of liquid lecithin as emulsifier. Catholyte, being the reduced fraction, accelerated separation, destroyed the emulsions stabilized by sodium carboxymethylcellulose or sodium alginate, and prevented gelation in the agar emulsion.

Aggregative stability of 30% oil emulsion. Low-calorie mayonnaises and sauces have the mass fraction of fat between 15 and 40%. We raised the fatty phase proportion to 30%. The hypothesis was that, by lowering the aqueous phase, we could affect the emulsion properties. This experiment involved only sodium carboxymethylcellulose because this polysaccharide had proved more active in stabilizing the oil-in-water system. The oil phase was prepared in line with the same method, and the mixing procedure also remained the same as described above, but the storage time was as long as 16 days (Fig. 3).

The catholyte-based sample had the best preservation effect on the emulsion with 30% of oil and sodium carboxymethylcellulose: no signs of separation were registered during the entire storage period. This effect was opposite to the one we observed when the oil phase was 20%. In that case, catholyte was more destructive. Anolyte also produced a different effect (Fig. 3), reducing the stable fraction to 30% of the total emulsion volume. The samples with water demonstrated the same kinetics at both oil concentrations.

By bringing up the oil phase proportion from 20 to 30%, we changed the conditions on the interfacial surface, i.e., surface tension, adsorption, coalescence, etc. Our results were consistent with the the effect of anolyte and catholyte on the structure and pattern of proteins and polysaccharides in water solutions reported in [30, 31].

A relatively high content of the dispersed medium might have affected the electrostatic interactions between the molecules of stabilizer biopolymers, their aggregation, and solvation. Probably, it also changed the properties of the hydrophilic molecules of the emulsifier on the interface between oil and water.

The first three days of storage saw no changes in the emulsion stability (Fig. 3) but the effective viscosity was significantly different. This indicator was 3706 mPa·s for the catholyte sample, 3544 mPa·s for the water sample, and 3316 mPa·s for the anolyte sample, respectively. The stability of each sample depended quite strongly on its effective viscosity. Therefore, the higher the initial effective viscosity value, the greater the stable component volume during storage.

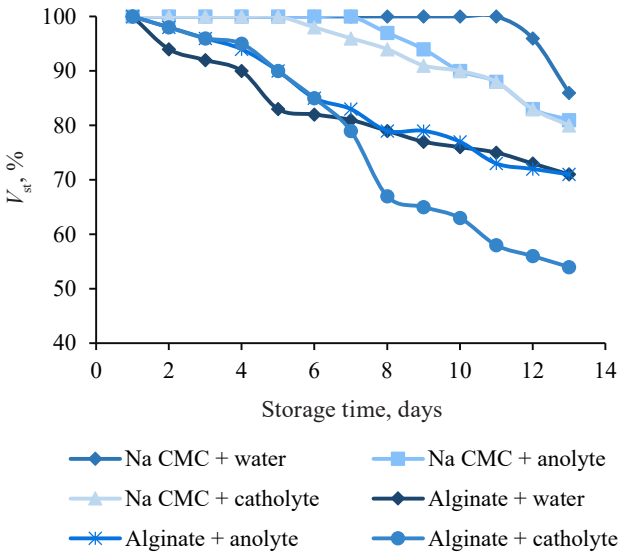

Figure 2 Stable component (V_{st}, %) vs. total emulsion (20% oil) stabilized with sodium carboxymethylcellulose or sodium alginate during 13 days at room temperature: water – softened tap water (redox potential +340 mV, pH 7.2); Na-CMC – sodium carboxymethylcellulose; anolyte – oxidized fraction of electrolyzed water solution (redox potential +800 mV, pH 4); catholyte – reduced fraction of electrolyzed water solution (redox potential –400, pH 9)

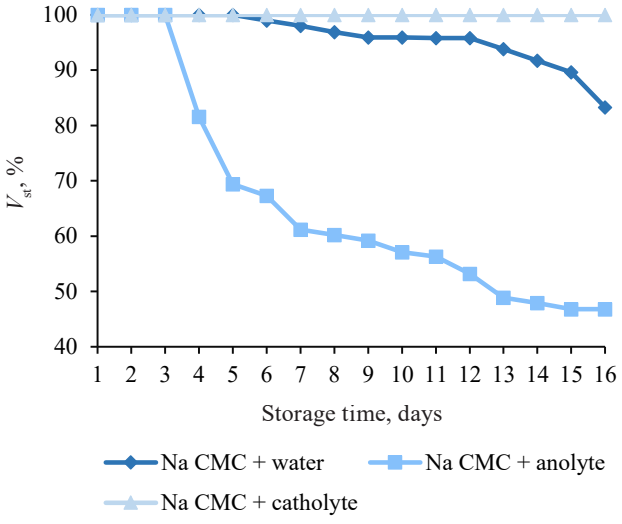

Figure 3 Stable component (V_{st}, %) vs. total emulsion (30% oil) stabilized with sodium carboxymethylcellulose or sodium alginate during 16 days at room temperature: water – softened tap water (redox potential +340 mV, pH 7.2); Na-CMC – sodium carboxymethylcellulose; anolyte – oxidized fraction of electrolyzed water solution (redox potential +800 mV, pH 4); catholyte – reduced fraction of electrolyzed water solution (redox potential –400, pH 9)

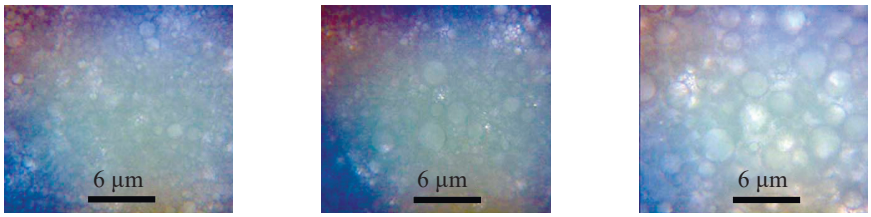

Figure 4 Emulsion (30% oil with lecithin) stabilized by sodium carboxymethylcellulose after 3 days of storage. The microphotographs were obtained in a light microscope at a magnification of 200×: A) emulsion based on softened water (redox potential +340 mV, pH 7.2); B) emulsion based on anolyte (redox potential +800 mV, pH 4); C) emulsion based on catholyte (redox potential –400, pH 9)

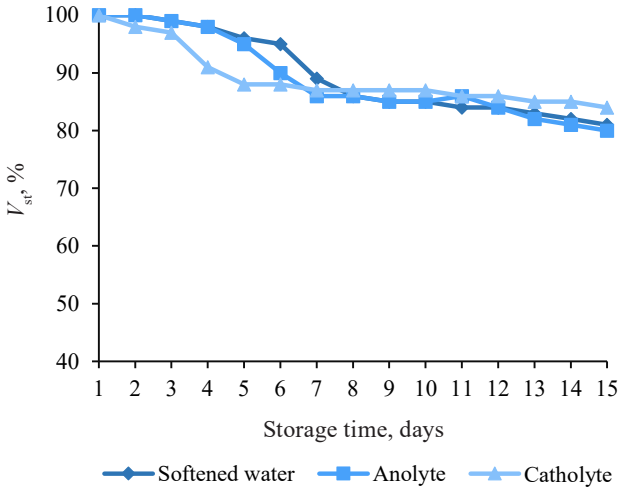

Figure 5 Stable component (V_{st}, %) vs. total emulsion (traditional mayonnaise) during 15 days at room temperature: softened tap water (redox potential +340 mV, pH 7.2), anolyte (redox potential +800 mV, pH 4), catholyte (redox potential –400, pH 9)

Under a light microscope, the samples with catholyte and anolyte demonstrated larger particles in the emulsion, compared to water-based emulsions. This trend was the strongest in the catholyte sample (Fig. 4).

This comparative experiment studied aggregative stability on model emulsions with 20 and 30% oil which were stabilized with plant polysaccharides. The aqueous phase consisted of softened water, anolyte, or catholyte.

Anolyte or catholyte affected the stability of the model system. The effect was multidirectional and depended on the concentration of oil.

Aggregative stability of mayonnaise. This part of the research featured emulsions that simulated low-energy mayonnaise or sauces and contained only plant ingredients. Such formulations follow the current trends in functional nutrition, e.g., developing new vegetarian fatty products. However, traditional mayonnaise is still in great demand.

Here, we studied the effect of replacing water with anolyte or catholyte on the aggregative stability of the emulsion system. Its composition was the same as in the target product, e.g., a popular Lyubitelsky mayonnaise (oil content of 46%) [32]. The samples were prepared according to the previously described procedure, which started by mixing together water-soluble ingredients.

The obtained dependence (Fig. 5) made it possible to evaluate the stability of mayonnaise prepared with anolyte or catholyte during 15 days of storage.

The similarity between the aggregation stability curves (Fig. 5) indicates that the fractions of electrolyzed water solution had almost no effect on the multicomponent mayonnaise. The mayonnaise was a complex system with a high oil concentration (46%) and an emulsifier in the form of animal phospholipids. All the mayonnaise samples remained highly and equally stable throughout the entire storage period.

The zeta potential of mayonnaise particles confirmed this conclusion. This parameter characterizes the stability of dispersed systems. The method of electrophoretic light scattering showed the following values: –40 ± 3 mV for the mayonnaise with softened water, –42 ± 3 mV for the mayonnaise with catholyte, and –38 ± 2 mV for the mayonnaise with anolyte.

Figure 6 shows microphotographs of emulsion particles in the traditional mayonnaise.

Figure 7 illustrates the morphometric analysis of the microphotographs in Fig. 6. The mayonnaise monolayer revealed that the particles were distributed heterogeneously. However, we detected no significant difference between the samples. These data indirectly confirmed the results of the stability experiment. The particles could be divided into three size groups. The most representative group consisted of droplets with the diameter of 0.6–0.9 µm.

The dynamic light scattering method confirmed the morphometric results. Figure 8 shows that each sample contained several groups of particles. The largest group consisted of droplets with the hydrodynamic diameter of 300–700 nm. The fraction of micron particles was also quite large, which also confirmed the morphometric data (Fig. 7). In addition, the research revealed an insignificant number of nanometer-sized particles that the light microscope could not identify because the method was limited in spatial resolution.

Considering that the anolyte or catholyte effect occurs at the interface between oil and water, we had to study the fine surface structure for an individual oil drop using the method of scanning electron microscopy.

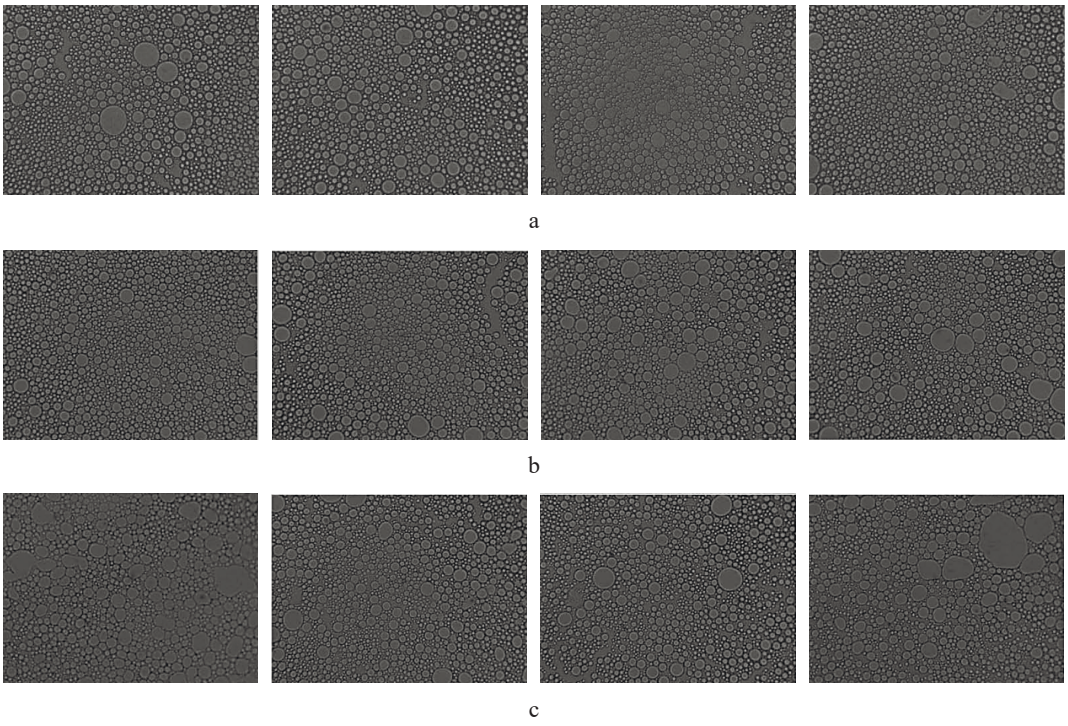

Figure 6 Monolayer of mayonnaise particles, microphotographs: samples prepared with water (a), anolyte (b), catholyte (c). The top side of all frames is 50 µm

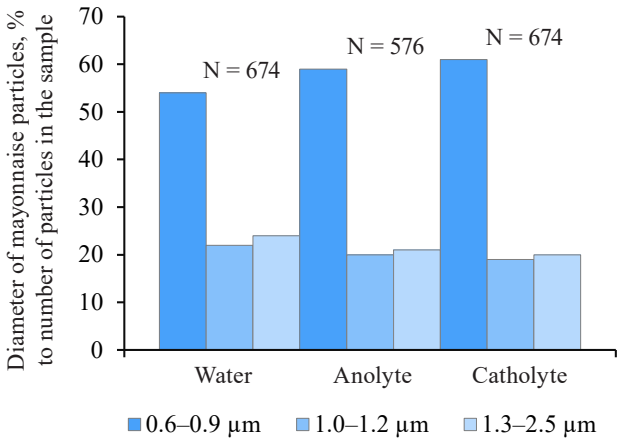

Figure 7 Diameter of mayonnaise particles after ImagoJ processing, % to number (N) of particles in the sample: softened water (redox potential +340 mV, pH 7.2), anolyte (redox potential +800 mV, pH 4), catholyte (redox potential –400, pH 9)

Figure 9 shows the results of the ultrastructural study. The microphotographs demonstrate the characteristic features of each mayonnaise sample.

The mayonnaise emulsion with water contained micron, submicron, and nano-sized particles. The high magnification revealed cavities that may point at fragmentation.

The ultrastructure of the emulsion particles with anolyte was similar to that of the previous sample but for larger particles of several microns in size. The catholyte sample also had similar visual characteristics but contained some large fat droplets which exceeded several microns.

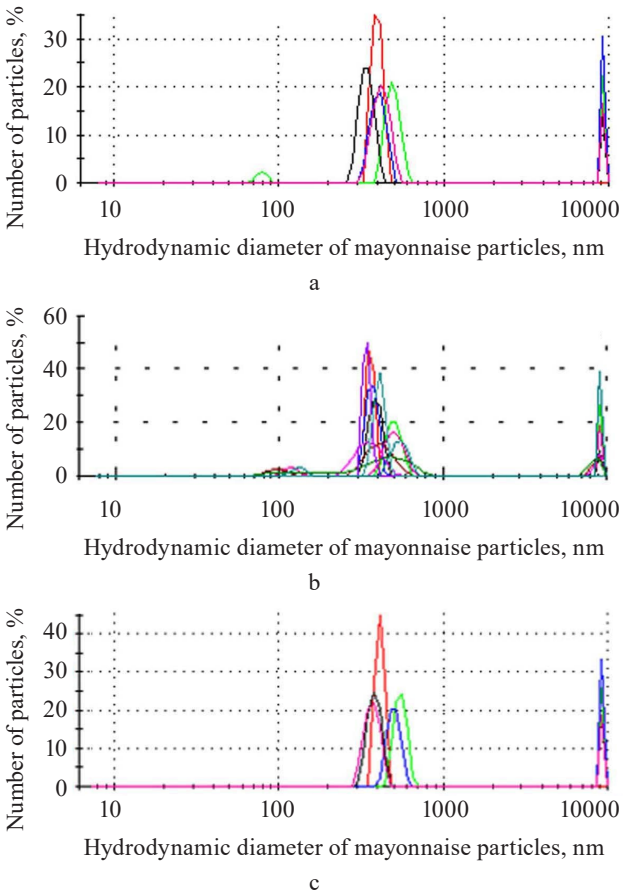

Figure 8 Hydrodynamic diameter (nm) of particles: samples prepared with softened water (a), anolyte (b), catholyte (c). The data were obtained by dynamic light, mead value from multiple tests for each sample

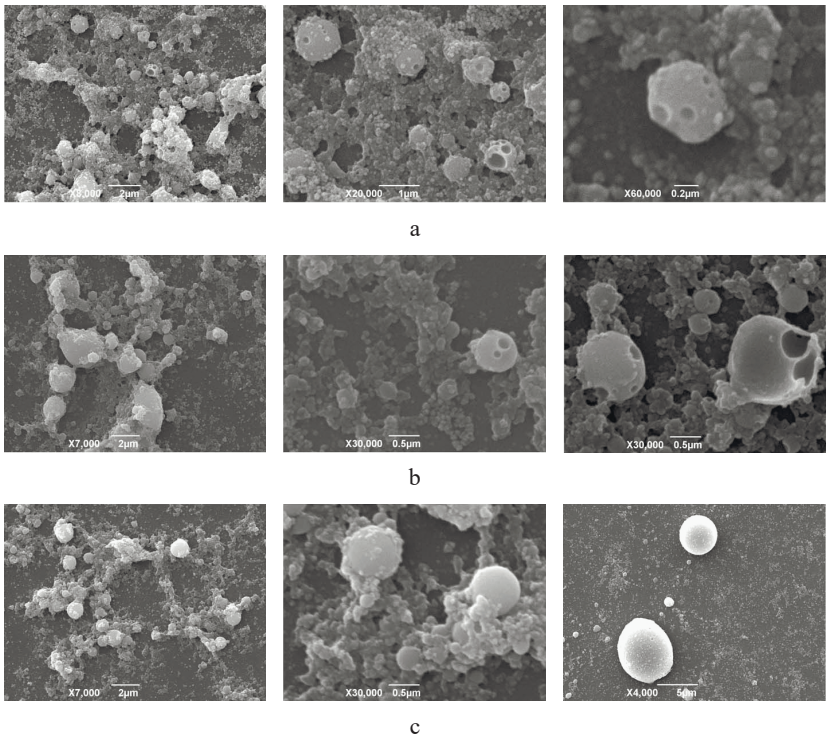

Figure 9 Mayonnaise emulsion obtained by scanning electron microscopy in the secondary electron mode. Microphotographs samples prepared with water (a), anolyte (b), catholyte (c)

These results were consistent with the micrographs and the dynamic light scattering method. Apparently, the pronounced effect of anolyte or catholyte on the aggregative stability of mayonnaise emulsion disappeared when the oil content was high and/or the mix involved a surfactant represented by phospholipids of animal origin. The effect of anolyte/catholyte on phospholipid emulsifier requires a more comprehensive study. Prospective research might include phosphatidylcholine or its derivatives, both fat-soluble and water-soluble. In addition, catholyte exhibited some antioxidant properties, which suggests that electrolyzed water solutions can protect emulsion-based products from oxidative processes during storage.

CONCLUSION

Electrolyzed water solution was able to serve as a hydrophilic phase in an oil-in-water model emulsified with fat-soluble lecithin and stabilized with polysaccharides of plant origin. It demonstrated a multidirectional effect depending on the oil phase proportion.

The catholyte fraction with a negative redox potential caused gradual separation in the emultions with a 20% share of sunflower oil stabilized by sodium carboxymethylcellulose or sodium alginate. A similar composition, but stabilized with agar, resulted in gelation, which did not occur when softened water was replaced with catholyte.

The samples with 30% of oil demonstrated an opposite effect: it was anolyte with high redox potential that destabilized the emulsion. After separation, the volume of the stable phase correlated with its initial effective viscosity. In other words, catholyte provided a more stable emulsion whereas anolyte lowered aggregation stability. Perhaps, the opposite results for the sample with 20 and 30% of oil could be explained by the changes in the interface conditions, i.e., surface tension, adsorption, coalescence, etc.

To sum it up, anolyte and catholyte fractions of electrolyzed water solution did not affect the stability of traditional high-fat mayonnaise emulsified by animal phospholipids.

Therefore, the practical application of anolyte and catholyte as means of reagent-free control of oil-in-water food emulsions is limited to low-fat products, e.g., mayonnaise sauces, emulsion drinks, and cocktails.

CONTRIBUTION

A.G. Pogorelov developed the research concept and wrote the manuscript. L.G. Ipatova interpreted the obtained data, designed the experiment, and performed the experimental studies. A.I. Panait prepared the samples for optical microscopy and processed the obtained results. A.A. Stankevich was responsible for electron microscopy. V.N. Pogorelova provided the physicochemical measurements and obtained the electrolyzed water solutions. O.A. Suvorov processed the materials and proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interests regarding the publication of this manuscript.

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Bibliometric analysis of scientific research on *Sclerocarya birrea* (A. Rich.) Hochst.

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Abstract:
Sclerocarya birrea (A. Rich.) Hochst. is an African wild tree found in many countries across the continent. Its leaves, bark, root, and fruits are used in food, cosmetics, and traditional medicine. However, no systematic bibliometric review on *S. birrea* have been performed so far.

This study covered publications made in 1986–2022 and investigated such bibliometric indicators as knowledge dynamics, links and structure, relevant journals, productive organizations, renowned authors, authoring patterns, national and international collaboration levels, etc. The bibliometric data on a total of 369 publications were downloaded from Scopus database and analyzed using the MS-Excel and VOSViewer software.

The Scopus publications on *S. birrea* showed a versatile range in the interdisciplinary areas of research, with agricultural and biological sciences predominating. The Republic of South Africa was responsible for the largest number of citations. All in all, 63 countries published research on *S. birrea* in the specified time period, and the share of African countries was 65.43% of the total global research. Co-authorship and international collaboration were registered for six clusters. A total of 1114 researchers and 160 institutions actively contributed to *S. birrea* studies, with 37 having a minimum of five publications. Mapping of knowledge structure using the keyword co-occurrence revealed the related research areas. The Journal of Ethnopharmacology took the lead in terms of average citation.

The co-citation analysis revealed some remarkable dynamics in *S. birrea* research. This bibliometric study provides a complete insight into *S. birrea* research development and publication patterns over the last 36 years.

Keywords: Bibliometric analysis, citation analysis, research-based data, *Sclerocarya birrea*, marula, Scopus, VOSViewer

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INTRODUCTION

Sclerocarya birrea (A. Rich.) Hochst. is a savannah tree from the family of *Anacardiaceae*. Its English name is marula. The tree requires sandy or alluvial soils; propagation is carried out with seeds or cuttings. It grows in a wide variety of soils but prefers well-drained soil. The marula survives at altitudes varying from sea level to 1800 m and at an annual rainfall range of 200–1500 mm [1–3]. The fruits are pale yellow and plum-like, with a plain tough skin and juicy, mucilaginous flesh [4].

Glew *et al.* reported that *S. birrea* seed contained relatively large amounts of copper (24.8 mg/g dry weiht), magnesium (4210 mg/g dry weight), and zinc (62.4 mg/g dry weight) [5]. The protein content of the pit was high (36.4% of dry weight); however, the protein fraction was low in leucine, phenylalanine, lysine, and threonine. The potential benefits of marula oil are due to its high content of antioxidants and some important nutrients. In addition, marula oil also contains monounsaturated and polyunsaturated fats, which are good for health [6].

Marula oil can enhance hair health by moisturizing and fighting dryness without leaving any greasy impact, especially when used in moderate amounts [6]. Marula oil can stimulate hair in cases of dandruff or infection as it moisturizes the scalp and relieves it from dead skin cells [7]. Marula oil contains healthy fatty acids. When used in cooking, it regulates the level of cholesterol in the body, which improves the health of the heart and blood vessels, thus reducing the possibility of circulatory diseases, e.g., stroke, heart attack, and atherosclerosis [8]. Marula oil was reported to help pregnant women with skin issues [9, 10]. Marula oil contains antioxidants. As a natural antimicrobial, it can serve as a wound sterilizer that prevents infection and germs from entering the body [11].

Marula leaves and fruit pulp can treat hypertension; the leaves are used against diabetes, dysentery, snake and scorpion bites, malaria, and inflammations. The plant is also utilized as a tonic, and the fruits are often fermented as a raw material for a refreshing drink. Chemical and pharmacological studies on the bark have reported antidiarrheal activity of the decoction. It is a source of phenolic compounds, such as procyanidins and (-)epicatechin-3-galloylester. No research has been done so far on the chemical composition of *S. birrea* leaves, although their extract was reported to exhibit the activity with Ca^{2+} mobilizing systems in muscle cells [12]. Methanolic extracts from different parts of the *S. birrea* tree were examined for antioxidant properties. They proved to contain phenolic compounds that inhibit the oxidation of linoleic acid and the subsequent bleaching of beta-carotene. The seed extract was more active, followed by extracts of roots, leaves, and bark [13].

S. birrea extract was tested against diabetes type II in an *in vitro* study, where it was found efficient in reducing blood pressure in diabetic rats [14]. *S. birrea* methanolic and aqueous extracts demonstrated antimalarial activities and were found effective against different plasmodia, the methanol extract being more active than the aqueous one [15]. In traditional African medicine, *S. birrea* treats hypertension, dysentery, infertility, schistosomiasis, constipation, abdominal cramps, stomachache, sore eyes, toothache, backache, body pains, and gastroenteritis. The lyophilized decoction of this plant showed great anti-diarrheic activity [16–21].

The bibliometric analysis and the literature review are tools of knowledge management, prospecting, and mapping [22]. Scientific research in medicinal plants is one of the richest fields of scientific exploration because it brings together many scientific fields, e.g., chemistry, pharmacology, veterinary sciences, botany, pharmaceutics, traditional knowledge, and computer science [23]. *S. birrea* is a wild tree that grows all over the African continent and is used in nutrition, cosmetics, and traditional medicine by many African nations. As a result, *S. birrea* is a popular research object and a source of numerous studies. However, it has acquired no bibliometric profile so far. This study fills in the gap by investigating into the patterns of scientific research and the most important researchers, universities, and countries that dealt with *S. birrea* in 1986–2022. Other factors involved research dynamics and development, collaboration, structure, and impact.

STUDY OBJECTS AND METHODS

Bibliometrics is a method of determining trends in publications and research in a certain field of study by analyzing data quantitatively [24, 25]. This study looks at author cooperation networks, scientific outputs and contributions, scientific impact, institutional partnerships, national shares, etc. The authors performed a complete analysis of data on involvement and collaborations among researchers worldwide, their affiliations and journals that publish articles on *Sclerocarya birrea* (A. Rich.) Hochst. The bibliometric study relied on the Scopus database. Such bibliometric studies are crucial for consistency and accuracy in data needed for analysis of prior works and identifying research prospects.

Selecting database and extracting bibliometric data. Scientific information can be retrieved from various databases, e.g., PubMed, Scopus, and Clarivate WOS [26]. We extracted 92, 346, and 302 publications from PubMed, Scopus, and Clarivate WOS, respectively. We chose Scopus because it is more comprehensive and contains much a greater number of research documents on the matter, compared to WOS and PubMed [27]. Also, PubMed does not contain scientific papers that are concerned with pure botanical research. Scopus is an Elsevier-produced platform that provides indexing and abstract database with research publication linkages. Scopus was founded in 2004 and now hosts over 70 million papers from a variety of disciplines [28]. It is popular among scholars and simple to use. Scopus also provides extensive data and insights on research papers across a wide range of fields, with life and natural sciences leading the way [29–31]. The database also includes detailed biographies of authors, their affiliations, funding sources, citation statistics, and number of publications, as well as the nations that lead in each study sector.

Selection criteria. The analysis involved only scientific articles and conference papers published after 1986. Books were excluded because they usually summarize research results published in scientific papers. Although the original sampling involved articles published in other languages, the research was limited to the English language and the existing indexing. We did not restrict the mechanism for selecting researchers and journals.

Search criteria. We limited the search to article titles that contained "*Sclerocarya birrea*". The search command was restricted to the following parameters: publication year (PUBYEAR > 1986 AND PUBYEAR January 11 2022) and document type (DOCTYPE Article OR Conference papers). The results of the Scopus database search instructions were exported as a CSV Microsoft Excel, RIS format, plain text in HTML format, and BibTex files. Figure 1 illustrates the approach we applied to this bibliometric analysis.

Bibliometric and bibliographic networks: constructing and visualizing. The VOSViewer software

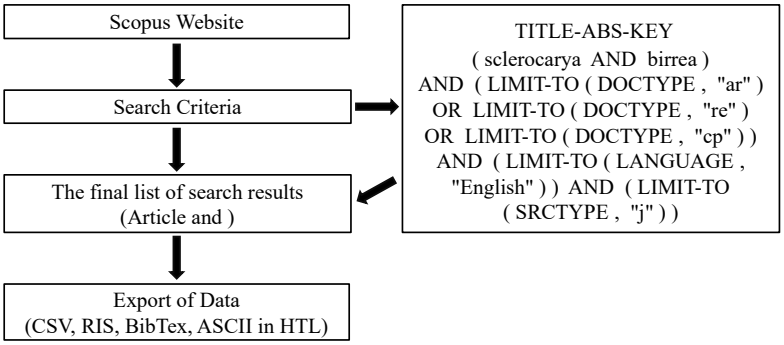


Figure 1 Search criteria

(Version 1.6.15, Leiden, Netherlands) is a program for creating and displaying bibliometric networks. These networks may be built via citation, bibliographic coupling, co-citation, or co-authorship relationships, and can comprise journal coupling, researchers, countries, institutional engagement, or individual articles. VOSViewer also has text mining capabilities, which may be used to build and display co-occurrence networks of key phrases retrieved from scientific literature [32, 33]. *S. birrea* proved to have yielded quite a lot of publications between 1986 and 2022.

RESULTS AND DISCUSSION

In this study, we set the time frame from 1986 to January 2022 because we found no alternative bibliometric studies on *Sclerocarya birrea* (A. Rich.) Hochst. for reference. The first paper on *S. birrea* was published in the *Botanical Journal of the Linnean Societ*y. All in all, we retrieved 369 research documents, 349 of which were original research papers, 25 belonged to review articles, and two could be classified as conference proceedings. Hover, Table 1 gives the total number of publications as 368, including articles (89.1%), reviews (7.0%), conference papers (2.6%), book chapters (1.0%), and editorials (0.3%). Such a substantial time frame made it possible to predict the research trends by analyzing its history.

International patterns. The time interval we identified for our analysis represented a milestone in the *S. birrea* research. The last fourteen years of the XX century (1986–2000) saw only 22 research papers (6.4%). In the new millennium, the publication rate reached 93.6% (n = 324) (Fig. 2). *S. birrea* prominence and potential cross-field applications may also be related to the prolific research output. The new publications revealed that every component of the *S. birrea* tree has some beneficial properties. The scientific community seems to have recognized its socioeconomic and medicinal potential [12, 17, 34–36]. Figure 2 shows that, despite the numerical increase in the research documents, the broken curve does not have a fixed frequency in the number of research papers for all years, with an average of 9.6 papers per year. The years 2008, 2011, 2016, and 2021 saw the largest numbers of publications with 20, 28, 20, and 21 documents, respectively. However, no scientific paper on *S. birrea* appeared in 1994.

Table 1 Types of Scopus publications on *Sclerocarya birrea* (A. Rich.) Hochst.

Type of documents	Number	Percentage, %
Article	328	89.1
Review	26	7.0
Conference Paper	10	2.6
Book Chapter	4	1.0
Editorial	1	0.3
Total records	368	100.0

Interdisciplinary areas of research, citations, and geographical distribution in 1986–2022. The *S. birrea*-related publications appeared to cover a versatile range of interdisciplinary research areas. All in all, the sampling demonstrated 22 fields of studies. Agricultural and biological sciences led in terms of research outputs, accounting for 35.8% of the overall number. Pharmacology, toxicology, and pharmaceutics (11.9%), medicine (10.5%), biochemistry, genetics and molecular biology (9.6%), as well as environmental science (9.1%) accounted for 41.1% (Fig. 3). Veterinary studies on *S. birrea* represented 1.7%. This result indicated the importance of *S. birrea* in African countries for treating and feeding domestic animals. It proved efficient against such endemic parasites as Trypanosoma, worms, and ticks [37–41]. Other ethnoveterinary treatments included the use of indigenous medicinal herbs to prevent and treat a variety of animal diseases. This application of *S. birrea* in farming can be termed traditional knowledge [42].

According to the VOSViewer analysis of worldwide research involvement by countries, 63 nations published original research articles or conference papers (Table 2). South Africa occupied the first place in the number of publications, i.e., 32.16%. Publications from outside the African continent were responsible for 34.57% with the highest number of citations being 3781. The United States, Germany, and the United Kingdom were the leading Western countries involved in the *S. birrea* studies. Kenya, the Republic of the Sudan, Zimbabwe, Burkina Faso, Nigeria, Namibia, and Cameroon participated with 3.14, 3.14, 3.14, 2.59, 2.59, 2.40, and 2.22%, respectively. African countries provided research representing 65.43% of the total global *S. birrea* research (Fig. 4) because Africa

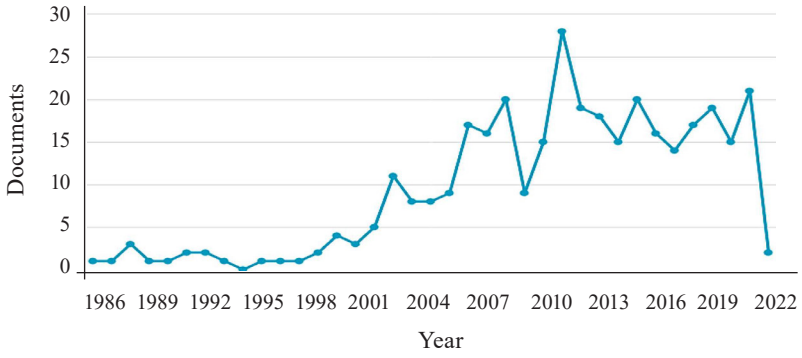


Figure 2 International pattern of publications

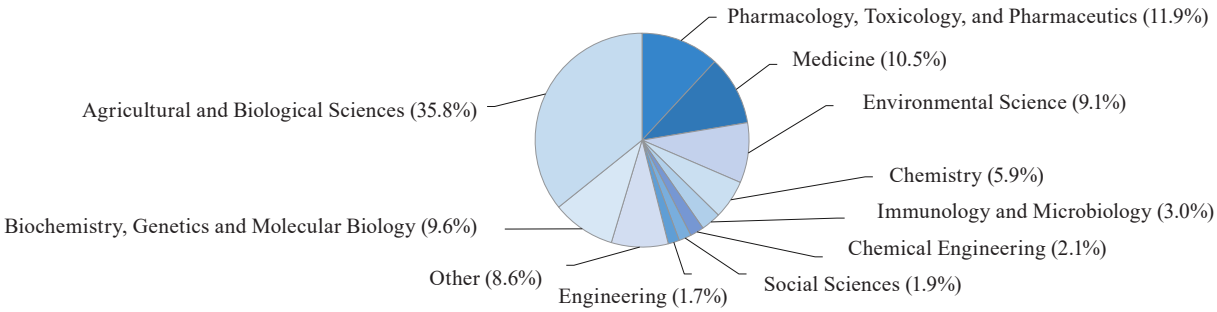


Figure 3 Distribution of Scopus-extracted documents related to *Sclerocarya birrea* (A. Rich.) Hochst. by subject area

Table 2 Worldwide research involvement, citation, and total link strength by countries

Country	Number of documents	Percentage	Citations	Average citation per publication	Total link strength
South Africa	174	32.16	3781	21.73	746
United States	24	4.44	753	31.38	148
Germany	21	3.88	527	25.10	120
United Kingdom	21	3.88	751	35.76	200
Kenya	17	3.14	669	39.35	115
Sudan	17	3.14	466	27.41	135
Zimbabwe	17	3.14	325	19.12	102
Burkina Faso	14	2.59	320	22.86	51
Nigeria	14	2.59	250	17.86	72
Namibia	13	2.40	279	21.46	193
Cameroon	12	2.22	264	22.00	115
Botswana	11	2.03	179	16.27	46
France	10	1.85	138	13.80	53
Denmark	9	1.66	173	19.22	61
Sweden	9	1.66	162	18.00	21
Benin	8	1.48	100	12.50	64
Malawi	8	1.48	155	19.38	88
Senegal	8	1.48	137	17.13	18
Spain	8	1.48	138	17.25	96
Australia	7	1.29	265	37.86	180
Belgium	7	1.29	220	31.43	61
Italy	7	1.29	272	38.86	62
Netherlands	7	1.29	194	27.71	44
Switzerland	7	1.29	119	17.00	54
Tanzania	7	1.29	219	31.29	29
China	6	1.11	64	10.67	67
India	6	1.11	110	18.33	17
Swaziland	6	1.11	60	10.00	22
Israel	5	0.92	46	9.20	32
Mali	5	0.92	177	35.40	40

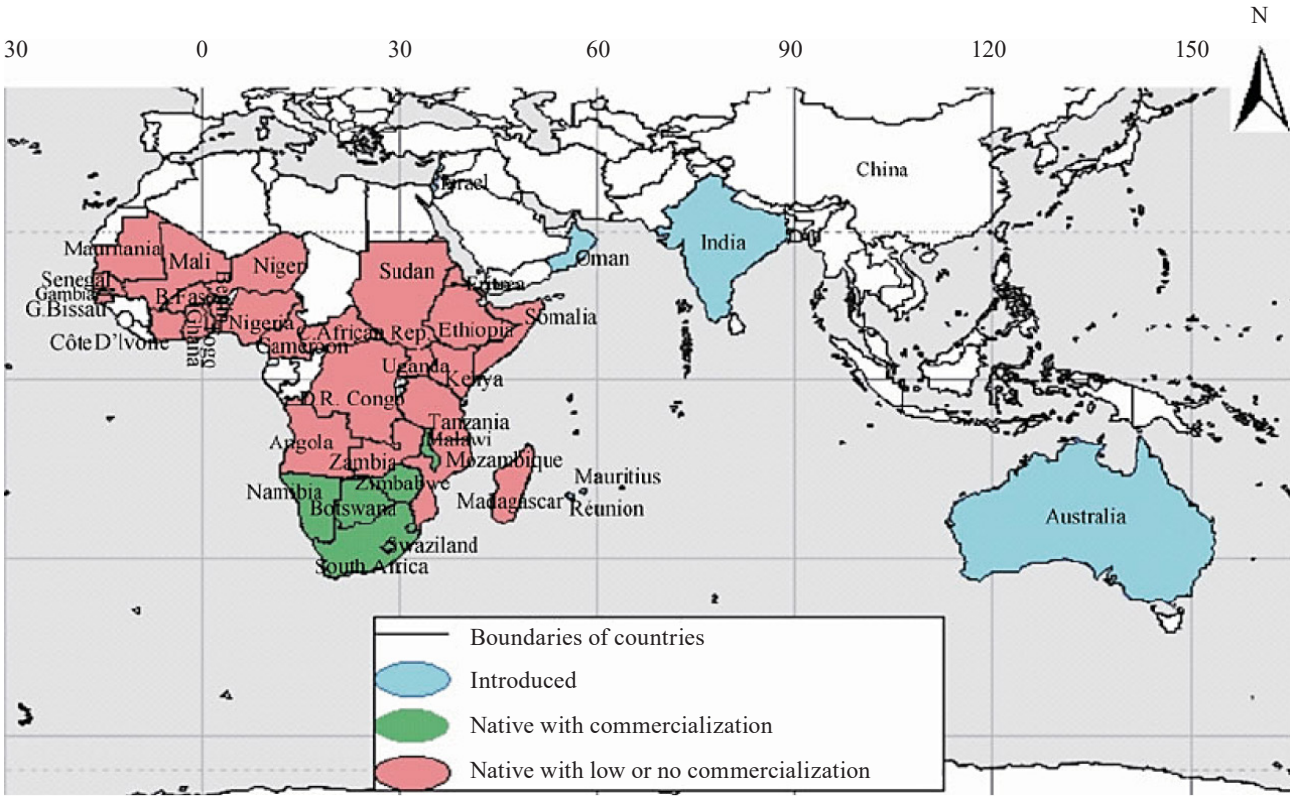


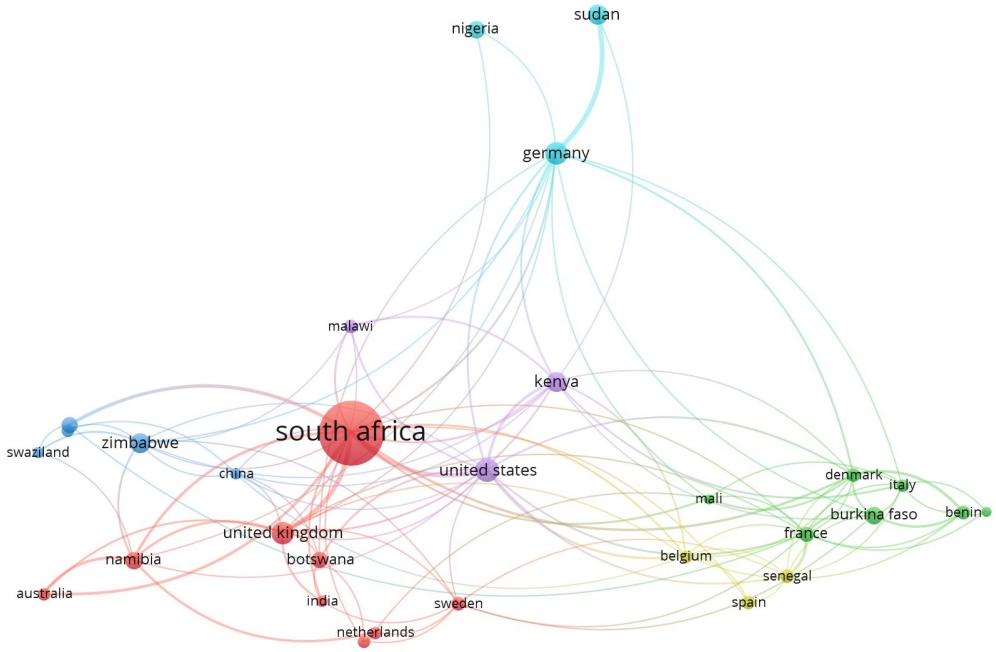
Figure 4 Geographical Distribution of *Sclerocarya birrea* (A. Rich.) Hochst. [43]

is its natural habitat with the tropical conditions it needs for growth [43]. In spite of the fact that marula also grows in India and Australia, these two countries contributed as little as 2.4% from the global research on *S. birrea*.

Citation analysis is a method of determining the relevance of an author or an article by counting the number of references to this author or article in other publications. National research assessments are conducted on a regular basis in numerous countries. They make it possible to compare citation indicators and peer ratings [44]. In this research, the Republic of South Africa appeared to have the largest number of citations, followed by the United States, Germany, the United Kingdom, Kenya, the Sudan, Zimbabwe, Burkina Faso, Nigeria, Namibia, Cameroon, Botswana, and France. The large number of citations obtained by Western countries indicated the global interest in *S. birrea* because of its medicinal and nutritional values [17, 34, 37, 38].

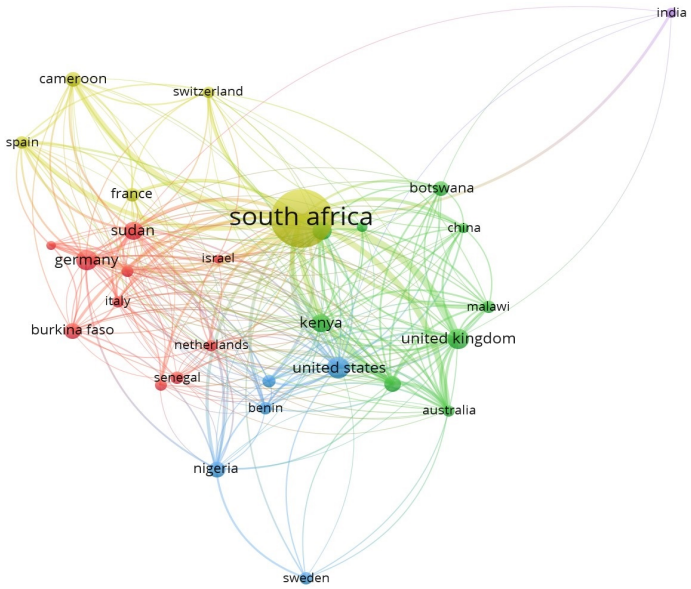
International collaboration and co-authorship links between countries. We analyzed the collaborative networks between various nations with a minimum of five documents published using VOSViewer (Fig. 5). The analysis revealed six clusters. We used the association method of normalization. Maximal iterations were 1000 steps, and the step size convergence was 0.001. Individual components (circled) were assembled to create a cluster and connected together by lines to represent partnerships or networks and their strength [25, 32, 33].

Among all the countries (n = 63) that published *S. birrea* research, only 30 countries fulfilled the criteria of five documents. We connected these groups by lines to represent the strength of co-authorship and scientific collaboration. South Africa had the largest network of research collaboration as presented in Cluster 1 with the total link strength of 56. In this cluster, South Africa had the highest number of documents, followed by the United Kingdom and the United States with link strengths of 11 and 10, respectively. This fact could be evidenced by the size of the dark red circle (Fig. 5). Cluster 2 (green) had 7 countries; France and Burkina Faso had the highest collaboration network with other countries in this cluster with total link strengths of 19 and 13, respectively. Cameroon, China, Swaziland, Switzerland, and Zimbabwe fell in Cluster 3 (blue). Zimbabwean researchers showed the highest strength in their international collaboration with total link strength and the number of documents of 12 and 17, respectively. Cluster 4 (gold) included three countries, namely Belgium, Spain, and Senegal. The importance of bibliometric analysis in understanding the role of impactful research is visible by the differences in the members of Cluster 4. Thus, Spain (3) and Senegal (12), regardless of their differences nominal GDP rankings, showed great variation in the total link strength indicator. The United States, Kenya, and Malawi were grouped together in Cluster 5 (purple) with total link strengths of 34, 21, and 9, respectively. Cluster 6 involved Germany, the Sudan, and Nigeria with 29, 17, and 14 publications, respectively. Although Nigeria had 14 publications, the total link strength showed that their international collaboration network was low, compared to other topmost



Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6

Figure 5 International collaborations with countries with a minimum of five publications in 1986–2022



Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5

Figure 6 Visual representation of citations with countries with a minimum of five citations in 1986–2022

productive countries in the same cluster, e.g., the Sudan. One of the co-authors of this article participated in the international collaboration between Sudan and Germany with a link strength of 12. This co-authorship was a product of joint research between the Max Rubner Institute, Germany, and the Sudan University of Science and Technology, which yielded 10 publications [4, 13, 45–52].

Figure 6 provide a visual representation of citations for countries with a minimum of five citations in 1986–2022. Five clusters were extracted using association as a method of normalization. Maximal iterations were 1000 steps; the step size convergence was 0.001. Cluster 1 (dark red) involved ten countries with a total link strength of 1526. This cluster included the Republic of

Table 3 Institutions with at least eight publications on *Sclerocarya birrea* (A. Rich.) Hochst.

Institution	Country	Total publications	Total citations
University of Pretoria	South Africa	29	757
University of the Witwatersrand	South Africa	28	563
University of KwaZulu-Natal	South Africa	20	444
University of Limpopo	South Africa	19	225
University of Fort Hare	South Africa	16	284
Sudan University of Science and Technology	Sudan	11	243
University of South Africa	South Africa	10	42
Tshwane University of Technology	South Africa	10	191
The Council for Scientific and Industrial Research	South Africa	10	383
University of Zululand	South Africa	10	305
Max Rubner Institute	Germany	10	227
Rhodes University	South Africa	9	350
University of Gezira	Sudan	9	200
University of Witwatersrand	South Africa	9	295
University of Cape Town	South Africa	8	232

the Sudan, Germany, Italy, Belgium, Burkina Faso, the Netherlands, Mali, Israel, Tanzania, and Senegal, with the Total Link Strength of 135, 120, 62, 61, 51, 44, 40, 32, 29, and 18, respectively. The Sudan and Germany were the most cited countries in this cluster. The greater number of citations for the Sudan gave it preference in this group, despite the greater number of documents for Germany. Clusters 2 (green), 3 (blue), 4 (gold), and 5 (purple) comprised 9, 5, 5, and 1, respectively. India was the only representative of Cluster 5.

Institutions and universities involved in *S. birrea* research in 1986–2022. A total of 160 institutions contributed to *S. birrea* studies, with 37 having at least five publications. Table 3 also shows the number of publications and citations for the universities with a minimum of eight documents. The number of universities and institutes from the Republic of South Africa represented about 70.05%, while the Sudan, Germany, and Namibia represented about 29.5%. The University of Pretoria, South Africa, had 29 publications and 757 total citations, which makes it university number one in the world in *S. birrea* research production and impact. Quantitative measures, such as citation counts, are often used to assess the research impact [53]. We used this index to compare the number of citations for the three universities that appeared to have the same number of scientific papers but differed in the number of citations. The University of South Africa, the Tshwane University of Technology, and The Council for Scientific and Industrial Research delivered 10 scientific papers, each with the number of citations as 42, 191, and 383, respectively (Table 3).

Mapping knowledge structure by keyword co-occurrence. The co-occurrence analysis provides an overview of the structure of the subjects researched, as assessed by the number of occurrences. The data are visualized as nodes of different size [54]. In the analysis of keyword co-occurrence, 362 out of 5617 keywords met the threshold of at least five occurrences, producing five clusters, 5532 links, and a total link strength of 16 413 (Fig. 7). The different colors indicate different general research foci. "*Sclerocarya birrea*" was the most popular key word (n = 262) with 153 links and a total link strength of 5532. In the analysis of the co-occurrence of authors' keywords, 24 out of 1134 keywords met the threshold with a minimum of five occurrences, producing six clusters, 45 links, and a total link strength of 80 (Fig. 8). The top occurrences were: *Sclerocarya birrea* (n = 66), marula (n = 29), and medicinal plants (n = 21). Figure 9 visualizes the co-occurrence of 4480 index keywords, i.e., those chosen by publishers and standardized using publicly accessible vocabularies. Unlike authors' keywords, indexed keywords take into consideration synonyms, spelling variations, and plurals [55]. VOSViewer extracted four clusters, 19 173 links, and a total link strength of 48 712. When we compared the number of all keywords (5617) with the index keywords (4480), we found out that the authors used all the indexed keywords.

Source of publications and bibliometric coupling. Between 1986 and 2022, a total of 187 periodicals published research works on *S. birrea*, i.e., original papers and conference proceedings. Table 4 focuses on journals with a minimum of five publications. Only ten journals satisfied this criterion, with *Ethnopharmacology* (Elsevier) having published 25 papers on *S. birrea*. Most of the journals are hosted by Elsevier, Springer, and Wiley Publishers. However, the *South African Journal of Science* became a standalone source. The *Journal of Ethnopharmacology* (Elsevier) and *Forests, Trees, and Livelihoods* (Taylor & Francis) took the lead in terms of average citation per journal. According to the data indexed in Scopus, agricultural and biological sciences led in terms of research outputs on *S. birrea*. The top three journals fell in the scope of this field. Figure 10 visualizes the analysis of coupling of all journals: 10 out of 187 journals met the threshold of at least five bibliometric couplings, producing three clusters, 42 links, and a total link strength of 1284. When two works in a bibliography refer to the same third work, it is known as bibliographic coupling.

Bibliographic coupling is a sign that the two works are likely to be about the same thing. When two documents share one or more citations, they are said to be bibliographically connected [56]. The *South African Journal of Botany* had the highest total link strength (531) followed by the *Journal of Ethnopharmacology* (521). The *Tropical Animal Health and Production* had the lowest total link strength (25).

Authors' contribution in *S. birrea* research in 1986–2022. A total of 1114 researchers were identified as *S. birrea* research authors. Only 21 of these co-authors satisfied the minimal requirement of five marula-related articles within the specified time frame. Table 5 depicts the most prolific *S. birrea* authors. With 11 total publications, Alfred Maroyi of the University of Fort Hare, South Africa, had the most prolific research output. Bertrand Matthäus from the Max Rubner Institute, Germany, had 11 publications. Finnie, van Staden, and Witkowski delivered 10 articles. The role of bibliometric analysis is to combine various data, such as citations and number of

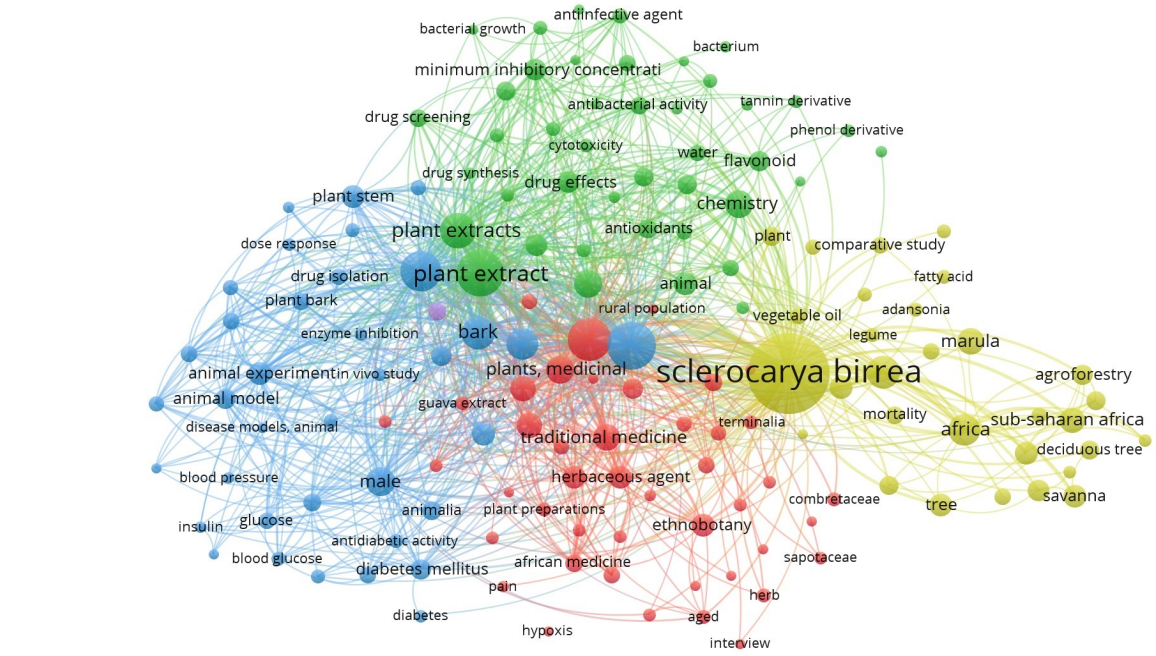


Figure 7 Visual representation of co-occurrence for all keywords with a minimum of five keywords in 1986–2022

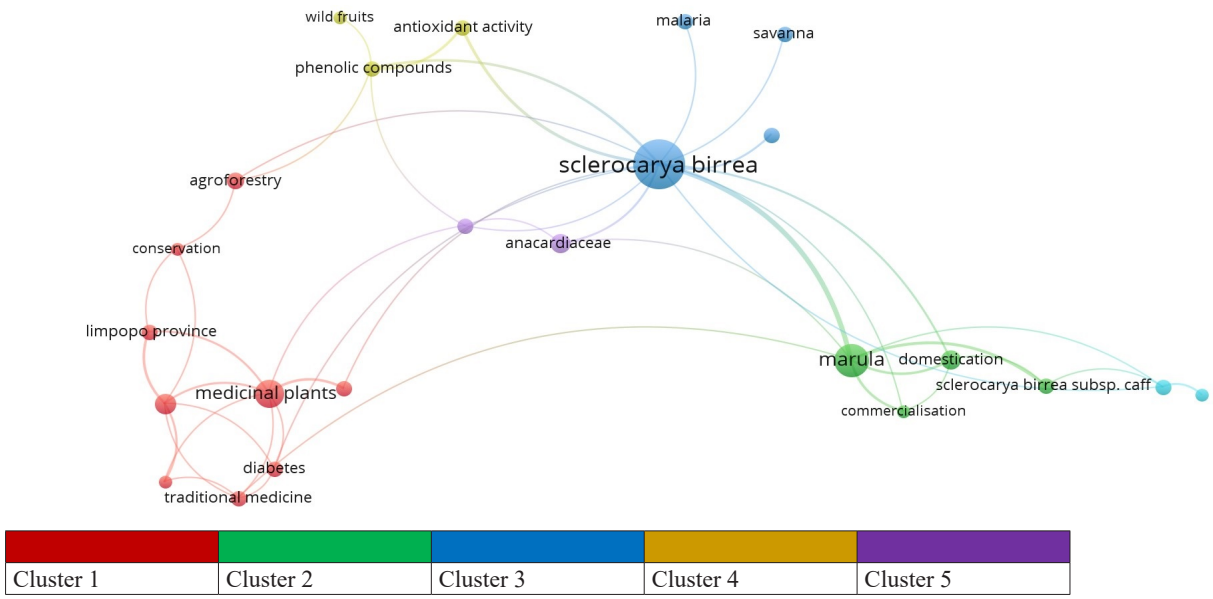


Figure 8 Visual representation of co-occurrence for authors' keywords with a minimum of five keywords in 1986–2022

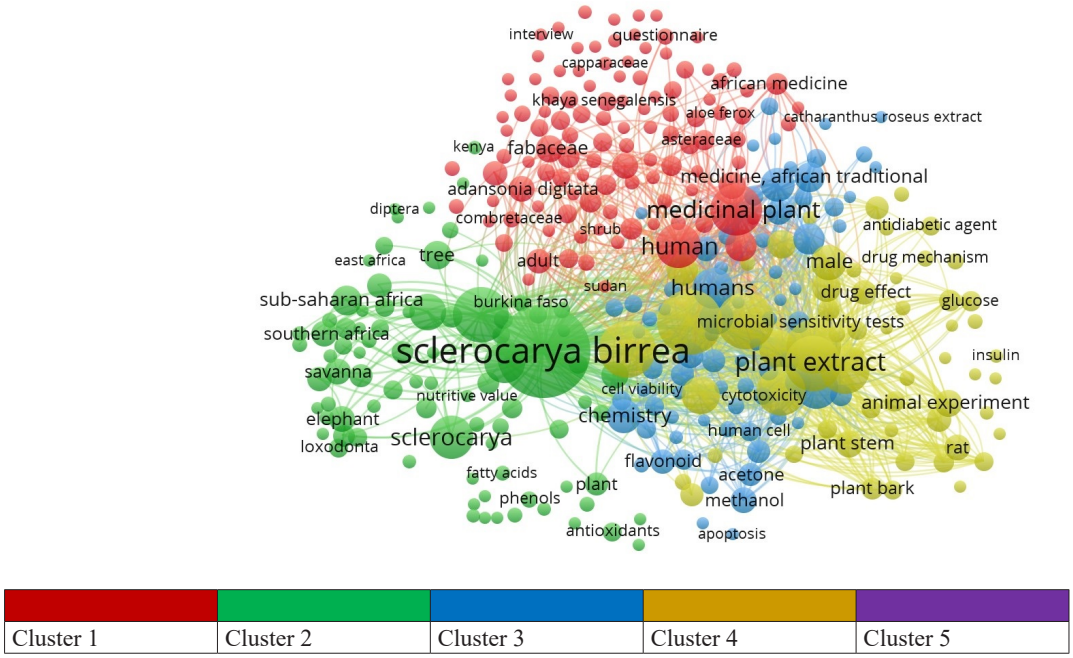


Figure 9 Visual representation of co-occurrence of index keywords with a minimum of five keywords in 1986–2022

Table 4 Journals with a minimum of five publications in 1986–2022

Source (Publisher)	Documents	Citations	Average citation per journal	Total link strength	ISI-impact factor
Journal of Ethnopharmacology (Elsevier)	28	1256	44.86	40	4.360
South African Journal of Botany (Elsevier)	21	361	17.19	41	2.315
Agroforestry Systems (Springer)	9	233	25.89	24	2.549
African Journal of Biotechnology (Academic Journals)	6	114	19.00	11	NA
African Journal of Ecology (Wiley)	6	85	14.17	11	1.426
Forest Ecology and Management (Elsevier)	6	146	24.33	26	3.558
Forests, Trees, and Livelihoods (Taylor & Francis)	6	266	44.33	20	NA
Phytotherapy Research (Wiley)	6	211	35.17	23	5.882
South African Journal of Science (Stand Alone)	5	65	13.00	5	2.197
Tropical Animal Health and Production (Springer)	5	12	2.40	1	1.559

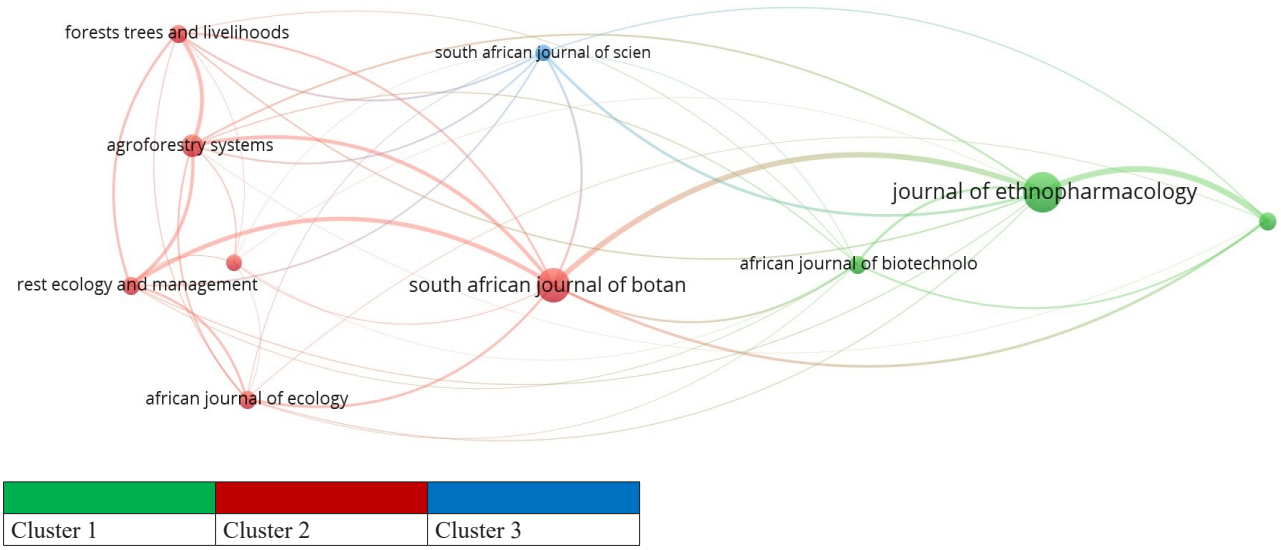


Figure 10 Visual representation of bibliographic coupling based on journals with a minimum of five keywords in 1986–2022

Table 5 Authors with a minimum of five publications

Author	Documents	Citations	Total link strength
van Staden, J.	13	319	37
Maroyi, A.	11	131	37
Matthäus, B.	11	247	59
Finnie, J.F.	10	270	33
Witkowski, E.T.F.	10	179	46
Hussein, I.H.	9	200	41
Mariod, A.	9	218	44
Moyo, M.	9	272	33
Semenya, S.S.	8	114	18
Van Vuuren, S.F.	8	289	45
Eichner, K.	7	182	37
Eloff, J.N.	7	205	31
Ojewole, J.A.O.	7	284	48
De Wet, H.	6	295	40
Ndip, R.N.	6	161	26
Dimo, T.	5	98	31
Helm, C.V.	5	116	40
Kamtchouing, P.	5	98	31
Lykke, A.M.	5	112	11
Shackleton, C.M.	5	191	45
Shackleton, S.	5	164	19

Table 6 Top ten cited articles

No.	Title	Number of citations	Journal	Reference
1	Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions	73	Malaria Journal	[59]
2	Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (*Anacardiaceae*) extracts	59	Food Chemistry	[60]
3	Antidiabetic screening and scoring of 11 plants traditionally used in South Africa	37	Journal of Ethnopharmacology	[61]
4	Chemical composition and antioxidant activity of phenolic compounds from wild and cultivated *Sclerocarya birrea* (*Anacardiaceae*) leaves	24	Journal of agricultural and food chemistry	[12]
5	Towards the development of Miombo fruit trees as commercial tree crops in Southern Africa	20	Forests, Trees and Livelihoods	[62]
6	Improving discrimination of Savanna tree species through a multiple-endmember spectral angle mapper approach: canopy-level analysis	20	IEEE Transactions on Geoscience and Remote Sensing	[63]
7	Ethnobotanical study of medicinal plants in the Blue Nile State, South-Eastern Sudan	18	Journal of Medicinal Plants Research	[64]
8	Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections	11	Journal of Ethnopharmacology	[65]
9	Polyphenol content and antioxidant activity of fourteen wild edible fruits from Burkina Faso	11	Molecules	[66]
10	Host plants and host plant preference studies for *Bactrocera invadens* (Diptera: Tephritidae) in Kenya, a new invasive fruit fly species in Africa	6	Annals of the Entomological Society of America	[67]

publications, and produce some indicators to evaluate the real knowledge impact of particular researchers [57, 58]. Therefore, Maroyi proved to have a greater number of publications than van Staden, but Maroyi had a greater impact on generating knowledge and research quality (Table 5). Table 6 sums up the top cited articles.

We determined the average number of publishing and active years of an author's outputs using a co-authorship analysis. Figure 11 shows a VOSViewer-based co-authorship analysis. The co-authorship network of authors was limited by a minimum of three publications made in 1986–2022. We extracted 28 clusters using the method

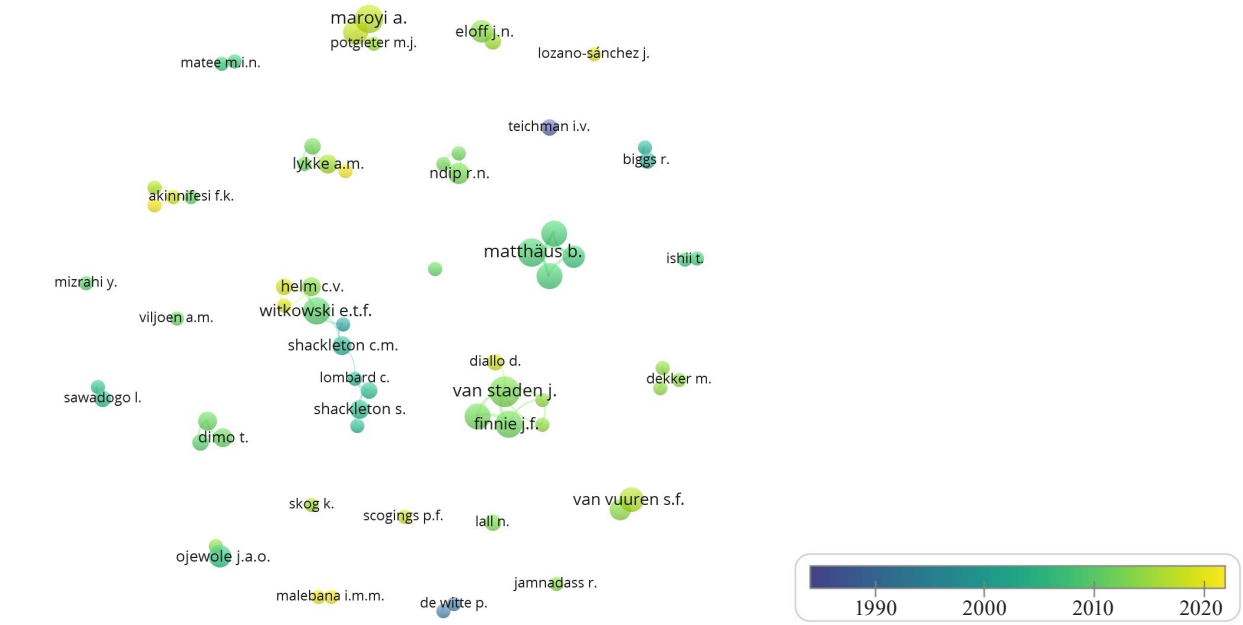


Figure 11 Co-authorship network of authors with a minimum of three publications in 1986–2022 based on average publications per year

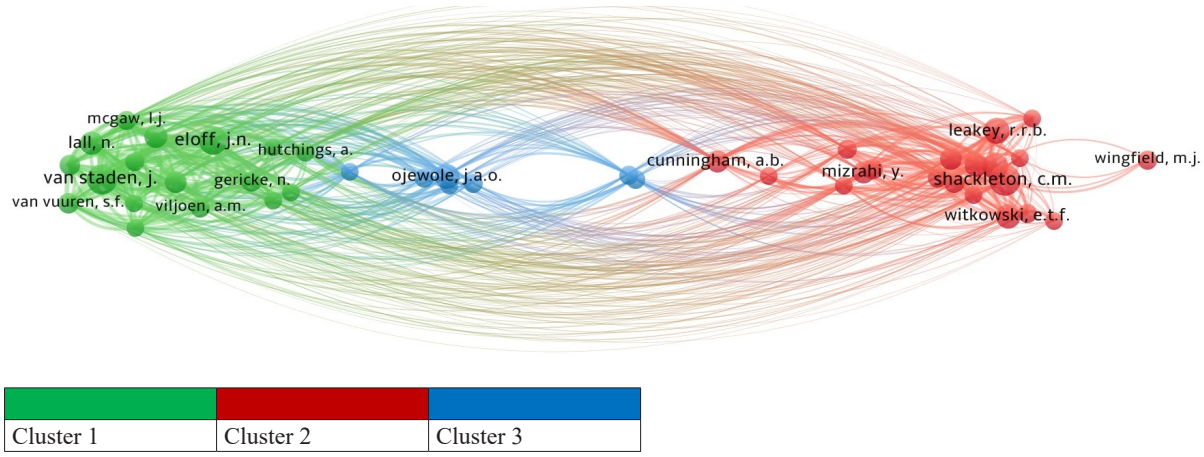


Figure 12 Co-citation network of authors with a minimum of 60 citations in 1986–2022

of co-authorship analysis based on author mapping with 67 items, 58 links, and a total link strength of 198. According to our research, Matthäus, Mariod, Eichner and Hussein belonged to the same cluster and worked closely as a research collaboration team. Their research scope was *S. birrea* kernel oil [4]. This team is presented by green nodes in Fig. 11. van Staden and other five authors fell in Cluster 2. These researchers shared the same interests in ethnobotany and bioactive properties of *S. birrea* [60, 68].

The co-citation analysis has become a leading tool of empirical insight into the structure of scientific communication. Unlike bibliographic coupling, which is retroactive, the co-citation analysis assesses document similarity in advance. Co-citation frequencies may alter in the future as a result of the development of an academic area [69]. Figure 12 shows clusters arranged by the VOSViewer software. The co-citation network of the cited authors had a total link strength of 37 949 for authors with a minimum of 40 citations, and only 44 authors met this threshold. We classified them into three clusters. van Wyk, Elof, Lall, and van Staden belonged to Cluster 1 (green) with a total link strength of 3651, 4078, 4830, and 7500, respectively. Cluster 2 (red) included Shackleton, who showed the highest total link strength of 3126. Shackleton is a full-time research professor at the Department for Environmental Science at Rhodes University, Grahamstown, South Africa. Matthäus proved to be the most prolific researcher in Cluster 3 (blue).

CONCLUSION

The leaves, bark, root, and fruit of *Sclerocarya birrea* (A. Rich.) Hochst., also known as marula, are used in nutrition and traditional medicine in many African

countries. Farmers eat its fruit raw, make marula wine, add seed juice to porridge, make jam, soda drinks, etc. The seed is surrounded by a delicate white kernel that is high in protein and oil. The oil, in its turn, is high in oleic, palmitic, myristic, and stearic acids, while the kernel protein is high in amino acids, with glutamic acid and arginine dominating. Extracts from various botanical parts of marula are known for their excellent total phenolic compounds, radical-scavenging capabilities, and antioxidant activities. Numerous studies have proved anti-diabetic, anti-inflammatory, analgesic, anti-parasitic, antibacterial, and antihypertensive properties of *S. birrea*. In addition, the tree provides shade in agricultural fields and is utilized to store maize plant remnants for animal feed later in the spring. Its parts serve as cattle forage. To sum it up, the numerous utility options and therapeutic properties of this tree led to scientific and research efforts that spread all over the world. It has become the focus of many scientific papers, books, conference proceedings, doctoral dissertations and Master's theses. Some of these works entered the Scopus database, from which the authors of this research retrieved 370 papers in English, French, Japanese, and Chinese.

This study represented the first bibliometric analysis of publications related to *S. birrea*. It covered worldwide publishing patterns, national and international partnerships, institutional engagement, the role of individual researchers and scientific journals, etc. The Republic of South Africa appeared to be the leading country in *S. birrea* research, followed by the United States, Germany, Britain, Kenya, the Republic of the Sudan, and Zimbabwe. In spite of the fact that the tree does not grow in Europe, the wide involvement of European countries is evidence of strong academic cooperations. Many postgraduate students from African countries completed their studies in Europe. Some researchers send their samples to European universities to be examined by advanced instruments.

The goal of any bibliometric analysis is to integrate diverse data, such as citations and the number of publications, as well as to develop certain indications that may be used to assess the true knowledge effect of various academics. In our study, Maroyi and van Staden appeared to have a lot of publications on the matter, but Maroyi proved to have a greater impact on creating knowledge and improving the overall quality of *S. birrea* research. Most publications were in agriculture and forestry (35.8%). However, many South-African researchers in the fields of pharmacy and medicine published their research materials in the *Journal of Ethnopharmacology*, which became the top journal on *S. birrea* with its 28 articles. Between 1986 and 2022, a total of 187 periodicals published marula-related articles. The prospects are such that *S. birrea* studies might one day become a bridge for research cooperation between Africa and the West. We expect that many scientific papers on the beneficial properties of marula will be published in the agricultural, environmental, and medical fields in the near future as researchers are urged to develop food and pharmaceutical products for commercialization.

CONTRIBUTION

All authors have contributed equally to this project.

CONFLICT OF INTEREST

The authors declared no conflict of interest regarding the publication of this article.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

There is no form of human subject involved in this manuscript; therefore, ethics approval is not needed.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Formulating edible films with red pitahaya extract and probiotic

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Abstract:
Preventing food spoilage and prolonging its shelf life are of great importance to meet the increasing food demand. Dietary fibers in red pitahaya are known to help maintain food freshness. Lactic acid bacteria have probiotic properties and can be a good alternative to additives in food production. Therefore, we aimed to investigate the potential use of gum-based edible films containing red pitahaya extract and probiotic as a coating material in the food industry.
Firstly, we determined the antimicrobial activity of red pitahaya peel and flesh extracts against pathogenic microorganisms and probiotic strains. Then, we employed the well diffusion method to determine the antimicrobial activity of the edible films containing red pitahaya extracts and *Limosilactobacillus fermentum* MA-7 used as a probiotic strain.
The largest inhibition zone diameters of peel and flesh extracts were 12.97 and 13.32 mm, respectively, against *Candida albicans* ATCC 10231. The inhibition of the growth of lactic acid bacteria was lower as the extract concentration decreased. The gum-based films with flesh extract and probiotic had the largest inhibition zone diameters of 21.63 and 21.52 mm, respectively, against Aeromonas hydrophila ATCC19570 and *C. albicans* ATCC 10231.
The edible films containing red pitahaya extract and *L. fermentum* MA-7 may have the potential to prevent spoilage caused by microorganisms in the food industry and to extend the shelf life of foods.

Keywords: *Hylocereus polyrhizus*, pitahaya, lactic acid bacteria, guar gum, coating material, antimicrobial activity, plant extracts

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INTRODUCTION

Today, the growing world population is increasing the demand for high-quality food. Extending the shelf life of foods is one of the ways to meet this demand. There is a lot of current research into packaging systems that prevent food spoilage. One of them, active packaging, is a system that maintains the product's quality and extends its shelf life through the interaction between packaging, the product, and the environment [1, 2]. This interaction has become of great importance recently due to the problem of hazardous waste and the environmental damage caused by non-biodegradable materials [3–5]. Coating processes are commonly used in various industries, such as food, agricultural, pharmaceutical, cosmetic, and textile industries. Products are generally coated for protective, decorative, or functional purposes [6, 7]. Edible film coatings, for example, have a number of advantages. They are biodegradable and therefore do not pollute the environment. They also serve as a nutritional supplement for consumers and as flavoring and dyeing agents for the product. Finally, edible film coatings exhibit antimicrobial and antioxidant properties due to essential oils, nisin, and plant extracts that they contain [8–11].

Edible film solutions can be prepared from gums of natural origin since they are inexpensive, biocompatible, non-toxic, and readily available [12]. Among natural biopolymers, guar gum is receiving a lot of attention in the field of food packaging due to its good film-forming and biological properties [13]. Guar gum is a hydrophilic nonionic macromolecule of polysaccharides with a high molecular weight. It is of low cost and has excellent biodegradability and biocompatibility [14]. Guar gum is one of the most essential thickeners and a flexible ingredient for a variety of food applications [15].

Pitahaya belongs to the genus *Hylocereus* of the *Cactaceae* family and is commonly known as the dragon fruit [16]. Fifteen years ago, the pitahaya fruit was unheard of, but today it has gained popularity in the European market and such countries as Colombia, Costa Rica, Vietnam, Mexico, the USA (Florida and California),

and Nicaragua [17]. In Turkey, pitahaya is grown in the Mediterranean region, especially in Mersin, Antalya and partially in Adana [18]. Pitahaya is considered a promising fruit with antioxidant, anticancerous, and antimicrobial properties, as well as prebiotic effects [19]. Dietary fibers in red pitahaya are important for maintaining the fruit's freshness. Therefore, red pitahaya can be potentially used to preserve food freshness [20].

Probiotics are non-pathogenic living microorganisms [21]. They can be found in various types of products such as foods, medicines, and dietary supplements [22]. Recently, probiotics have been increasingly used as a biocontrol agent in the food industry. In particular, lactic acid bacteria are excellent biocontrol agents due to their probiotic potential. Various methods have been developed to preserve the biological activities of probiotics during food processing and storage [23]. One of them is the use of edible films and coatings as potential carriers for probiotics [24]. The inclusion of probiotics in edible film solutions or coatings promotes the survival of these microorganisms [25]. This can also contribute to better food stability and safety due to the antimicrobial activity of probiotics against spoilage or pathogenic bacteria [26].

Unlike synthetic additives, new natural coating materials can inhibit the growth of pathogenic and food spoilage microorganisms without having any negative effects on health. In this regard, we aimed to study the potential use of gum-based edible films containing red pitahaya extracts and the probiotic candidate strain *Limosilactobacillus fermentum* MA-7 in the food industry. First, we investigated the antimicrobial activity of red pitahaya extracts against pathogenic test microorganisms. Then, the extracts were tested on the probiotic candidate strains. Finally, we determined the antagonistic effect of the film solutions prepared with red pitahaya extracts and *L. fermentum* MA-7 as natural biocontrol agents against pathogenic test microorganisms.

STUDY OBJECTS AND METHODS

Preparation of red pitahaya methanol extracts. Red pitahaya fruits were obtained from Kumluca (Antalya, Turkey) in October 2021 (Fig. 1). Then, their flesh was separated from the peel, and they were left to dry. After grinding, the powder from red pitahaya peel and flesh (10 g) was extracted with 99.7% methanol (30 mL) in two repetitions for two days. For this, we sonicated the mixes on ice for 10 min every day using a sonication device (Hielscher, 30 kHz, 100% amplitude). The crude red pitahaya methanol extracts were stored at 4°C until used.

Microorganisms and growth conditions. *Candida albicans* ATCC 10231 was cultured at 30°C for 24 h in YPD (Yeast Peptone Dextrose). *Aeromonas hydrophila* ATCC 19570 and *Listeria monocytogenes* ATCC 7644 were cultured at 37°C for 24 h in NB (Nutrient Broth) and TSB (Tryptic Soy Broth). *Yersinia ruckeri* and *Vibrio anguillarum* A4 were cultured in TSB and TSB/NaCl medium at 25°C for 24 h. *Limosilactobacillus fermentum* MA-7, *Lactobacillus gasseri* MA-1, *Limosilactobacillus vaginalis* MA-10, and *Lactobacillus delbrueckii* MA-9 were cultured at 37°C in MRS (Man, Rogosa, and Sharpe) for 24 h. *Streptococcus thermophilus* MAS-1 was cultured at 37°C in M17 broth medium for 24 h.

a b c

Figure 1 Pitahaya: (a) production greenhouse and (b, c) red pitahaya

Disc diffusion susceptibility test. The disc diffusion susceptibility test was used to determine the inhibitory effect of the red pitahaya peel and flesh methanol extracts against pathogenic test microorganisms and probiotic lactic acid bacteria. The prepared culture suspension (adjusted to 0.5 McFarland) was inoculated on an agar medium using the spread method and sterile discs (6 mm in diameter) were placed on the agar. The red pitahaya methanol extracts dissolved in dimethyl sulfoxide were dripped onto the sterile discs. Kanamycin (K; 30 µg/disc) and Ampicillin (AM; 10 µg/disc) antibiotic discs were used as controls for pathogenic microorganism strains, and Fluconazole (FCA; 25 µg/disc) was used for yeast. The culture dishes were incubated for 24 h at the suitable temperatures indicated previously. Then, the inhibition zone around the discs was measured using a caliper.

Micro-dilution assay. The micro-dilution assay was used to determine minimum inhibitory concentrations, as well as minimum fungicidal or bactericidal concentrations of the red pitahaya extracts. For this, the extracts were added to the growth medium and diluted by a two-fold serial dilution method to obtain a final concentration of 80–5 µg/µL. The culture suspension (0.5 McFarland) was added to each tube and then incubated under the conditions required for each microorganism as mentioned above. After incubation, the extract's concentration in the tube without microbial growth was determined according to turbidity and the lowest concentration was recorded as a minimum inhibitory concentrations value. Minimum bactericidal or fungicidal concentrations values were determined by inoculating samples from the mixture onto an agar medium. The culture dishes were incubated at the appropriate temperature for 24 h. The lowest concentration without growth at the end of incubation was defined as minimum bactericidal or fungicidal concentrations values.

Microbial and physicochemical characterization of edible film solutions containing red pitahaya and *L. fermentum* MA-7. *Preparation of edible film solutions.* An edible film formulation was designed by modifying

a b c d e f

Figure 2 Preparation of edible film solutions: (a) guar gum; (b) guar gum + *Limosilactobacillus fermentum* MA-7; (c) guar gum + pitahaya flesh extract; (d) guar gum + pitahaya peel extract; (e) guar gum + *Limosilactobacillus fermentum* MA-7 + pitahaya flesh extract and (f) guar gum + *Limosilactobacillus* MA-7 + pitahaya peel extract

the methods of Kılınç *et al.* and Bambace *et al.* from commercially available guar gum, the pitahaya methanol extract, and the human milk-originated probiotic candidate strain *L. fermentum* MA-7 [27, 28]. This study included a control group and three different experimental test groups. The control group contained guar gum (1%, w/v) adjusted to the final volume with distilled water. The edible film formulation test groups included: guar gum (1%, w/v) with *L. fermentum* MA-7, guar gum (1%, w/v) with red pitahaya extract (10%, w/v), and guar gum (1%, w/v) with red pitahaya extract (10%, w/v) and *L. fermentum* MA-7. Glycerol (3%, w/v) was used as a plasticizer in all the groups. First, we determined the antimicrobial activity of the edible film solutions. Then, the solutions were dried in a Pasteur oven until they reached constant weight, to be used in characterization tests (Fig. 2).

Antimicrobial activity of edible film solutions. The antifungal and antibacterial activities of the edible film formulation test groups were determined using the well diffusion assay. The test microorganisms included *C. albicans* ATCC 10231, *A. hydrophila* ATCC 19570, *L. monocytogenes* ATCC 7644, *Y. ruckeri*, and *V. anguillarum* A4. The culture suspensions (0.5 McFarland, 100 µL) were spread on the surface of the medium. Then, 100 µL of the mixture from the test groups and the control were added to each well (6 mm diameter, 0.1 cm^3 volume). The experiment was carried out in triplicate. After incubation of the petri dishes for 24 h at appropriate temperatures, the inhibition zone diameters were obtained using a caliper.

Thickness and density of edible film solutions. The thickness of the films was determined with a digital micrometer. The density of the films was reported as the ratio of the cut mass of the film to its volume (Thickness × Surface Area) [29].

Moisture content of edible film solutions. The moisture content, %, was determined using the oven-drying method by differential weighing of a film sample before and after drying. Three different film samples from each group were oven-dried at 90°C to constant weight. The film content was calculated using Eq. (1):

$$\text{Moisture content} = \frac{w_i - w_d}{w_i} \times 100 \quad (1)$$

where w_i is the initial weight of the film sample, g; w_d is the weight of the oven-dried film sample, g. Each of the groups was tested in triplicate [30].

Transparency and light transmission of edible film solutions. The transparency and light transmission values were determined using a UV-VIS spectrophotometer (Beckman Coulter, USA) by reading the absorbance of the number of films at wavelengths in the range of 200–800 nm [30]. The film samples were cut into three strips (0.7×3 cm). Each of the strips was placed in a quartz cuvette and its absorbance was read against an empty cuvette. The relative transparency, A_{600}/mm, of the film strip was measured at 600 nm and calculated using Eq. (2):

$$\text{Transparency} = \frac{A_{600}}{X} \quad (2)$$

where A_{600} is the absorbance value at 600 nm and X is the film thickness, mm. Triplicate readings were made for each film formulation.

The light transmittance, %, of the film groups was recorded by making spectrophotometric readings at

50 nm intervals at 200–800 nm and calculated using the Lambert-Beer equation:

$$\text{Lidht transmittance} = antilog_{10}(2 - A) \quad (3)$$

where A is the absorbance value of the film strip.

Water solubility of edible film solutions. The film samples were cut into square pieces in triplicate. The films were weighed in glass Petri dishes and then 30 mL of distilled water was added. After immersion at room temperature (~ 25°C) for 24 h, the residues were filtered and weighed to determine the degree of swelling or dried in an oven at 70°C to constant weight to determine their solubility [30]. The solubility in water, %, was calculated using Eq. (4):

$$\text{Solubility in water} = \frac{w_i - w_d}{w_i} \times 100 \quad (4)$$

where w_i is the initial weight of the film sample, g; w_d is the weight of the oven-dried film sample, g. Triplicate readings were made for each of the film solution groups.

Statistical analysis. The analysis of variance (one-way ANOVA) was performed using the SPSS program (GNU) to determine significant differences between antimicrobial activity values. Tukey's post-hoc test was used for multiple comparisons between different groups with 5% statistical significance ($p < 0.05$).

RESULTS AND DISCUSSION

Increasing antibiotic resistance in the world has led researchers to look for plant-based natural alternatives to control pathogenic microorganisms instead of synthetic preservatives [31]. In this study, we determined the biological activity of red pitahaya extracts against food-borne, fish, and yeast microorganisms by using the disc diffusion susceptibility and micro-dilution methods (Table 1). The largest inhibition zone diameters in the peel and flesh extracts were determined as 12.97 and 13.32 mm, respectively, against *Candida albicans* ATCC 10231. The smallest inhibition zone diameters in the peel and flesh extracts were determined as 9.09 and 10.62 mm, respectively, against *Listeria monocytogenes* ATCC 7644. The difference in antimicrobial activity between the *C. albicans* ATCC 10231 and *L. monocytogenes* ATCC 7644 strains was statistically significant ($p < 0.05$) in both extracts.

In our previous study, where we investigated the biological activity of fruit and peel methanol extracts from white pitahaya, the inhibition zone diameters were determined against *L. monocytogenes* ATCC 7644 (6.30 and 6.35 mm, respectively) and against *C. albicans* ATCC 10231 (11.66 and 13.15 mm, respectively) [32]. The differences in the phenolic content, especially betalain, of fruits may cause different results in antimicrobial activity against the same test microorganisms [33, 34].

Antimicrobial agents may have a static or cidal effect. The static effect has the ability to prevent the growth or reproduction of microorganisms, while the cidal effect has the ability to kill microorganisms [35]. The disc diffusion assay alone is not sufficient to determine whether the antimicrobial activity is a static or a cidal effect [36]. For this reason, it is necessary to determine minimum inhibitory concentrations, as well as bactericidal or fungicidal concentrations of the extracts. The micro-dilution assay results for red pitahaya extracts are presented in Table 2. As can be seen, the minimum inhibitory

Table 1 Biological activity of red pitahaya extracts

Microorganism strains	Inhibition zone diameters, mm ± SD				
	Extracts		Antibiotics		
	Red pitahaya peel methanol	Red pitahaya flesh methanol	Kanamycin	Ampicillin	Fluconazole
Candida albicans ATCC 10231	12.97 ± 0.46[a]	13.32 ± 0.51[a]	n.d.	n.d.	21.85 ± 1.76
Listeria monocytogenes ATCC 7644	9.09 ± 0.65[b]	10.62 ± 0.30[b]	14.77 ± 0.05	30.60 ± 0.11	n.d.
Aeromonas hydrophila ATCC19570	12.93 ± 0.21[a]	12.77 ± 0.84[a]	25.40 ± 1.30	29.57 ± 0.10	n.d.
Yersinia ruckeri	10.65 ± 1.06[b]	12.26 ± 0.44[a]	18.90 ± 0.05	18.70 ± 0.12	n.d.
Vibrio anguillarum A4	9.64 ± 0.64[b]	10.76 ± 0.33[b]	12.10 ± 0.13	15.13 ± 0.15	n.d.

n.d. – is the no inhibition zone diameter. Different letters in the same column show significance ($p < 0.05$)

Table 2 Micro-dilution assay of red pitahaya methanol extracts

Microorganism strains	Minimum inhibitory concentrations, μg/μL		Minimum bactericidal or fungicidal concentrations, μg/μL	
	Red pitahaya peel methanol	Red pitahaya flesh methanol	Red pitahaya peel methanol	Red pitahaya flesh methanol
Candida albicans ATCC 10231	40	40	80	80
Listeria monocytogenes ATCC 7644	40	40	80	> 80
Aeromonas hydrophila ATCC19570	40	20	80	> 80
Yersinia ruckeri	40	40	40	80
Vibrio anguillarum A4	20	40	40	80

concentrations value for the peel extract was determined as 40 µg/µL against all the test microorganisms, except *V. anguillarum* A4 (20 µg/µL). The flesh extract had a minimum inhibitory concentrations value of 40 µg/µL against all the test microorganisms, except *A. hydrophila* ATCC19570 (20 µg/µL). The minimum bactericidal concentrations values were determined in the range of 40–80 µg/µL in the peel extract and 80 and higher µg/µL in the flesh extract. The minimum fungicidal concentrations value was 80 µg/µL for both extracts.

Lactic acid bacteria are an important group of probiotic microorganisms. One of them is *Limosilactobacillus fermentum*, a generally recognized as safe bacterium used for food fermentation [25–38]. Table 3 shows the inhibition zone diameters of red pitahaya extracts against the probiotic candidate lactic acid bacteria strains at 1 and 2 mg/disc concentrations. As can be seen, the inhibitory zone diameters of 6.23 and 6.36 mm were determined in 1 mg/disc peel extract against *L. fermentum* MA-7 and *Lactobacillus delbrueckii* MA-9, respectively. Similarly, low inhibition activities against *L. fermentum* MA-7 (6.35 mm), *Lactobacillus gasseri* MA-1 (6.31 mm), and *L. delbrueckii* MA-9 (6.65 mm) were observed for 1 mg/disc flesh extract. At a concentration of 1 mg/disc, both extracts had a statistically insignificant antibacterial effect against the tested lactic acid bacteria ($p > 0.05$). As the extract's concentration decreased, its inhibitory activity against the lactic acid bacteria also decreased (Table 3).

A study by Siregar and Julianti showed no antibacterial activity of water, ethanol, and ethyl acetate extracts of red pitahaya peel against *Lactobacillus acidophilus* [39]. The differences in antimicrobial activity may be due to the environmental conditions in which pitahaya is grown, as well as the solvent, extraction method, and microorganism strains.

In our study, the minimum inhibitory and bactericidal concentrations values of the extracts against the tested lactic acid bacteria strains were determined in the range of 20 to > 80 µg/µL (Table 4). The highest minimum bactericidal concentrations value in the flesh extract was > 80 µg/µL against *L. fermentum* MA-7. The high minimum inhibitory and bactericidal concentrations values indicate that the red pitahaya extracts may have lower inhibitory activity against the lactic acid bacteria strains tested.

Edible film coatings with antimicrobial properties have been developed to provide consumers with foods that preserve high quality, do not spoil easily, keep microorganism growth under control, and have a long shelf life [40]. Literature has reported that *L. fermentum* strains produce various food-preservative antimicrobial peptides (fermenticins) and bacteriocins that can be used as an alternative to antibiotics [41]. These natural compounds are involved in antimicrobial activity in food bio-preservation and biomedicine [42]. For this reason, we used *L. fermentum* MA-7, which meets the criteria for being a good probiotic, to develop an edible film solution [43]. In addition, the tested red pitahaya extracts had relatively high minimum inhibitory and bactericidal concentrations values against *L. fermentum* MA-7.

Table 5 shows the biological activity of gum-based edible film solutions containing red pitahaya extracts and *L. fermentum* MA-7 (GEL) against the test microorganism. In most of the GEL groups, the antimicrobial activity was higher than in the other test groups, indicating a synergistic effect of pitahaya extracts with the probiotic. The antimicrobial activity of the GEL groups was statistically significant when compared to the gum-based film solutions without red pitahaya extract or *L. fermentum* MA-7 G ($p < 0.05$).

Table 3 Disc diffusion values for red pitahaya peel and flesh methanol extracts against lactic acid bacteria

Microorganism strains	Red pitahaya peel methanol		Red pitahaya flesh methanol	
	1 mg/disc	2 mg/disc	1 mg/disc	2 mg/disc
Limosilactobacillus fermentum MA-7	6.23 ± 0.13[a]	8.20 ± 0.15[a]	6.35 ± 0.07[a]	8.37 ± 0.19[a]
Lactobacillus gasseri MA-1	n.d.[b]	8.26 ± 0.09[a]	6.31 ± 0.46[a]	8.18 ± 0.07[a]
Limosilactobacillus vaginalis MA-10	n.d.[b]	6.27 ± 0.13[b]	n.d.[b]	7.22 ± 0.20[b]
Lactobacillus delbrueckii MA-9	6.36 ± 0.08[a]	7.62 ± 0.35[c]	6.65 ± 0.68[a]	8.92 ± 0.35[c]
Streptococcus thermophilus MAS-1	n.d.[b]	6.12 ± 0.07[b]	n.d.[b]	n.d.[d]

n.d. – is the no inhibition zone diameter. Different letters in the same column show statistical significance ($p < 0.05$)

Table 4 Micro-dilution assay of red pitahaya methanol extracts against probiotic strains

Microorganism strains	Minimum inhibitory concentrations, µg/µL		Minimum bactericidal concentrations, µg/µL	
	Red pitahaya peel methanol	Red pitahaya flesh methanol	Red pitahaya peel methanol	Red pitahaya flesh methanol
Limosilactobacillus fermentum MA-7	40	80	40	> 80
Lactobacillus gasseri MA-1	40	40	40	40
Limosilactobacillus vaginalis MA-10	40	40	40	40
Lactobacillus delbrueckii MA-9	40	40	40	40
Streptococcus thermophilus MAS-1	80	20	80	20

Table 5 Biological activity of edible film solutions containing red pitahaya extracts

Microorganism strains	Inhibition zone of edible film solutions containing red pitahaya peel and flesh extracts, mm					
	Gum	Gum + *L. fermentum* MA-7	Peel		Flesh	
			Gum + extract	Gum + extract + *L. fermentum* MA-7	Gum + extract	Gum + extract + *L. fermentum* MA-7
Candida albicans ATCC 10231	n.d.[a]	13.41 ± 0.45[b]	n.d.[a]	11.36 ± 0.76[c]	n.d.[a]	21.52 ± 0.08[d]
Listeria monocytogenes ATCC 7644	n.d.[a]	3.52 ± 0.32[b]	n.d.[a]	4.07 ± 0.28[b]	2.62 ± 0.09[c]	9.24 ± 0.46[d]
Aeromonas hydrophila ATCC19570	n.d.[a]	5.33 ± 0.77[b]	3.36 ± 0.53[c]	14.62 ± 0.61[d]	3.99 ± 0.97[b,c]	21.63 ± 0.28[e]
Yersinia ruckeri	n.d.[a]	4.74 ± 0.90[b]	n.d.[a]	8.74 ± 0.42[c]	n.d.[a]	11.00 ± 0.65[d]
Vibrio anguillarum A4	n.d.[a]	6.18 ± 0.47[b]	n.d.[a]	7.91 ± 0.33[c]	n.d.[a]	11.42 ± 0.75[d]

n.d. – is the no inhibition zone diameter. Different letters on the same line show statistical significance ($p < 0.05$)

Table 6 Physicochemical characterization of edible films containing red pitahaya peel extracts and *Limosilactobacillus fermentum* MA-7

Film groups	Thickness, mm	Density, g/cm^3	Moisture content, %	Transparency, A_{600}/mm	Solubility in water, %
Gum	0.14 ± 0.02[a]	0.73 ± 0.26	94.84 ± 0.02[a]	0.27 ± 0.16[a]	76.45 ± 0.61[a]
Gum + *L. fermentum* MA-7	0.24 ± 0.20[b]	2.92 ± 3.08	90.31 ± 0.27[b]	1.95 ± 0.06[b]	86.00 ± 1.06[b]
Gum + extract	0.18 ± 0.02[a,b]	0.74 ± 0.04	94.50 ± 0.08[a]	3.21 ± 0.50[c]	81.15 ± 0.79[c]
Gum + extract + *L. fermentum* MA-7	0.30 ± 0.04[c,b]	1.06 ± 0.33	89.28 ± 0.11[c]	5.67 ± 0.76[d]	79.36 ± 0.89[e]
F(Sig)	17.178(0.001)	1.359(0.323)	994.712(0.000)	71.321(0.000)	65.383(0.000)

Different letters on the same line show statistical significance ($p < 0.05$)

The GEL film solutions containing red pitahaya peel and flesh extracts showed inhibition zone diameters of 11.36 and 21.52 mm, respectively, against *C. albicans* ATCC 10231. Yeasts are important contaminants that enter the food chain during food processing, storage, and transportation [44]. Restricting yeast and fungi growth in food remains of high priority in the food and agricultural industries [45–47]. Edible films containing antimicrobials have the potential to prevent food spoilage caused by yeasts [48]. We found that our GEL film formulation has the potential to be used in preventing yeast-induced spoilage.

The GEL group showed statistically significant ($p < 0.05$) antimicrobial activity against *A. hydrophila* ATCC 19570, with inhibition zone diameters of 14.62 and 21.63 mm for the samples with the peel and flesh extracts, respectively. The use of antimicrobial film coatings in meat, fish, and seafood shows promising results for maintaining microbial stability during storage and ultimately increasing shelf life [49]. Our study indicated that the film formulations containing red pitahaya extract and *L. fermentum* MA-7 may had the potential to extend the shelf life of meat, fish, and seafood.

Qin *et al.* determined the antimicrobial activity of different film formulations obtained from red pitahaya peel extract against *Staphylococcus aureus*, *L. monocytogenes*, *Escherichia coli*, and *Salmonella* by the well diffusion method [50]. They found that the film solutions with large inhibition zones had the potential to be used not only as active packaging to extend the shelf life of foods, but also as smart packaging to preserve the freshness of protein-rich animal foods. Further, in a more recent study, edible films containing 0.5 and 1% concentrations of red pitahaya peel extract showed inhibition zone diameters of 1.24 and 1.69 mm, respectively, against *S. aureus* [51].

Table 6 shows the physicochemical characterization of the films with the red pitahaya peel extract. As can be seen, the thickness and density of the control group were the lowest compared to the films with *L. fermentum* MA-7 and the films with both the peel extract and the probiotic ($p < 0.05$). The difference between the control group and the group with the peel extract was not statistically significant ($p > 0.05$). The moisture contents of the control films and the ones with the extract were 94.84 and 94.50%, respectively. These values were higher compared to the films with *L. fermentum* MA-7 or the films with a combination of the red pitahaya peel extract and the probiotic. The addition of the extract and the probiotic changed the moisture content of the film by 5.56%, as well as decreased its transparency. The water solubility of the control group was 76.45%, while the group with *L. fermentum* MA-7 had the highest water solubility among the test groups ($p < 0.05$).

Table 7 presents the physicochemical characterization of the films containing the red pitahaya flesh extract. The highest thickness was detected in the films with *L. fermentum* MA-7. The films with both the flesh extract and the probiotic had a higher density (1.93 g/cm^3) compared to the control group (0.73 g/cm^3), whereas their moisture content was lower compared to the control group ($p < 0.05$). We found a statistically significant ($p < 0.05$) difference in transparency between the control films and the ones containing the flesh extract and

Table 7 Physicochemical characterization of edible films containing red pitahaya flesh extracts and *Limosilactobacillus fermentum* MA-7

Film groups	Thickness, mm	Density, g/cm^3	Moisture content, %	Transparency, A_{600}/mm	Solubility in water, %
Gum	0.14 ± 0.02^a	0.73 ± 0.26	94.84 ± 0.02^a	0.27 ± 0.16^a	76.45 ± 0.61^a
Gum + *L. fermentum* MA-7	0.24 ± 0.02^b	2.92 ± 3.08	90.31 ± 0.27^b	1.95 ± 0.06^b	86.00 ± 1.06^b
Gum + extract	0.18 ± 0.02^{a,b}	1.29 ± 0.04	92.76 ± 0.03^c	1.62 ± 0.21^b	61.63 ± 1.94^c
Gum + extract + *L. fermentum* MA-7	0.20 ± 0.02^{a,b}	1.93 ± 0.06	90.36 ± 1.04^b	3.44 ± 0.02^c	80.89 ± 1.55^d
F(Sig)	8.128(0.008)	1.139(0.390)	48.660(0.000)	156.996(0.000)	171.774(0.000)

Different letters on the same line show statistical significance ($p < 0.05$)

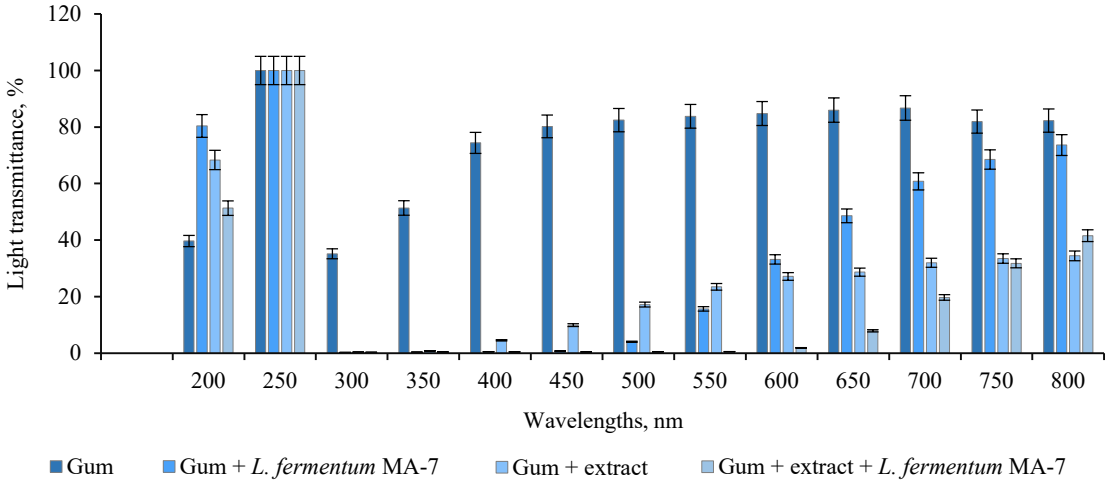

Figure 3 Light transmittance of films with red pitahaya peel extract

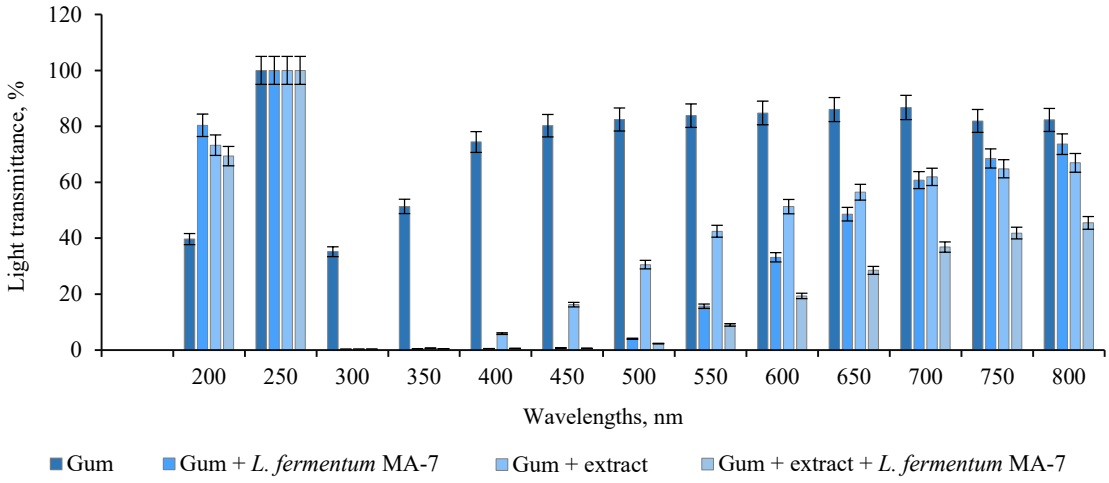

Figure 4 Light transmittance of films with red pitahaya flesh extract

L. fermentum MA-7. The lowest water solubility was determined as 61.63% in the films with the extract. The difference in water solubility among the film test groups was statistically significant ($p < 0.05$).

In a study by López-Díaz *et al.*, the films with red pitahaya had thickness values between 0.037 and 0.060 mm, whereas their moisture content ranged between 21.3 and 32.4% [52]. In a study by Azlim *et al.*, the films with red pitahaya peel extract had a moisture content between 0.24 and 0.28%, while their water solubility varied between 30.63 and 52.73% [53].

Thickness is one of the properties of edible films that affects the shelf life and biological structure of foods. The optimal thickness for edible films or coatings is ≤ 0.25 mm [54, 55]. In our study, the films with red pitahaya flesh and *L. fermentum* MA-7 were 0.20 mm thick, which was 0.05 mm thicker than the desired value stated in literature. High moisture content is a desirable

criterion for coating foods. In our study, the moisture content of the films with red pitahaya extracts and *L. fermentum* MA-7 was found to be higher than in the study by Šuput *et al.* [56]. High-resolution films are materials that dissolve easily but do not have the ability to hold water [55]. High water resistance is preferable for coatings, since water sensitivity of some products may lead to a loss of quality. For this reason, edible films need high solubility and rapid dissolution in water [57]. In our study, the films with red pitahaya extracts and *L. fermentum* MA-7 had high solubility in accordance with literature.

Figures 3 and 4 present the light transmittance of the films containing the red pitahaya peel and flesh extracts. As can be seen, the light transmittance of the control group (82.28–39.68%) was higher than in the test groups with the extract and/or *L. fermentum* MA-7 (51.31–41.57%).

The light transmittance of the extract-containing films ranged between 73.26 and 66.94%, while in the films with both the extract and *L. fermentum* MA-7, it varied between 69.34 and 45.47%. We found that light transmittance increased as the wavelength increased.

Socaciu *et al.* reported the light transmittance of films in the range of 0.01 to 70.65% [30]. The appearance of a product is important for presenting its quality and appeal to the consumer. Therefore, the transparency of films should not change the appearance, taste, or smell of the food [58]. The interaction of food with light depends on the relationship between packaging material and light. In this respect, it is important to know the optical properties of the packaging material. The interaction between the food material and light may cause unwanted photochemical reactions in the food depending on its composition [59]. In our study, the addition of red pitahaya extract and *L. fermentum* MA-7 to the films reduced their light transmittance.

CONCLUSION

We investigated the use of extracts from red pitahaya grown in Turkey as a natural antimicrobial agent and the potential use of edible films prepared with these extracts as a coating material in the food industry. Consumers are concerned about the potential dangers of synthetic preservatives for human health. Therefore, there is an increasing tendency toward using natural antimicrobial agents. According to our results, the gum-based edible films containing red pitahaya extract and *Limosilactobacillus fermentum* MA-7 had a cidal/static effect against pathogenic microorganism strains. These film solutions had large inhibition zones against the bacteria and yeast. Thus, the use of edible film formulations with antimicrobial effects as a coating material can be an alternative solution to prevent the deterioration of foods and extend their shelf life. Our study proved that the gum-based film formulations with red pitahaya extract and *L. fermentum* MA-7 have high biological activity and may be used as a coating material in the food industry. Since literature offers limited studies on pitahaya, there is a need for more research and our results study can be used in further *in vivo* studies. However, since literature offers limited studies on pitahaya, there is a need for more research and our results study can be used in further *in vivo* studies.

CONTRIBUTION

All authors have contributed equally to this project.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the authorship and publication of this article.

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Pomegranate leaves, buds, and flowers: phytochemical, antioxidant, and comparative solvent analyzes

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Abstract:
Punica granatum L. possesses significant nutritional and medicinal potential. Its pharmacological activities have been investigated, but no comparative evaluation has been reported regarding the effect of different extraction solvents on the phytochemical content and antioxidant activity of its leaf, bud, and flower extracts.

This research involved seven various solvents, namely methanol, ethanol, water, acidified methanol, acidified ethanol, acidified water, and hexane. A set of experiments made it possible to define the effect of each of these solvents on the contents of phenolics, flavonoids, flavanols, flavonols, anthocyanins, and tannins, as well as on the antioxidant activity of pomegranate leaf, bud and flower tissues. The research objective was to identify the optimal solvent for the most effective extraction of the abovementioned functional compounds. The antioxidant activity tests involved DPPH free radical scavenging, metal chelating, iron (III) reducing power, and CUPRAC assays.

The aqueous extract of *P. granatum* leaves demonstrated the highest total phenolic content (192.57 mg GAE/g extract) while the greatest flavonoid content belonged to the acidified methanol extract of *P. granatum* buds (73.93 mg RE/g extract). The HPLC analysis detected such significant phenolic compounds as punicalagin in buds and flowers, as well as gallic acid in leaves. All the extracts showed good antioxidant activity; however, the bud extracts had a better antioxidant profile than the extracts from leaves and flowers.

The pomegranate leaf, bud, and flower extracts demonstrated excellent phytochemical and antioxidant properties, which makes it possible to recommend these plant tissues as raw materials to be used in pharmaceutical, food, nutraceutical, and cosmetic industries.

Keywords: *Punica granatum* L., pomegranate buds, pomegranate leaves, pomegranate flowers, antioxidant activity, phytochemical analysis, pharmacological potential

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INTRODUCTION

Reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO·), comprise both free radical and non-free radical species formed by the partial reduction of oxygen [1, 2]. Reactive oxygen species are a result of mitochondrial respiration. It involves a variety of enzymes, e.g., nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, nitric oxide synthase, and peroxisomal constituents [3]. However, environmental toxins, ultraviolet and ionizing radiation, quinone compounds, inflammatory cytokines, tobacco smoke, and some pharmaceutical agents can also trigger reactive oxygen species production [3, 4].

Antioxidants and their functions in physiological cell processes can normally balance reactive oxygen species. At high concentrations, reactive oxygen species become pathological and cause oxidative stress [2–5]. Oxidative stress is defined as an imbalance between oxidants and antioxidants caused by excessive production of reactive oxygen species [3, 6]. Oxidative stress is responsible for such cardiovascular diseases as atherosclerosis, ischemia, hypertension, and cardiomyopathy. It is known to cause cancer, kidney diseases, food allergies, and other

allergic conditions, e.g., asthma, rhinitis, atopic dermatitis, etc. Oxidative stress is also associated with such neurological diseases as depression, memory loss, amyotrophic lateral sclerosis, Parkinson disease, Alzheimer disease, etc. [7, 8]. Natural antioxidants maintain the delicate balance between oxidants and antioxidants, thus protecting the cell from the harmful effect of reactive oxygen species [8].

Synthetic antioxidants include butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tert-butyl hydroquinone. They can also be used to avoid the harmful effects of oxidative stress. However, a long-term intake has been found to increase the risk of cancer [9, 20]. Herbs, species, seeds, fruits, vegetables, and edible mushrooms are natural sources of antioxidants [11].

The pomegranate (*Punica granatum* L.) is one of the world's oldest cultivated and consumed fruit [12]. This plant belongs to the *Lythraceae* family [13]. Pomegranates are cultivated in Turkey, Morocco, Spain, Afghanistan, Egypt, Italy, India, China, Pakistan, Africa, Israel, Japan, the USA, Russia, Australia, and Saudi Arabia [14]. Pomegranates are valued for their peel, seeds, arils, flowers, and leaves. They are an important source of phytochemicals with proven antioxidant properties [15–20]. Different parts of pomegranate cure a variety of diseases in Islamic, Ayurvedic, Persian, Unani, and Chinese folk medicines. Pomegranate bark and root treat diarrhea, dysentery, and ulcers; seeds are used to treat anorexia and dyspepsia; flowers are applied in case of gingival inflammation, gomphosis, and other dental ailments [21]. In addition, pomegranate extracts demonstrate important pharmacological activities. Leaf extracts exhibit anticancer, antibacterial, anti-diabetic, anti-inflammatory, anticholinesterase, and antigenotoxic effects [19, 22–25]. Flower extracts demonstrate anti-diabetic, anti-inflammatory, analgesic, antibacterial, hepatoprotective, and antispasmodic properties [26–31]. Bud extracts show antihistaminic and antiproliferative activities [32, 33].

The pharmacological properties of pomegranate leaf, flower, and bud extracts attract scientific attention due to their antioxidant activity and phytochemical content. In plants, phytochemical components and antioxidant properties depend on such factors as genotype, climate, maturity, cultivation practice, region, and extraction solvent [34–36]. The extraction solvent is a significant factor that affects both quality and quantity [37]. Wakeel *et al.* demonstrated the importance of solvent type and polarity [38]. In their study of *Isatis tinctoria* L., methanol-ethyl acetate proved to be the most efficient solvent in terms of total phenolic content in leaves while methanol and acetone-water were the most effective solvents in flower extracts. Elfalleh *et al.*, who studied *P. granatum* flowers, leaves, seeds, and peels, reported methanol to be more effective solvent for polyphenols than water [39].

Therefore, each plant has its own most effective extraction solvent. According to the literature that we reviewed, pomegranate buds are low in antioxidant activity. This research featured the effect of seven different extraction solvents on the phytochemical content and antioxidant activity of pomegranate leaves, flowers, and buds. We also investigated the relationship between their phytochemical content and antioxidant activity.

STUDY OBJECTS AND METHODS

Chemicals and equipment. The research included trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), 1,1′-diphenyl-2-picryl hydrazyl (DPPH), α-tocopherol (α-TOC), ferrous chloride, sodium carbonate, sodium acetate, aluminum chloride, 4-(dimethyamino)cinnamaldehyde (DMAC), and neocuprine. These chemicals were purchased from Sigma, USA. Ethanol, methanol, glacial acetic acid, ferric chloride, ammonium acetate, copper (II) chloride, and formic acid came from Merck, Germany. Gallic acid and n-hexane were purchased from Riedel-de Haën. All other reagents and solvents were of analytical grade. The research involved such equipment as a lyophilizer (Christ Alpha 1–2 LD Plus), a heating mantle (Daihan WHM 12013), a rotary evaporator (Laborota 4000-efficient Heidolph), a spectrophotometer (Shimadzu UV-1601), a shaking water bath (Clifton 100–400 rpm), a centrifuge (Nüvefuge CN180), a vortex (Fisons), a pH meter (Jenco 6177), and a high-performance liquid chromatographer (Shimadzu) with a diode array detector (DAD).

Plant materials. Buds, flowers, and leaves of *Punica granatum* L. were picked up manually in Adıyaman (Kâhta, southeast territory of Anatolia) in 2020. A voucher specimen (ZT1001) was identified by Professor Turan Arabacı (İnönü University). It was stored at the herbarium of the Pharmacy Department of İnönü University, Malatya. Each of the samples was dried in a freeze dryer for two days and then pulverized**.**

Preparing *P. granatum* extracts. We extracted 20.0 g of each sample, i.e., leaves, flowers, and buds, with different solvents in the following order: methanol, ethanol, water, n-hexane, 1.0% formic acid in methanol, 1.0% formic acid in ethanol, and 1.0% formic acid in water. The procedure involved a Soxhlet extractor and lasted for 4 h. The extract was concentrated at 40°C in a rotary evaporator. All the extracts were kept in an airtight container and stored at 4°C in a refrigerator.

Determining total phenolic content. We used the Folin-Ciocalteu assay with slight modifications to estimate the total phenolic content in all extracts [40]. In brief, 50 µL of extract solutions were mixed with 450 µL of distilled water and 250 µL of 10-fold diluted 2.0 N Folin-Ciocalteu reagent. We vortexed the mix and incubated it for 5 min before adding 2500 µL of 2.0% Na_2CO_3 and vortexing it once more. After incubating, all the samples remained at room temperature for 2 h. After that, their absorbance was measured at 765 nm using an ultraviolet-visible spectrophotometer. The calibration curve was constituted using gallic acid (GA). Finally, we determined the total phenolic content using the calibration line of gallic acid and expressed it as mg GAE/g extract, i.e., 1 mg gallic acid equivalent per 1 g of extract.

Determining total flavonoid content. We used the aluminum chloride colorimetric method to define the total flavonoid content in all extracts [41]. We incubated a mix of 500 µL of extract solution, 4500 µL distilled water, and 300 µL $NaNO_2$ (1:20) at room temperature for 5 min before adding 300 µL of $AlCl_3$ (1:10). After 6 min of incubation, we added 2000 µL of NaOH (1.0 M) and 2400 µL of distilled water and vortexed the resulting mix. Its absorbance was registered at 510 nm with a spectrophotometer. We sued a calibration curve for rutin to determine the total flavonoid content, which we expressed as mg RE/g extract, i.e., 1 mg of rutin equivalent (RE) per 1 g of extract.

Determining total flavonol content. The total flavonol content was determined as described by Kumaran & Karunakaran [42]. According to the procedure, we mixed 1000 µL extract solution with 1000 µL $AlCl_3$ (1:50) and 3000 µL CH_3COONa (1:20). After incubating the mix at room temperature for 30 min, we measured the absorbance at 440 nm using a spectrophotometer. The total flavonol content was calculated based on the quercetin calibration curve and expressed the results as mg QUE/g extract, i.e., 1 mg of quercetin equivalent (QUE) per 1 g extract.

Determining total flavanol content. This procedure involved the dimethylacetamide (DMAc) reagent, as recommended by McMurrough & McDowell [43]. In this method, the reaction mix comprised 1.0 mL of extract and 3.0 mL dimethylacetamide (1:100). We vortexed the mix and allowed it to settle for 20 min. The absorbance was registered at 640 nm using an ultraviolet-visible spectrophotometer. We used the catechin calibration curve and expressed the results as mg CE/g extract, i.e., 1 mg catechin equivalent per 1 g extract.

Determining total anthocyanin content. This experiment involved a spectrophotometer and the pH differential method [44]. We used two buffer systems: potassium chloride buffer with pH of 1.0 (25.0 mM) and sodium acetate buffer with pH of 4.5 (0.4 M). We mixed 0.4 mL extract solution with 3.6 mL of each buffer and incubated the resulting mix at room temperature for 15 min. The absorbance was registered at 510 and 700 nm using an ultraviolet-isible spectrophotometer. The total anthocyanin amount was demonstrated as equivalents for cyanidin-3-glucoside (mg Cy3G/kg extract).

Determining total tannin content. We used the vanillin-HCl staining method as proposed by Broadhurst & Jones [45]. This method relies on the ability of tannin to react with vanillin in the presence of HCl, giving a pink-red color [46]. We added 1.0 mL of extract, 3.0 mL vanillin reagent (1:25), and 1.5 mL concentrated HCl to tubes wrapped in aluminum foil. After vortexing, we incubated the mix in the dark at room temperature for 15 min. The absorbance was registered at 500 nm. The outcomes were given as epicatechin equivalent (mg ECE/g extract).

HPLC analysis for individual phenolic compounds. To identify the phenolic composition of *P. granatum* extracts, we used a high-performance liquid chromatographer with a diode array detector. The chromatographic separation included an ACE column (5 µm, C18, 250×4.6 mm) heated at 30°C. Mobile phase A comprised 70.0% acetonitrile and 30.0% distilled water while mobile phase B comprised 2.0% acetic acid in water. The experimental conditions were as follows: 5:95 A: B for 0–3 min, 15:85 for 4–40 min, 70:30 for 41–50 min, 90:10 for 50.01–55 min, 95:5 for 56–60 min. The flow rate was 1 mL/min. The chromatograms were taken at 250, 280, 320, and 360 nm.

Determining DPPH free radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity was measured as described by Blois [47]. We took 3.0-mL extract solutions at concentrations of 12.5, 25.0, 37.5, 62.5, and 125.0 µg/mL and added 1.0 mL of DPPH solution. After vortexing the mix and incubating it in the dark at room temperature for 30 min, we measured the absorbance of the solution at 517 nm using an ultraviolet-visual spectrophotometer. We appealed to butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *α*-tocopherol (*α*-TOC) as reference standards. The outcomes were given as percentage of DPPH radical scavenged by the extracts.

Determining ferrous ion chelating activity. We adopted the method described by Carter to specify the metal chelating activity [48]. In line with the procedure, we mixed the extract solutions at concentrations of 12.5, 25.0, 37.5, 62.5, and 125.0 µg/mL with 0.10 M sodium acetate buffer (pH 4.9) and 0.01% iron (II) chloride. After vortexing the mix and incubating it for 15 min, we added 40.0 mM ferrozine. After 10 min, we registered the absorbance at 562 nm, using ethylenediaminetetraacetic acid (EDTA) as positive control. The outcomes were given as percentage of inhibition of ferrozine-Fe^{2+} complex.

Determining reducing power. We used the method described by Oyaizu to measure the reducing power ability of *P. granatum* extracts [49]. We added 0.20 M phosphate buffer (pH 6.60) and 1.0% potassium ferricyanide to 1.0 mL extract solutions at the concentrations of 5.88, 14.70, 29.41, and 44.11 µg/mL. After vortexing, we incubated the resulting mix in a water bath at 50°C for 20 min, followed by centrifuging at 6000 rmp for 10 min. Subsequently, we transferred 1250 µL supernatants to another tube, adding 1250 µL of distilled water and 500 µL of iron (III) chloride. After vortexing, we registered the absorbance at 700 nm, using butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *α*-tocopherol (*α*-TOC) as reference standards.

Determining total antioxidant capacity. This procedure involved the CUPRAC (cupric reducing antioxidant capacity) method as described by Apak *et al.* [50]. We added 1000 µL of 10.0 mM $CuCl_2$, 1000 µL 7.5 mM neocuproine, 1000 µL 100.0 mM ammonium acetate buffer (pH 7.00), 100 µL extract solutions, and 1000 µL distilled water in the test tubes. After vortexing the mix and incubating it in the dark at room temperature for 30 min, we measured the absorbance at 450 nm using an ultraviolet-visible spectrophotometer. The outcomes were presented as µmol Trolox equivalents (µmol TRE/g extract).

Statistical analysis. All tests were carried out in triplicate. The results were given as mean ± standard deviations. We also performed the Tukey's Honestly Significance Difference (HSD) test to compare significant differences between means.

RESULTS AND DISCUSSION

Pomegranate leaf, bud, and flower extract yield. *Punica granatum* L. leaves, buds, and flowers were extracted using solvents with different polarities. Acidified solvents were used to extract more phenolic compounds, depending on the nature of the polyphenols in the structure of the plant materials, characterized using the HPLC method. Acidified organic solvent was used to obtain phenolic extracts with high anthocyanin content. According to the yield results (Table 1), acidified methanol proved to be the most effective extraction solvent. However, the yields ranged from 0.91–40.75, 1.77–58.76, and 0.36–37.45% for leaf, bud, and flower extracts, respectively. The highest extraction yield was found in the bud extract.

In general, the bud extract registered the best yield in acidified methanol and methanol (58.76 ± 1.11 and 51.61 ± 1.59% respectively), followed by leaves (40.75 ± 0.46) and flowers (37.45 ± 0.64) in acidified methanol. Trabelsi *et al.* used six different solvents to extract *P. granatum* leaves: hexane, chloroform, ethyl acetate, ethanol, aqueous, and acetone/water [51]. The highest extract yield of 20.07% belonged to the aqueous leaves extract. In another study, Kaur *et al.* applied a Soxhlet extractor to flowers and obtained a 23.51% yield with 95.0% ethanol [52].

These differences in yield can be due to the method of extraction and the region where the plant came from. Likewise, the extraction yield depends on temperature, solvent, solvent/sample ratio, and extraction time, as well as on the particle size and chemical nature of plant tissues. Under the same extraction time and temperature, solvent and chemical composition remain the two most important factors [53, 54].

Total phenolic content in different pomegranate parts. To specify the total phenolic content of the leaf, bud, and flower extracts, we applied a regression equation of calibration curve ($y = 0.0096x + 0.0742$) and expressed the result as mg GAE/g extract, i.e., 1 mg gallic acid equivalent per 1 g of extract (Table 2). The amount of total phenolic extracted from leaves, buds, and flowers in different solvents ranged from 10.64 ± 0.07 to 192.57 ± 0.02 mg GAE/g extract. Among all the extracts, the aqueous extract of leaves had the highest phenolic content (192.57 ± 0.02 mg GAE/g extract), followed by the methanol and the acidified methanol extracts of buds (187.97 ± 0.03 and 187.16 ± 0.07 mg GAE/g extract), respectively.

According to our data, methanol proved to be the most effective solvent for extraction of phenolic compounds from pomegranate leaves, buds, and flowers. Also, the pomegranate bud extracts were rich in phenols. In our study, the total phenolic content in the leaf extract appeared to be higher than that reported by

Table 1 Extraction yield in different *Punica granatum* L. tissues

Extraction solvents	Yield, %		
	Leaves	Buds	Flowers
Methanol	30.79 ± 0.24^{e}	51.61 ± 1.59^{b}	28.57 ± 1.66^{ef}
Ethanol	26.22 ± 1.19^{fg}	29.28 ± 0.42^{ef}	26.31 ± 0.35^{fg}
Water	16.08 ± 2.64^{j}	27.93 ± 0.89^{ef}	23.36 ± 0.66^{gh}
Acidified methanol	40.75 ± 0.46^{c}	58.76 ± 1.11^{a}	37.45 ± 0.64^{cd}
Acidified ethanol	21.60 ± 0.57^{hi}	36.89 ± 1.29^{d}	16.79 ± 0.34^{j}
Acidified water	16.07 ± 0.79^{j}	26.26 ± 0.43^{fg}	18.54 ± 0.21^{ij}
n-Hexane	0.91 ± 0.10^{k}	1.77 ± 0.07^{k}	0.36 ± 0.06^{k}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

Table 2 Total phenolic content in different parts of *Punica granatum* L.

Extraction solvents	Total phenolic content, mg GAE/g extract		
	Leaves	Buds	Flowers
Methanol	173.91 ± 0.07^{g}	187.97 ± 0.03^{b}	174.74 ± 0.06^{f}
Ethanol	124.70 ± 0.04^{o}	172.40 ± 0.05^{h}	153.09 ± 0.06^{l}
Water	192.57 ± 0.02^{a}	156.51 ± 0.03^{j}	122.87 ± 0.04^{p}
Acidified methanol	150.78 ± 0.07^{m}	187.16 ± 0.07^{c}	175.52 ± 0.58^{e}
Acidified ethanol	80.30 ± 0.05^{q}	170.88 ± 0.11^{i}	154.54 ± 0.07^{k}
Acidified water	178.12 ± 0.05^{d}	147.91 ± 0.05^{n}	123.18 ± 0.35^{p}
Hexane	10.64 ± 0.07^{t}	41.51 ± 0.04^{r}	21.57 ± 0.04^{s}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

Bekir *et al.* and Elfalleh *et al.* [24, 39]. However, the phenolic content in the flower extract was lower than that reported by Zheng *et al.* [55]. In addition, the pomegranate bud extract in our study contained more total phenolics than that reported by Attanayake *et al*., which was 110.37 mg GAE/g [56]. Our results for total phenolic content were in agreement with those published by Orgil *et al.* who reported that pomegranate buds had more phenolics than leaves and flowers [33]. Such differences may be explained by the different extraction solvents and pomegranate cultivars.

Total flavonoid content in different pomegranate parts. To calculate the total flavonoid content in pomegranate leaf, bud, and flower extracts, we appealed to a regression equation of calibration curve ($y = 0.005x + 0.0217$) and expressed the obtained results as mg RE/g extract, i.e., 1 mg of rutin equivalent (RE) per 1 g of extract (Table 3). The amount of total flavonoid extracted from leaves, buds, and flowers in different solvents varied from 8.83 ± 0.29 to 73.93 ± 0.09 mg RE/g extract. Table 3 shows that the highest total flavonoid content (73.93 ± 0.09 mg of rutin in 1 g of extract) belonged to the acidified methanol bud extract whereas the lowest one (8.83 ± 0.29 mg rutin in 1 g of extract) belonged to the hexane leaf extract. Our results seem to be much higher than the total flavonoid content in the leaf and flower extracts recorded by Mekni *et al.* and Elfalleh [39, 57].

These differences result from the cultivar diversity. In addition, the acidified solution of methanol was more efficient than the other solvents used for flavonoid extraction. Kopjar *et al.* reported that acidified methanol extract had the highest total flavonoid content, which supports the results of our study [58].

Total flavonol content in different pomegranate parts. To specify the total flavonol content in the pomegranate leaf, bud, and flower extracts, we used a regression equation of calibration curve ($y = 0.0148x + 0.0278$) and expressed the results as mg QUE/g extract, i.e., 1 mg of quercetin equivalent (QUE) per 1 g extract (Table 4). In our case, the acidified methanol extract of pomegranate buds had higher flavonol content than the other extracts. The highest flavonol amount belonged to the pomegranate bud extracts.

Cheurfa *et al.* examined the antioxidant and antidiabetic properties of pomegranate leaves [19]. They reported the total flavonol contents in aqueous and hydroalcoholic extracts as 9.20 ± 2.80 and 7.68 ± 0.60 mg QUE/g extract, respectively. Although the abovementioned research gives an important amount of data about pomegranate, very little information is available regarding the total flavonol content of pomegranate leaves, buds, and flowers.

Total flavanol content in different pomegranate parts. To measure the total flavanol content in pomegranate leaf, bud, and flower extracts, we used a regression equation of calibration curve ($y = 0.1317x + 0.0021$) and expressed the obtained results as mg CE/g extract, i.e., 1 mg catechin equivalent per 1 g extract (Table 5). We detected some significant difference in flavanol content between extraction solvents and plant tissues. The

Table 3 Total flavonoid content in different parts of *Punica granatum* L.

Extraction solvents	Total flavonoid content, mg RE/g extract		
	Leaves	Buds	Flowers
Methanol	69.83 ± 0.13^{c}	72.53 ± 0.05^{b}	49.33 ± 0.21^{j}
Ethanol	55.57 ± 0.09^{h}	60.57 ± 0.13^{g}	43.37 ± 0.17^{l}
Water	72.73 ± 0.09^{b}	67.67 ± 0.09^{d}	46.57 ± 0.13^{k}
Acidified methanol	69.50 ± 0.08^{c}	73.93 ± 0.09^{a}	62.30 ± 0.22^{f}
Acidified ethanol	39.43 ± 0.13^{m}	66.27 ± 0.13^{e}	54.63 ± 0.33^{i}
Acidified water	60.00 ± 0.08^{g}	69.70 ± 0.08^{c}	60.00 ± 0.14^{g}
Hexane	8.83 ± 0.29^{o}	11.17 ± 0.13^{n}	9.03 ± 0.21^{o}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

Table 4 Total flavonol content in different parts of *Punica granatum* L.

Extraction solvents	Total flavonoid content, mg QUE/g extract		
	Leaves	Buds	Flowers
Methanol	8.12 ± 0.05^{de}	8.49 ± 0.07^{cd}	7.30 ± 0.32^{f}
Ethanol	6.75 ± 0.08^{gh}	6.67 ± 0.05^{h}	5.47 ± 0.06^{j}
Water	8.97 ± 0.07^{b}	7.10 ± 0.11^{fg}	7.20 ± 0.07^{f}
Acidified methanol	7.05 ± 0.07$^{f–h}$	9.44 ± 0.05^{a}	8.79 ± 0.06^{bc}
Acidified ethanol	6.02 ± 0.12^{i}	5.57 ± 0.13^{j}	7.33 ± 0.19^{f}
Acidified water	8.08 ± 0.05^{e}	8.22 ± 0.05^{de}	7.91 ± 0.05^{e}
Hexane	0.41 ± 0.02^{l}	0.69 ± 0.05^{l}	1.18 ± 0.04^{k}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

Table 5 Total flavanol content in different parts of *Punica granatum* L.

Extraction solvents	Total flavonoid content, mg CE/g extract		
	Leaves	Buds	Flowers
Methanol	0.79 ± 0.07^{ab}	$0.64 \pm 0.07^{b–f}$	$0.59 \pm 0.03^{b–f}$
Ethanol	$0.77 \pm 0.03^{a–c}$	$0.63 \pm 0.03^{b–f}$	$0.54 \pm 0.03^{c–f}$
Water	0.88 ± 0.02^{a}	$0.60 \pm 0.01^{b–f}$	$0.53 \pm 0.04^{d–f}$
Acidified methanol	$0.74 \pm 0.06^{a–d}$	$0.62 \pm 0.08^{b–f}$	$0.54 \pm 0.02^{c–f}$
Acidified ethanol	$0.73 \pm 0.09^{a–e}$	$0.57 \pm 0.03^{b–f}$	0.50 ± 0.03^{ef}
Acidified water	$0.70 \pm 0.02^{a–f}$	$0.56 \pm 0.04^{c–f}$	0.48 ± 0.03^{f}
Hexane	0.18 ± 0.09^{g}	0.12 ± 0.03^{g}	0.13 ± 0.02^{g}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

Table 6 Total anthocyanin content in different parts of *Punica granatum* L.

Extraction solvents	Total anthocyanin content, mg Cy3G/kg extract		
	Leaves	Buds	Flowers
Methanol	0.11 ± 0.02^{i}	2.97 ± 0.09^{e}	3.36 ± 0.14^{e}
Ethanol	0.14 ± 0.00^{i}	1.17 ± 0.03^{g}	1.86 ± 0.28^{f}
Water	0.26 ± 0.01^{i}	0.48 ± 0.31^{hi}	0.94 ± 0.09^{gh}
Acidified methanol	0.31 ± 0.01^{i}	5.52 ± 0.22^{c}	7.88 ± 0.18^{a}
Acidified ethanol	0.42 ± 0.01^{hi}	3.01 ± 0.45^{e}	6.51 ± 0.27^{b}
Acidified water	0.50 ± 0.01^{hi}	1.26 ± 0.11^{g}	4.40 ± 0.15^{d}
Hexane	0.03 ± 0.00^{i}	0.04 ± 0.01^{i}	0.12 ± 0.03^{i}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

amount of total flavanol extracted from leaves, buds, and flowers in different solvents varied from 0.12 ± 0.03 to 0.88 ± 0.12 mg CE/g extract. The aqueous leaf extract showed the highest amount of flavanol compounds whereas the hexane bud extract gave the lowest result.

In our research, methanol proved to be the most effective solvent for flavanol extraction. Lee *et al.*, who examined the anti-inflammatory activity and phytochemical content in pomegranate peel, reported the total amount of flavanols as 257.0 ± 19.6 μg CE/mg [59]. The difference may be due to the different plant tissues used. As far as we know, no study has reported the total flavanol content in pomegranate leaves, buds, and flowers so far.

Total anthocyanin content in different pomegranate parts. We defined the total anthocyanin content in *P. granatum* leaf, bud, and flower extracts using the pH differential method. The flower extract in acidified methanol demonstrated the highest content, i.e., 7.88 ± 0.18 mg Cy3G/kg extract (cyanidin-3-glucoside equivalent). The total anthocyanin content varied from 0.03 ± 0.00 to 7.88 ± 0.18 mg Cy3G/ kg extract. The anthocyanin content in the flower extract was significantly higher than those in the leaf and the bud extracts (Table 6).

Our results were in agreement with those reported by Elfalleh *et al.* who revealed that pomegranate flowers had more anthocyanin than leaves [37]. In the same study, the acidified methanol was found to be more effective in extracting anthocyanin than methanol, water, ethanol, acidified ethanol, acidified water, and hexane. Similar results were also reported by Castañeda-Ovando *et al.* [58].

Total tannin content in different pomegranate parts. To identify the total tannin content in pomegranate leaf, bud, and flower extracts, we appealed to a regression equation of calibration curve ($y = 0.0075x + 0.0179$) and expressed the results as mg ECE/g extract (epicatechin equivalent). The values varied between 2.99 ± 0.08 and 16.07 ± 0.09 for different pomegranate tissues (Table 7). The highest amount of total tannin compounds was found in the methanol flower extracts.

Mekni *et al.* examined the phytochemical contents and antioxidant properties of leaves, flowers, and bark of four different pomegranate cultivars [57]. In their study, the total tannin content in Nabli and Gapsi flowers was higher than in other samples. On the other hand, Trabelsi *et al.* showed that the aqueous extract of pomegranate leaves contained more tannin than chloroform, ethyl acetate, ethanol, and acetone/water extracts [51]. In our research, methanol proved to be the best extraction solvent for tannin compounds. These results were in agreement with other studies, where methanol extracts of different plants were reported to contain more tannins [61, 62].

High-performance liquid chromatography (HPLC) of individual polyphenol compounds in different pomegranate parts. The identification and quantification of polyphenol compounds in pomegranate leaves, buds, and flowers were conducted using a HPLC analysis. Figure 1 illustrates the chromatograms of the solutions of all 18 standard phenolic compounds acquired at 250, 280, 320, and 360 nm.

Table 7 Total tannin content in different parts of *Punica granatum* L.

Extraction solvents	Total tannin content, mg ECE/g extract		
	Leaves	Buds	Flowers
Methanol	7.80 ± 0.06^{e}	5.72 ± 0.08^{j}	16.07 ± 0.09^{a}
Ethanol	7.26 ± 0.04^{f}	4.13 ± 0.05^{m}	6.30 ± 0.04^{i}
Water	6.52 ± 0.03^{hi}	4.45 ± 0.02^{l}	9.99 ± 0.10^{c}
Acidified methanol	6.88 ± 0.13^{g}	5.04 ± 0.04^{k}	12.74 ± 0.08^{b}
Acidified ethanol	6.65 ± 0.07^{gh}	4.40 ± 0.08^{l}	8.57 ± 0.05^{d}
Acidified water	5.80 ± 0.04^{j}	3.77 ± 0.05^{n}	5.84 ± 0.07^{j}
Hexane	3.07 ± 0.12^{o}	2.99 ± 0.08^{o}	3.18 ± 0.04^{o}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test

Values are given as mean ± standard deviation

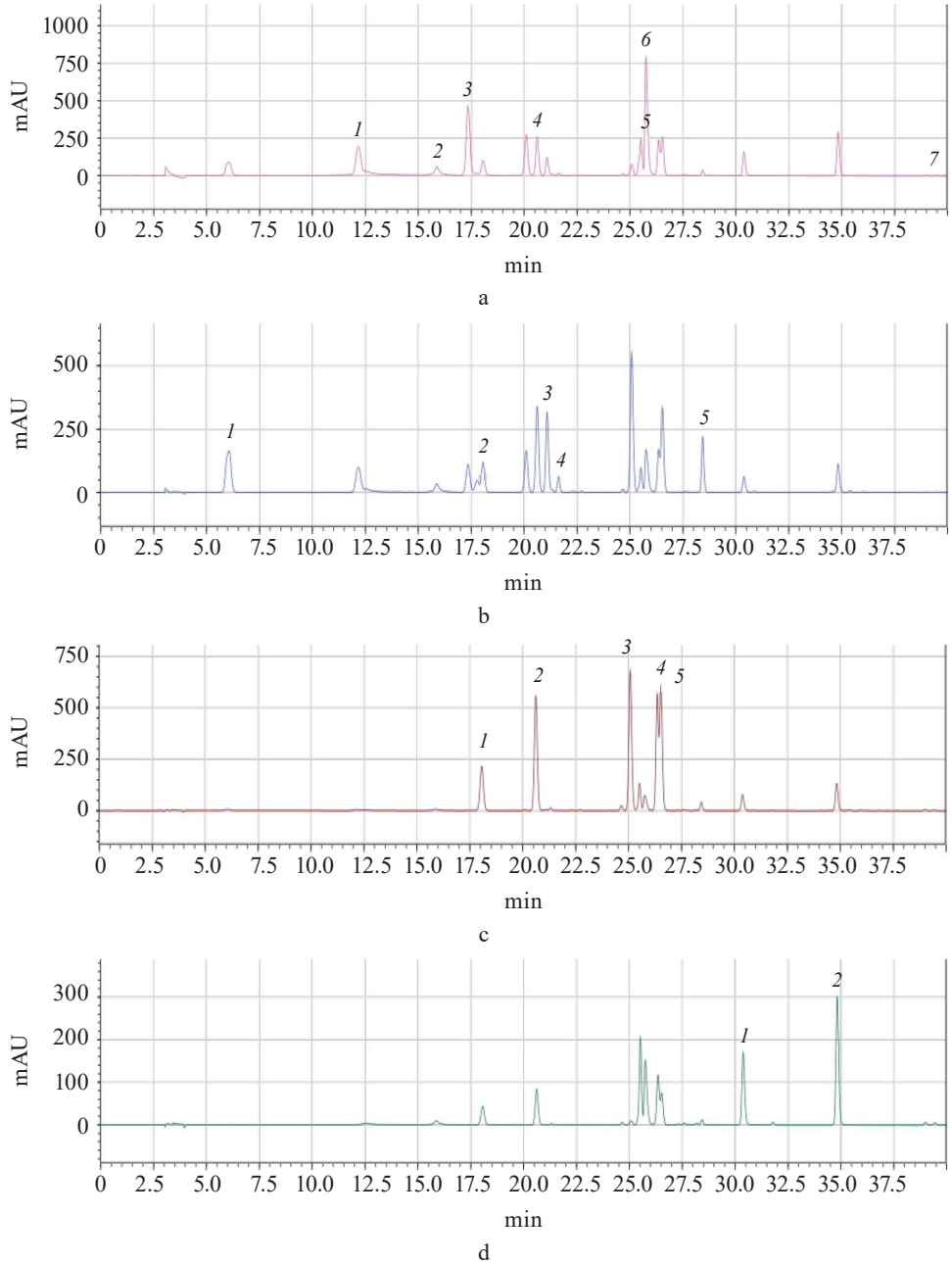

Figure 1 High-performance liquid chromatograms at: (a) 250 nm: protocatechuic acid (*1*), punicalagin (*2*), p-OH benzoic acid (*3*), vanillic acid (*4*), rutin (*5*), ellagic acid (*6*); (b) 280 nm: gallic acid (*1*), catechin (*2*), syringic acid (*3*), epicatechin (*4*), naringin (*5*); (c) 320 nm: chlorogenic acid (*1*), caffeic acid (*2*), p-coumaric acid (*3*), sinapic acid (*4*), trans-ferulic acid (5); and (d) 360 nm: myricetin (*1*), quercetin (*2*)

The HPLC analysis detected some major phenolic compounds, e.g., punicalagin in buds and flowers, as well as gallic acid in leaves. Gallic acid and ellagic acid were determined in all the methanol extracts of pomegranate leaves, buds, and flowers. However, punicalagin and protocatechuic acid were detected only in the methanol extracts of buds and flowers while punicalagin was more abundant than protocatechuic acid (Table 8).

We also tested the methanol extracts for snapic acid, trans-ferulic acid, caffeic acid, chlorogenic acid, myricetin, quercetin, naringin, p-coumaric acid, (-)-epicatechin, (+)-catechin, syringic acid, p-OH benzoic acid, rutin, and vanillic acid but detected none of them. In general, our results, except those obtained for the bud extracts, coincided with those reported by other authors [33, 51, 63, 64]. We found some studies that used HPLC to identify phenolic compounds in various buds but we found no article that reported pomegranate buds [65, 66].

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity in different pomegranate parts. We used the DPPH method to identify the free radical scavenging activity in different solvent extracts of pomegranate leaves, buds, and flowers (Figs. 2–4). As a rule, 2,2-diphenyl-1-picrylhydrazyl is employed as a substrate that reduces oxidative stress and hinders cellular ageing [67]. It helps evaluate the antioxidative activity of antioxidant substances. The assay relies on the reduction of methanolic or ethanolic DPPH solution in the

Table 8 Phenolic compounds identified in different parts of *Punica granatum* L.

Chemical identity	Retention time, min	Chemical constituents, mg/kg extract		
		Leaves	Buds	Flowers
Punicalagin	16.59	n.d.	19 005.31	6687.36
Protocatechuic acid	12.86	n.d.	3190.87	908.35
Ellagic acid	26.28	1444.60	11 427.82	4698.24
Gallic acid	6.41	7255.42	3190.85	4032.21

n.d. – not detected

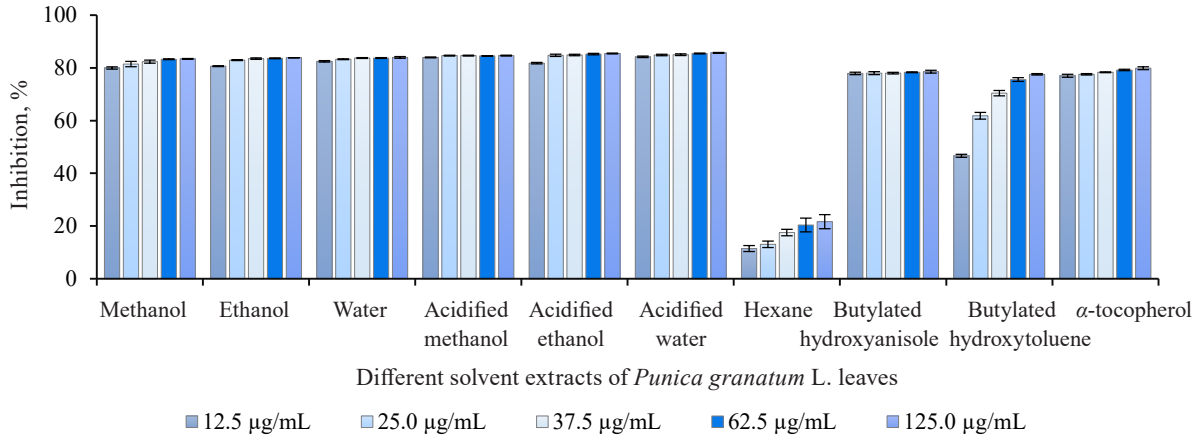

Figure 2 DPPH free radical scavenging activity in *Punica granatum* L. leaves

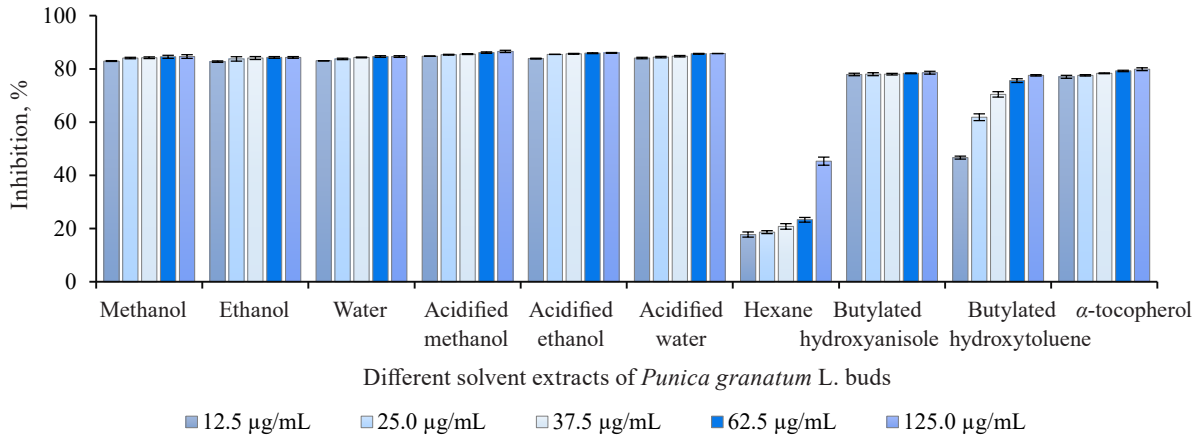

Figure 3 DPPH free radical scavenging activity in *Punica granatum* L. buds

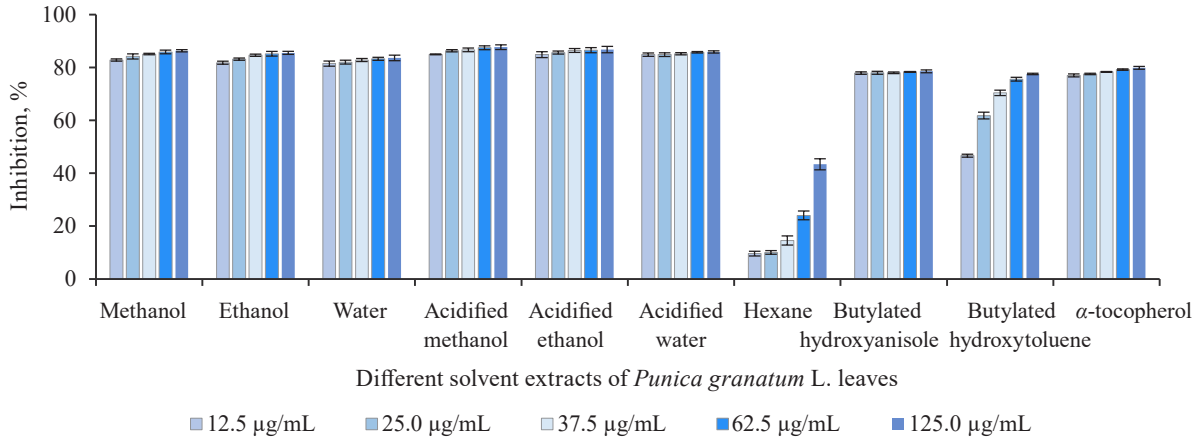

Figure 4 DPPH free radical scavenging activity in *Punica granatum* L. flowers

presence of a hydrogen atom from the antioxidant. The reaction forms DPPH-H, a non-radical form of diphenylpicrylhydrazine [68]. In our research, all the extracts were able to reduce the stable radical DPPH to its yellow-stained non-radical form. The inhibition values of leaves, buds, and flowers ranged from 11.46 ± 1.13 to 85.68 ± 0.16%, from 17.74 ± 0.97 to 85.98 ± 0.16%, and from 9.60 ± 0.89 to 87.68 ± 0.89%, respectively.

While the acidified methanol extract of *P. granatum* flowers had the highest radical scavenging activity of 87.68 ± 0.89% at 125.0 µg/mL, the hexane extract of *P. granatum* flowers showed the lowest radical scavenging activity. All the extracts but the hexane one showed higher antioxidant activity than the synthetic antioxidant compounds used as standards, namely butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol (α-TOC). At 12.5 µg/mL, which was the lowest concentration, the antioxidant activity of the acidified methanol bud extract was 1.8 and 4.7 times as high as that of butylated hydroxyanisole and the hexane bud extract. At 12.5 µg/mL, the pomegranate flower extract in acidified methanol showed approximately 8.8-fold higher activity than the hexane extract. All the extracts, excluding the hexane ones, exhibited a greater radical scavenging ability than the standard antioxidant compounds, even at the lowest concentration. No statistically significant differences were observed. We found several articles that featured the DPPH• free radical scavenging activities of *P. granatum* leaves, buds, and flowers [23, 29, 69].

The DPPH radical scavenging activity of pomegranate leaves ranged from 16.90 ± 0.46% at 20 µg/mL to 79.13 ± 0.37% at 120 µg/mL, probably, due to the diverse concentrations of methanol extract [23]. Yu *et al.* reported that the DPPH radical scavenging activity of hydro-methanolic extracts of pomegranate leaves was 4–200 times higher than the leaf extracts of *Salvia officinalis* L., *Rosmarinus officinalis* L., *Olea europaea* L., *Ruta graveolens* L., *Mentha piperita* L., and *Petroselinum crispum* Mill. [69]. In the study of Abdolahi *et al.*, the radical scavenging activity in the pomegranate flower ethanol extract was 91.04% at 100 µg/mL [29]. This variation in the DPPH scavenging activity could be explained by the difference in plant varieties, growing environmental, and extraction conditions.

Ferrous ion chelating activity in different pomegranate parts. We used the assay described by Carter to determine the chelating activity of ferrous ions in different pomegranate extracts [48]. Metal chelating is frequently excogitated as the most accepted and widespread antioxidant method [70]. Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, and the purple staining of the complex decreases. By measuring the color reduction, researchers are able to estimate the chelating activity of the co-existing chelator [71]. In this method, both pomegranate extracts and ethylenediaminetetraacetic acid (EDTA) as chelating agent hindered the formation of ferrous complex with the reagent ferrozine, suggesting that pomegranate extracts possessed chelating activity and could capture the ferrous ion before ferrozine.

The absorbance of Fe^{2+}-ferrozine complex went down in a dose-dependent manner in leaf, bud, and flower extracts as 12.5, 25.0, 37.5, 62.5, and 125.0 µg/mL, respectively. Butylated hydroxyanisole, butylated hydroxytoluene, and α-tocopherol showed less metal chelating activity than the pomegranate extracts and ethylenediaminetetraacetic acid. Figure 5 illustrates the metal chelating activity in the *P. granatum* leaves, butylated hydroxyanisole, butylated hydroxytoluene, α-tocopherol, and ethylenediaminetetraacetic acid at different concentrations.

The metal chelating activity of pomegranate leaf extracts varied from 4.71 ± 0.30 to 76.52 ± 0.89%. Statistically, the aqueous extracts had the highest chelating activity, followed by acidified water, methanol, acidified methanol, ethanol, acidified ethanol, and hexane extracts. The hexane leaf extracts displayed the lowest chelating activity. Figure 6 shows the metal chelating activity in *P. granatum* bud extracts, standard antioxidants, and ethylenediaminetetraacetic acid at different concentration.

The metal chelating activity of *P. granatum* bud extracts ranged from 0.14 ± 0.10 to 74.15 ± 1.01%. The

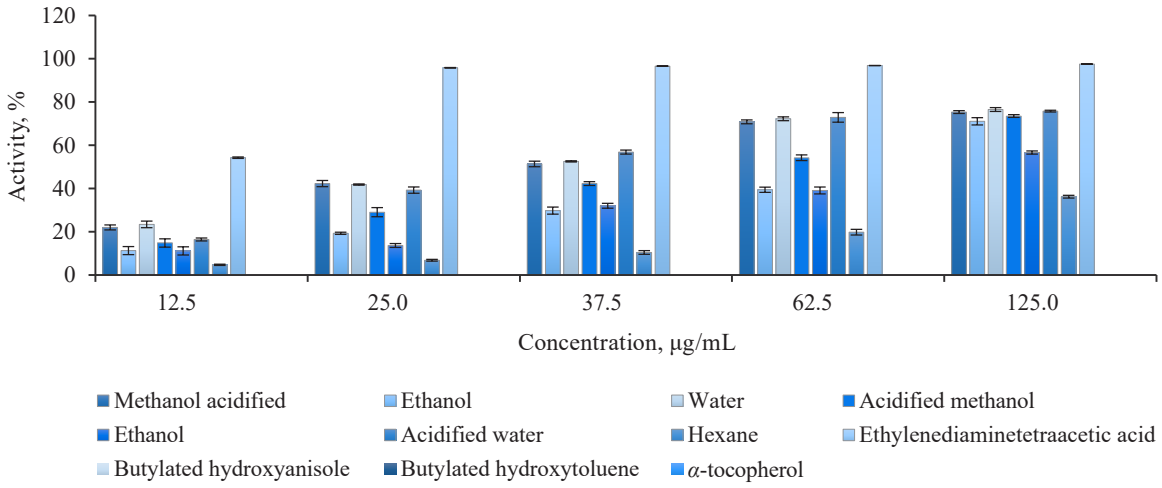

Figure 5 Metal chelating activity in different *Punica granatum* L. extracts

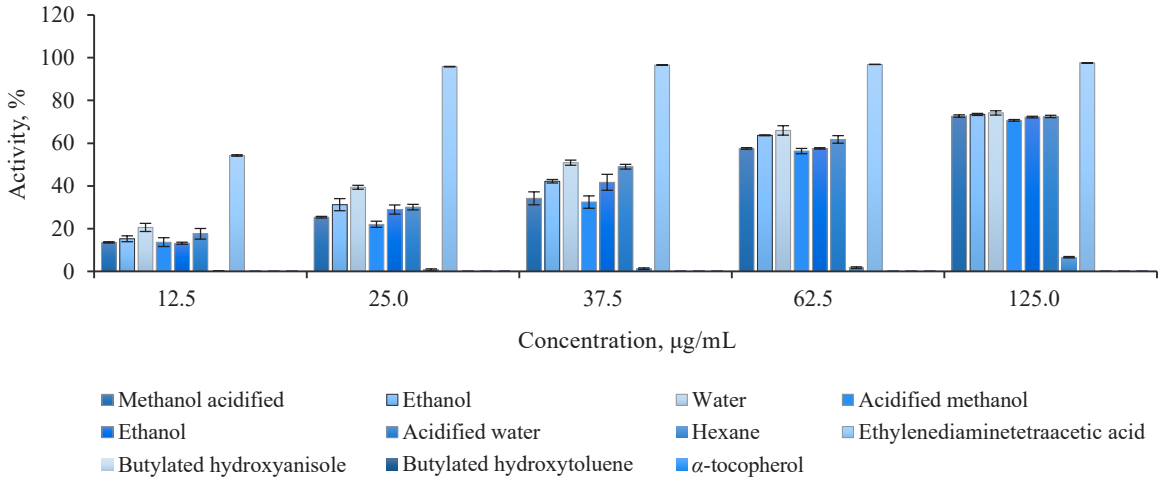

Figure 6 Metal chelating activity in different extracts of *Punica granatum* L. buds

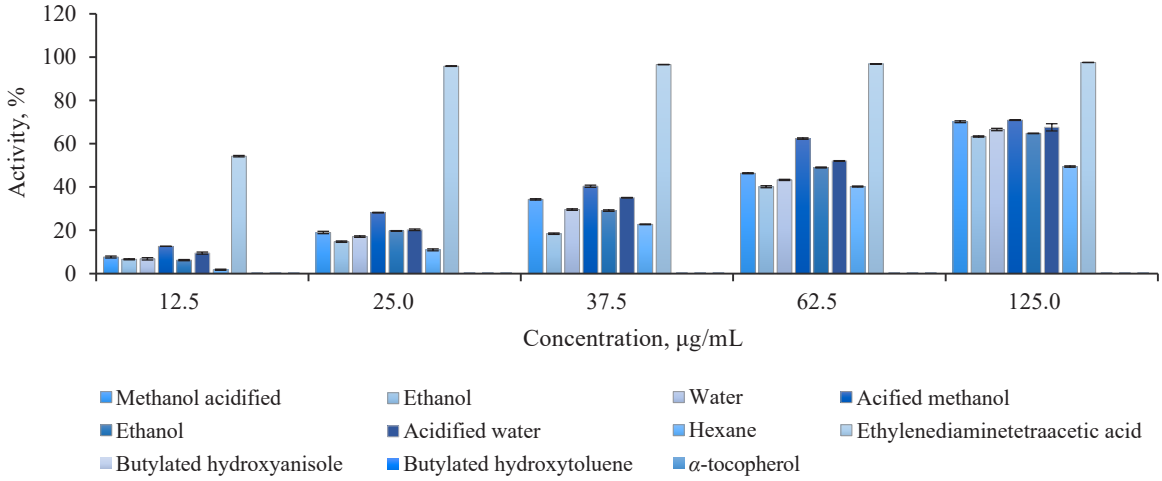

Figure 7 Metal chelating activity in different extracts of *Punica granatum* L. flowers

aqueous bud extract exhibited the greatest metal chelating activity compared to other bud extracts. At 125.0 µg/mL, all the pomegranate flower extracts were highly capable of metal chelating, irrespectively of the solvent applied (Fig. 7).

The acidified methanol flower extracts showed the maximal metal chelating activity, compared to other solvents. The metal chelating activity of *P. granatum* flower extracts varied from 1.79 ± 0.28 to 70.90 ± 0.15%. Our metal chelation assay data were similar to other reports. For instance, Rummun *et al.* assessed the metal chelating properties of pomegranate leaves, peel, stems, seeds, and flowers to report that flowers exhibited higher activity than leaves [72]. We found no published reports describing metal chelating activity for *P. granatum* buds.

Reducing power ability in different pomegranate parts. We used the reducing power method to evaluate the reduction potential of different pomegranate extracts because this method comprises the reduction of ferricyanide ion to ferrocyanide ion by electron donation from phenolic compounds. Therefore, the reducing capability of a compound can function as an important indicator of its potential antioxidant activity. All extracts of pomegranate leaves, buds, and flowers clearly exhibit a dose-dependent reducing power. Figures 8, 9, and 10 depict the reductive effects of different pomegranate extracts compared to butylated hydroxyanisole, butylated hydroxyxytoluene, and α-tocopherol. Similar to metal chelating activity, the reducing power of *P. granatum* leaf, bud, and flower extracts increased together with concentration.

The aqueous extracts of leaves demonstrated a higher reducing power compared to other solvents and synthetic antioxidant compounds. It was 2.06 ± 0.07 while that of butylated hydroxytoluene was 1.02 ± 0.02 at 44.11 µg/mL. The range of reducing power in *P. granatum* bud extracts varied from 0.11 ± 0.00 to 2.17 ± 0.03 at 5.88 and 44.11 µg/mL, respectively. In general, *P. granatum* bud extracts exhibited a higher reducing power than the antioxidant compounds like butylated hydroxytoluene and alpha-tocopherol. The highest reducing power value belonged to the acidified water extract, followed by the acidified methanol extract. The reducing power in all the *P. granatum* flower extracts but the hexane one was higher than that of butylated hydroxytoluene and alphatocopherol at 29.41 and 44.11 µg/mL (Fig. 10). The reducing power value ranged from 0.11 ± 0.02 at 5.88 µg/mL to 2.13 ± 0.04 at 44.11 µg/mL with the highest reducing power measured in the acidified methanol extracts.

Consequently, bud and flower extracts exhibited higher activities than leaf extracts. In addition, no significant difference was observed between the acidified water, water, methanol, and acidified methanol extracts of pomegranate leaves, buds and flowers. Our results were similar to those reported by other research teams [29, 39, 73]. For instance, Zhang *et al.* wrote that the reducing power of different parts of pomegranate flowers increased steadily following the increase in the extract concentration [73].

Total antioxidant activity in different pomegranate parts. In this research we used the CUPRAC method, i.e., cupric reducing antioxidant capacity. It is based on the ability of antioxidants to reduce the cupric neocuproine complex Cu (II)-neocuproine in the presence of ammonium acetate to the cuprous form Cu (I)-neocuproine chelate, which shows the maximal light absorption at 450 nm [50]. The leaf extracts gave antioxidant activity that ranged from 517.27 ± 1.40 to 3509.42 ± 0.40 µmol TRE/g extract (Trolox equivalent). In various bud extracts, this variable ranged from 385.60 ± 0.42 to 3323.09 ± 1.78 µmol TRE/g extract. In different flower extracts, it was between 350.66 ± 3.84 and 3282.58 ± 1.31 µmol TRE/g extract (Table 9).

Statistically, we determined that the total antioxidant activity of pomegranate buds exceeded that of the leaves and flowers. In our research, water proved to be the best extraction solvent in terms of the cupric reducing antioxidant capacity of pomegranate. Our results were similar to those obtained by some other researchers. For example, Ghazzawi *et al.* examined the effects of different solvents on the antioxidant capacity and content in nine seasonal fruits, including pomegranates [74]. In their study, water appeared to be the best extraction

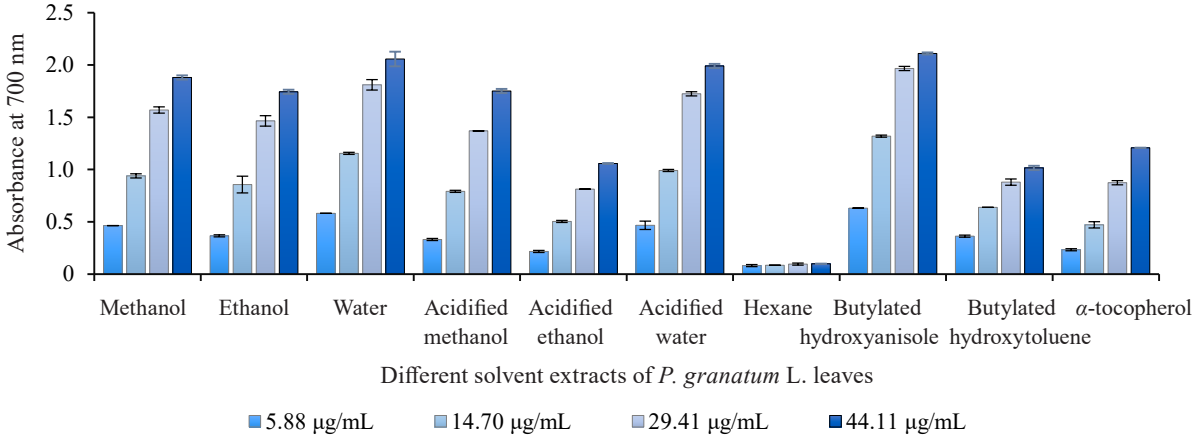

Figure 8 Reducing power ability in different solvent extracts of *Punica granatum* L. leaves

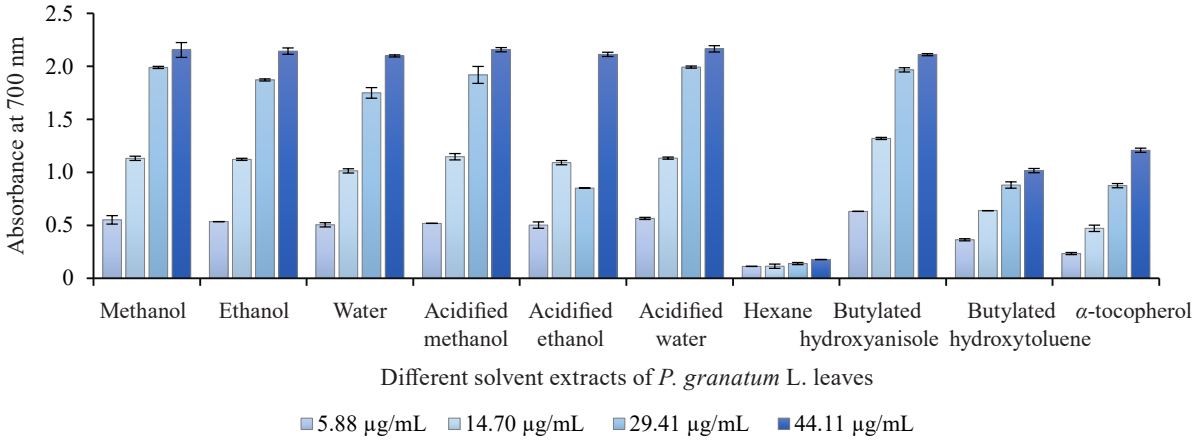

Figure 9 Reducing power ability in different solvent extracts of *Punica granatum* L. buds

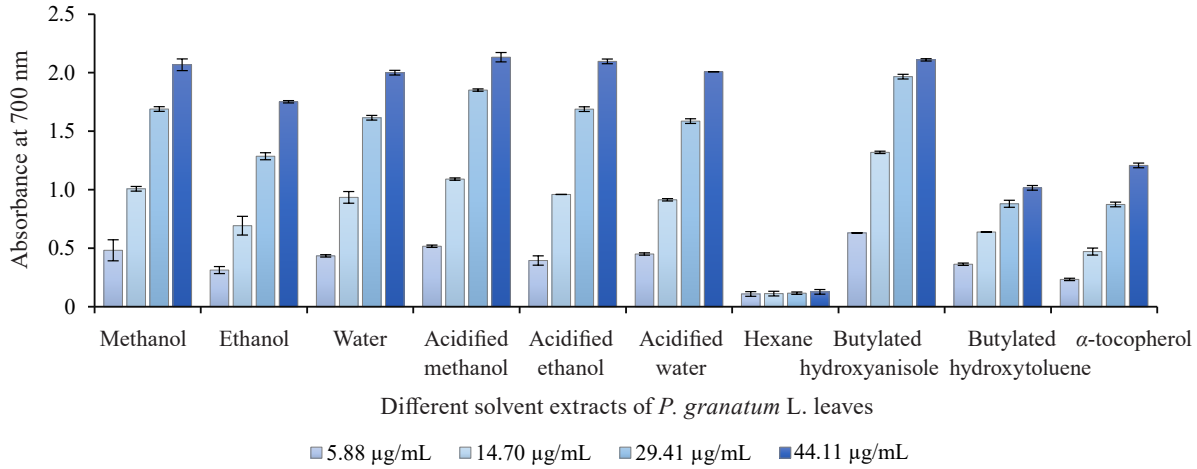

Figure 10 Reducing power in different solvent extracts of *Punica granatum* L. flower

Table 9 Total antioxidant activity in different *Punica granatum* L. parts

Extraction solvents	Total antioxidant activity, µmol TRE/g extract		
	Leaves	Buds	Flowers
Methanol	3121.12 ± 0.61^{h}	3060.81 ± 1.75^{i}	2869.08 ± 5.91^{m}
Ethanol	3341.01 ± 1.64^{b}	2947.23 ± 2.09^{k}	1559.17 ± 1.75^{q}
Water	3509.42 ± 0.40^{a}	3216.79 ± 1.59^{f}	2883.73 ± 2.65^{l}
Acidified methanol	2587.57 ± 0.00^{n}	3323.09 ± 1.78^{c}	3282.58 ± 1.31^{e}
Acidified ethanol	2346.82 ± 1.42^{o}	3192.32 ± 1.48^{g}	2049.60 ± 3.12^{p}
Acidified water	3018.83 ± 2.72^{j}	3310.16 ± 0.61^{d}	3198.95 ± 3.98^{g}
Hexane	517.27 ± 1.40^{r}	385.60 ± 0.42^{s}	350.66 ± 3.84^{t}

Different letters indicate significantly different levels according to Tukey's Honestly Significance Difference test
Values are given as mean ± standard deviation

solvent for the antioxidant capacity of bananas, figs, white grapes, and jujube fruits. Sanda *et al.* also reported that the aqueous extract of *D. pontica* leaves exhibited the highest cupric reducing power capacity [75]. Alsataf *et al.* explored the antioxidant, anti-diabetic, and antimicrobial activities of pomegranate peel, mesocarp, and seed, as well as pomegranate juices [76]. In their research, the highest cupric reducing capacity belonged to pomegranate peel (954.4 µmol trolox/g). The researchers also reported that pomegranate tissues had a significant effect on anti-α-glucosidase activity and exhibited different antimicrobial activities against pathogens. Our values exceeded those obtained by Alsataf *et al.* [76]. This difference could be explained by the different pomegranate tissues involved. Uysal *et al.* performed the CUPRAC test on nine different fruit tree leaves [77]. The

list included avocado, walnut, mulberry, fig, carob, lemon, pomegranate, grape, and loquat. In their study, the pomegranate leaf extracts had the highest CUPRAC absorbance values at the 0.1 mg/mL concentration. As far as we know, no study reported cupric reducing power activity of pomegranate buds and flowers so far.

CONCLUSION

In this research, which featured pomegranate leaves, buds, and flowers, the phenolic content and antioxidant activity of plant extracts depended on the plant tissue and the extraction solvent involved. *Punica granatum* L. buds demonstrated the greatest polyphenol content, as well as antioxidant activity. The flower extracts were rich in tannin and anthocyanin. *P. granatum* leaves demonstrated higher flavanol content than other tissues. The *P. granatum* bud extract showed higher antioxidant activity than leaves and flowers. Pomegranate buds, leaves, and flowers possessed beneficial polyphenol and antioxidant profiles, which made them potential raw material sources for pharmaceutical preparations, functional foods, nutraceuticals, and cosmetics.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for any potential cases of plagiarism.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Oral tobacco-free nicotine products: Quality and safety during storage

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Abstract:
Oral non-tobacco nicotine products have gained enormous popularity in recent years. The countries of the Eurasian Economic Union also produce and sell this type of innovative but poorly studied goods. As a result, the safety profile and quality of such products as nicotine poaches require urgent comprehensive research. This study featured the changes in quality of nicotine poaches during storage, i.e. nicotine content, water activity, and microbiological index.

The research featured nicotine poaches of several popular brands. The authors used standard research methods; the experiments were performed in the laboratory for chemistry and quality control, Institute of Tobacco, Makhorka, and Tobacco Products, Krasnodar, and at the Department of Bioorganic Chemistry and Technical Microbiology, Kuban State Technological University, Krasnodar.

The water activity was 0.8911–0.9502 A_w at the initial stage and remained stable in most samples even after six months of storage. Velo Freeze was the only brand to show significant variations in water activity. The nicotine content was 10.115–12.950 mg/g at the initial stage. Only four samples maintained the initial values after six months of storage. The fluctuations of nicotine content were also mentioned by the manufacturer. The microbiological profile remained stable during the six months of storage and met the requirements for similar products, i.e., chewing gum and unglazed caramel.

The project needs further research because the qualitative characteristics of nicotine poaches provided rather unambiguous results. Our study will help develop state standards for oral nicotine products. The results obtained will be used to formulate proposals to the organizations responsible for the future Technical Regulations of the Eurasian Economic Union for nicotine products.

Keywords: Oral non-tobacco nicotine products, smokeless tobacco, water activity, nicotine, snus, safety

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INTRODUCTION

Oral tobacco-free nicotine products have gained global popularity in recent years, and the Eurasian Economic Union is no exception.

The government of Sweden subjected the production and use of snus to the Food Act. A special technical specification controls its quality and safety. Sweden developed a special standard for oral non-tobacco nicotine products. In the late 1990s, this standard was used to develop a voluntary quality standard for Swedish snus called GothiaTek®. It is GothiaTek® that was adopted by the trade organization of the European Smokeless Tobacco Council (ESTOC). Eventually, it became the industry standard for all smokeless tobacco products in Europe [1]. Unlike Sweden, Russia offers domestic consumers both tobacco and non-tobacco oral nicotine products.

Currently, Armenia's Ministry of Economy is developing Technical Regulations for nicotine products for all Eurasian Economic Union counties. The draft regulations establish safety requirements for heated tobacco products, electronic nicotine delivery liquids, and oral non-tobacco nicotine products, e.g., nicotine poaches.

All such products, except nicotine poaches, are consumed as aerosol, which appears when the substance is heated. Nicotine poaches, however, are consumed orally, which means that the safety of this type of nicotine-containing products should be strictly controlled.

Oral non-tobacco nicotine products have an obvious advantage over conventional smoking: its consumption is individual and produces no tobacco smoke with its harmful effect on the so-called passive smokers.

In Russia, technical regulations for tobacco products can be found in Federal Law No. 268-FZ (December 22, 2008). However, the law standardizes neither nicotine content nor microbiological indicators, and such approach poses certain health risks for consumers.

Although the demand for these products keeps growing, consumers receive very little information about the properties, quality profile, and quantitative characteristics of oral nicotine products, not to mention their potential adverse impact on human health. In the absence of state standard, these products remain beyond the scope of any regulatory documents.

However, some domestic studies have been going on in this sphere; for example, Duruncha *et al.* developed a method for determining the mass fraction of nicotine in oral non-tobacco nicotine products [2].

What is even more important, the early years of nicotine poaches on the Russian market saw some comprehensive studies of nicotine content. Pankov *et al.* commented upon the absence of standards for nicotine poaches that were visioned to replace traditional cigarettes [3]. The authors reported that the nicotine content in nicotine poaches varied from 29.22 to 62.83 mg/g, and high nicotine content could harm consumer's health.

Other early publications indicated that the lacking governmental regulation led to illegal homemade production of oral nicotine products [4]. This research again mentioned the major fluctuations in nicotine content in these products, which, in turn, could pose potential harm to consumers.

As a result, the qualitative characteristics and safety indicators of oral nicotine products are a prospective and relevant research area. Our study featured the quality profile of nicotine poaches during storage: nicotine content, water activity, and microbiological indicators. Our conclusions could be of great use to the future quality and safety standard of oral nicotine products.

STUDY OBJECTS AND METHODS

Samples of nicotine poaches were provided by British American Tobacco, Belarus (Figs. 1–4). Other experimental materials of four brands came from BAT Pécsi Dohánygyár (2–8 Dohany St., Pecs, 7622, Hungary) (Table 1).

The research was conducted in the laboratory of chemistry and quality control, the All-Russian Research Institute of Tobacco, Makhorka, and Tobacco Products, Krasnodar, and included the following methods:

– the mass fraction of nicotine in oral non-tobacco nicotine products was measured using the method of gas chromatography, certificate No. 025-01.00281-2013-2020;
– another method of nicotine determination followed CORESTA CRM 62: *Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis*;

Figure 1 Velo Violet Frost Medium nicotine poaches

Figure 2 Velo Ice Cool nicotine poaches

Figure 3 Velo Freeze nicotine poaches

Figure 4 Velo Tropic Breeze nicotine poaches

Table 1 Research samples of nicotine poaches

Sample	Brand	Manufacture date – use-by date	Number of poaches/total weight	Labelled nicotine content
1	Velo Violet Frost Medium	Sept.23, 2022 – Sept.23, 2023	20 poaches, 10 g	6 ± 2 mg
2	Velo Ice Cool Strong	Nov.24, 2022 – Nov.24, 2023	20 poaches, 14 g	8 ± 2 mg
3	Velo Freeze X-Strong	Nov.14, 2022 – Nov.11, 2023	20 poaches, 14 g	9 ± 2 mg
4	Velo Tropic Breeze X-Strong	Nov.17, 2022 – Nov.17, 2023	20 poaches, 14 g	9 ± 2 mg

Table 2 Water activity in oral nicotine products

No.	Brand	Storage conditions	Water activity					
			Initial		After three months of storage		After six months of storage	
			Replications		Replications		Replications	
			1	2	1	2	1	2
1	Velo Violet Frost	Natural, indoors	0.9314	0.9427	0.9410	0.9410	0.9453	0.9389
2	Velo Ice Cool		0.8986	0.9081	0.9053	0.9118	0.9083	0.9045
3	Velo Freeze		0.9046	0.8911	0.9266	0.9282	0.9207	0.9138
4	Velo Tropic Breeze		0.9502	0.9459	0.9530	0.9590	0.9546	0.9513
5	Velo Violet Frost	Air-conditioned room (t = 22 ± 2°C; φ = 60 ± 5%)	0.9314	0.9427	0.9482	0.9466	0.9419	0.9419
6	Velo Ice Cool		0.8986	0.9081	0.9203	0.9240	0.9161	0.9183
7	Velo Freeze		0.9046	0.8911	0.9044	0.9151	0.9138	0.9174
8	Velo Tropic Breeze		0.9502	0.9459	0.9523	0.9545	0.9507	0.9504
9	Velo Violet Frost	Refrigeration chamber (t = +4°C)	0.9314	0.9427	0.9386	0.9385	0.9426	0.9362
10	Velo Ice Cool		0.8986	0.9081	0.9076	0.9038	0.9179	0.9163
11	Velo Freeze		0.9046	0.8911	0.9203	0.9204	0.9385	0.9306
12	Velo Tropic Breeze		0.9502	0.9459	0.9592	0.9547	0.9634	0.9509

– the water activity was studied based on CORESTA CRM 88: *Determination of Water Activity of Tobacco and Tobacco Products* using an Aqualab TDL2 device with a tunable diode laser;
– the moisture content test relied on a 3-h standard method with a drying oven, where the samples were dried at 95°C for 3 h, as in State Standard 3935-2000.

The microbial tests were conducted at the Department of Bioorganic Chemistry and Technical Microbiology, Kuban State Technical University, Krasnodar, and involved the following guidelines: State Standard 10 444.15-94, State Standard 31747-2012, and State Standard 10 444.12-2013.

RESULTS AND DISCUSSION

We needed three stages to study the safety factors of oral nicotine products.

The first stage involved such variables as sample weight, filling volume, and type (encased/not encased), as well as their effect on water activity. These experiments included an Aqualab TDL2 device.

The sample weight and filling volume did not affect the water activity, provided that the sample stayed below the internal upper mark of the Aqualab bowl.

The second stage featured environmental variables, i.e., natural conditions, air-conditioning, and refrigeration chamber, as well as their impact on the water activity and nicotine content in four different samples of nicotine poaches, i.e., Velo Violet Frost, Velo Ice Cool, Velo Freeze, and Velo Tropic Breeze, after three and six months of storage (Table 2).

Table 2 shows that seven samples out of twelve (No. 1, 2, 4, 8, 9, 10, and 12) maintained stable water activity after three months of storage. Under different environmental conditions, these samples demonstrated insignificant fluctuations (≤ 0.012) in water activity indicator.

Seven samples (No. 1, 2, 4, 5, 8, 9, and 12) maintained stable water activity after six months of storage: even under different environmental conditions, fluctuations did not exceed 0.012 A_w.

The Velo Freeze sample (No. 3, 7, and 11) demonstrated a gradual increase in water activity under all storage conditions. As a result, these samples received special attention in the further studies because growing water activity may indicate an early change in the microbiological status of the product. Such products needed further monitoring after nine and twelve months of storage.

As for the nicotine content (Table 3), five samples (No. 1, 3, 4, 5, and 6) yielded stable values after three months of storage. Under different storage conditions, nicotine fluctuations were insignificant and stayed below 0.7 mg/g.

Four samples (No. 2, 5, 7, and 11) maintained the same nicotine content after six months of storage. Under different storage conditions, nicotine fluctuations were insignificant and stayed below 0.7 mg/g. Further monitoring is necessary after nine and twelve months of storage.

The third stage featured the microbiological parameters. This part of our research involved direct methods to test the samples for the presence or absence of microbiological contamination. The samples spent six months under various storage conditions.

The domestic food industry knows no microbiological standards for nicotine poaches. However, these products are similar in consumption to unglazed caramel and chewing gum, so we adopted the permissible levels of microorganisms from the microbiological safety standards stipulated by Technical Regulations of Customs Union TR CU 021/2011 (Clause 1.4: Sugar and Confectionery Products, Appendix 2: Microbiological Safety Standards) (Table 4).

Tables 5 and 6 illustrate the results of the microbiological experiments.

Table 5 shows that all samples, regardless of storage conditions, revealed no microbiological changes after three months of storage. Table 6 shows that almost all samples, regardless of storage conditions, revealed no changes in microbiological status after six months of storage. However, we registered a slight increase in mesophilic aerobic and optionally anaerobic microorganisms after six months of refrigerated storage in the Velo Violet Frost and Velo Freeze samples.

These samples had rather high humidity (44–50%), which could potentially trigger the development of unwanted microflora. However, the microbiological indicators remained below the permissible content level of 500 CFU/g because the product formulation included preservatives.

Table 3 Nicotine content in oral nicotine products

No.	Brand	Storage conditions	Nicotine, mg/g					
			Initial		After three months of storage		After six months of storage	
			Replications		Replications		Replications	
			1	2	1	1	2	1
1	Velo Violet Frost	Natural, indoors	10.423	10.115	10.000	10.480	9.040	9.071
2	Velo Ice Cool		10.440	10.146	11.680	10.560	10.612	10.817
3	Velo Freeze		11.360	11.287	11.280	11.640	10.461	10.321
4	Velo Tropic Breeze		12.950	12.768	13.680	12.760	11.819	11.617
5	Velo Violet Frost	Air-conditioned room (t = 22 ± 2°C; φ = 60 ± 5%)	10.423	10.115	10.188	10.143	9.776	9.438
6	Velo Ice Cool		10.440	10.146	10.568	10.339	11.231	10.234
7	Velo Freeze		11.360	11.287	12.273	11.271	10.676	10.773
8	Velo Tropic Breeze		12.950	12.768	14.119	13.767	12.193	11.430
9	Velo Violet Frost	Refrigeration chamber (t = +4°C)	10.423	10.115	10.411	8.916	10.852	12.042
10	Velo Ice Cool		10.440	10.146	11.837	11.515	12.045	11.126
11	Velo Freeze		11.360	11.287	12.961	12.004	11.310	10.806
12	Velo Tropic Breeze		12.950	12.768	9.550	16.236	10.287	20.818

Table 4 Acceptable microbiological safety standards for chewing gum and unglazed caramel as products similar in consumption to nicotine poaches

Indicator	Permissible level	Notes
Quantity of mesophilic aerobic and facultative anaerobic microorganisms, CFU/g	≤ 500	Chewing gum and unglazed caramel
Coliforms not allowed in food products per g (L)	1.0	
Mold, CFU/g	≤ 50	
Yeasts, CFU/g	≤ 50	

Table 5 Microbiological profile of oral nicotine products after three months of storage

No.	Brand	Storage conditions	Quantity of mesophilic aerobic and facultative anaerobic microorganisms, CFU/g	Yeasts and molds, CFU/g	Coliforms/1 g product
1	Velo Violet Frost	Natural, indoors	≤ 1.0×10	≤ 1.0×10	n. d.
2	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
3	Velo Freeze		≤ 1.0×10	≤ 1.0×10	n. d.
4	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.
5	Velo Violet Frost	Air-conditioned room (t = 22 ± 2°C; φ = 60 ± 5%)	≤ 1.0×10	≤ 1.0×10	n. d.
6	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
7	Velo Freeze		≤ 1.0×10	≤ 1.0×10	n. d.
8	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.
9	Velo Violet Frost	Refrigeration chamber (t = +4°C)	≤ 1.0×10	≤ 1.0×10	n. d.
10	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
11	Velo Freeze		≤ 1.0×10	≤ 1.0×10	n. d.
12	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.

n. d. – not detected

Table 6 Microbiological profile of oral nicotine products after six months of storage

No.	Brand	Storage conditions	Quantity of mesophilic aerobic and facultative anaerobic microorganisms, CFU/g	Yeasts and molds, CFU/g	Coliforms/1 g product
1	Velo Violet Frost	Natural, indoors	≤ 1.0×10	≤ 1.0×10	n. d.
2	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
3	Velo Freeze		≤ 1.0×10	≤ 1.0×10	n. d.
4	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.
5	Velo Violet Frost	Air-conditioned room (t = 22 ± 2°C; φ = 60 ± 5%)	≤ 1.0×10	≤ 1.0×10	n. d.
6	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
7	Velo Freeze		≤ 1.0×10	≤ 1.0×10	n. d.
8	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.
9	Velo Violet Frost	Refrigeration chamber (t = +4°C)	4.5×10^2	≤ 1.0×10	n. d.
10	Velo Ice Cool		≤ 1.0×10	≤ 1.0×10	n. d.
11	Velo Freeze		3.6×10^2	≤ 1.0×10	n. d.
12	Velo Tropic Breeze		≤ 1.0×10	≤ 1.0×10	n. d.

n. d. – not detected

Our results suggest that, even if water activity exceeds 0.7, oral nicotine products do not have to be banned from sale because they still comply with the permissible microbiological safety standards given in Table 4.

The All-Russian Research Institute of Tobacco, Makhorka, and Tobacco Products submitted a proposal to standardize the water activity indicator for nicotine poaches as part of the future Technical Regulations of the Eurasian Economic Union for nicotine-containing products.

The technical regulation draft also establishes that the nicotine mass fraction in a nicotine poach is not to exceed 11 mg per product.

Microbiological monitoring should be repeated after nine and twelve months of storage, taking into account the expiry date and water activity dynamics.

CONCLUSION

This research obtained important experimental data on the effect of various storage conditions on nicotine poaches after three and six months of storage, e.g., their water activity indicator, nicotine content, and microbiological parameters.

Samples of Velo Violet Frost and Velo Freeze revealed slight microbiological contamination with mesophilic aerobic and facultative anaerobic microorganisms after refrigerated storage at t = +4°C, but this indicator stayed well within the permissible microbiological safety standards of 500 CFU/g.

Since the poaches were refrigerated in original sealed packaging, the increase in mesophilic aerobic and facultative anaerobic microorganisms may indicate contamination at the production stage.

Some samples demonstrated a slight increase in water activity. Minor fluctuations in nicotine content did not exceed the permissible error. However, Velo Tropic Breeze poaches stored in a refrigerator at t = +4°C showed a nicotine content of 9.550–16.236 mg/g after three months of storage and 10.287–20.818 mg/g after six months. Such fluctuations were most likely caused by mixing errors during production.

The ambiguous quality characteristics means that the research needs to be continued. Eventually, these studies will help to develop a state standard for oral nicotine tobacco-free products. Now we are preparing a number of proposals to be included in the future Technical Regulations of the Eurasian Economic Union for nicotine products.

CONTRIBUTION

N.A. Pankov, E.V. Gnuchikh, and V.G. Lobanov supervised the project. N.A. Pankov, A.Yu. Lushnikova, and T.V. Vanitskaya provided the research data. N.A. Pankov, E.V. Gnuchikh, and T.A. Perezhogin collected the data and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interests regarding the publication of this article.

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The impact of lead, cadmium, and mercury on the reproduction of mountain hares (*Lepus timidus* L., 1758) in the north of Krasnoyarsk Krai

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Abstract:
Reproduction is key to the survival and development of a species. Anthropogenic activities release significant amounts of toxic pollutants into the environment. In this study, we aimed to determine effects of heavy metals on some reproductive parameters of the mountain hare.

Female mountain hares (n = 41) were hunted in the reference and industrially polluted areas of Krasnoyarsk Krai during four seasons. Their skeletal muscles, liver, and kidneys were subjected to atomic absorption spectrometry to determine concentrations of lead, cadmium, and mercury.

The contents of lead, cadmium, and mercury were significantly higher in the hares from the contaminated areas compared to the reference sites. According to the results, the exposure to lead, cadmium, and mercury had an impact on the reproductive potential of the female mountain hares. In particular, we established correlations between numbers of embryos and corpora lutea and contents of lead in the kidneys and liver, as well as cadmium in the kidneys. The number of corpora lutea and embryonic losses in the female hares from the contaminated areas were higher than those in the hared from reference areas. However, the numbers of embryos did not differ significantly between the compared areas.

Our study showed that about 40% of the liver samples and 100% of the muscle tissue samples obtained from the hares in the impact zone contained high concentrations of lead and cadmium. Therefore, hunting in these industrially polluted areas may pose a toxic hazard to the indigenous peoples living there. Further research is needed to assess potential and actual fertility, offspring survival, and other important parameters of mountain hare populations exposed to different levels of chemical pollution.

Keywords: Heavy metals, cadmium, lead, mercury, reproduction, warm-blooded animals, corpus luteum, embryo, liver, kidneys, skeletal muscles

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INTRODUCTION

Species can only sustain their natural populations if they are able to reproduce under unfavorable conditions, especially in the areas of anthropogenic impact [1]. The development of industry and farming has led to environmental pollution, with toxic elements entering the food chains [2, 3]. Anthropogenic pollutants include a wide range of synthetic organic compounds and heavy metals. Commonly found in low concentrations, they can accumulate in feed, water, and air, inevitably harming herbivores and other animals. While a one-time intake of chemicals may have a low toxic load and therefore not cause any physiological abnormalities, some com-

pounds accumulate over time, producing a cumulative effect. Moreover, pollutants can additively disrupt body processes and functions, often targeting reproductive organs. Numerous studies point to the complexity of effects that chemical compounds can have on the endocrine system of mammals. As a result, scientists find it difficult to identify general patterns and extrapolate the data.

Under certain conditions, anthropogenic pollutants can have a significant impact on the reproductive system of animals, reducing their reproductive potential. Studies have identified potential effects of some toxic pollutants on warm-blooded animals. However, there is insufficient information on the factors that determine the rate of these effects or the impact of combined pollutants on physiological systems. These factors are key to identifying risks to the productivity and well-being of populations. Heavy metals, particularly cadmium, lead, and mercury are among the most dangerous pollutants, or ecotoxicants.

Cadmium is a common environmental pollutant from industrial and agricultural activities throughout the world. It enters the body of animals through contaminated feed and water, as well as through cadmium-containing smoke or dust. Humans absorb cadmium from food in relatively low concentrations (3–5%). However, once cadmium reaches the kidneys, it can remain there for 10 to 30 years, causing necrosis of tubular cells [4, 5]. In the liver, cadmium provokes necrosis, apoptosis of hepatocytes, and cessation of autophagy [6]. Intoxication with cadmium salts can cause a disease called "itai-itai", which originated in Japan. This disease was caused by the contamination of the Jinzu River and its tributaries with cadmium salts and other heavy metals from mining waste dumped into the river. Those residents who consumed fish from this river and used contaminated water for drinking or irrigation developed pathologies, including joint pain, hypotension, muscle hypotrophy, bone fractures and deformations, anemia, pneumonia, as well as gastrointestinal and kidney diseases [7, 8].

Cadmium poses a serious public health risk. It is especially dangerous for the reproductive system due to its impact on the hormonal status. This is why cadmium is called an endocrine disruptor [9, 10]. In particular, it disrupts the rate of steroidogenesis in the ovaries and placenta, interfering with the secretion of female sex hormones [11].

Cadmium can cause spontaneous abortion in early pregnancy. High levels of this metal in the placenta increase the expression of metallothioneins. These are low-molecular-weight proteins with a high cysteine content that prevent toxic metals from penetrating the placenta [12]. Cadmium reduces the bioavailability of zinc and can replace it in some fetal tissues, affecting normal growth and development of the fetus, as well as disrupting cell division and differentiation [13]. Moreover, cadmium reduces the synthesis of leptin, increases the concentration of corticosterone, and interrupts the production of progesterone in the placenta [14].

High concentrations of cadmium were studied in the reproductive organs of rats, rabbits, and Japanese quails [15–17]. The administration of cadmium to laboratory animals caused stromal proliferation in the ovaries, an increase in the number of atretic follicles, and degeneration of the corpus luteum [18, 19]. The effect of cadmium on ovarian follicles is associated with changing levels of gonadotropin hormones and lower levels of follicle-stimulating and luteinizing hormones.

Exposure to cadmium significantly induced oxidative stress in the ovaries of rats. This resulted in increased levels of malondialdehyde (a lipid oxidation product) coupled with decreased levels of the antioxidant enzyme catalase [20]. Agarwal *et al.* also reported that cadmium can cause hormonal imbalance or oxidative stress, which can provoke miscarriage [21].

At moderate or high concentrations, cadmium affects the synthesis of steroids in the reproductive organs [22]. At low concentrations, cadmium can act like estrogen or androgen by binding directly to their receptors [23]. Johnson *et al.* found that cadmium can cause disturbances in the reproductive system of some wild animals and contribute to hormone-related cancer [24].

Lead is one of the most dangerous toxic metals that disrupts the functioning of all organs and systems, especially the kidneys and hematopoietic, nervous, and reproductive systems. Lead has a toxic effect on the developing fetus. High concentrations of lead cause acute poisoning in humans, while chronic lead-poisoning promotes severe consequences and death [25]. Lead enters the body through inhalation of lead-containing dust or fumes, as well as through the oral route. It enters the bloodstream and accumulates in the bones and soft tissues, mainly in the brain, liver and kidneys, for a long time, often for life. Bones are believed to contain up to 95% of all lead in the body [26]. Pregnancy increases the need for calcium. Accumulated in the bones, lead can replace calcium and circulate in the bloodstream, becoming an endogenous cause of poisoning [27, 28]. Through the bloodstream, lead penetrates the placenta and disrupts the development of the fetus [29–32]. In pregnant women, lead intoxication can cause spontaneous abortion, premature birth or rupture of fetal membranes, arterial hypertension, preeclampsia, or gestational diabetes mellitus. Moreover, it slows down the growth and development of the fetus and promotes the birth of underweight babies [33–40].

Lead exposure is associated with hormonal imbalances that cause disruption in the reproductive system. Its accumulation has an adverse effect on the functioning of the endocrine glands. In particular, it affects the hypothalamic-pituitary axis, causing changes in the secretion of thyroid-stimulating hormone, growth hormone, as well as follicle-stimulating and luteinizing hormones. Nkomo *et al.* reported that women with an increased level of lead in the blood had higher concentrations of follicle-stimulating and luteinizing hormones [41].

An experiment with rats showed that higher concentrations of lead promoted persistent vaginal estrus after

a period of normal estrus, the development of ovarian follicular cysts, and a decrease in the number of corpora lutea [42]. Chronic doses of lead administered orally caused atresia at all stages of folliculogenesis and decreased the development of follicles [43]. High doses of lead induced more noticeable changes such as swelling and necrosis of the ovarian follicles [44]. An *in vitro* study showed the effects of lead on the total antioxidant status and superoxide dismutase activity [45]. Vigeh *et al.* reported adverse effects of lead acetate at a concentration of 10 μg/dL on the reproductive parameters of men [46].

Mercury is a natural trace element that can leach from geological sediments into aquatic ecosystems or is emitted into the air from volcanic eruptions, forest fires, or hot springs [47]. Mercury is also a by-product of human activities such as gold mining, fuel combustion, cement production, and chemical industry [47–49]. Volatile mercury can be transported through the air to locations distant from its sources [50]. This makes mercury pollution a global problem. While natural emissions of mercury into the atmosphere have remained relatively stable over the past 150 years, its anthropogenic emissions have increased sharply. This has led to higher stress, especially in aquatic ecosystems [47]. Exposure to mercury varies depending on its concentrations in the environment and in different foods [25, 51].

Mercury is most toxic when converted to methylmercury, primarily by bacteria in marine and freshwater ecosystems. Methylmercury bioaccumulates in the tissues of organisms and passes from one trophic level to another. As a result, predators are exposed to the highest concentrations of methylmercury and suffer from its negative neurological, immunological, and reproductive effects. This is most pronounced in aquatic food systems [47, 48]. Elemental mercury, like organic mercury, penetrates the placental barrier and causes fetal development defects [52].

Mercury and its compounds cause a wide range of toxic effects depending on their chemical form, dose, and level of exposure [53]. In women, mercury can accumulate in the ovaries and cause reproductive problems, including infertility. High doses of mercury in experimental animals have increased the potential incidence of reproductive disorders, such as infertility, stillbirth, congenital malformations, or spontaneous abortions [48]. Exposure to mercury causes imbalance in the female hormonal system, causing infertility. The progesterone/estrogen ratio changes in favor of estrogens, inhibiting the release of follicle-stimulating and luteinizing hormones from the anterior pituitary gland [54]. Mercury can also cause infertility by increasing the secretion of prolactin. This is similar to the effect of dopamine on the pituitary gland and midbrain, which affects lactopoiesis and reproductive organs [55].

Ma *et al.* observed a positive relationship between ovarian mercury accumulation and the incidence of follicular atresia in laying hens [56]. In their study, progesterone levels decreased significantly in all mercury-treated groups. On the other hand, the levels of follicle-stimulating and luteinizing hormones showed an inverse correlation with mercury doses. The experimental groups also showed a significant decrease in the activity of catalase, superoxide dismutase, and glutathione reductase, as well as in the content of glutathione.

In a study by Lundholm, poultry treated with daily doses of methylmercury (5 mg for 6 days and 1 mg for 50 days) experienced significant thinning and deformation of the eggshell, as well as a decrease in egg production [57]. The *in vitro* assays demonstrated the inhibitory effect of mercury on gastrointestinal calcium absorption and/or bone marrow mobilization.

Altunkaynak *et al.* determined the effects of exposure to mercury vapor on the reproductive parameters of rats [58]. In their study, the rats exposed to higher concentrations of mercury exhibited prolonged estrous cycles and morphological changes in the corpus luteum. Their ovaries had various histomorphometric changes, with a significant increase in the volume of atretic follicles. The authors concluded that exposure to mercury vapor alters the estrous cycle but does not have a significant effect on ovulation, implantation, or pregnancy.

Another *in vitro* study determined the effect of mercury on the secretion activity of progesterone and insulin-like growth factor-I (IGF-I) by analyzing porcine ovarian granulosa. The study confirmed the direct effect of mercury on the release of the steroid hormone progesterone, as well as the interference of mercury in steroidogenesis and apoptosis [59].

Koli *et al.* studied the effect of mercury on myometrial activity in Wistar rats treated with 5, 50, and 500 μg/L of mercury chloride in drinking water for 28 days [60]. The authors concluded that low doses of mercury had a detrimental effect on myometrial activity by altering calcium entry into smooth muscle and/or calcium release from intracellular stores [60]. Their study also confirmed that mercury has a concentration-dependent uterotonic effect. Nakade *et al.* reported inflammation of the endometrium and myometrium in the animals exposed to mercury [61].

Most bioavailable compounds of lead, cadmium, and mercury are not destroyed in the soil, water, plants, and animals, exposing them to their toxic, carcinogenic, and mutagenic effects. The progressive emission of heavy metals into the atmosphere, soil, and water can increase their concentrations in animals and humans to critical values, with potentially irreversible consequences for both individual species and whole populations [25, 40]. Most researchers see the highest risk in the toxic effects of heavy metals on reproduction [1].

The above fully applies to mammals such as lagomorphs that live in the conditions of chemical environmental pollution. Studies have shown that heavy metals can have a significant negative impact on the reproductive systems of humans and laboratory or farm animals, as well as the development of their offspring. However, we lack scientific data on their effects on the reproduction processes in wild mammals and birds. This is due to certain difficulties in conducting field studies of

free-living populations compared to laboratory experiments, where dose-related toxic effects can be accurately assessed. Field studies should consider a number of additional factors, including the movement of animals along a pollution gradient, the mosaic nature of impact fields, diet variability, etc. [1].

Literature lacks information on effects of cadmium, lead, and mercury on the natural populations of mountain hares [62–66]. Therefore, more research is needed to manage these populations in pessimal environmental conditions more effectively and to ensure safety of resulting food products.

STUDY OBJECTS AND METHODS

Materials were collected in 2011–2014 in Krasnoyarsk Krai (Russia), particularly in the middle reaches of the Agapa river (71°638611 north, 87°881650 east) (n = 22) contaminated by industrial activities, as well as in the reference areas near Kheta village, Kresty village (the Khatanga river), and Kataryk village (the Kheta river) (71°319090 north, 99°312443 east) (n = 19).

In particular, we used internal organs (ovaries, liver, kidneys) and skeletal muscles (forelimb muscles undivided into separate muscles) of adult female mountain hares (n = 41) selected in the late spring-summer period, from the 20th of May to the 20th of June. The hares were hunted with snares by local hunters from among the indigenous peoples of the North, who are allowed to hunt in the spring and summer season by law (Article 19 of Russian Federal Law No. 209-FZ of July 24, 2009 "On hunting and conservation of hunting resources").

To obtain biomaterial, linear routes (at least 10 km long), or trapping paths, were laid in the floodplains of the rivers in the reference and contaminated areas. This method is highly efficient and provides intact organs and tissues. In addition, it avoids contamination with lead, unlike shooting with lead-containing bullets.

The captured animals were marked with tags placed around their necks. The tags had been prepared and provided to the hunters in advance. The carcasses were frozen in an icebox at –18°C and packaged separately in new food-grade plastic bags.

Once every ten days, the carcasses were transported by air to a veterinary laboratory in the city of Dudinka. There, they were measured and weighed by the authors in partnership with veterinary experts from the Russian Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor) in Krasnoyarsk Krai. Next, the samples of organs and tissues were taken for microelement analysis. Also, we counted the number of corpora lutea in the ovaries and the number of embryos in the uterus of every animal.

The material was then packaged in food-grade polyethylene and delivered to a chemical laboratory at the Rosselkhoznadzor Reference Center in Krasnoyarsk. There, Sollar (TJA Solution, USA) and Varian (Agilent Technologies, USA) atomic absorption spectrophotometers were used to determine the contents of lead, cadmium, and mercury in the muscles, liver, and kidneys expressed in terms of natural moisture (69–73, 74–78, and 70–73%, respectively).

The number of corpora lutea of pregnancy was an indicator of potential fertility, while the number of viable embryos characterized actual fertility. The viability of embryos was assessed visually based on morphometric characteristics, linear dimensions, and the absence of signs of tissue resorption or degeneration.

Total embryonic losses were calculated as a difference, %, between the number of corpora lutea of pregnancy and the number of viable embryos, according to [1]. Female hares with more corpora lutea than viable embryos were considered to have embryonic losses. Females with identical numbers of corpora lutea and viable embryos were considered to have no embryonic losses.

The number of lost embryos, or a simple difference between corpora lutea and viable embryos, was also considered in comparative analysis. The percentage of female hares with embryonic losses was calculated in relation to all the females in the sample. Average fertility rates were based on the data for all the females that had corpora lutea and/or embryos.

All the females in the sample were of reproductive age, with their precise age not determined. Neither was it possible to determine the stage of embryogenesis, since there has been little research into the reproductive processes of mountain hares. Moreover, their rutting season is quite lengthy.

The statistical analysis was carried out with standard methods using MS Excel (Office 2019) and Statgraphics (19-X64) [67]. The samples were described by calculating the mean (M), error of the mean (m), standard deviation (SD), median (Med), as well as 25 and 75% percentiles.

Since the distribution of some part of the sample differed from normal distribution, nonparametric analysis was used in addition to the standard methods of variability statistics. In particular, the Mann-Whitney test (U) and the Kruskal-Wallis test (H) were employed to compare the significance of differences, while the Spearman rank correlation method was used to determine relationships between different parameters. Statistical significance was set at $p \leq 0.05$.

According to the Ministry of Ecology and Rational Management of Natural Resources in Krasnoyarsk Krai, the man-made impact on the environment has remained the same over the last few years [68]. Therefore, our experimental data are still quite relevant and can be used for comparative environmental monitoring in the study areas both in the short and long terms.

RESULTS AND DISCUSSION

First, we determined the number of corpora lutea in the ovaries of, and the number of embryos in, female mountain hares sampled from the reference and contaminated areas (Fig. 1).

As can be seen, the number of corpora lutea found in the ovaries of female hares from the contaminated areas averaged 8.72 ± 1.63, which was significantly ($p = 0.00$)

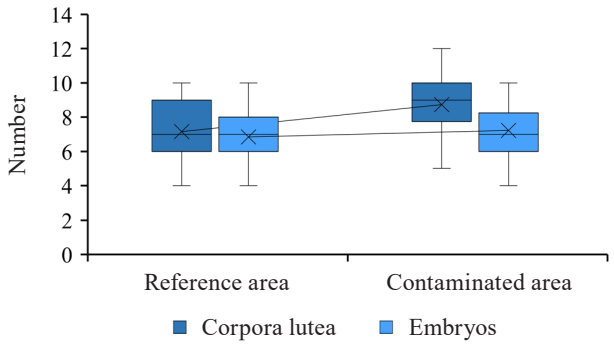
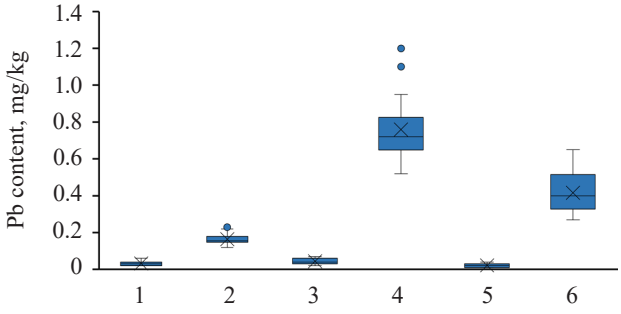
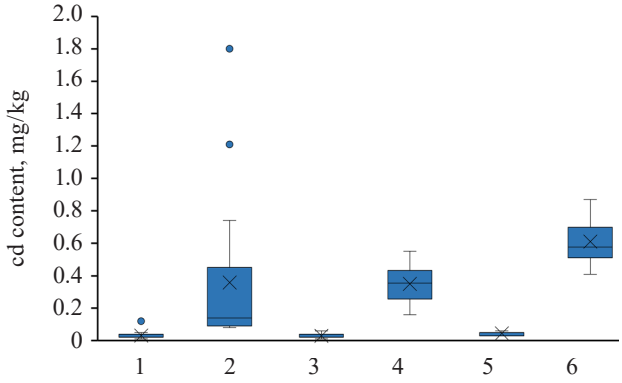


Figure 1 The numbers of corpora lutea and embryos in female mountain hares from reference and contaminated areas in the north of Krasnoyarsk Krai



1 – Muscles, reference area
2 – Muscles, contaminated area
3 – Liver, reference area
4 – Liver, contaminated area
5 – Kidneys, reference area
6 – Kidneys, contaminated area

Figure 3 Concentrations of lead in the muscles, liver, and kidneys of female mountain hares from reference and contaminated areas, north of Krasnoyarsk Krai



1 – Muscles, reference area
2 – Muscles, contaminated area
3 – Liver, reference area
4 – Liver, contaminated area
5 – Kidneys, reference area
6 – Kidneys, contaminated area

Figure 4 Concentrations of cadmium in the muscles, liver, and kidneys of female mountain hares from reference and contaminated areas, north of Krasnoyarsk Krai

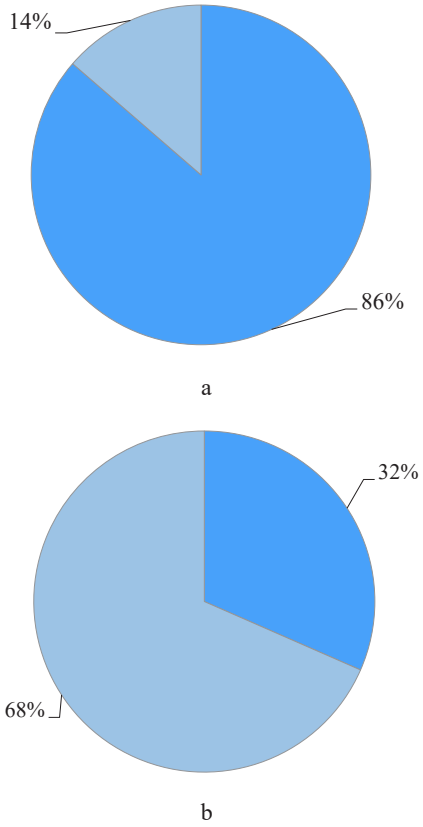


Figure 2 Proportion of female mountain hares with embryonic losses (in blue): a in the contaminated areas, north of Krasnoyarsk Krai; b in the reference areas, north of Krasnoyarsk Krai

larger (1.21 times) than in the reference areas. However, the numbers of embryos did not differ significantly between the areas.

Correlational analysis established a strong positive correlation between the number of corpora lutea and the number of embryos in the female hares, both in the reference ($r = 0.95$, $p = 0.00$) and contaminated ($r = 0.76$, $p = 0.00$) areas.

Figure 2 shows the proportion of female hares with embryonic losses in the contaminated and reference areas.

The contents of heavy metals in the organs and tissues of mountain hares were previously reported by us in [66].

The average concentrations of lead in the muscles, liver, and kidneys of female hares in the contaminated areas were 0.16 ± 0.03, 0.75 ± 0.16, and 0.41 ± 0.10 mg/kg, respectively (Fig. 3). These values were significantly ($p = 0.00$) higher than those for female hares in the reference areas, namely 5.33, 18.75, and 20.5 times as high, respectively.

The average concentrations of cadmium in the muscles, liver, and kidneys of female hares in the contaminated areas were 0.35 ± 0.46, 0.35 ± 0.10, and 0.61 ± 0.11 mg/kg, respectively (Fig. 4). These values were significantly ($p = 0.00$) higher than those for female hares in the reference areas, particularly 11.66, 17.50, and 15.25 times as high, respectively.

Table 1 Statistically significant correlations ($p \leq 0.05$) of lead and cadmium concentrations in female mountain hares from reference and contaminated areas in Krasnoyarsk Krai

Pairs	Correlation	*p*
Pb in reference muscles/ Pb in reference liver	0.49	0.03
Pb in contaminated muscles/ Pb reference liver	–0.54	0.02
Pb in contaminated muscles/ Pb in contaminated liver	0.58	0.00
Pb in reference liver/ Pb in reference kidneys	0.81	0.00
Pb in contaminated liver/ Pb in contaminated kidneys	0.73	0.00
Cd in reference muscles/ Cd in contaminated muscles	0.55	0.01
Cd in reference muscles/ Cd in reference liver	0.55	0.01
Cd in contaminated muscles/ Cd in contaminated liver	0.68	0.00
Cd in contaminated muscles/ Cd in reference kidneys	–0.46	0.04
Cd in contaminated muscles/ Cd in contaminated kidneys	0.49	0.02
Cd in contaminated liver/ Cd in contaminated kidneys	0.54	0.01

Having performed correlational analysis, we found a close positive correlation between lead concentrations in the liver and kidneys of the hares from the reference ($r = 0.81$, $p = 0.00$) and contaminated ($r = 0.73$, $p = 0.00$) areas, an average positive correlation between lead concentrations in the muscles and liver in the reference ($r = 0.49$, $p = 0.03$) and contaminated ($r = 0.58$, $p = 0.00$) areas, and an average negative correlation between lead concentrations in the liver of the hares from the reference areas and in the muscles of the hares from the contaminated areas ($r = –0.54$, $p = 0.02$) (Table 1).

As for cadmium, we established a positive correlation between its concentrations in the muscles and liver of the hares from the contaminated ($r = 0.68$, $p = 0.00$) and reference ($r = 0.55$, $p = 0.01$) areas. We also found an average positive correlation between cadmium concentrations in the liver and kidneys ($r = 0.54$, $p = 0.01$), as well as in the muscles and kidneys ($r = 0.49$, $p = 0.02$), in the contaminated areas. Finally, there was an average negative correlation between cadmium concentrations in the muscles of the hares from the contaminated areas and in the kidneys of the hares from the reference areas ($r = –0.46$, $p = 0.04$) (Table 1).

Table 2 shows correlations between lead and cadmium concentrations in the organs and tissues of the hares in the areas with different levels of contamination. In both the reference and contaminated areas, we found a correlation between cadmium concentrations in the muscles and lead concentrations in the liver ($r = –0.51$, $p = 0.02$ and $r = 0.50$, $p = 0.02$, respectively), as well as between cadmium concentrations in the muscles and lead concentrations in the kidneys ($r = –0.51$, $p = 0.02$ and $r = 0.50$, $p = 0.02$, respectively). In the contaminated areas, an average correlation was established between cadmium and lead concentrations in the muscles ($r = 0.43$, $p = 0.04$).

Table 2 Statistically significant correlations ($p \leq 0.05$) between lead and cadmium concentrations in the organs and tissues of female mountain hares from reference and contaminated areas in Krasnoyarsk Krai

Pairs	Contaminated area		Reference area	
	Correlation	*p*	Correlation	*p*
Cd in muscles/ Pb in liver	0.50	0.02	–0.51	0.02
Cd in muscles/ Pb in kidneys	0.50	0.02	–0.51	0.02
Cd in muscles/ Pb in muscles	0.43	0.04	–	–

The content of mercury in the muscles, liver, and kidneys of the hares in the contaminated areas varied between 0.01 and 0.025 mg/kg and was significantly higher than in the reference areas.

Next, we analyzed the relationships between reproductive indicators and the contents of heavy metals in the internal organs of the mountain hares in the contaminated areas. As a result, average positive correlations were established between the number of embryos and the lead concentration in the kidneys ($r = 0.43$, $p = 0.04$), the number of corpora lutea and the lead concentration in the liver ($r = 0.50$, $p = 0.01$), the number of corpora lutea and the cadmium concentration in the kidneys ($r = 0.44$, $p = 0.04$), as well as the number of corpora lutea and the lead concentration in the kidneys ($r = 0.46$, $p = 0.03$). However, there was no correlation between reproductive indicators and mercury concentrations in the hares' organs and tissues.

The liver and kidneys of mammals are known to accumulate lead, cadmium, and mercury. Therefore, these organs can be used to determine concentrations of these metals in the body. Although skeletal muscles are not as indicative of metal concentrations, they are quite significant in environmental and toxicological studies due to a high nutritional value [69].

Our results showed a clear relationship between the environment and the contents of heavy metals in the mountain hares. The sampling area is affected by the Norilsk industrial region, with its large metallurgical and mining enterprises accounting for 1.9% of the Russian GDP. About 2.5 million tons of pollutants are released into the atmosphere of Norilsk annually, according to the Federal Statistics Service and the Ministry of Ecology and Rational Management of Natural Resources in Krasnoyarsk Krai. A significant proportion of these pollutants are heavy metals [68]. Moreover, many of them spread through the air over a distance of 250–400 km from the point of release, polluting water sources, bottom sediments, and soil, as well as causing vegetation degradation [70–72]. Emissions from the enterprises in the cities of Kayerkan and Norilsk spread in the northern

and northwestern directions, accelerating during winter snowstorms [73, 74]. Accumulating in depressions, contaminated snow delivers large amounts of trace elements to shrubs and herbaceous plants during the growing season, as well as contaminating their shoots and bark. And these plants are what mountain hares feed on.

Previous studies have reported the impact of chemical pollution on wild animals in the north of Krasnoyarsk Krai. Increased concentrations of lead, cadmium, and mercury were detected in the organs and tissues of the wild reindeer, mountain hare, and ptarmigan [66, 75, 76]. Histological and morphological studies found abnormal changes in the main body systems of micromammals, such as mouse-like rodents, living in the Norilsk industrial region [77]. Numerous pathologies were identified in the organs and tissues of small mammals, including the hematopoietic organs and endocrine system. This undoubtedly affects the reproductive potential of the local animals. Obviously, similar processes can occur in other plant-feeders in these and adjacent areas.

In our study, the average lead contents in the liver and kidneys of mountain hares in the contaminated area (Krasnoyarsk Krai) were 0.75 ± 0.16 and 0.41 ± 0.10 mg/kg, respectively. These values were significantly higher in the internal organs of lagomorphs inhabiting polluted areas in Pakistan and Turkey, but only slightly higher in the liver of the brown hare (*Lepus europaeus* L.) in Serbia and Poland [78–81]. Yet, the lead contents in our study were similar to those found in the liver and kidneys of mountain hares in Norway, Finland, and the Kirov region in Russia [62–65]. The concentrations of lead in the reference area in Krasnoyarsk Krai were similar to those for lagomorphs in uncontaminated regions of Europe [82–84].

The highest concentrations of cadmium (averaging 0.61 ± 0.11 mg/kg) were detected in the kidneys of mountain hares at a contaminated landfill in the north of Krasnoyarsk Krai. However, these values were significantly lower than similar indicators in the industrial and mining regions of Canada, Poland, Turkey, and Serbia [79–81, 85, 86]. Yet, they were comparable to cadmium concentrations in lagomorphs and other species from non-industrial areas [80, 83, 87–92]. Also, the contents of cadmium in the liver and kidneys of mountain hares in the reference area of Krasnoyarsk Krai were lower than similar indicators for lagomorphs in European non-industrial regions. This may be because the main sources of cadmium in the environment are agricultural, energy, and transport enterprises.

Thus, the contents of lead and cadmium in the female mountain hares from the reference areas of Krasnoyarsk Krai appeared quite low compared to these indicators for lagomorphs from other countries. At the contaminated landfills in Krasnoyarsk Krai, these contents were comparable to the values for contaminated areas in other regions. Our study did not detect abnormally high concentrations of these heavy metals, unlike numerous studies of wild animals in other industrialized countries. However, a significant part of the liver samples and all the samples of hare meat obtained in the middle reaches of the Agapa river exceeded the hygienic standards for lead and cadmium concentrations (The unified sanitary, epidemiological, and hygienic requirements for goods that are subject to sanitary and epidemiological supervision (control)). This undoubtedly indicates an increased toxic impact on the population of mountain hares and its well-being.

The numbers of corpora lutea in the ovaries of female mountain hares (potential fertility) in the contaminated and reference areas of Krasnoyarsk Krai were 8.72 and 7.15, respectively, with a significant ($p = 0.00$) difference of 19.16%. The number of embryos was also larger in the contaminated areas. In particular, the average number of viable embryos per female (maximum actual fertility) was 7.22 in the contaminated areas, compared to 6.84 in the reference areas. Yet, the difference between them of 5.27% was not significant. However, the survival rate of embryos was higher in the reference areas than in the contaminated areas (95.6 and 82.8%, respectively).

The female hares with embryonic losses accounted for 31.57% in the reference areas, which is close to the average for other species. This indicator reached 86.36% in the contaminated areas. Noteworthily, the females in the reference areas lost only one embryo, while those in the contaminated areas, an average of 1.73 embryos ($p = 0.02$). According to Fig. 5, 42% of the latter lost one embryo, 42% lost two embryos, and 16% lost three embryos.

Many researchers consider the average number of corpora lutea per female as an indicator of potential fertility. Its natural increase is one of the adaptation mechanisms enabling the population to survive in unfavorable conditions. Toxic pollution appears to stimulate potential fertility, which partially compensates for losses induced by chemical exposure. We can also assume that the frequency of embryo resorption may increase in contaminated areas, especially under harsh climatic conditions.

Previous studies have shown changes in the reproductive parameters of female small rodents and other species caused by increasing toxic load. In particular,

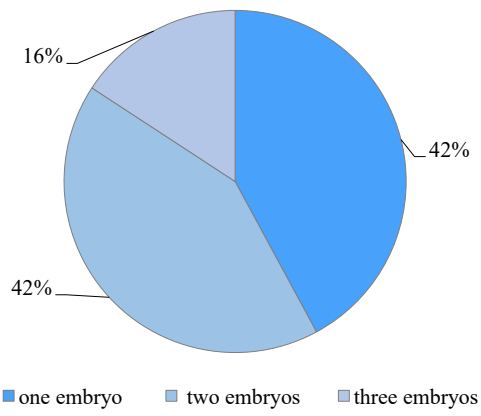


Figure 5 Embryonic losses in female mountain hares from contaminated areas, north of Krasnoyarsk Krai

the density, biomass, and diversity of micromammals in Murmansk region and the Middle Urals increased with distance from the impact zone around large metal works [93–96]. The proportion of pregnant red-backed voles (*Clethrionomys rufocanus*) was significantly associated with a distance from the source of pollution [97]. The embryo resorption near the source of pollution was 7.7 times as high as in the control group. However, the embryo survival rate was 96.4–95.8% in the reference areas, 92.0–81.9% in the buffer zone, and just over 60% in the impact zone, i.e., 4 km away from the source of pollution [98]. Considering the level and nature of contamination, similar values are typical for the mountain hare in the north of Krasnoyarsk Krai. However, our study showed no decrease in the actual fertility of the hares, compared to this indicator for mouse-like rodents in the above studies.

Mukhacheva studied the reproduction of the bank vole (*Clethrionomys glareolus*) in an industrial pollution gradient [99]. The author reported significantly reduced levels of toxicants in the organs, tissues, and developing embryos due to the system of blood-tissue barriers in the female bodies. This decrease in toxicants was detected alongside their increased contents in the contaminated feeding areas. At certain development stages, the embryos are highly resistant to the effects of heavy metals. The study showed no significant effects of their contents on potential fertility or embryonic death during intrauterine development. Noteworthily, all breeding females in the reference and contaminated areas had similar numbers of corpora lutea of pregnancy. However, corpora lutea were more numerous in the group of sexually mature females in the impact zone, which is consistent with our results. Also, the number of viable embryos was higher in the bank voles in contaminated areas [99].

It should be noted that the number of living embryos does not indicate the quality of the offspring. Studies rarely identify deviations from normal development related to the location of the placenta, the size or weight of the embryo, etc. However, chronic exposure to heavy metals during pregnancy can cause up to 30 types of serious pathological changes in the embryos of small mammals. These pathologies can lead to the birth of weakened offspring or its death [100–102].

Weight is the most important indicator of offspring's life potential. Benitez *et al.* established an inverse relationship between the weight of embryos and/or newborns and the concentrations of toxicants in the placenta [103]. According to Salomeina and Mashak, chronic exposure to pollutants during pregnancy and lactation can decrease the weight of embryos by 6–10%, and in some cases by up to 25%, compared to the control group [101].

A study in the Middle Urals found that increased exposure to lead and cadmium in bank vole embryos by the end of the prenatal period caused weight loss, compared to the embryos from reference areas [1]. This inevitably weakened the offspring and increased their mortality in the early postnatal period. There is an opinion that the weight of embryos is more indicative of changes in the reproductive potential of a population than the level of embryonic losses.

A number of objective reasons prevented us from establishing relationships between the sex, age, and size of a population and its reproduction in a chemically polluted area [1]. Therefore, this should become the object of further research.

CONCLUSION

Our study showed significantly higher concentrations of lead, cadmium, and mercury in the liver, kidneys, and muscles of mountain hares in the industrially polluted areas compared to the reference sites.

The relationships between the reproductive indicators and the contents of heavy metals in the internal organs of the mountain hare revealed an average positive correlation between the number of embryos and the concentration of lead in the kidneys ($r = 0.43$, $p = 0.04$), the number of corpora lutea and the concentration of lead in the liver ($r = 0.50$, $p = 0.01$), the number of corpora lutea and the concentration of cadmium in the kidneys ($r = 0.44$, $p = 0.04$), and the number of corpora lutea and the concentration of lead in the kidneys ($r = 0.46$, $p = 0.03$). The significant correlations between individual metals in the organs and tissues may indicate common sources of their entry into the environment of the study area.

The average number of corpora lutea per female mountain hare was 8.72 in the contaminated areas, which was 19.16% higher than in the reference areas ($p = 0.00$). However, the numbers of embryos did not differ significantly in the reference and contaminated areas.

The proportion of female hares with embryonic losses was 31.57% in the reference areas, which is close to the average for other species, and 86.36% in the contaminated areas. Moreover, they lost only one embryo in the reference areas and an average of 1.73 embryos ($p = 0.02$) in the contaminated areas. The females that lost one, two, and three embryos in the contaminated areas accounted for 42, 42, and 16%, respectively.

According to our results, the levels of cadmium, mercury, and lead detected in the bodies of the mountain hares in the north of Krasnoyarsk Krai do not pose a risk of acute poisoning. Although significant differences were found in the potential fertility of female hares in the reference and contaminated areas, their actual fertility was not affected. The increase in corpora lutea of pregnancy, or potential fertility, apparently compensated for greater embryonic losses induced by exposure to pollutants.

Environmental pollutants caused by anthropogenic activities enter the food chain. Due to their toxicity, these pollutants, even at low concentrations, can have an adverse effect on animals in case of prolonged exposure. This has been shown by previous studies on numerous species of vertebrates in the European part of Russia [104–109]. Pollutants affect the structure of animal communities, their territorial distribution, aggregation, population density, and reproductive indicators

(fertility, survival, embryonic mortality, etc.). This was confirmed by our study, where we compared the samples from reference and industrially contaminated areas in the north of Central Siberia.

Our results are also indicative of the well-being of the mountain hare population in Krasnoyarsk Krai, mainly its ability to produce healthy offspring at the required level.

Further research is needed to assess potential and actual fertility, offspring survival, and other important parameters of mountain hare populations exposed to chemical pollution compared to those in reference areas.

Our study showed that about 40% of the liver samples and 100% of the muscle tissue samples obtained from the hares in the impact zone contained high concentrations of lead and cadmium. Therefore, hunting in these industrially polluted areas may pose a toxic hazard to the indigenous peoples living there.

CONTRIBUTION

All the authors contributed equally to the study and are equally responsible for the information published in this article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Quercetin isolated from *Hedysarum neglectum* Ledeb. as a preventer of metabolic diseases

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Abstract:
Diseases associated with metabolic disorders seem to affect more and more people worldwide. Biologically active supplements may prevent or relieve metabolic disorders. Quercetin is known for its potential to inhibit metabolic syndrome. This paper introduces an *in vivo* experiment on rodents. It featured hypoglycemic, hypocholesterolemic, and hepatotoxic properties of quercetin.
Quercetin was obtained from the hairy root extract of *Hedysarum neglectum* Ledeb. Two doses (50 and 100 mg/kg) were used to evaluate its hypoglycemic potential. Rats with induced diabetes were tested for body weight, glucose, and cholesterol while mice with induced hypercholesterolemia were checked for blood cholesterol changes. Potential biochemical and pathological changes in the liver were also studied on rats.
Quercetin treatment caused neither significant health problems nor death in the model animals. It had no effect on body weight, even in the animals with induced diabetes. In addition, quercetin did not increase glucose and cholesterol in the blood and triggered no pathological changes in the liver.
Quercetin isolated from *H. neglectum* hairy root extract demonstrated no hepatotoxicity. Unfortunately, it showed no beneficial effect on cholesterol and glucose levels and had no efficacy against metabolic syndrome. Further research is needed to assess the effect of quercetin on other metabolic markers, e.g., genes associated with the metabolism of lipids, carbohydrates, etc.

Keywords: Quercetin, *in vivo*, *Hedysarum neglectum*, metabolic syndrome, hepatotoxicity, hypercholesterolemic activity, hypoglycemic activity

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INTRODUCTION

Metabolic syndrome is a cluster of metabolic conditions that lead to dyslipidemia, hyperglycemia, insulin resistance, oxidative stress, inflammations, and, ultimately, to non-alcoholic fatty liver disease, obesity, diabetes mellitus, cardiovascular diseases, etc. [1]. Figure 1 illustrates the risk factors most closely tied to metabolic syndrome as evaluated by the All-Russian Association of Cardiologists [2, 3].

The diseases in Fig. 1 pose a threat to healthcare worldwide: for instance, cardiovascular diseases are the leading cause of death globally [4]. The number of people newly diagnosed with metabolic disorders has increased in the recent years (Fig. 2) [5–7].

On the one hand, metabolic conditions, e.g., diabetes and hypertension, accelerate age-related pathologies as they facilitate inflammatory and oxidative processes [9–11]. On the other hand, metabolic syndrome itself is age-related. According to Dominguez & Barbagallo, it is global population aging that stands behind the high incidence of metabolic syndrome because senior citizens often experience cardiovascular diseases, diabetes, and metabolic conditions all together (Fig. 3) [12].

Preventive measures against metabolic disorders involve healthy lifestyle combined with functional foods

and biologically active additives that contain metabolites of plant origin with antioxidant and anti-inflammatory properties [13, 14]. Such additives reduce cholesterol and glucose in the blood. For example, Sotiropoulou *et al.* and Serba *et al.* reported some effective biologically active substances of plant origin that improve human metabolism [15, 16].

Shabbir *et al.* highly praised quercetin, curcumin, and resveratrol as substances that inhibit inflammatory reactions, reduce insulin resistance, and counteract pathogenic and opportunistic strains that harm gastrointestinal microbiota [17]. All these beneficial properties make them potential preventers of metabolic diseases. Xu *et al.* studied berberine (*Berberis aristata*, *Berberis*

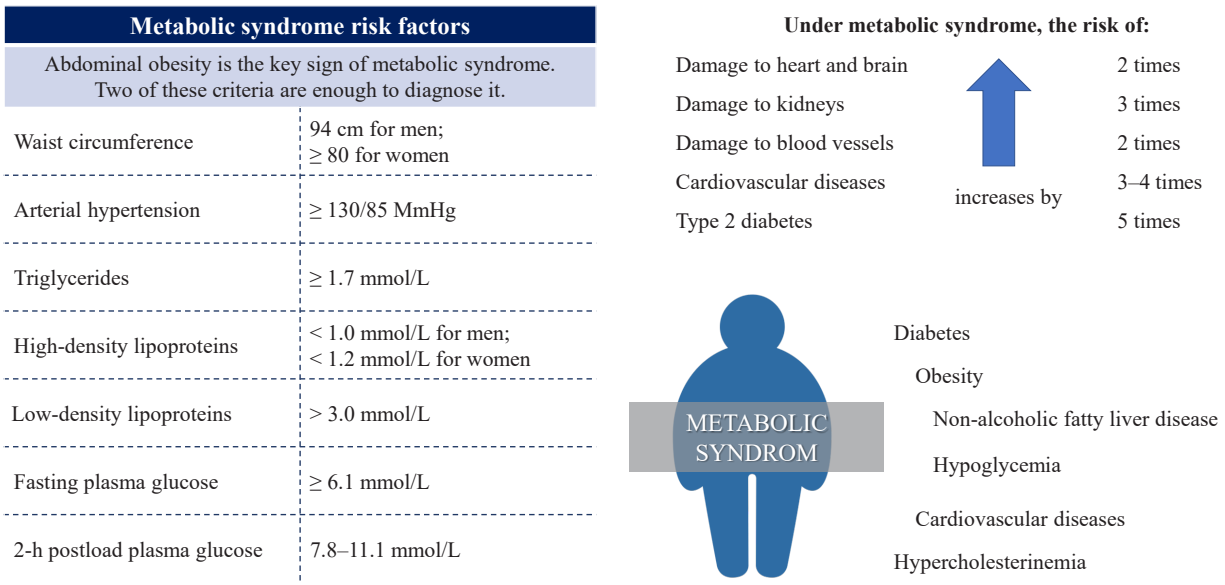

Figure 1 Risk factors of metabolic syndrome

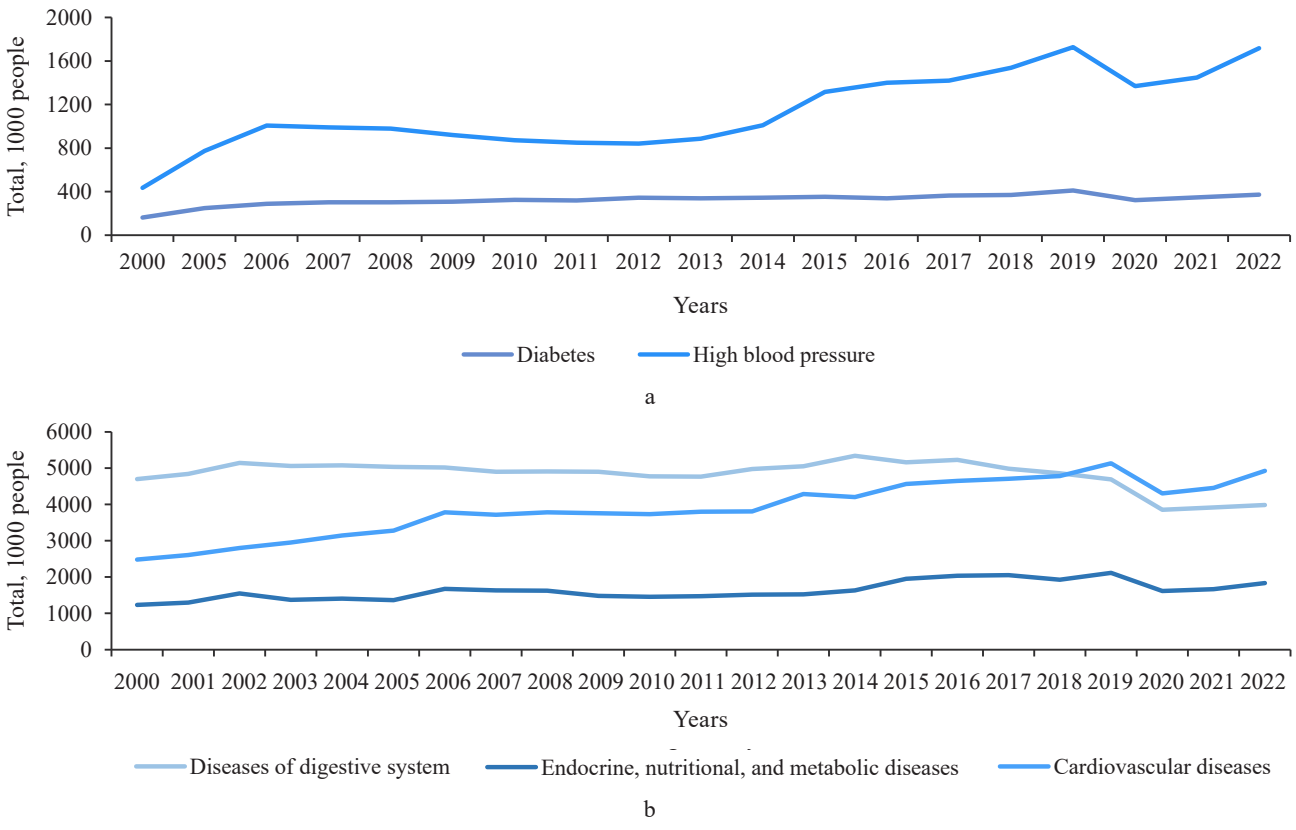

Figure 2 Morbidity statistics in Russia; patients newly diagnosed with: a) socially significant diseases, b) other major classes of diseases [8]

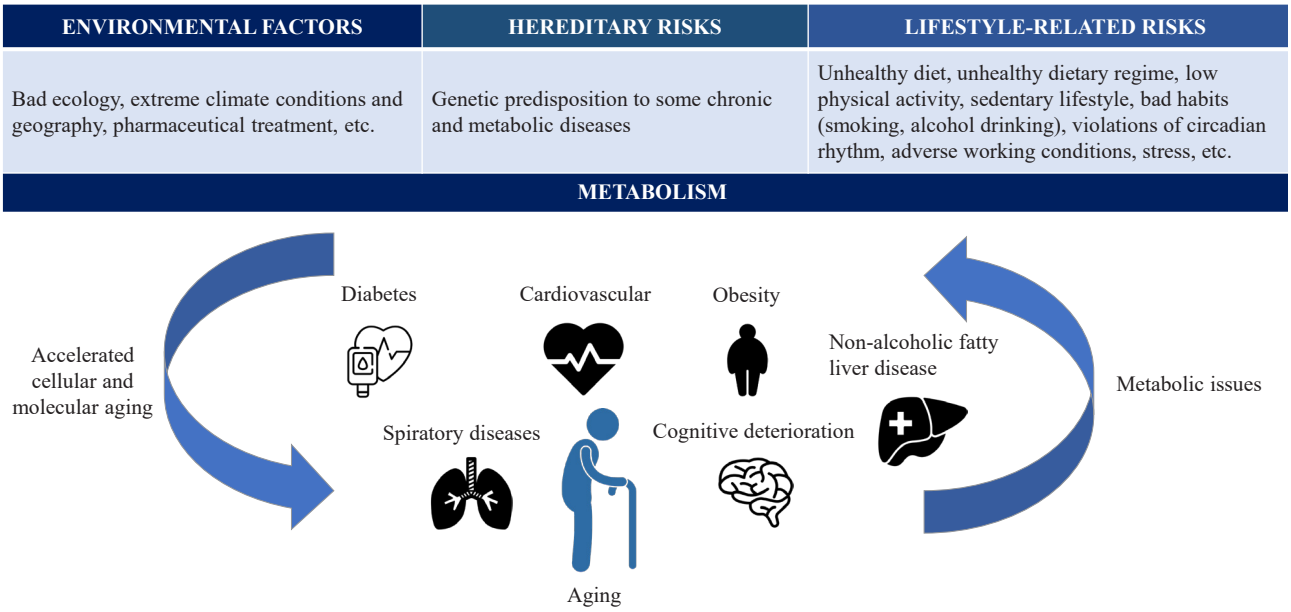

Figure 3 Correlation between aging and metabolic diseases

vulgaris, *Coptis chinensis*), an alkaloid found in many medicinal plants of India and China [18]. Berberine proved able to stimulate insulin secretion, facilitate insulin resistance, inhibit lipogenesis, and prevent adipose tissue fibrosis. In addition, it reduced liver steatosis and improved gastrointestinal microbiota.

Quercetin is a wide-spread plant flavonoid with antioxidant, anti-inflammatory, hypoglycemic, hypolipidemic, and hepatoprotective properties that have good potential against metabolic syndrome [17, 19, 20].

Belovol *et al.* gave quercetin to patients with arterial hypertension [4]. The additive improved the effectiveness of antihypertensive therapy, e.g., reduced cholesterol and triglycerides. Other studies *in vivo* reported quercetin as able to remove free radicals. It also regulated enzymatic defenses by affecting such enzymes as superoxide dismutase, catalase, glutathione peroxidase, etc. Quercetin had a positive impact on such properties of red blood cells as deformability, nitric oxide production, and osmotic resistance. It regulated metabolic dysfunction of glucose and lipids while exhibiting hepatoprotective properties, thus preventing metabolic syndrome [15, 21, 22]. Quercetin owes this activity to the effect that it exerts on the expression of some genes associated with inflammatory reactions, e.g., *SIRT1*, *NF-κB* p65, *iNOS*, etc., as well as on lipid and carbohydrate metabolism, e.g., *PON1*, *PPARG*, *ALDH1B1*, *APOA4*, etc. [15].

As their systematic intake causes few or no side effects, herbal biologically active substances are popular as remedies against metabolic disorders. Such substances as stanines or biguanides often cause gastrointestinal problems, liver dysfunction, weight gain, and other effects that restrict healthy life activities [18].

In our previous study, we established that the hairy root extract of *Hedysarum neglectum* Ledeb., also known as sweet vetch, exhibits cardioprotective potential. We used soil nematode *Caenorhabditis elegans* to test the extract, and the model organisms demonstrated better survival under oxidative stress and accumulated less lipid fractions [23]. We linked this activity to quercetin metabolite and its ability to prevent metabolic syndrome. However, as part of dietary supplements and functional foods, the extracts' efficacy depended on the extraction conditions, the initial composition of the raw materials, and the eventual variability of the quantitative and qualitative composition. Therefore, we extracted individual components from the extracts to be used in dietary supplements and functional foods. Thus, quercetin we isolated from the hairy root extract of *H. neglectum* increased the survival rate of nematodes under oxidative stress and reduced the accumulation of lipid inclusions. In addition, quercetin also increased the expression of the antioxidant defense gene *SOD-3* [24].

In this *in vivo* study, we evaluated the bioactivity of quercetin isolated from the *H. neglectum* hairy root extract, i.e., its ability to reduce cholesterol and glucose, protect the liver, and prevent metabolic syndrome.

We tested its effect on hypoglycemic properties, hypocholesterolemic activity, and hepatotoxicity.

STUDY OBJECTS AND METHODS

The research featured 95% quercetin obtained as a secondary metabolite of *Hedysarum neglectum* Ledeb. hairy roots. We described the cultivation, extraction, and purification processes in our previous publication [24].

All studies *in vivo* were conducted on the premises of Ifar Company, Tomsk. For the study, we used rodents that are similar to humans in physiological, cellular, and other functions. They were chosen for their small size, high reproduction rate, low maintenance, and short average life expectancy [4, 25].

For the hypoglycemic tests, we used 50 male rats (*Rattus* sp., 12 weeks old, 219–272 g) with induced diabetes mellitus. The model organisms were recognized as healthy and free from pathogenic microflora. The hypocholesterolemic experiments involved 29 male mice (*Mus musculus*, 19 weeks old, 40.8–49.6 g) with induced hypercholesterolemia. The animals were healthy and unaffected by pathogenic microflora. The hepatotoxicity tests involved 15 male rats (*Rattus* sp., 28 weeks old, 623–755 g). The model organisms were recognized as healthy and free from pathogenic microflora.

Male podens are preferable for laboratory experiments because they have no estrous cycle that could affect the susceptibility to etiological factors in females (Guidelines for preclinical studies of drugs. Part 1).

A bioethical committee proved that the animal test conditions complied with the Policy for Working with Laboratory Animals in Ifar Company and state standards of Humane Care and Use of Laboratory Animals (Guidelines for preclinical studies of drugs. Part 1, State Standard 33215-2014, State Standard 33216-2014, State Standard R ISO 10993-2-2009, SP 2.2.1.3218-14).

The hypoglycemic and hypocholesterolemic experiments followed similar procedures:

– we took blood samples from the tail vein, obtained serum, and measured the concentration of glucose and total cholesterol;

– we took blood samples from the inferior vena cava, obtained serum, and measured the concentration of total cholesterol.

In the hepatotoxicity experiment, we took blood samples from the jugular vein, obtained serum, and measured alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, and alkaline phosphatase, as well as total protein, albumin, and globulin. The latter was determined as the difference between the concentrations of total protein and albumin. The experiment involved an incomplete necropsy to study liver tissues. After the euthanasia, the liver was weighed, and its weight was compared to the body weight as percentage. The obtained liver tissues underwent a histological analysis.

In the hypoglycemic experiment, the male rats were anaesthetized by CO_2 inhalation with further cervical dislocation. As part of the hypocholesterolemic experiment, the mice were anesthetized with CO_2 for blood collection and euthanized. The male rats that participated in the hepatotoxicity tests were euthanized by exsanguination.

The blood biochemistry measurements involved a Minitecno LIND 126 biochemical analyzer (I.S.E. Srl, Italy) with commercial kits purchased from Vector-Best, Novosibirsk. The histological analysis of the liver microslides involved an Axio Lab A1 binocular light microscope with an Axiocam 105 digital camera (Carl Zeiss, Germany).

The hypoglycemic tests of 50 and 100 mg/kg quercetin were performed on male rats with diabetes mellitus induced by alloxan. The tests followed the protocol described in Table 1. Purified water served as negative control substance and medium for the test substances. Glibenclamide (Ozon, Russia), a glucose-lowering substance, was used as positive control.

This experiment involved 50 rats, which were divided into five groups of ten animals in each (Guidelines for preclinical studies of drugs. Part 1). The animals in groups 2–5 remained without food for 16 h, upon which we induced diabetes mellitus with a single intraperitoneal injection of alloxan solution at a dose of 150 mg/kg per 1 mL in line with experimentally selected conditions [26]. To confirm the development of diabetes mellitus, we measured the body weight and the concentration of glucose in the blood 48 h before and after the alloxan injection. Only animals with ≥ 11 mmol/L blood glucose stayed in the experiment. The test substances were administered two weeks later to let the animals stabilize. They received the test substance once a day during the last week of the experiment. We measured the fasting body weight and the concentration of glucose and total cholesterol in the blood once a week for 21 days. The hypoglycemic agent glibenclamide served as positive control at an effective dose of 5 mg/kg, which was administered intragastrically as often as the test substance [27].

The hypocholesterolemic tests involved 50 and 100 mg/kg of quercetin. The male mice received a lipoprotein lipase inhibitor to develop hypercholesterolemia. Table 2 shows the research protocol. Purified water served as a negative control substance and a medium for the substances under analysis. Alloxan (Diaem LLC, Russia) and Poloxamer P 407 (Koliphor) (BASF, USA) were used as pathology inducers.

The intact group included five animals while the experimental groups included eight animals each (Guidelines for preclinical studies of drugs. Part 1). To simulate hypercholesterolemia, the animals in groups 7–9 were intraperitoneally injected three times a week (Monday, Wednesday, and Friday) for two weeks with aqueous solution of a lipoprotein lipase inhibitor at 400 mg/kg

Table 1 Hypoglycemic test protocol

Group	Number of animals	Diabetes	Active substance
1	10	–	–
2	10	+	Purified water
3	10	+	Quercetin, 50 mg/kg
4	10	+	Quercetin, 100 mg/kg
5	10	+	Glibenclamide, 5 mg/kg

Table 2 Hypocholesterolemic test protocol

Group	Number of animals	Hypercholesterolemia	Active substance
6	5	–	–
7	8	+	Purified water
8	8	+	Quercetin, 50 mg/kg
9	8	+	Quercetin, 100 mg/kg

per 1 mL. Poloxamer P 407 (Koliphor) disrupted the clearance of lipoproteins. The intact animals in group 6 received no substances. The test substances and the negative control substance were injected into stomachs daily for two weeks in a volume of 1 mL. After the last administration, the animals were anesthetized for blood collection and euthanized. After that, we determined the concentration of total cholesterol in the blood serum.

To study hepatotoxicity, quercetin was administered intragastrically to male rats at 50 and 100 mg/kg daily for 14 days. Purified water served as negative control and medium for the test substances. Table 3 illustrates the research protocol.

The animals were monitored daily; the body weight and stool consistency tests took place once a week. The animals were euthanized 24 h after the last administration and fasting. The histological analysis made it possible to assess the potential toxic damage to the liver. The weight coefficient was determined as the percentage of liver weight to the body weight. The blood serum was tested in a semi-automatic biochemical analyzer for markers of damage to hepatocytes and biliary tract, i.e., alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, alkaline phosphatase, and total protein and albumin content. The globulin concentration was represented as the difference between the concentrations of total protein and albumin.

The literature review made it possible to determine the experimental doses as 50 and 100 mg/kg [19, 28].

Each group consisted of ≤ 10 animals, so we applied the nonparametric Mann-Whitney test to compare the indicators from different groups [29]. In the experiment on hypoglycemic activity, we used the Grubbs criterion as in [31] to define outliers (State Standard R ISO 16269-4-2017). Differences were considered statistically significant at $p < 0.05$. The data obeyed the normal distribution law, so we used the Shapiro-Wilk test to assess the hypoglycemic and hypocholesterolemic activity. The final results were presented as the mean of trait X and the mean error SE.

RESULTS AND DISCUSSION

In this research, quercetin caused neither severe health problems nor death in the model animals. During the entire experiment, the animals demonstrated no health problems associated with any potential toxic effects of quercetin. No changes in stool consistency were recorded. Thus, intragastric 50 and 100 mg/kg quercetin caused no major health problems after 14 days of treatment.

Hypoglycemic activity test results. We tested the rats' body weight after oral administration of quercetin and glibenclamide (Table 4).

The body weight changes in the male rats with induced diabetes after 50 and 100 mg/kg of quercetin were almost the same as in the intact and control groups ($p > 0.05$). The diabetic rats treated with 5 mg/kg of glibenclamide for 7 days showed body weight results similar to the intact and control groups ($p > 0.05$). Thus, a single intraperitoneal injection of 150 mg/kg alloxan and intragastric administration of 50 and 100 mg/kg quercetin did not affect the body weight in the experimental animals.

After oral administration of quercetin and glibenclamide, we also assessed the blood biochemistry of the rats (Table 5).

Before alloxan was administered, the concentration of glucose in the blood serum of the intact animals (5.264 ± 0.374 mmol/L, n = 10) and the grouping pool (5.254 ± 0.163 mmol/L, n = 80) conformed to standards. These indicators were similar ($p > 0.05$).

The rats developed diabetes 48 h after the intra-peritoneal injection of alloxan solution at a dose of 150 mg/kg ($p < 0.05$). These animals had a higher concentration of glucose and cholesterol in the blood serum than the intact animals. The effect persisted in experimental animals throughout the experiment ($p < 0.05$).

The rats received quercetin into the stomach for 7 days at doses of 50 and 100 mg/kg, which did not reduce the concentration of glucose and cholesterol in the blood serum ($p > 0.05$). Glibenclamide administered at a dose of 5 mg/kg reduced the concentrations of glucose and cholesterol in the blood serum, but their levels still exceeded those in the intact group ($p < 0.05$).

Table 3 Hepatotoxicity test protocol

Group	Number of animals	Active substance
10	5	Purified water
11	5	Quercetin, 50 mg/kg
12	5	Quercetin, 100 mg/kg

Table 4 Mean body weight of rats after oral administration of quercetin and glibenclamide (X ± SE)

Time	Body weight, g				
	Inactive rats	Purified water (control)	Quercetin		Glibenclamide, 5 mg/kg
			50 mg/kg	100 mg/kg	
Before alloxan	242 ± 4 (n = 10)	243 ± 1 (n = 80)			
48 h after alloxan	243 ± 4 (n = 10)	246 ± 4 (n = 10)	243 ± 2 (n = 10)	243 ± 3 (n = 10)	247 ± 3 (n = 10)
1 week after alloxan	247 ± 4 (n = 10)	248 ± 2 (n = 9)	246 ± 3 (n = 8)	248 ± 3 (n = 9)	248 ± 5 (n = 9)
2 weeks after alloxan	249 ± 3 (n = 10)	251 ± 4 (n = 7)	248 ± 3 (n = 6)	249 ± 2 (n = 7)	248 ± 4 (n = 7)
3 weeks after alloxan	258 ± 3 (n = 10)	260 ± 4 (n = 6)	258 ± 2 (n = 6)	259 ± 2 (n = 6)	257 ± 4*# (n = 6)

* statistically significant differences with intact group ($p < 0.05$)

\# statistically significant differences with control ($p < 0.05$)

Table 5 Mean blood biochemistry in rats after oral administration of quercetin and glibenclamide (X ± SE)

Time	Intact animals	Purified water (control)	Quercetin		Glibenclamide, 5 mg/kg
			50 mg/kg	100 mg/kg	
Glucose, mmol/L					
48 h after alloxan	4.883 ± 0.301 (n = 10)	22.618 ± 0.792* (n = 10)	22.015 ± 2.349* (n = 10)	21.563 ± 1.259* (n = 10)	21.475 ± 0.620* (n = 10)
1 week after alloxan	4.335 ± 0.227 (n = 10)	24.457 ± 0.700* (n = 9)	24.148 ± 0.800* (n = 8)	24.043 ± 0.776* (n = 9)	25.134 ± 0.579* (n = 9)
2 weeks after alloxan	4.533 ± 0.285 (n = 10)	23.372 ± 0.721* (n = 7)	23.068 ± 0.534* (n = 6)	23.446 ± 0.761* (n = 7)	22.861 ± 0.319* (n = 7)
3 weeks after alloxan	4.370 ± 0.378 (n = 10)	20.094 ± 0.398* (n = 6)	20.513 ± 0.401* (n = 6)	20.815 ± 0.413* (n = 6)	13.022 ± 0.584*# (n = 6)
Cholesterol, mmol/L					
1 week after alloxan	1.612 ± 0.063 (n = 10)	2.592 ± 0.090* (n = 9)	2.693 ± 0.059* (n = 8)	2.638 ± 0.114* (n = 8)	2.583 ± 0.043* (n = 9)
2 weeks after alloxan	1.544 ± 0.088 (n = 10)	2.584 ± 0.074* (n = 7)	2.415 ± 0.133* (n = 6)	2.445 ± 0.118* (n = 7)	2.554 ± 0.066* (n = 7)
3 weeks after alloxan	1.580 ± 0.074 (n = 10)	2.437 ± 0.073* (n = 6)	2.615 ± 0.077* (n = 6)	2.616 ± 0.059* (n = 6)	1.927 ± 0.042*# (n = 6)

* statistically significant differences with intact group ($p < 0.05$)

\# statistically significant differences with control ($p < 0.05$)

Table 6 Mean blood biochemistry in mice after oral administration of quercetin

Indicator	Intact animals (n = 5)	Purified water (control) (n = 8)	Quercetin	
			50 mg/kg (n = 7)	100 mg/kg (n = 8)
Cholesterol, mmol/L	1.921 ± 0.199	9.923 ± 1.274*	9.746 ± 0.912*	9.575 ± 0.840*

* statistically significant differences with intact group ($p < 0.05$)

Table 7 Mean body weight of rats during intragastric quercetin treatment, g

Time	Purified water	Quercetin	
		50 mg/kg	100 mg/kg
Before treatment	681 ± 25	683 ± 7	700 ± 22
After 1 week of treatment	676 ± 26	695 ± 10	713 ± 20
Body-weight increase	–5 ± 7	11 ± 8	13 ± 4
After two weeks of treatment	692 ± 34	713 ± 8	730 ± 20
Body-weight increase	16 ± 9	18 ± 2	17 ± 1

Therefore, 50 and 100 mg/kg of quercetin produced no hypoglycemic activity under these experimental conditions.

Hypocholesterolemic activity test results. Table 6 shows the biochemistry results of the blood of mice that received quercetin orally in doses of 50 and 100 mg/kg.

Poloxamer P 407 (Koliphor) is a lipoprotein lipase inhibitor that disrupts the clearance of lipoproteins. The mice received it intraperitoneally as a 400 mg/kg aqueous solution for 14 days three times a week (Monday, Wednesday, and Friday). As a result, they developed hypercholesterolemia. The experimental mice were intragastrically injected with 50 and 100 mg/kg of quercetin for 14 days, but the concentration of cholesterol in the blood serum did not go down.

Thus, 50 and 100 mg/kg of quercetin had no hypocholesterolemic effect under the experimental conditions.

Hepatotoxicity activity test results. Table 7 illustrates the body weight of animals during the intragastric treatment with quercetin.

During the entire treatment, the animals that received quercetin demonstrated the same body-weight increase as the control animals ($p > 0.05$).

In this research, 14 days of intragastric 50–100 mg/kg quercetin did not affect the body weight of the test animals and, therefore, had no impact on their general health condition.

Table 8 shows the blood biochemistry indicators at the end of the intragastric treatment.

The animals that received 50 mg/kg of quercetin demonstrated a significantly lower gamma-glutamyltransferase activity, but this indicator remained the same in the animals that received 100 mg/kg. Both quercetin doses reduced the aspartate aminotransferase activity.

High alanine aminotransferase and aspartate aminotransferase activities are a characteristic biochemical sign of hepatotoxicity. Therefore, the low liver enzyme activity could not be interpreted as hepatotoxicity, and we observed no critical liver damage in this experiment. The decrease in gamma-glutamyltransferase,

Table 8 Mean indicators of blood biochemistry in rats at the end of intragastric treatment with quercetin

Indicator	Purified water	Quercetin	
		50 mg/kg	100 mg/kg
Alanine aminotransferase, U/L	39.24 ± 5.43	36.78 ± 2.63	36.92 ± 3.44
Aspartate aminotransferase, U/L	166.50 ± 5.20	109.06 ± 14.08*	107.44 ± 13.86*
Gamma-glutamyltransferase, U/L	1.485 ± 0.104	0.724 ± 0.222*	1.529 ± 0.182
Alkaline phosphatase, U/L	155.04 ± 15.06	157.88 ± 14.07	129.57 ± 16.96
Total protein, g/L	63.97 ± 9.05	74.68 ± 8.91	71.81 ± 9.45
Total albumin, g/L	22.82 ± 0.85	13.42 ± 0.82*	23.33 ± 3.47
Total globulin, g/L	41.15 ± 9.18	61.25 ± 9.61	48.47 ± 9.37

* statistically significant differences with control ($p < 0.05$)

Table 9 Mean values of absolute and relative liver weight at the end of intragastric treatment quercetin

Indicator	Purified water	Quercetin	
		50 mg/kg	100 mg/kg
Body weight, g	681 ± 25	683 ± 7	700 ± 22
Liver, g	23.312 ± 1.729	23.645 ± 0.756	24.869 ± 1.232
Liver, %	3.432 ± 0.215	3.432 ± 0.146	3.503 ± 0.121

Table 10 Individual data of histological tests

Group	Animal No.	Morphological changes
10	1	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	2	Congestion with red blood cells, stromal edema, focal hydropic degeneration of hepatocytes
	3	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	4	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	5	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
11	1	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	2	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	3	Congestion with red blood cells, stromal edema, focal hydropic degeneration of hepatocytes
	4	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	5	Congestion with red blood cells, stromal edema, focal hydropic degeneration of hepatocytes
12	1	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	2	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	3	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes
	4	Congestion with red blood cells, stromal edema, focal hydropic degeneration of hepatocytes
	5	Congestion with red blood cells, stromal edema, focal granular degeneration of hepatocytes

alanine aminotransferase, and aspartate aminotransferase proved that quercetin was not hepatotoxic.

To sum it up, 50 and 100 mg/kg of intragastric quercetin had no effect on blood biochemistry that would signal of hepatotoxicity after 14 days of treatment.

The necropsy revealed no pathological changes. Therefore, 14 days of intragastric treatment with 50–100 mg/kg of quercetin caused no pathological changes in the liver.

Table 9 demonstrates the absolute and relative liver weight at the end of the intragastric treatment with quercetin.

We detected no statistically significant differences in body and liver weight between the intact group and the test group. Therefore, 14 days of treatment with 50–100 mg/kg of quercetin caused no pathological damage to liver tissues.

Table 10 demonstrates the histological results.

All experimental animals revealed congestion accompanied by red blood cell stasis, stromal edema, and focal granular or hydropic degeneration of hepatocytes. These phenomena occurred in all groups, including the control, and were caused by the acute circulatory disorders during euthanasia. Thus, 14 days of intragastric treatment with 50 and 100 mg/kg of quercetin triggered no morphological changes in liver tissues.

Our results contradicted those published by Hosseini *et al.*, who reported the positive effect of quercetin on glucose, triglycerides, cholesterol, and weight [3]. Vessal *et al.* gave 10 and 15 mg/kg of quercetin to rats with streptozotocin-induced diabetes [30]. After 10 days, glucose, triglycerides, and cholesterol went down whereas some liver enzymes became more active. Kılıçarslan & Dönmez also experimented on rats with streptozotocin-induced diabetes [31]. They administered 15 mg/kg of quercetin for 28 days and observed low activity of liver enzymes, lipid peroxidation, and increased expression of *SOD* and *GSH*, i.e., genes responsible for antioxidant cell defense. Zhang *et al.* experimented on mice with genetic obesity and diabetes [32]. The

animals received quercetin in doses of 50–200 mg/kg for 35 days and demonstrated a decrease in blood glucose, triglycerides, and total cholesterol. Presumably, this effect was associated with increased insulin secretion. Bhaskar *et al*. injected female rabbits with 25 mg/kg of quercetin: after 90 days of hypercholesterolemic diet, their lipid profile significantly improved [33]. Rivera *et al.* assessed the effect of 2 and 10 mg/kg of quercetin on Zucker rats [34]. The treatment relieved symptoms of dyslipidemia, hypertension, and hyperinsulinemia; 10 mg/kg of quercetin promoted weight loss. Zhao *et al.* studied Wistar rats on a high-fat diet [35]. The animals received resveratrol at a dose of 120 mg/kg/day and quercetin at a dose of 240 mg/kg/day. The combination of quercetin and resveratrol reduced insulin resistance and inflammation in adipose tissue.

Our data differed from those reported by other authors because we used different model objects, quercetin doses, and markers of metabolic syndrome.

In general, our results do not refute other studies. After administering 50 and 100 mg/kg of quercetin, we observed no increase in body weight, blood glucose, or cholesterol in male rats with alloxan-induced diabetes, nor did we detect anything like that in male mice with hypercholesterolemia induced by a lipoprotein lipase inhibitor.

CONCLUSION

Our *in vivo* experiments on male rats and mice featured the hypoglycemic, hypocholesterolemic, and hepatotoxic properties of quercetin isolated from the hairy root extract of *Hedysarum neglectum* Ledeb. The results could be summarized as follows:

1. Treatment with quercetin in doses of 50 and 100 mg/kg caused neither serious health problems nor death, which means that quercetin produced no toxic effect on the test subjects.

2. The same treatment caused no changes in the body weight of the model animals with induced diabetes;

3. It did not affect the levels of glucose and cholesterol in the blood of the model animals with induced diabetes. Therefore, quercetin exhibited no hypoglycemic activity: it did not reduce the high blood glucose levels that caused diabetes. However, quercetin did not increase serum glucose and cholesterol either.

4. Administration of 50 and 100 mg/kg of quercetin had no impact on the cholesterol in the blood of the model animals with induced hypercholesterolemia. Therefore, quercetin demonstrated no hypocholesterolemic activity as it did not reduce the concentration of cholesterol in the blood serum. Still, the treatment did not increase the serum cholesterol levels in the hypercholesterolemic animals.

5. The treatment inhibited the activity of aspartate aminotransferase; at 50 mg/kg, quercetin led to a statistically significant decrease in the activity of gamma-glutamyltransferase.

6. Administration of 50 and 100 mg/kg of quercetin neither affected blood biochemistry nor caused any pathological changes in liver tissue.

This *in vivo* research on rodents showed that quercetin can be safely used in dietary supplements because it has no toxic effect on liver cells. However, quercetin demonstrated no preventive activity against metabolic syndrome, i.e., it did not reduce cholesterol or blood glucose. Further research is needed to assess the effect of quercetin on other markers of hypoglycemia and hypocholesterolemia, such as antioxidant defense genes or those involved in carbohydrate and lipid metabolism, e.g., *PON1*, *PPARG*, *ALDH1B1*, *APOA4*, etc.

CONTRIBUTION

All authors have contributed equally to this project.

CONFLICT OF INTEREST

The authors declared no potential conflict of interests regarding the publication of this article

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Iranian grape syrup used as a prebiotic and its effect on the physicochemical, microbiological, and sensory properties of probiotic yogurt

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Abstract:
Yogurt is known as a suitable carrier of probiotics. Its supplementation with Iranian grape syrup used as a prebiotic can enhance its sensory and physicochemical properties, as well as improve the viability and growth of probiotics. Therefore, we aimed to investigate the effect of Iranian grape syrup on stirred probiotic yogurt's rheological, physicochemical, and microbial properties.
Probiotic yogurt samples were fortified with 3, 6, and 9% of Iranian grape syrup and evaluated in terms of pH, acidity, syneresis, viscosity, total phenolic and anthocyanin contents, as well as probiotic bacterial counts during 21 days of storage in a refrigerator at 4°C.
The results revealed that increasing concentrations of grape syrup inversely affected the yogurt's pH, so the lowest and highest pH levels were recorded in the samples with the highest syrup concentration and the control (without syrup), respectively. No general trend was observed in acidity despite significant differences in acidity among the syrup-supplemented yogurts and the control ($p \leq 0.05$). Syneresis demonstrated an inverse correlation, while viscosity exhibited a direct relationship, with a grape syrup concentration. Monitoring microbial changes in the samples throughout storage revealed a better growth in microbial colonies in the yogurts with higher grape syrup concentrations.
According to consumer preferences and physicochemical qualities, the optimal concentration of Iranian grape syrup was found to be 9%. Supplementing yogurt with grape syrup enhances its probiotic viability and metabolic activity. Considering its positive impact on both consumer preferences and product properties, Iranian grape syrup can be utilized as a prebiotic in future research to develop functional and symbiotic yogurts.

Keywords: Fermentation, grape syrup, probiotics, prebiotics, yoghurt, bacterial count

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INTRODUCTION

Yogurt is a widely consumed dairy product with high nutritional value. It is produced through the fermentation of milk by various bacteria, mainly lactic acid bacteria and bifidobacteria [1, 2]. The consumption of yogurts containing probiotic bacteria has significantly increased in recent years. In general, yogurt and other dairy products are referred to as suitable carriers of probiotic microorganisms. Recently, attention has been drawn to various compounds that can positively affect yogurt's bacterial, rheological, and physicochemical properties and increase its nutritional value.

Grape syrup is a highly viscose honey-like product that is widely used in Iran. It is usually produced by heating the juice from grapes that are not suitable for fresh consumption. In some cases, unfermented grape syrup, which has a °Brix value of about 68, is used as a natural sweetener instead of sugar. According to the classical method of grape syrup production, white soil is added to the grape juice, and the mixture is boiled, cooled, and filtered. The filtered liquid is further boiled and concentrated to obtain syrup. Iranian grape syrup contains iron, phosphorus, calcium, and potassium. It is also rich in organic acids, vitamins A, C, B_1, and B_2, as well as

some antioxidants, including flavonoids [3]. In some communities, Iranian grape syrup is used as a dessert – alone or in combination with other foods such as yogurt. Adding prebiotic compounds, such as syrup or fruit extracts, can lead to better consumer acceptance of dairy products, especially yogurt, as well as increases the viability of probiotic bacteria. The Iranian Askari grape is a common type of grape in Iran that is cultivated for fresh consumption and preparing Iranian grape syrup. Previous studies have revealed that adding grape extract to probiotic yogurt can increase the product's nutritional value and improve its rheological, microbial, and sensory properties [4, 5].

When adding different compounds to probiotic yogurt, attention should be paid to the subsequent changes in the product's physical, chemical, and sensory properties as an essential factor in consumer acceptance [4, 6]. In this study, we aimed to evaluate the effect of Iranian grape syrup used as a prebiotic at different concentrations (3, 6, and 9%) on the rheological, physicochemical, and microbial properties of stirred probiotic yogurt throughout storage.

STUDY OBJECTS AND METHODS

Probiotic cultures. The probiotic cultures *Lactobacillus rhamnosus*, *Bifidobacterium animalis* subsp. lactis BB-12 and *Lactobacillus acidophilus* La-5 in the freeze-dried form were obtained from Chr. Hansen (Denmark).

Preparing Iranian grape syrup. To prepare 4 kg of Iranian grape syrup, Asgari grapes purchased from the local market in Qazvin (Qazvin province, Iran) were washed and cleaned with distilled water. Then, we prepared grape juice (3 kg), transferred it to cooking utensils, added 100 g of white yogurt. The cooking utensils were heated to the boil and then left to reach room temperature. After filtering the juice through filter paper and measuring its pH, we heated it further until grape syrup was extracted at a high concentration [3].

Preparing yogurt. Fresh milk needed to prepare yogurt was obtained from the dairy workshop at the Department of Food Science and Technology (University of Tehran, Karaj, Iran). It had a pH of 6.4 and contained 3% of fat, 0.906% of ash, and 13% of total acids. The initial culture containing *Streptococcus thermophiles* and *Lactobacillus delbrueckii* spp. *bulgaricus* in the freeze-dried form (YO-FAST-88) was purchased from Chr. Hansen (Denmark). The milk was heated at 80°C for 15 min and then cooled to 42 ± 1°C to add the starter. After the temperature decreased, Iranian grape syrup was added to the milk in a ratio of 3:9 (v/v). Then, the samples were incubated at 42 ± 1°C and transferred to plastic containers. The incubation process continued until the pH of the samples reached 4.6. Both the control and experimental (with Iranian grape syrup) samples were stored at 4°C until used in the experiment.

Measuring the total titratable acidity and pH. The value of total titratable acidity was calculated on the °Dornic scale by adding 9 g of distilled water to a 9-g yogurt sample and titrating it with 0.1 N NaOH to the endpoint with the phenolphthalein detector [7]. The pH was measured by the direct electrode immersion method and the pH meter (WTW inoLab® 720, Weilheim, Germany).

Measuring the total phenolic content. To measure the total phenolic content of the samples, we employed a method described by Latifi *et al.* with some modifications [8]. First, we mixed 100 µL of the test solution and 100 µL of Folin-Ciocalteu's phenol reagent (1 N solution) and allowed the mixture to react at room temperature for 3 min. Then, we added 300 µL of 1 N Na_2CO_3 to each tube and incubated them for 90 min at room temperature. Finally, 1 mL of distilled water was added to the tubes to complete the reaction. The absorbance was determined at 725 nm using a spectrophotometer. The results were expressed as mg of gallic acid equivalents per 10 g sample.

Microbiological examination of yogurt. To determine bacterial count in the yogurt, 10 g of a sample was homogenized in 90 mL of peptone water (0.1% peptone) in a Stomacher 400 homogenizer (Type BA7021, Seward Medical, UK) for a minimum of 2 min. The samples were diluted in peptone water and inoculated in other containers of 0.1 mL. The plate count method was employed for *S. thermophiles* and *L. delbrueckii* subsp. *bulgaricus* on M17 agar and MRS agar, respectively (Merck, Iran). The samples were incubated for two days at 37°C (aerobic) and three days at 43°C (anaerobic) with anaerocult sachets (Merck). The *L. acidophilus* LA-5 and *Bifidobacterium* BB-12 were counted on MRS-Sorbitol agar (1% sorbitol) and MRS-NNLP agar (100 mg neomycin sulfate, 15 mg nalidixic acid, and 3 g LiCl), respectively. The results of counting plates containing 20–200 colonies were expressed in colony-forming units per gram of sample (CFU g^{-1}) [9].

Measuring the total anthocyanin content. The pH differential method described by Jaafar *et al.* was employed to determine the total anthocyanin content with a UV-601spectrophotometer (Shimadzu, Japan) [10]. The wavelengths of 520 and 700 nm were used for the samples in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) to measure the anthocyanin content (A – (A λ520–A λ700) pH 1.0 – (A λ520–A λ700) pH 4.5). Accordingly, the anthocyanin content of each sample was obtained in mg of malvidin-3-O-glucoside equivalent per kg of the sample.

Measuring the syneresis. The Syphon method was applied to calculate the spontaneous whey separation of yogurt. For this, three tubes containing 5 g of yogurt each were weighed before and after syneresis [11]. The amount of spontaneous whey separation, %, was calculated using the following equation:

$$\text{Spontaneous whey separation} = \left(\frac{W_i - W_f}{W_i - W_c}\right) \times 100$$

where W_i is the initial weight of the tube containing yogurt, g; W_f is the final weight of the tube containing yogurt after syneresis, g; W_c is the weight of the empty tube, g.

Table 1 The pH values of the yogurt samples during storage

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	4.69 ± 0.01^{c}	4.45 ± 0.01^{c}	4.38 ± 0.01^{c}	4.38 ± 0.01^{c}	4.39 ± 0.01^{c}
3	4.63 ± 0.02^{b}	4.39 ± 0.01^{b}	4.29 ± 0.01^{a}	4.29 ± 0.01^{b}	4.29 ± 0.01^{b}
6	4.62 ± 0.02^{b}	4.36 ± 0.01^{a}	4.32 ± 0.02^{b}	4.27 ± 0.01^{ab}	4.28 ± 0.01^{b}
9	4.58 ± 0.01^{a}	4.35 ± 0.01^{a}	4.29 ± 0.01^{a}	4.25 ± 0.03^{a}	4.21 ± 0.01^{a}

The similar letters in each column indicate that there is no significant difference

Viscosity. A cylindrical programmable viscometer (Rheomat RM 100, France) was employed to calculate the viscosity. The shear velocity was 70.8 rpm. Accordingly, the viscosity of each sample was obtained in cP/Poises.

Sensory evaluation. A 7-point scale was used to specify the sweetness and color of the samples, with 1 and 7 being the lowest and highest scores, respectively (ISO 4121, 2003). In the evaluation of sweetness, monochromatic illumination was applied to hide the color differences. The samples (6°C) were evaluated by a test panel of 15 panelists aged 22–35. According to ISO 8589 (2007), the evaluations were performed in a standardized test room. According to ISO 8587 (2006), yogurt acceptance was evaluated by a ranking test from 1 (lower tendency) to 3 (highest tendency) by 60 students aged 22–26.

Statistical analysis. The samples were randomly selected in all the experiments with three repetitions. The means were classified based on a significance level of 0.05 using a one-way ANOVA test with SPSS software (Version 19). The graphs were drawn using Excel 2010 software.

RESULTS AND DISCUSSION

pH changes. The evaluation of pH changes in the samples with Iranian grape syrup and the control showed significant differences at each of the evaluation points ($p \leq 0.05$) (Table 1). The mean pH of all the samples significantly decreased from day 1 to day 3, followed by a gentler decline until day 7. Afterwards, the sample with 3% Iranian grape syrup, along with the control, showed constant pH values until day 14, while the samples with 6 and 9% Iranian grape syrup continued to decrease (Fig. 1). The lowest pH (4.21 ± 0.01) was recorded in the sample with 9% Iranian grape syrup throughout storage, while the highest pH (4.69 ± 0.01) was registered in the control sample on day 1 (Table 1). The other samples ended the storage period on day 21 with an increase or no change in pH values. The trend of pH changes showed that the concentration of Iranian grape syrup and the storage time were in inverse relation to pH (Fig. 1).

Our results did not agree with some previous studies. In particular, Sarwar *et al.* found that the introduction of inulin alongside *Saccharomyces boulardii* demonstrated no notable impact on the alteration of pH or acidity in synbiotic yogurt [13]. This observation suggests that neither the prebiotic (inulin) nor yeast (*S. boulardii*) exerted a discernible influence on the acid production capacity of the starter lactic acid bacteria in yogurt.

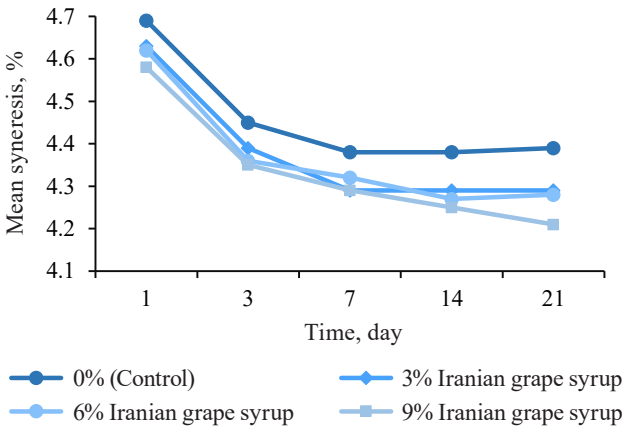

Figure 1 The pH values of the yogurt samples stored at 4°C

However, numerous studies showed similar trends to the ones we observed in our study. For example, Ghasempour *et al.* reported a decrease in pH during the storage of functional Manuka honey yogurts containing probiotics [14]. The authors attributed the decreasing trend to post-acidification, resulting from the continuous production of organic acids by the starter culture bacteria throughout the product's shelf life.

Shahein *et al.*, who examined probiotic-fermented camel milk fortified with date syrup, reported a significant decrease in pH values and a concurrent increase in acidity across all treatments during the 15-day storage period [15]. The authors attributed these results to the influence of camel milk on microorganism growth impacting pH values over time. Another study, which formulated probiotic yogurt with basil seed gum and red beet extract, found a more conspicuous trend of pH reduction in the samples characterized by an elevated quantity of basil seed gum at 0.4%, particularly when supplemented with 0.1% red beet extract [14]. In a study fortifying probiotic yogurt with almond milk, higher concentrations of almond milk significantly attenuated pH reduction, which was attributable to the absence of its buffering capacity [16]. Similarly, the incorporation of passion fruit juice into set yogurt during the initial fermentation phase was found to induce a decline in pH, effectively inhibiting the proliferation of diverse bacterial strains [14].

Acidity changes. The changes in titratable acidity of the samples during storage indicated significant differences between the samples with Iranian grape syrup and the control at each of the evaluation points ($p \leq 0.05$)

Table 2 Measured acidity of the yogurt samples during storage

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	80.10 ± 0.05^{c}	88.80 ± 0.52^{d}	96.60 ± 0.52^{b}	97.20 ± 0.05^{c}	98.37 ± 0.46^{d}
3	82.80 ± 0.05^{b}	96.12 ± 0.10^{a}	100.50 ± 0.52^{a}	105.30 ± 0.05^{a}	108.00 ± 0.10^{a}
6	82.8 ± 0.1^{b}	90.0 ± 0.2^{c}	91.8 ± 0.1^{d}	98.1 ± 0.1^{b}	103.5 ± 0.2^{b}
9	83.7 ± 0.1^{a}	92.10 ± 0.52^{b}	94.80 ± 0.52^{c}	97.20 ± 0.01^{c}	100.83 ± 0.10^{c}

The similar letters in each column indicate that there is no significant difference

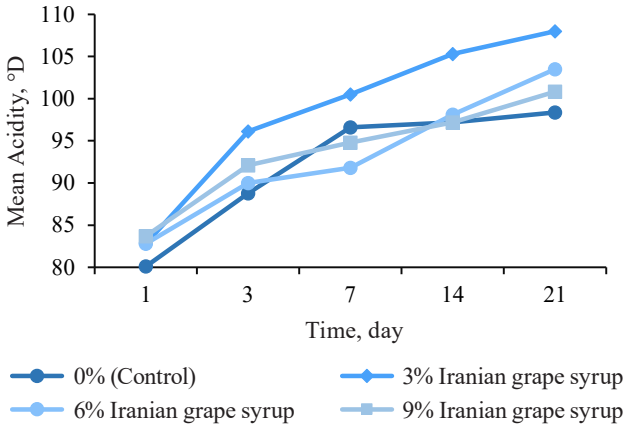

Figure 2 Acidity changes in the yogurt samples stored at 4°C

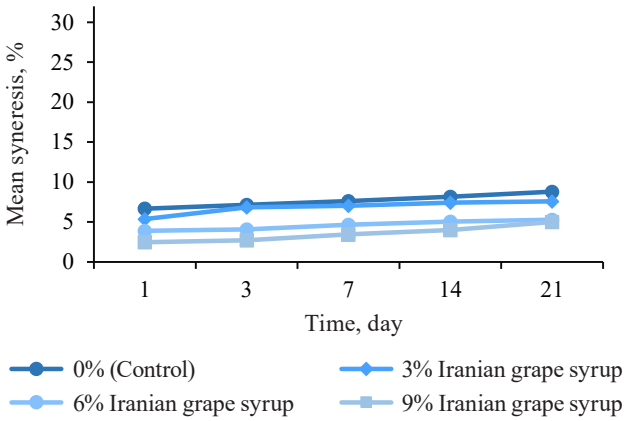

Figure 3 Syneresis changes in the yogurt samples stored at 4°C

Table 3 Syneresis changes in the yogurt samples during storage

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	6.67 ± 0.29^{d}	7.17 ± 0.29^{c}	7.63 ± 0.58^{c}	8.17 ± 0.29^{c}	8.80 ± 0.17^{d}
3	5.37 ± 0.29^{c}	6.83 ± 0.29^{c}	7.03 ± 0.29^{c}	7.43 ± 0.25^{c}	7.59 ± 0.20^{c}
6	3.90 ± 0.10^{b}	4.07 ± 0.12^{b}	4.67 ± 0.29^{b}	5.04 ± 0.17^{b}	5.29 ± 0.10^{b}
9	2.47 ± 0.06^{a}	2.73 ± 0.06^{a}	3.47 ± 0.06^{a}	4.00 ± 0.05^{a}	5.01 ± 0.12^{a}

The similar letters in each column indicate that there is no significant difference

(Table 2). The highest increase in average acidity was observed from day 1 to day 3, followed by different slopes for each sample until the end of the storage period (Fig. 2). The sample containing 3% Iranian grape syrup consistently had the highest acidity throughout storage, reaching 108.0 ± 0.1°D on day 21. The control sample showed the lowest acidity at the end of storage (98.37 ± 0.46°D) and on the first day of storage (80.10 ± 0.05°D). A study consistent with our research showed a rise in titratable acidity of camel milk fermented with date syrup compared to the control during storage. This rise was attributed to the conversion of lactose to lactic acid by the yogurt starter culture, coupled with a stimulating effect of date syrup's composition (simple sugars, glucose and fructose, and nutrients) on bacterial activity [15]. Nevertheless, this elevation in acidity was not tangible due to the same reason in a study by Mohan *et al.* on functional manuka honey. The authors reported that inherent sweetness and supplementary flavor constituents in the stingless bee honey proficiently obscured the heightened acidity generated throughout fermentation [12].

A number of previous studies were consistent with our results. In the study of synbiotic yogurt containing inulin and *S. boulardii*, all the samples exhibited a slight decrease in pH and an increase in titratable acidity during storage [13]. In the study that fortified probiotic yogurt with almond milk, an elevation in milk concentration induced a surge in acidity [16]. In another study, the addition of apple peel-derived phenolic extract marginally increased the acidity of probiotic yogurt compared to the control [18].

Syneresis changes. The syneresis changes among the samples during storage indicated significant differences between the samples with Iranian grape syrup and the control at each of the evaluation points ($p \leq 0.05$) (Table 3). Furthermore, we found an inverse relationship between the concentration of Iranian grape syrup in the sample (3, 6, and 9%) and its syneresis percentage during the storage period (Fig. 3). According to these results, the samples with 9% Iranian grape syrup consistently recorded lower syneresis percentages than the other samples throughout storage, with the lowest value

Table 4 Viscosity values of the yogurt samples during storage

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	349.67 ± 1.53[a]	351.67 ± 2.89[a]	328.67 ± 0.58[a]	325.33 ± 0.58[a]	317.67 ± 0.58[a]
3	368.00 ± 2.65[b]	370.67 ± 0.50[b]	348.00 ± 1.00[b]	342.00 ± 1.73[b]	335.00 ± 1.00[b]
6	393.33 ± 2.08[c]	393.33 ± 2.89[c]	368.33 ± 2.89[c]	363.00 ± 1.73[c]	356.00 ± 1.73[c]
9	407.33 ± 2.52[d]	408.67 ± 0.58[d]	388.33 ± 1.53[d]	380.33 ± 0.58[d]	371.33 ± 2.30[d]

The similar letters in each column indicate that there is no significant difference

on day 1 (2.47 ± 0.06%). In contrast, the control samples consistently showed higher percentages of syneresis than the Iranian grape syrup yogurts, with the highest value on day 21 (8.80 ± 0.17%). In the study of probiotic yogurt fortified with manuka honey, the cohesiveness values exhibited no significant differences among the sweetened samples over the storage period and remained lower than those of the unsweetened control sample [12]. Notably, the unsweetened samples displayed a pronounced visual syneresis, a phenomenon associated with elevated cohesiveness readings in yogurts.

Karaca *et al.* concluded that larger amounts of apricot fiber in yogurt increased its water-holding capacity, whereas prolonging the storage period caused a decrease in this capacity [19]. Some studies have also reported similar findings. For example, introducing higher concentrations of inulin into yogurt to create a synbiotic product reduced its syneresis due to its enhanced water-holding capacity [13]. Similarly, lower syneresis resulted from incorporating polymerized whey protein with xanthan gum and pectin as gelation agents into a symbiotic almond yogurt alternative [20]. In another study, adding apple peel-derived phenolic extract to probiotic yogurt decreased the product's syneresis while enhancing its firmness and viscosity. The study also underscored the potential for syneresis control through the augmentation of total solid contents in the yogurt formulation [18].

Viscosity changes. Evaluating viscosity changes throughout the storage period revealed that the samples containing Iranian grape syrup and the control sample had significant differences at each of the evaluation points ($p \leq 0.05$) (Table 4). We also found a direct relationship between the concentration of Iranian grape syrup in the samples (3, 6, and 9%) and their average viscosity at all evaluation points (days 1, 3, 7, 14, and 21). During the entire storage period, the yogurt with 9% grape syrup and the yogurt without grape syrup (control) showed the highest and lowest viscosity values, respectively. On the whole, after a slight increase in the samples' viscosity on day 3, the viscosity trend for all the samples was downward until the end of the storage period (Fig. 4). Accordingly, the highest viscosity (408.67 ± 0.58 cP/Poises) was recorded in the sample with 9% Iranian grape syrup on day 3, while the lowest viscosity (317.67 ± 0.58 cP/Poises) was observed in the control sample on day 21.

The results of some studies entirely or partly align with our findings. In particular, incorporating inulin into yogurt augmented its viscosity by effectively binding

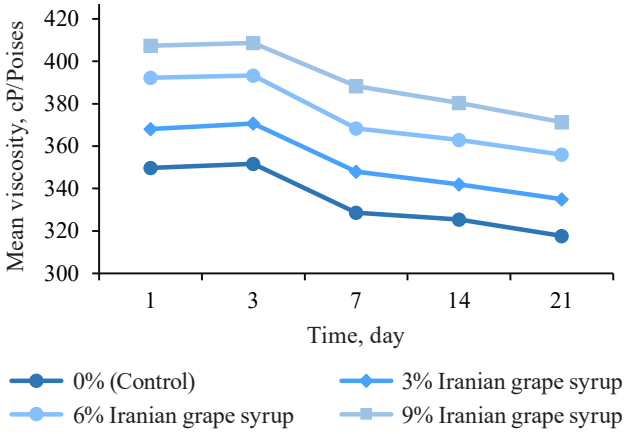

Figure 4 Viscosity changes in yogurt samples stored at 4°C

and orienting water molecules that did not integrate into the protein network. This consequently impeded wheying off and fostering robust aggregation of casein micelles [13]. Conversely, adding invert syrup to yogurt resulted in markedly higher viscosity compared to manuka honey samples at equivalent concentrations, with a discernible reduction observed over the storage period [12]. Furthermore, introducing date syrup to camel milk yielded synbiotic products with significantly higher viscosity, possibly due to the presence of oligosaccharides in date syrup [15]. In the study that fortified yogurt with basil seed gum and red beet extract, the interplay between basil seed gum and storage time significantly influenced yogurt viscosity, with concentration-dependent effects (higher gum levels corresponded to higher yogurt viscosity) [14]. Similarly, a symbiotic almond yogurt-like product formulated with polymerized whey protein as a gelation agent alongside pectin and xanthan gum exhibited non-significant variations in viscosity throughout storage [20]. In another study, addition of apple peel-derived phenolic extract to probiotic yogurt demonstrated a direct relationship between yogurt viscosity and extract concentration, with higher concentrations leading to notably elevated viscosity [18].

Total polyphenol and anthocyanin contents. The total polyphenol contents of the probiotic yogurt samples supplemented with Iranian grape syrup are presented in Table 5. As can be seen, the total polyphenol content was influenced by the concentration of Iranian grape syrup (Table 5). The yogurt fortified with 9% Iranian grape syrup had a higher polyphenol content (128.6 mg gallic

Table 5 Total phenol content of the yogurts during storage, mg gallic acid equivalents/mL

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	15.10 ± 0.03[d]	13.20 ± 0.01[d]	10.50 ± 0.01[d]	5.30 ± 0.02[d]	3.70 ± 0.01[d]
3	60.40 ± 0.04[c]	59.10 ± 0.01[c]	57.90 ± 0.02[c]	55.20 ± 0.01[c]	52.10 ± 0.02[c]
6	95.10 ± 0.04[b]	93.20 ± 0.03[b]	91.50 ± 0.02[b]	89.10 ± 0.04[b]	86.50 ± 0.04[b]
9	128.60 ± 0.05[a]	127.10 ± 0.04[a]	125.30 ± 0.05[a]	121.10 ± 0.06[a]	117.20 ± 0.05[a]

The similar letters in each column indicate that there is no significant difference

Table 6 Total anthocyanin content of the yogurts during storage, mg malvidin-3-O-glucoside equivalents/mL

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	6.50 ± 0.01[d]	5.30 ± 0.02[d]	4.50 ± 0.01[d]	2.90 ± 0.01[d]	1.70 ± 0.01[d]
3	12.10 ± 0.02[c]	11.20 ± 0.01[c]	10.50 ± 0.01[c]	8.90 ± 0.02[c]	6.80 ± 0.01[c]
6	18.20 ± 0.02[b]	17.50 ± 0.01[b]	16.80 ± 0.02[b]	13.90 ± 0.01[b]	11.10 ± 0.01[b]
9	23.30 ± 0.03[a]	22.40 ± 0.03[a]	21.70 ± 0.02[a]	19.10 ± 0.02[a]	16.40 ± 0.03[a]

The similar letters in each column indicate that there is no significant difference

Table 7 Logarithmic values of probiotic bacteria in yogurts during storage

Iranian grape syrup, %	Days				
	1	3	7	14	21
0 (control)	8.43 ± 0.02[a]	8.51 ± 0.01[a]	8.31 ± 0.01[a]	8.21 ± 0.02[a]	7.94 ± 0.03[a]
3	8.50 ± 0.02[b]	8.57 ± 0.01[b]	8.69 ± 0.01[b]	8.57 ± 0.02[b]	8.45 ± 0.03[b]
6	8.48 ± 0.01[b]	8.60 ± 0.01[c]	8.70 ± 0.01[b]	8.68 ± 0.01[c]	8.61 ± 0.02[c]
9	8.53 ± 0.01[c]	8.60 ± 0.02[c]	8.71 ± 0.01[b]	8.70 ± 0.00[c]	8.65 ± 0.01[d]

The similar letters in each column indicate that there is no significant difference

acid equivalents/mL) than the other samples on day 1 ($p < 0.01$). Generally, phenolic compound levels in the yogurt samples decreased during 21 days of storage.

Table 6 presents total anthocyanin contents of the yogurts containing different concentrations of Iranian grape syrup and the control sample. As can be seen, the highest anthocyanin content (23.3 mg malvidin-3-O-glucoside equivalents/mL) was recorded in the sample with 9% Iranian grape syrup on day 1, while the lowest anthocyanin content (1.7 mg malvidin-3-O-glucoside equivalents/mL) was observed in the control sample on day 21.

Numerous studies have indicated that phenolic compounds can enhance probiotics' ability to stick to surfaces and endure conditions resembling the digestive tract. Phenolic compounds also seem to positively influence the composition of gut microbiota, benefiting markers and risks related to chronic illnesses. Additionally, metabolites produced by gut microbes (including probiotics) from phenolic compounds offer health advantages surpassing the original compounds [21]. Our findings demonstrated that increased amounts of Iranian grape syrup in the yogurt samples led to higher total anthocyanin contents. We also found that increasing the storage time over 14 days reduced the anthocyanin contents. Thus, storage time had a significant effect on the polyphenol and anthocyanin contents of the yogurt samples.

Silva *et al.* reported that goat yogurt formulated with fiber- and phenolic-rich ingredients from the integral valorization of Isabel grape had a positive effect on gut microbiota and human health [22]. Our findings were consistent with the results obtained by Fuhrman *et al.*, who determined total phenolic and anthocyanin contents in red and white grape varieties during storage [23]. According to Jackman *et al.*, phenolic and anthocyanin compounds are readily degradable and form colorless or brown-colored compounds [24]. The stability of anthocyanin is influenced by pH, microbial activity, storage temperature, enzymes, and metallic ions in the environment [24]. Okogeri and Tasioula-Margari also reported that phenolic compounds degraded during storage in virgin olive oils [25]. Similarly, we found significant decreases in polyphenol and anthocyanin contents in the yogurt samples.

Microbial changes. Evaluating microbial changes in the yogurts showed significant differences in the samples containing Iranian grape syrup and the control sample at each of the evaluation points ($p \leq 0.05$) (Table 7). As can be seen, the average number of bacterial colonies increased from day 1 to day 3. This trend continued up to day 7 for all the samples except the control, with the highest number of colonies (8.71 ± 0.01 CFU/g) recorded in the sample containing 9% Iranian grape syrup. After day 7, the number of bacterial colonies in the 3, 6, and 9% samples had a decreasing trend until the end of the storage period. The control sample had the lowest number of colonies (7.94 ± 0.03 CFU/g) on day 21. Throu-

ghout the entire storage period, the control yogurt showed consistently lower numbers of colonies, while the 9% Iranian grape syrup yogurt showed consistently higher numbers of bacterial colonies than the other samples at all the evaluation points (Fig. 5).

A number of studies align with some of our findings. Sarwar *et al.* reported that the survivability of *S. boulardii* and lactic acid bacteria in synbiotic yogurt notably decreased over a four-week storage period, while inulin was a crucial factor that preserved the viability of the yeast in the yogurt matrix [13]. In the study of manuka honey-added yogurt, the total counts of viable probiotics exhibited a decline over a three-week refrigerated storage period, with an exception noted in the yogurt containing invert syrup. Nevertheless, the viable counts of probiotics remained consistently above the recommended minimum dosage of 6 log CFU/mL throughout storage [12]. Another study, where date syrup was added to camel milk, observed a significant increase in total bacterial and bifidobacterial counts, with the degree of augmentation correlating with the quantity of date syrup added. This augmentation was potentially attributed to certain components in date syrup, particularly oligosaccharides, which can act as prebiotics and stimulate bacterial growth [15]. Additionally, a study focusing on almond milk added to probiotic yogurt revealed that while the content of fermentable sugars in almond milk supported probiotic bacterial growth, it was insufficient for metabolic activity. Increasing the amount of almond milk substantially elevated the viability of probiotics, suggesting its potential as a substrate for diverse functional product development [16]. Finally, incorporating apple peel polyphenol extract as a prebiotic ingredient into yogurt not only resulted in viable counts above the minimum requirement for probiotics but also demonstrated the highest viable counts throughout fermentation and a subsequent 21-day refrigerated storage period, indicating an enhancement in probiotic survival [18].

Sensory changes. Evaluating sweetness values in the yogurts with Iranian grape syrup and the control revealed significant difference ($p \leq 0.05$). We found a direct relationship between the concentration of Iranian grape syrup in the sample and its sweetness, with the highest sweetness (5.97) recorded in the 9% Iranian grape syrup yogurt. We also observed significant differences in the color of the Iranian grape syrup samples and the control ($p \leq 0.05$). Based on the scores given by the panelists, there was no significant difference between the samples containing 3, 6, and 9% Iranian grape syrup. In the ranking test for sample acceptance, the total scores for the control sample and the samples containing 3, 6, and 9% Iranian grape syrup were equal to 105, 120, 131, and 149, respectively. According to Table 8, the values did not differ significantly ($p \geq 0.05$), and the acceptance of the samples by the panelists was identical.

Our results were consistent with some previous studies. In particular, introducing date syrup into camel milk resulted in a marked enhancement of sensory scores (flavor, consistency, appearance, and overall evaluation) in

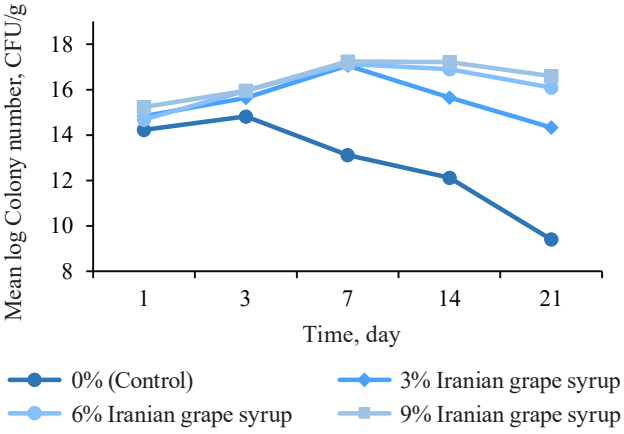

Figure 5 Logarithmic changes in probiotic bacteria in the yogurts stored at 4°C

Table 8 Sensory evaluation of sweetness, color, and acceptability of the yogurt samples

Iranian grape syrup, %	Sensory criteria		
	Sweetness	Color	Overall acceptability
0 (control)	3.4[d]	5.9[5]a	105
3	4.5[c]	4.8[b]	120
6	5.41[b]	4.71[b]	131
9	5.97[a]	4.65[b]	149

Values with different letters in each row are significantly different at the level of $p \leq 0.05$

the resulting synbiotic products [15]. Furthermore, the yogurts fortified with apple peel extract showed notable distinctions in taste and color compared to the control yogurt throughout the storage period [18]. The addition of inulin to yogurt was found to enhance mouthfeel and taste, while the incorporation of the probiotic yeast *S. boulardii* resulted in decreased scores for yogurt texture. This reduction in texture scores was attributed to the production of alcohol and carbon dioxide, contributing to an overall improvement in flavor and taste [13]. In contrast, the sensory evaluation of manuka honey-fortified synbiotic yogurts containing *Lactobacillus reuteri* DPC16 showed no statistically significant differences in consumer ratings for color, appearance, mouthfeel, and smoothness. However, overall acceptance appeared to be dependent on consumer preferences for sweetness and sourness levels in the yogurts [12]. Additionally, higher levels of red beet extract and basil seed gum in the yogurts led to pronounced antioxidant activity but a decline in the sensory scores during storage [14].

CONCLUSION

Our study demonstrated that high concentrations of Iranian grape syrup could increase the number of probiotic bacterial colonies in yogurt. According to our findings, higher concentrations of Iranian grape syrup increased the viscosity and decreased the syneresis of the yogurt, so the amount of grape syrup has a significant impact on achieving the desired rheological properties.

The activity of bacteria affected by different concentrations of Iranian grape syrup can lead to changes in the physicochemical properties of yogurt. Our study indicated that the acidity and sweetness of the probiotic yogurt containing Iranian grape syrup and fermented with a mixture of starter cultures are directly related to the concentration of Iranian grape syrup, whereas the product's syneresis and pH are in inverse relation to Iranian grape syrup concentration. Particularly, higher concentrations of Iranian grape syrup showed a decrease in syneresis and pH but an increase in acidity and sweetness. We found that the yogurt containing 9% of grape syrup received the highest consumer's ratings and had the best physicochemical properties. Our study also showed that supplementing yogurt with Iranian grape syrup can improve the viability and metabolic activity of probiotic bacteria. Thus, Iranian grape syrup can be used as a prebiotic in further research to develop functional and symbiotic yogurts.

CONTRIBUTION

F. Abdolmaleki wrote the manuscript. R. Rezaei Mokarram conceived and designed the analysis. M. Daneshniya and M.H. Maleki collected and analyzed the data.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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