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Editorial
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Editor's column

In recent years, scientists have focused on studying the properties and possible applications of natural bioactive compounds (alkaloids, flavonoids, carotenoids, anthocyanins, and sterols) derived from various types of plant materials.

Medicinal plants have been traditionally used, alone or as dietary supplements, in daily diets or in functional foods to promote health and prevent disease. Plant metabolites have a wide range of functional effects on the human body. Their antioxidant, anti-inflammatory, and antimicrobial properties can normalize the metabolism of lipids, carbohydrates, vitamins, and other compounds.

Bioactive substances derived from medicinal plants play a significant role in preventing many metabolic diseases, such as cardiovascular diseases, obesity, type 2 diabetes, and others. Rich in phytochemicals, medicinal plants are also important in treating acute respiratory and viral diseases. Thanks to their antioxidant properties, plant-based bioactive compounds are widely applied in the food, pharmaceutical, cosmetic, and other industries.

One of the consequences of the COVID-19 pandemic has been a world-wide change in people's attitudes to their health and disease prevention. To strengthen their immune system and prevent various diseases, people most commonly take bioactive supplements (vitamins, polyphenols, antioxidants, etc.). There is an increased demand for herbal medicines and nutritional supplements based on medicinal plants. The demand for functional foods is also on the rise. Russia, for example, has doubled its production of functional foods in the last five years. Experts predict an upward trend in this market, which may exceed 350 billion rubles by 2024.

According to the FAO/WHO's recommendations, the Russian Strategy for Improving Food Quality until 2030 and the Russian Doctrine of Food Security, this market should aim towards providing good nutrition, preventing disease, improving the quality of life, and stimulating production.

In the current economic situation, Russian manufacturers of dietary supplements are having problems with the supply of ingredients produced in Western countries. Therefore, they are looking for new suppliers who can potentially come from Central Asia, India, Latin America, and, of course, Russia itself.

Russia's Pharma-2030 Program aims to ensure import independence and increase the domestic production of innovative drugs, including those based on natural compounds. The National Technological Initiative's Healthnet Roadmap intends to involve more regions in cultivating medicinal plants and to create a medicinal plant sector.

Of particular interest are plants growing in the Siberian Federal District. They include not only traditional plants registered in the State Pharmacopoeia, but also those which may be new to the professional medical community. Many Siberian medicinal plants have a wide range of useful properties and are a valuable source of geroprotectors (quercetin, baicalin, kaempferol, chlorogenic and caffeic acids). They include Baikal skullcap (Scutellaria baicalensis), maral root (Rhaponticum carthamoides), rose root (Rhodiola rosea), Chinese magnolia vine (Schisandra chinensis), as well as Eleutherococcus and Dioscorea nipponica.

The use of local plants as a source of bioactive substances can ensure import independence and promote the production of high quality domestic dietary supplements. Therefore, we should continue our search for promising medicinal plants and cultivate them using optimal methods. We need to develop ways of isolating active substances from plant cell cultures, study their properties and effects on the human body, and give evidence-based recommendations for their rational use.

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Pig adipose tissue of two different breeds and locations: morphology and Raman studies

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Abstract:
According to the recent data, there are 4–5-local pig breeds left in Russia by now. Livni is among them. This breed is characterized by high fat content. Back fat has been analyzed earlier. We aimed to assess fat morphometrics from other localizations in pigs.
Sacral, axillary, and perirenal fat samples from 6-month-old Duroc and Livni pig breeds were analyzed using morphological and Raman-based techniques.
Livni adipocytes were characterized by dense packing with a polyhedron-like structure. In Duroc fat, they were more rounded (spherical). A "two-phase" cell disperse was identified in all samples. Fat cells in Livni pigs were bigger than those in the Duroc breed: 70–102, 15–18, and 26% for sacral, axillary, and perirenal locations. Differences in the intensity of the Raman signal between the samples were found: in the samples of subcutaneous adipose tissue, more intense peaks were observed, which are responsible for unsaturation; the samples of Livni axillary fat were characterized by greater unsaturation than sacral fat.
Livni and Duroc adipocytes differ from each other in form and size and the difference depends on location. Pork fat from local breeds is expected to have potentially more health protecting (for animals) and health promoting (for consumers) properties.

Keywords: Fat, pigs, Duroc, Livni, histology, adipocytes, morphometry, Raman spectroscopy

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INTRODUCTION

Worldwide, there are around 950–960 million pigs (according to various sources), of which 80% are in Asia and Europe. The domestic pig (*Sus scrofa*) was domesticated in China and Europe about 9000 years ago, and there are now about 350 breeds [1, 2].

In the USSR, the number of pigs in 1980 exceeded 70 million and 22 local breeds were registered, including the Ukrainian Steppe White, Siberian Northern, Livni, Kemerovo, Mirgorod, Urdjum, Semirechensk, etc., which accounted for one third of the total number of livestock. Although pigs in Russia were descended from imported breeds, they were subsequently adapted to local conditions, and breeds with unique characteristics were developed. Before the beginning of the 20th century, the number of livestock totalled about 20 million [3].

The crisis of the 1990s led to a reduction in the number of livestock to 13 million [4]. There has been a significant growth in pig breeding since 2010 as a result of the state sectorial target programme "Development of pig breeding in the Russian Federation for 2010–2012", approved by Order № 567 of the Ministry of Agriculture of Russia of 30 November 2009. Since then, pig farming in Russia has been gaining momentum: between 2016 and 2020, the number of pigs increased from 23.3 to 26.9 million.

Modern pig production is based on the hybridisation of three pure breeds: Yorkshire or Large White, Landrace, and Duroc [5]. In Russia, the reduction in the number of local breeds is due to the global trend towards the industrial use of imported cosmopolitan breeds as they are well adapted to intensive production systems [6].

Local breeds, which have unique variability, are deemed, together with their wild relatives, a valuable genetic resource. They are characterized by greater individual variability, strength of constitution, greater stress and weather resistance, and the highest quality meat [6–9]. However, they have a lower growth potential and protein deposition [10].

As of the beginning of 2021, the pig breeding stock in Russia is represented by eight breeds of pigs, including the Large White (53.4%), Yorkshire (21.4%), Landrace (18.9%), and Duroc (5.2%). Other domestic breeds account for 0.8% of the stock [11].

All species of the *Suidae* (swine/pigs) family have well-developed subcutaneous as well as visceral adipose tissue, but their structure has very specific features due to both genetic and paratypical factors. One of the main objectives of the continuous improvement of pork production is to regulate the amount of fat.

All pig breeds can be divided into three groups according to lean meat yield per carcass: bacon – above 58% (Urdjum, Latvian White, Hampshire, Estonian Bacon); lard – less than 50% (Large Black, Breitovo, Berkshire, Mangalica) and universal, which occupy an intermediate position (Polish-Chinese, Lithuanian White, Kemerovo, Ukrainian Steppe White, North Caucasian, Livni) [12].

It should be noted that domestic breeds are characterised by a significant proportion of stored fat on the carcass, relatively low early maturity and associated low feed conversion ratio. This is also seen as an advantage for meat products from foreign breeds. There are also differences in fat quality; for example, the back fat of Irish-bred pigs is more malleable than that of domestic pigs, due to the ratio of saturated/unsaturated fatty acids, which in turn affects the stress tolerance of the animals [13].

Adipose tissue is the largest and most dynamic energy reservoir – most energy is stored as triglycerides in fat cells (adipocytes), and also an active metabolic and endocrine organ that secretes several bioactive peptides (adipokines). There are four fat depots with varying anatomical locations: visceral, subcutaneous, intermuscular, and intramuscular [14, 15]. Each fat depot has specific morphological and metabolic properties [16].

Morphological studies of (mainly) back fat of pigs of different breeds have been conducted by several authors [17–19]. However, there is very little information on adipose tissue in other locations. Lard and universal breeds generally have larger adipose tissue cells than bacon pigs of the same body weight. For example, Meishan lard pigs have larger back fat adipocytes than Landrace bacon pigs [19]. In addition, local pig breeds are characterized by an earlier maturation (development) of adipose tissue [20].

Breed is also known to have a direct impact on the quality and fatty acid composition of adipose tissue [21]. Significant anatomical differences in fatty acid composition in pigs have been reported [16]. Raman spectroscopy is a proven method for determining the concentration of major fatty acid groups [22]. The use of Raman spectroscopy for fat estimation has a significant practical advantage as it does not require complex extraction or purification processes and can be used both for rapid screening to control fat quality and for fundamental research into the factors affecting its polymorphic transitions and stability.

This means that it is imperative that the adipose tissue of pigs of different breeds and different locations is studied, as this will provide an understanding of the potential of lipogenesis and allow us to assess the quality of these adipose tissues as a valuable raw material for the meat industry, especially in pigs with varying degrees of body fat. In this regard, the aim of the study was to investigate and compare the morphological features of adipose tissue of different locations in pigs of two breeds with different productivity levels: Duroc (bacon) and Livni (universal).

STUDY SUBJECTS AND METHODS

In our experiment, we analyzed 6-month-old pigs of two breeds, namely Livni (n = 6, private farm, Livensky district, Orel region) and Duroc (n = 8, LLC Breeding-Hybrid Center, Voronezh, under the conditions of the experimental farm of the L.K. Ernst Federal Science Center for Animal Husbandry). The following subcutaneous (fat) and internal adipose tissue samples were taken from the pigs: sacral fat, subcutaneous axillary, and internal perirenal.

The thickness of the subcutaneous fat was measured with a flexible ruler, accurate to 0.5 mm.

Morphological (histological) examination. To study adipose tissue morphology, the samples were preserved in a 10% neutral buffered formalin solution for 72 h at room temperature. Then a 1.5×1.5×0.5 cm slice was taken from each sample, washed with cold running water for 4 h and compacted in gelatin at an ascending concentration (12.5 and 25%) (AppliChem GMBH, Germany) at 37°C for 8 h each using a thermostat (SPU, Russia). 14-µm thick sections were prepared on a MIKROM-HM525 cryostat (Thermo Scientific, USA). The obtained sections were placed on Menzel Glaser slides (Thermo Scientific, USA) and stained with Erlich haematoxylin and a 1% aqueous-alcoholic eosin solution (BioVitrum, Russia) according to conventional methods [23]. The sections were encased in glycerol-gelatin. The histological preparations were studied and photographed using an AxioImaiger A1 light microscope (Carl Zeiss, Germany) and an AxioCam MRc 5 video camera (Carl Zeiss, Germany).

To calculate the area of adipocytes, 14 µm-thick sections were obtained from formalin-fixed samples, placed on slides, embedded in a drop of a saline solution, and immediately examined using the AxioVision 4.7.1.0 image analysis system (Carl Zeiss, Germany) (modified technique) [24]. At least three slices were made for each specimen. The adipocyte area was measured interactively with an accuracy of ± 1.0 µm^2.

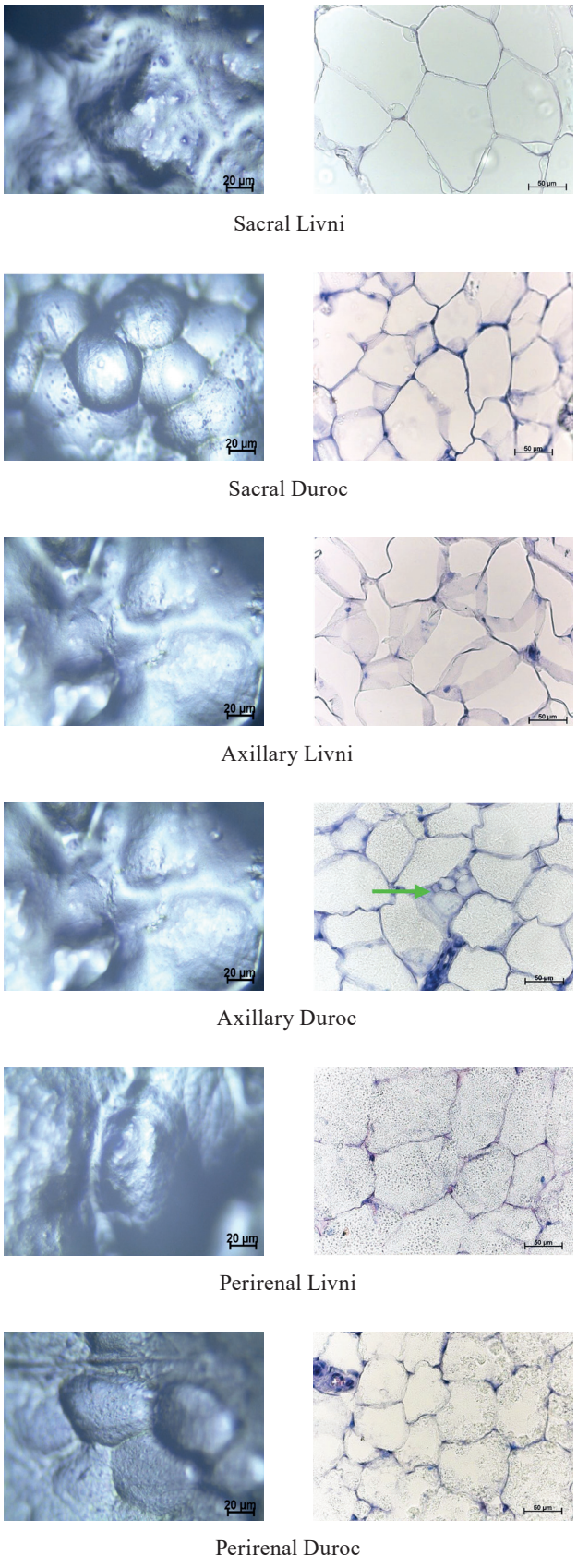


Figure 1 Representative photographs of adipose tissue samples from Livni and Duroc pigs: (a) structure without staining (20×); (b) hematoxylin and eosin staining (40×). The arrow shows a beige adipocyte

For subcutaneous adipose tissue samples, cell areas of the outer (just under the skin) and inner (closer to the muscle) layers were counted of at least 100 for each layer.

Raman spectroscopy. Raman spectroscopy was used to evaluate the total fatty acid profile of the studied samples [22, 25]. The spectra were collected on a Renishaw model inVia Reflex confocal Raman dispersive spectrometer (Renishaw plc, Wotton-under-Edge, UK) using a 785-nm laser. The spectrometer was calibrated by recording the Raman spectrum of a silicon crystal wafer at 520 cm^{-1} (exposure time 1 s, laser power 10 mW, 1 scan). Raman spectra were obtained directly from the fat samples by placing them under the spectrometer's microscope, an L50× magnification was used to focus the laser on the surface of the samples. The laser power was 100 mW, exposure time 10 s, 3 scans (accumulations). Measurements were recorded in the detection range of 700–1800 cm^{-1}. The laser power and integration time were carefully optimized to avoid the photodegradation of fat samples. An average of six spectra was used for each sample.

The raw Raman spectra included noise, fluorescence background, and spectral overlap of adjacent bands, making them difficult to identify visually. The entire spectral analysis and pre-processing was performed using Renishaw WiRE 5.2 software (Renishaw plc, Wotton-under-Edge, UK). Pre-processing included cosmic ray removal, baseline correction using intelligent polynomial algorithms, smoothing using the Savitzky-Golay algorithm, and normalization.

Statistical processing of the results. Statistical analysis of the data was performed using the STATISTICA software package, version 10.0 (StatSoft, Inc., USA). The results were presented as a mean (Mean), standard error of the mean (± SE), minimum and maximum [MIN MAX] interquartile range (R 25/75). The geometric mean (GeoMean) was used to analyze the spectral intensities. The differences were considered to be significant and the association between the indicators was considered to be at a level of probability not more than 0.05.

Correlation matrices (correlation matrices in the form of square tables, with row and column headings representing the variables to be processed, and the intersection of the rows and columns displaying the correlation coefficients for the corresponding pair of features) were generated in the RStudio IDE using the R programming language with the *psych* function. The raw data in *.xls format was converted to csv format (comma delimiters). The libraries ggplot2, tidyverse, ggthemes, RColorBrewer, grid, and gridExtra were used for data visualization. A scatter plot, representing the values of two variables as points on the Cartesian plane, was also obtained.

RESULTS AND DISCUSSION

On visual inspection, the fat samples had a white to pale pink colour and soft consistency, and were odorless. The average thickness of the subcutaneous fat samples

Table 1 Results of the morphometric analysis of adipose tissue samples of different locations

Locus	Layer	Adipocyte area, μm² (Mean ± SE [MIN MAX])	
		Livni	Duroc
Sacral	External	7063.29 ± 166.66 [3928.44–2107.29]	4139.92 ± 49.68 [2605.71–6022.94]
	Internal	9432.96 ± 152.47 [5725.98–14255.27]	4677.37 ± 63.74 [2851.51–6761.85]
Axillary	External	4172.14 ± 61.48 [1550.5–6684.84]	3534.79 ± 50.53 [2579.32–4522.09]
	Internal	5404.43 ± 74.35 [3419.95–10012.89]	4691.24 ± 77.99 [3304.18–6188.9]
Perirenal		5328.14 ± 64.33 [2931.98–8825.86]	4215.01 ± 23.15 [2884.72–313.18]

was: sacral Livni – 39.8 ± 1.6 mm; sacral Duroc – 16.70 ± 0.47 mm; axillary Livni – 8.80 ± 0.37 mm; and axillary Duroc – 5.5 ± 0.5 mm.

Microstructure analysis revealed that in the subcutaneous fat samples (sacral and axillary), adipocytes were presented as polygonal cells, with slightly rounded edges. The adipocytes of Livni pigs were tightly packed in the shape of a polyhedron, while in Duroc pigs the cells were more rounded (spherical) in shape. A large fat droplet was located in the central part of the cells, displacing the oval nucleus to the periphery of the cell. Thin and sparse collagen fibres were located between the cells in Livni pigs, while in Duroc pigs thicker intercellular layers of connective tissue with frequent collagen fibres were present. This structure is characteristic of mature white adipocytes. The adipocytes of internal body fat (perirenal) in both breeds differed in their more rounded shape (Fig. 1).

In the axillary adipose tissue samples from Duroc pigs, single adipocytes were found to be polygonal in shape and smaller in size, having a rounded nucleus closer to the centre of the cell and several fat droplets of different sizes (from 3 to 20 μm in diameter). This cell structure is characteristic of beige adipocytes [26]. The presence of beige adipocytes in pig adipose tissue has been described, for example, by Zhao *et al.* and is of interest in terms of evaluating the therapeutic and prophylactic potential of pig fat [27]. No such cells were detected in samples from other locations or in all samples from Livni pigs.

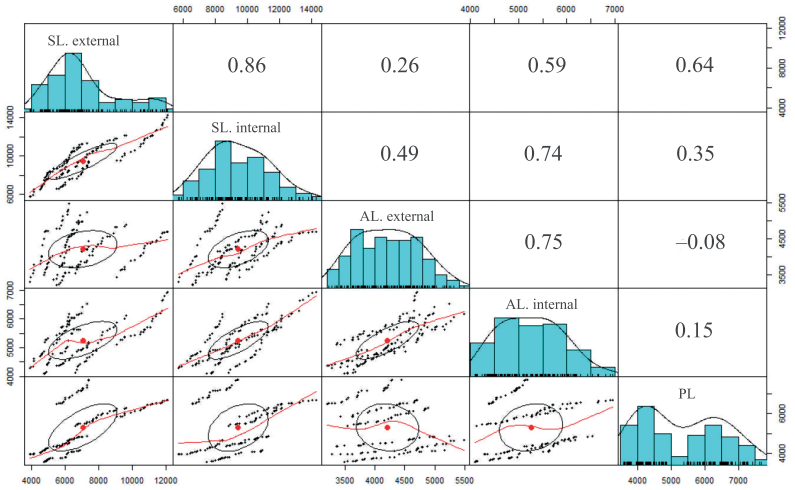
The results of the morphometric analysis of adipose tissue cells of different locations from Duroc and Livni pigs are shown in Table 1. Adipose tissue growth is known to result from hypertrophy (increased size of adipocytes) and hyperplasia (increased number of adipocytes) [14]. Adipocytes increase in number and size with increasing weight and age of animals, which affects the thickness of the fat. Early in life, adipose tissue in pigs grows mainly through hyperplasia, and after a significant increase in cell number, adipocytes begin to hypertrophy through triglyceride accumulation. A biphasic distribution of adipose cells into small and large cells was observed in all adipose tissue samples, which is consistent with the results of other studies. According to [19], small backfat adipocytes accounted for 15–19% of the total population in 5-month-old Meishan and Landrace pigs. In our study, the proportion of small adipocytes was 8% in the sacral Livni samples, 11% in the sacral Duroc samples, 8% in the axillary Livni samples, 14% in the axillary Duroc samples, 9% in the perirenal Livni samples, and 10% in the perirenal Duroc samples.

Correlation matrices of fat cells are shown in Fig. 2. Above the main diagonal are correlation coefficients for the area of adipocytes of different locations. Below the main diagonal is a visualization of the correlation between fat cells of different locations. The main diagonal contains cell density distribution diagrams. According to the data obtained, the area of adipocytes in the external and inner layers of subcutaneous fat correlated well with each other: the correlation coefficient was 0.86 for the sacral Livni samples, 0.75 for the axillary Livni samples, 0.75 for the sacral Duroc samples, and 0.95 for the axillary Duroc samples. On the contrary, there is no linear correlation between the adipocytes of perirenal and other locations on carcasses of Livni breed pigs, unlike pigs of the Duroc breed.

Figure 3 presents a dot plot of fat cell dispersion as a function of area. Along the main diagonal, there are fat cells of various locations (sacral, axillary, and perirenal), below is a visualization of the adipocytes of the Livni (green dots) and Duroc (red dots) breeds using a scatter diagram, while the correlation coefficients are at the top of the main diagonal. The difference in cell area distribution between the breeds and locations is clearly visible. However, the correlation is low between inner perirenal and axillary fat, and the dots are chaotically arranged and not clustered in the diagram.

The results of the morphological study showed that there were differences in the cellular structure between the adipose tissue of pigs of different breeds, which is consistent with the published works of other authors [17, 24]. In our study, the adipocytes of fat samples from Livni pigs were the largest compared with samples from the Duroc breed: the cell area in the sacral Livni samples exceeded that of sacral Duroc adipocytes by an average of 70 and 102% ($p \leq 0.01$), in the axillary Livni samples by 18 and 15% for the external and internal layers respectively; and in the perirenal Livni samples by 26%.

The adipocyte area of subcutaneous fat was found to differ as a function of its location on the animal's body – larger cells were detected in the samples of the sacral fat of both breeds compared to the axillary samples. At the same time, there were larger adipocytes in the internal layers of the fat than in the external layers: for sacral



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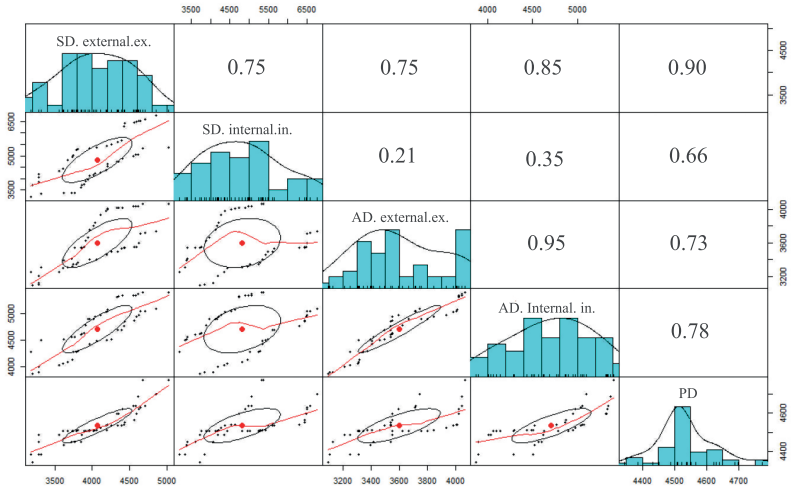

b

Figure 2 Correlation matrices of fat cells of various locations: (a) for the Livni breed; (b) for the Duroc breed

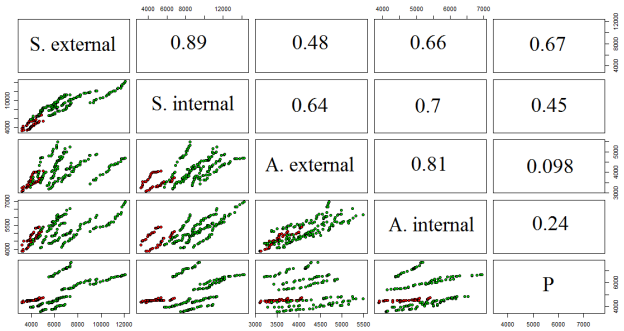

Figure 3 Scatter diagram and correlation coefficients of fat cells of various locations of the Livni (green dots) and Duroc (red dots) breeds. S, A, and P are acral, axillary, and perirenal fat, respectively

Livni by 33.5%; for sacral Duroc by 13%; for axillary Livni by 29.5%; and for axillary Duroc by 32.7%. The large size of adipocytes likely contributes to a greater increase in sacral fat thickness in Livni pigs compared to Duroc pigs. This means that pigs of the Livni breed were characterised by a higher development of adipose tissue at all loci.

The Raman spectra of lipids are mainly represented by bands due to oscillations of hydrocarbon chains (saturated and unsaturated structures) [28, 36]. The Raman spectra of adipose tissue samples contain essentially the same signals (Fig. 4), whose characteristics are given in Table 2. Differences in Raman signal intensity were identified between the samples from the same breed but having different locations on the carcass and between pig breeds.

As the difference between individual fatty acids lies in the length of carbon chains and the number and arrangement of double bonds, they have similar Raman spectra [31, 32]. This means that the problem of overlapping peaks from different fatty acids arises when analyzing adipose tissue samples [33, 34]. To determine the relative content of unsaturated fatty acids in each sample, we calculated the ratio of the intensity

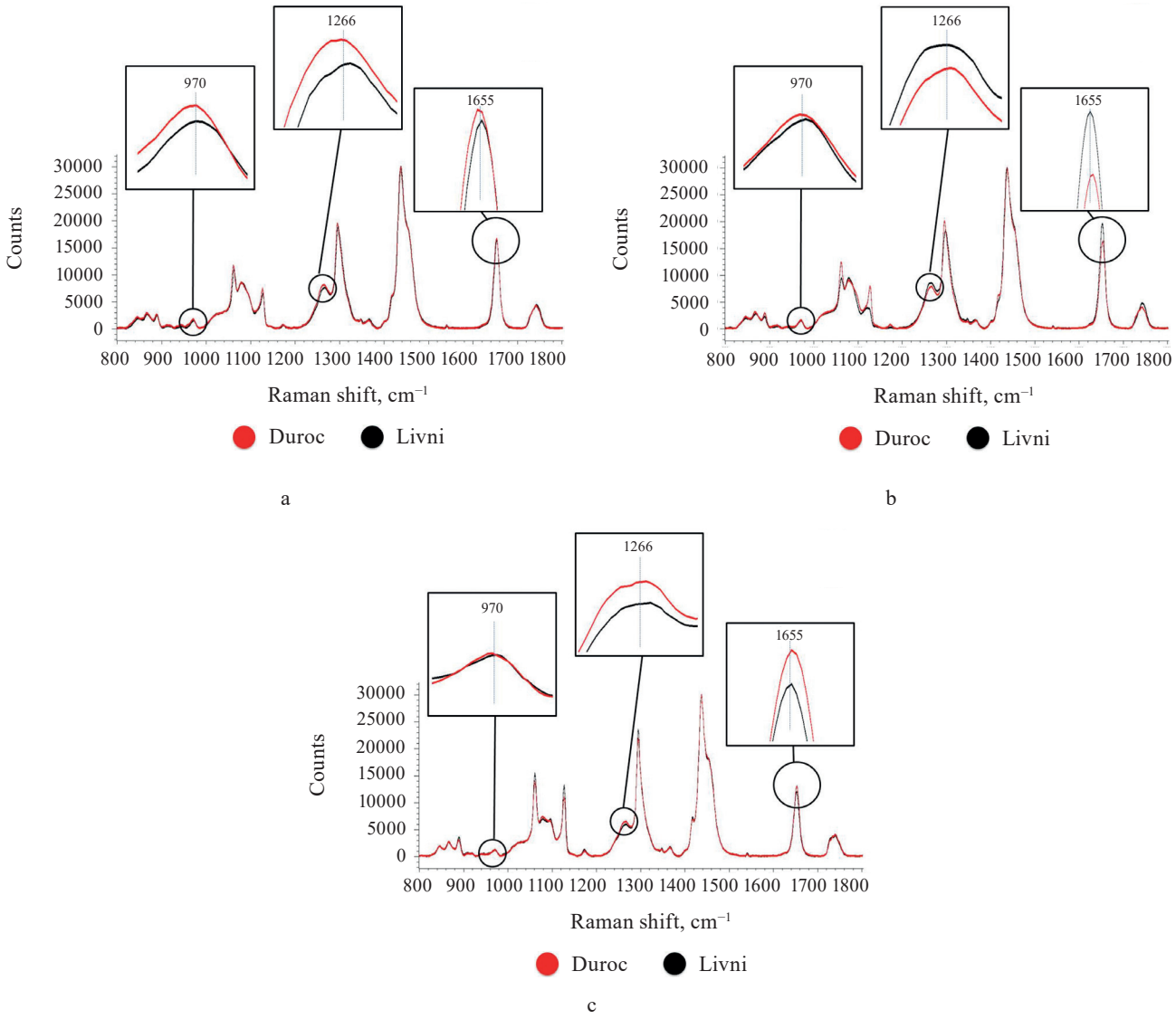

Figure 4 Raman spectra of adipose tissue samples: (a) sacral fat; (b) subcutaneous axillary; (c) internal perirenal

Table 2 Characteristics and intensity of obtained Raman spectra of adipose tissue signals from two breeds of pig

Band number	Band position, cm^{-1}	Mode of vibration [25, 29]	Livni			Duroc		
			Sacral	Axillary	Perirenal	Sacral	Axillary	Perirenal
1	868	C-C stretching	2665.18	2771.44	2683.69	2881.81	3130.60	2694.85
2	970	=C-H out-of-plane bend *cis* isomer	1447.37	1535.33	1207.89	1771.12	1608.46	1227.42
3	1061/1068	C-C aliphatic out-of-phase stretching	10945.40	9692.98	15466.90	11711.90	12470.8	13913.00
4	1080	C-C aliphatic stretching	8056.34	9091.33	6927.31	7998.18	8474.42	7347.22
5	1127	C-C aliphatic in-phase stretching	6440.39	4301.32	13255.10	7408.41	7992.15	11057.00
6	1266/1 272	=C-H symmetric rock *cis* isomer	7440.65	8533.44	5761.50	7995.07	7695.84	6345.80
7	1297/1 301	CH_2 twisting	19402.40	18106.70	23546.30	20242.40	20372.4	21913.2
8	1368	CH_3 symmetric deformation (umbrella)	1670.61	1618.70	1736.24	1495.95	1532.85	1624.32
9	1430/1 460	CH_2 symmetric deformation (scissoring)	30466.10	30569.60	29897.70	30510.40	30128.40	29999.60
10	1655	C=C stretching	16372.50	19602.30	12018.20	16667.70	16403.80	13022.90
11	1735/746	C=O stretching	4363.810	4708.97	3897.12	4222.31	4020.36	4117.51

Table 3 Unsaturated fatty acid content in adipose tissue samples from two pig breeds

Intensity (I) ratio of Raman signals	Livni			Duroc		
	Sacral	Axillary	Perirenal	Sacral	Axillary	Perirenal
I_{970}/I_{1297}	0.074597	0.084793	0.051299	0.087496	0.078953	0.056013
I_{970}/I_{1430}	0.047508	0.050224	0.040401	0.058050	0.053387	0.040915
I_{970}/I_{1735}	0.331676	0.326044	0.309944	0.419467	0.400079	0.298098
I_{1266}/I_{1297}	0.383491	0.471286	0.244688	0.394967	0.377758	0.289588
I_{1266}/I_{1430}	0.244227	0.279148	0.192707	0.262044	0.255435	0.211529
I_{1266}/I_{1735}	1.705081	1.812167	1.478399	1.893530	1.914217	1.541174
I_{1655}/I_{1297}	0.843839	1.082599	0.510407	0.823405	0.805197	0.594295
I_{1655}/I_{1430}	0.537401	0.641235	0.401977	0.546296	0.544463	0.434102
I_{1655}/I_{1735}	3.751882	4.162757	3.083867	3.947531	4.080182	3.162810
GeoMean	0.408874	0.461877	0.312392	0.448982	0.434953	0.334639

of Raman signals corresponding to unsaturated bonds to those of saturated or ester bonds (Table 3). The peaks at 970, 1266/1272, and 1655 cm^{-1} reflect the degree of unsaturation, while the signals at 1297/1301, 1430/1460, and 1735/1746 cm^{-1} correspond to saturated bonds or ester groups [35]. So we used nine intensity ratios to calculate, namely I_{970}/I_{1297}, I_{970}/I_{1430}, I_{970}/I_{1735}, I_{1266}/I_{1297}, I_{1266}/I_{1430}, I_{1266}/I_{1735}, I_{1655}/I_{1297}, I_{1655}/I_{1430}, and I_{1655}/I_{1735}. The relative amount of unsaturated fat in the sample was determined by calculating the geometric mean (GeoMean) of the ratios obtained.

Internal perirenal fat samples from both breeds showed a more intense peak of 1297/1301 cm^{-1} and less intense peaks of 970, 1266/1272, and 1655 cm^{-1}. The peak of 1430/1460 cm^{-1} showed no significant difference than subcutaneous fat samples. The geometric mean ratio of intensities was also minimal: 0.312392 for the perirenal Livni samples and 0.334639 for the perirenal Duroc samples. This may indicate a lower proportion of double-bonded fatty acids and a higher proportion of saturated fatty acids in internal fat. This result is in agreement with the work of Monziols *et al.* and Bee *et al.*, where it was shown that the degree of unsaturated fat deposition in pigs follows a negative gradient from outside to inside [16, 35]. The metabolic reasons for the preferential deposition of unsaturated fatty acids in subcutaneous fat are presumably related to low lipid metabolism. Internal fat exhibits greater *de novo* lipogenesis, with the result that feed-derived unsaturated fatty acids are diluted with more endogenous fatty acids than in subcutaneous fat.

The subcutaneous adipose tissue samples showed more intense peaks responsible for unsaturated bonds, while signals from saturated bonds did not show a uniform trend: 1297/1301 cm^{-1} was lower in all samples, 1430/1460 cm^{-1} with no significant difference, 1735/1746 cm^{-1} in the Livni breed samples was higher in subcutaneous fat (both locations), while in the Duroc breed it was higher in sacral fat and lower in axillary fat than in perirenal fat. The axillary Livni samples were characterized by more intense signals from unsaturated bonds than the sacral Livni samples, 0.461877 and 0.408874 respectively, while the axillary Duroc fat, on the contrary, showed less intense signals than the sacral Duroc samples, 0.434953 and 0.448982 respectively.

In a comparative analysis of the spectra of the samples from Duroc and Livni pigs, sacral Duroc showed more intense signals from unsaturated bonds, whereas the signals from saturated bonds varied – 1297/1301 cm^{-1} higher and 1735/1746 cm^{-1} lower than in sacral Livni, while axillary Duroc was more saturated than axillary Livni.

CONCLUSION

Taken together, the findings confirm that in pigs, the adipose tissue of different locations differs in morphology, lipid content and composition. Our results show that there are differences in cellular structure between adipose tissue of different locations (subcutaneous and internal) of Duroc and Livni pigs, and that adipocyte hypertrophy contributes most to the greater deposition of subcutaneous fat in Livni pigs. Both breeds showed a biphasic distribution of adipocyte size at all loci, suggesting that adipocytes are in their growth and fat accumulation phase.

In the Raman spectroscopy analysis, the inner perirenal adipose tissue showed less intense signals from unsaturated bonds compared to the subcutaneous tissue irrespective of the pig breed. The axillary fat samples of the Livni breed were characterised by more intense peaks responsible for unsaturated bonds than the sacral fat. The sacral fat from the Duroc breed was more unsaturated than that of the Livni breed. Further studies of adipose tissue of different locations, including using Raman spectroscopy, are needed to establish the lipogenesis potential of these adipose tissues, especially in pigs with different grades of body fat.

In the future, pig adipose tissue will be studied in greater depth. Most of the published studies have been

conducted with the subcutaneous adipose tissue of pigs, but the morphology of inter- and intramuscular fat also needs study, as its content has a significant influence on the consumer characteristics of pork. So, a comparative study of adipocytes from other anatomical loci of pigs, their development and composition, as well as their impact on the quality and structural and functional properties of raw meat would be of interest.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Bioaccumulation of trace elements in vegetables grown in various anthropogenic conditions

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Abstract:
Hazardous compounds accumulate in plants and animals as a result of anthropogenic impact. Trace elements, such as heavy metals, move up in the system of snow – soil – water – plant – animal. When contaminants accumulate in plants that serve as animal feed, they eventually accumulate in the animals that consume the feed because heavy metals usually enter living organisms via digestive tract, i.e., with food.
In 2003–2021, we studied fodder plants grown and harvested by urban zoological organizations, e.g., zoos, nature corners, etc. This research covered the Central Federal District represented by the cities of Moscow, Ivanovo, Yaroslavl, and Uglich. The empirical part of the study relied on a combination of modern ecological, biochemical, and statistical methods. A KVANT-2AT atomic absorption spectrometer was used to define the trace elements and their quantities.
Broccoli proved to be the most resistant feed vegetable to all the toxic elements in this study. Kohlrabi, sweet potato, and dill had low content of lead and cadmium, while garlic was highly resistant to cadmium and arsenic. Spinach, fennel, potatoes, beets, and bell peppers, which were used as fodder in metropolis conditions, exceeded the maximal permissible concentration of heavy metals. The samples obtained from the Moscow Zoo contained by 1.98 times more zinc, by 1.06 times more copper, and by 89.47 times more lead than average. The samples from Ivanovo accumulated the greatest extent of iron, which exceeded the average level by 3.26 times. The vegetables from Uglich and Ivanovo had the lowest concentration of zinc, which was by 67.86 and 62.70% below the average, respectively. The samples from Yaroslavl contained by 33.08% less copper. In 2003–2021, feed vegetables grown in the Central Federal District had an average increase in zinc, copper, and lead by 1.13, 1.45, and 2.80 times, respectively. The level of iron stayed almost the same throughout 2018–2021, while that of arsenic gradually decreased in concentration. The accumulation level of zinc, copper, iron, and arsenic in feed vegetables appeared to depend on the concentration of their water-soluble metal forms in the soil.
Therefore, forage agriculture in urban areas requires constant chemical and toxicological tests to prevent contaminated feed from entering animal diet.

Keywords: Vegetables, trace elements, heavy metals, arsenic, migration, deposit media, pollution

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INTRODUCTION

The growing anthropogenic impact increases the content of hazardous compounds in feed and animals that are subject to veterinary supervision. Accumulation of heavy metals has been the focus of scientific attention for many years, and the mechanisms of their biogeochemical behavior are clear. However, the jury is still out on the maximal permissible concentration of heavy metals in various environmental objects. As a result, foreign and domestic researchers are busy studying the content of hazardous macro- and microelements in water, soil, and atmosphere [1–7]. Most studies of depositing environments involve urban areas with a high anthropogenic load, e.g., metal-processing industrial wastes, industrial emissions, fuel combustion products, vehicle exhaust fumes, pesticides, phosphate and organic fertilizers, etc.

The content of trace elements in the environment depends on the anthropogenic load on the particular territory. Contaminants follow the snow – soil – water – plant – animal chain to enter food of plant origin [8]. Unfortunately, very few comprehensive studies feature

the accumulation and migration of trace elements and heavy metals from the surface soil to plant products. The most prospective research directions in this area include the following topics: the range of chemical contaminants; their content in food and organisms vs. their background concentrations in the soil; their transition to the aqueous soluble phase; their accumulation in plants; etc.

Chemical and toxicological tests are an obligatory stage according to all standardization documents and technical regulations. Such documentation is usually based on foreign and domestic studies on the maximal permissible concentration of toxic elements in objects of veterinary supervision [9, 10]. Yet, such issues as antagonism and synergy of composite pollutants, the migration of metals, and their cumulation in plant products receive very little scientific attention [11]. Therefore, the complex nature of pollution should underlie all comprehensive environmental studies, i.e., interrelations of heavy metals, their migration, the negative impact of their excessive level on environmental objects, etc.

The accumulation of contaminants in feed plants trigger their accumulation in animals, since most hazardous chemical elements enter the body through the alimentary route, i.e., with food [12–14]. Almost all animal diets center on plants or at least include them as additives. Urban zoological institutions, e.g., zoos, circuses, petting zoos, stations for young naturalists, etc., usually use roughage, vegetables, fruits, grain, etc.

Modern physical and chemical methods make it possible to monitor the content of chemical elements in animal diet and identify their residual amounts. Such studies reveal the general trend in heavy-metal contamination, which may be used to update regulatory documents on animal feed safety. Essential elements in animal feed also require a thorough control because they affect their vital activity [11, 15–17].

The present research objective was to determine the content of some trace elements, including heavy metals, in feed vegetables, as well as to study the effect of anthropogenic load on their accumulation and migration ability.

STUDY OBJECTS AND METHODS

This research lasted from 2003 to 2021 and covered some urban areas of the Central Federal District with different anthropogenic load, namely the cities of Moscow, Ivanovo, Yaroslavl, and Uglich. The study relied on an integrated approach which combined modern environmental, biochemical, and statistical methods. The vegetable samples were obtained from the subsidiary farms of zoos in Moscow, Ivanovo, and Yaroslavl, as well as from a station for young naturalists in Uglich.

The sampling followed the procedure specified by the recommended practice for Moscow zoos and animal stations MosMR 2.3.2.006-03.

The research featured the microelement composition of tubers (potatoes, sweet potatoes), roots (carrots, beets, turnips, daikon, celery), cabbages (white cabbage, broccoli, kohlrabi), lettuce and spinach cultures (endive, spinach), spice plants (parsley, dill, fennel), bulbs (onion, garlic, lettuce, leek), fruit vegetables (tomatoes, cucumbers, bell peppers, eggplant), and cucurbits (pumpkin, zucchini).

The samples were tested for iron, copper, lead, cadmium, zinc, and arsenic by acid mineralization using nitric acid. The sampling procedure followed State Standard 26929-94, while the tests themselves followed State Standards 30178-96 and 51766-2001 [2]. The heavy-metal test involved a KVANT-2AT atomic absorption spectrometer.

The samples weighed 20 g. They were charred on an electric stove with concentrated nitric acid and potassium or magnesium oxide, depending on the element. The charring lasted until the smoke emission ceased, which depended on the moisture content. Then, the samples were heated up to 250°C in a muffle furnace for 30 min, and the temperature was maintained at the same level for another 30 min. After that, the temperature was gradually increased by 50°C every 30 min and maintained so for 30 min. The maximal temperature was 450°C (500°C for cereals). After that, the crucibles were taken out, and samples with black ash were returned in the muffle furnace for another combustion cycle. Samples with white ash were processed in the spectrometer. The crucibles were cooled, and a base solution was added to dissolve the ash. The dissolved and filtered sample was poured into a 100-mL volumetric flask with a blue-ribbon filter. The base solution went in until the sample reached 100 mL. After that, the sample was tested in the spectrometer.

The experiment included 107 samples and 1764 measurements.

All the tests were performed in triplicates with intermediate precision. The obtained data underwent metrological processing according to State Standard R ISO 5725-6-2002.

The migration in the snow – soil – water – plant system was assessed based on the contingency between traits and the distribution of compatibility data by the Shapiro-Wilk W test. To determine the interdependence between two or more samples, we used regression analysis and the Spearman's Rank correlation coefficient. The significance level was 5% for all types of statistical analysis. The primary research materials entered the databases of Microsoft Office Excel 2010, Statistica version 10.0, Windows XP.

RESULTS AND DISCUSSION

The results obtained were compared with the maximal permissible levels specified in Technical Regulation of the Customs Union TR TS 021/2011 "On food safety" and the interim hygienic standards for certain chemical elements in basic food products No. 2450-81, as well as with the temporary maximal

Table 1 Trace elements in feed vegetables in zoological institutions of the Central Federal District

Product	Trace elements, mg/kg					
	Zn	Cu	Fe	Pb	Cd	As
Maximal permissible concentration in vegetables	10.00	5.00	50.00	0.50	0.03	0.20
Tubers						
Potato	4.18 ± 1.42	0.90 ± 0.71	13.05 ± 1.63	0.60 ± 0.19	0.02 ± 0.02	0.02 ± 0.01
Sweet potato	1.07 ± 0.01	0.71 ± 0.04	0.61 ± 0.02	0	0	0.02 ± 0.00
Roots						
Carrot	1.65 ± 0.93	0.79 ± 0.47	15.04 ± 1.31	0.25 ± 0.15	0.01 ± 0.01	0.02 ± 0.00
Beet	3.61 ± 1.83	0.82 ± 0.77	24.31 ± 3.09	0.13 ± 0.07	0.03 ± 0.01	0.05 ± 0.06
Turnip	2.35 ± 0.01	0.89 ± 0.01	4.76 ± 0.02	0.19 ± 0.01	0	0.01 ± 0.00
Celery	0.65 ± 0.00	0.28 ± 0.00	0.07 ± 0.01	0	0	0.01 ± 0.00
Daikon	3.55 ± 0.03	1.72 ± 0.01	2.98 ± 0.02	0.36 ± 0.01	0	0.01 ± 0.00
Cabbages						
White cabbage	0.93 ± 0.38	1.04 ± 0.59	6.96 ± 3.95	0.06 ± 0.02	0.01 ± 0.01	0.01 ± 0.00
Broccoli	4.48 ± 0.15	0.10 ± 0.01	1.19 ± 0.03	0	0	0
Kohlrabi	2.29 ± 0.00	0.59 ± 0.01	2.96 ± 0.02	0	0	0.01 ± 0.00
Lettuce and spinach						
Spinach	13.69 ± 0.06	2.29 ± 0.02	10.66 ± 0.01	0.63 ± 0.07	0.02 ± 0.00	0.01 ± 0.00
Endive	5.55 ± 0.04	2.42 ± 0.05	6.42 ± 0.07	0.38 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
Spice plants						
Dill	3.96 ± 0.04	0.28 ± 0.01	0.13 ± 0.00	0	0	0.01 ± 0.00
Parsley	3.27 ± 0.08	0.37 ± 0.00	0.03 ± 0.01	0	0.01 ± 0.00	0.01 ± 0.00
Fennel	3.80 ± 0.01	0.65 ± 0.02	3.91 ± 0.03	0.52 ± 0.01	0	0.02 ± 0.00
Bulbs						
Onion	0.70 ± 0.55	0.34 ± 0.04	26.92 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Leek	1.03 ± 0.02	0.22 ± 0.02	2.06 ± 0.09	0	0.03 ± 0.00	0.01 ± 0.00
Lettuce	3.39 ± 0.00	0.70 ± 0.02	5.53 ± 0.00	0.39 ± 0.02	0	0.02 ± 0.00
Garlic	7.07 ± 0.02	0.07 ± 0.02	5.97 ± 0.02	0.01 ± 0.00	0	0
Cucurbits						
Zucchini	0.68 ± 0.55	0.44 ± 0.26	7.29 ± 6.59	0.07 ± 0.09	0.01 ± 0.01	0.02 ± 0.01
Pumpkin	2.13 ± 0.01	0.46 ± 0.03	0.24 ± 0.02	0.03 ± 0.01	0	0.01 ± 0.00
Fruit vegetables						
Bell pepper	0.84 ± 0.44	1.20 ± 1.02	12.44 ± 4.43	0.15 ± 0.05	0.04 ± 0.03	0.01 ± 0.01
Cucumbers	1.08 ± 0.02	0.29 ± 0.01	7.68 ± 0.26	0.05 ± 0.00	0	0
Eggplants	0.88 ± 0.00	0.20 ± 0.02	3.07 ± 0.18	0.01 ± 0.00	0.01 ± 0.00	0
Average	2.52 ± 1.51	0.78 ± 0.15	9.34 ± 1.03	0.19 ± 0.12	0.01 ± 0.00	0.02 ± 0.01

permissible level for certain chemical elements and gossypol in farm animal feeds and feed additives and with some scientific publications [2].

Table 1 presents the main results of the study. The average accumulation levels of trace elements in the feed samples decreased as Fe > Zn > Cu > Pb > As > Cd, which was consistent with the available publications on this matter [5, 15].

Monitoring results from other regions of Russia showed that the degree of contamination in the areas covered by this research had a much lower average heavy-metal content: Cu – by 2–4 times, Zn – by 4–12 times, Pb – by 12 times, Cd – by 2 times, and As – by 2–8 times [15, 19–21]. Iron, copper, zinc, and manganese also were in lower concentrations compared to the average Russian data (Table 1).

However, we registered higher-than-average concentrations for some heavy metals. Tubers contained 3.81 ± 1.33 mg/kg of Zn and 0.01 ± 0.00 mg/kg of Cd. Rootshad 15.02 ± 1.31 mg/kg of Fe and 0.01 ± 0.00 mg/kg of Cd. Lettuce and spinach demonstrated 0.01 ± 0.00 mg/kg of Cd. Spice vegetables had 3.68 ± 0.32 mg/kg of Zn. Lettuce and spinach vegetables had a high content of Zn and Cu: 9.62 ± 1.46 and 8.54 ± 2.33 mg/kg, respectively. Tubers had 0.51 ± 0.01 mg/kg of Pb while lettuce and spinach vegetables had 0.53 ± 0.12 mg/kg. Fruit vegetables contained a lot of Cd (0.02 ± 0.00 mg/kg). Cucurbits demonstrated the lowest accumulation level of all trace elements but Cd. Cabbages were also low in all the toxic elements but Cu (Fig. 1), which confirmed the results obtained by an Iranian research team [5].

The content of Pb in tubers, as well as in lettuce and spinach plants, exceeded the maximal permissible concentration by 1.06 and 1.02 times, respectively. Probably, the concentration was so high because the samples were obtained from such a big industrial metropolis as Moscow. However, the results were consistent with those obtained in Serbia [22]. Other

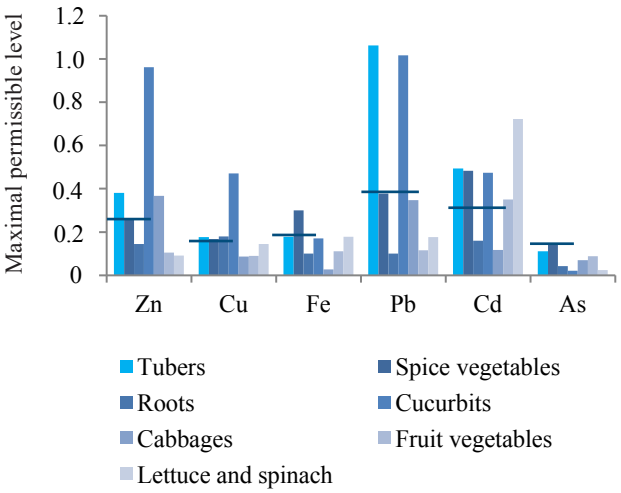

Figure 1 Accumulation of trace elements and heavy metals in feed vegetables in zoological institutions of the Central Federal District

Table 2 Intercorrelation of trace elements in feed vegetables of the Central Federal District: a correlation analysis, mg/kg

Element	Cu	Fe	Pb	Cd	As
Zn	0.21**	–0.02	0.12	–0.15	–0.12
Cu	–	0.52***	0.53***	0.04	0.46***
Fe		–	0.38***	0.54***	0.42***
Pb			–	0.12	0.06
Cd				–	0.25*

* significant difference ($p < 0.05$)

** significant difference ($p < 0.01$)

*** significant difference ($p < 0.001$)

groups revealed no excess of maximal permissible concentration, but Zn in the lettuce and spinach samples had an upward trend.

We performed a pairwise correlation analysis to check the possible interdependent accumulation of metals (Table 2). Cu and Fe had a direct relationship ($R = 0.52$ at $p < 0.001$), and so did Cu and Pb ($R = 0.53$ at $p < 0.001$), Cu and As ($R = 0.46$ at $p < 0.001$), Fe and Pb ($R = 0.38$ at $p < 0.001$), Fe and Cd ($R = 0.54$ at $p < 0.001$), and Fe and As ($R = 0.42$ at $p < 0.001$). Zn and Cu ($R = 0.21$ at $p < 0.01$) and Cd and As ($R = 0.25$ at $p < 0.05$) demonstrated a trend to symbatic accumulation level. The pairs Cd-As and Cu-Fe showed a mutual dependency similar to research conducted on other territories [15, 20].

We registered a significant increase in the content of all the elements in the samples from Moscow, compared with the average indicators: Zn – by 1.98 times, Cu – by 1.06 times, and Pb – by 89.47 times. On the contrary, Fe had the highest accumulation level in the samples from Ivanovo, the textile capital of Russia, where it exceeded the average content by 3.26 times. The lowest level of Zn belonged to the samples from Uglich and

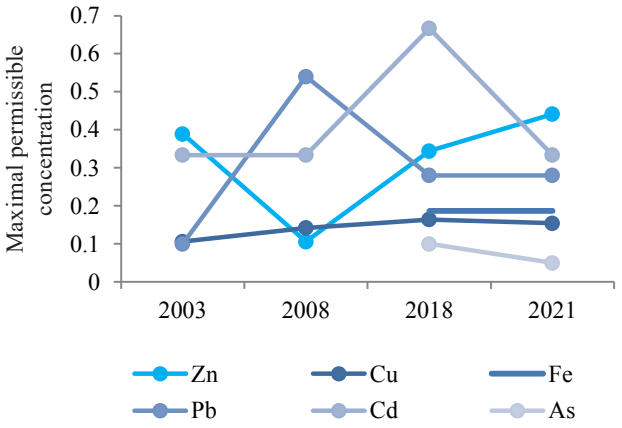

Figure 2 Accumulation of heavy metals and other trace elements in the Yaroslavl Region, 2003–2021

Ivanovo: they were by 67.86 and 62.70% below the average, respectively. The lowest content of Cu was in the samples from Yaroslavl: it was by 33.08%) below the average level (Tables 1 and 3).

A comparative analysis for 2003–2021 in the Central Federal District revealed an increase in the content of Zn, Cu, and Pb by 1.13, 1.45, and 2.80 times, respectively (Table 4). The content of Fe was approximately at the same level in 2018–2021, while As demonstrated a downward trend (Fig. 2). These data were found consistent with the general trend registered for the content of these elements in the Central Federal District and with our previous studies [2, 3].

A comparative analysis for 2003–2021 revealed a significant decrease in the content of Zn in the samples obtained from the rural and industrial territories of the Yaroslavl Region by 4.58 and 4.22 times, respectively. The content of Pb and Cd in Uglich samples increased by 3.09 and 4.02 times, while in the Yaroslavl samples it increased by 1.10 and 1.50 times, respectively. The accumulation level of Cu in the Yaroslavl samples increased by 1.10 times, while in Uglich it remained the same.

In 2008, Zn showed a significant accumulation decrease while Pb kept increasing. The highest level of Cd was registered in 2018.

The Central Federal District covers a huge territory, which varies greatly in geographical features and anthropogenic load. Consequently, this fact affects the accumulation level of pollutants in vegetables obtained from different environments. To establish the factors that affect the accumulation of chemical elements in vegetables, we studied the relationship between the content of trace elements and their level in environmental objects, i.e., snow, soil, and natural water bodies. We also investigated their migratory properties along the snow – soil – water – plant system.

The experiments revealed a competition in the transfer from the soil through water-soluble forms of Fe – Zn ($R = –0.27$), Cu – Fe, Cu – Cd, Cu – As

Table 3 Heavy metals and arsenic in feed vegetables from areas with different anthropogenic load

Location	Trace elements, mg/kg					
	Zn	Cu	Fe	Pb	Cd	As
Moscow	4.98 ± 1.29**	0.83 ± 0.28*	5.28 ± 0.61**	0.17 ± 0.12	0.01 ± 0.00	0.01 ± 0.00**
Yaroslavl	1.14 ± 0.15**	0.60 ± 0.16*	12.30 ± 1.84**	0.31 ± 0.17	0.01 ± 0.00	0.02 ± 0.01**
Ivanovo	0.94 ± 0.14**	1.05 ± 0.03*	30.47 ± 1.86**	0.25 ± 0.14	0.01 ± 0.00	0.02 ± 0.00**
Uglich	0.81 ± 0.32**	0.78 ± 0.19*	12.40 ± 3.12**	0.14 ± 0.02	0.01 ± 0.00	0.02 ± 0.01**

* significant difference ($p < 0.05$)

** significant difference ($p < 0.001$)

Table 4 Average content of the trace elements in feed vegetables of the Central Federal District in 2003–2021

Year	Trace elements, mg/kg					
	Zn	Cu	Fe	Pb	Cd	As
2003	3.89 ± 1.25	0.53 ± 0.31	–	0.05 ± 0.02	0.01 ± 0.00	–
2008	1.06 ± 0.54	0.71 ± 0.14	–	0.27 ± 0.16	0.01 ± 0.00	–
2018	3.44 ± 0.54	0.82 ± 0.27	9.34 ± 1.03	0.14 ± 0.02	0.02 ± 0.01	0.02 ± 0.00
2021	4.41 ± 0.58	0.77 ± 0.16	9.33 ± 1.24	0.14 ± 0.08	0.01 ± 0.00	0.01 ± 0.00

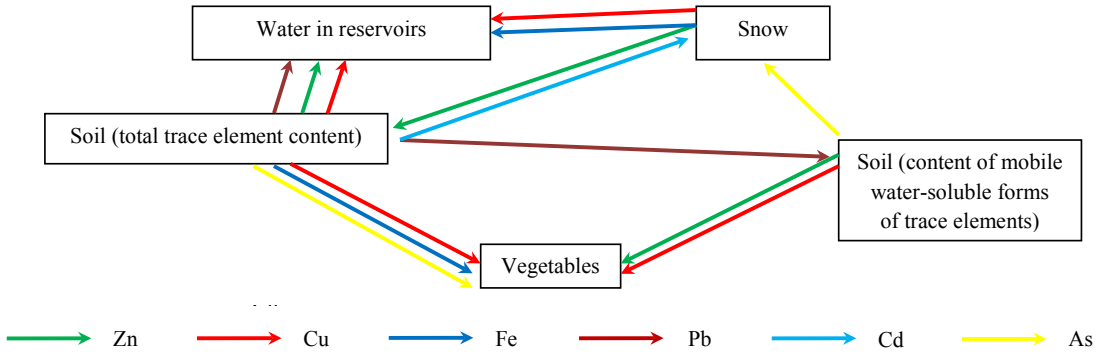

Figure 3 Intermedia transition of trace elements in the snow – soil – water – plant system in the Central Federal District

(R = –0.52; R = –0.46; R = –0.30, respectively), as well as Pb – Cu and Pb – Fe (R = –0.37 and R = –0.49, respectively). No direct correlation occurred between the chemical composition of snow and water and the accumulation of chemical elements in feed vegetables (Fig. 3).

Zn demonstrated good migration ability in the snow – soil – water – plant system. The level of Zn was low in the deposition media, i.e., soil and snow, as well as in drinking water and food. We also registered a weak and medium direct relationship between the concentrations of Zn in vegetables and environmental objects. The concentration of water-soluble metal forms in the soil affected the level of microelement content in the vegetables. Cu had good snow – water and soil – plants migration ability, which allowed Cu to accumulate in plants in high concentrations. The concentration of metal in vegetables was most affected by the type of soil-forming material and the amount of its water-soluble forms in the soil. These results were found consistent with those reported for the Altai Region [23].

Fe usually entered vegetables from its aqueous solution in the soil, as evidenced by the weak correlation. We revealed no clear migration between environmental objects and plants, which indicates the natural origin of the metal.

The migration tests revealed a strong effect of the As content in the soil, especially its total accumulation, on the As concentration in feed vegetables.

The migratory ability of Pb and Cd had a loose dependance on their content in vegetables, and the intermedia transition was not obvious. We established a high content of Pb in depositing media, i.e., soil and snow, which may indicate its anthropogenic origin.

CONCLUSION

Broccoli turned out to be the most resistant vegetable to the accumulation of all the toxic elements in this research. Kohlrabi, sweet potato, and dill were highly resistant to toxic heavy metals, namely Pb and Cd, while garlic did not accumulate Cd and As. Spinach, fennel, potatoes, beets, and bell peppers cultivated as feed for animals in the Moscow Zoo exceeded the

maximal permissible level of some microelements. Compared with the average indicators, vegetables from the Moscow Zoo demonstrated a significant increase in the content of Zn (by 1.98 times), Cu (by 1.06 times), and Pb (by 89.47 times), but no increase was registered for Fe. The samples from the city of Ivanovo had the greatest accumulation of Fe, which exceeded the average content level by 3.26 times. In the samples from Uglich and Ivanovo, the content of Zn was by 67.86 and 62.70% below the average, respectively, which was the lowest level in this research. The content of Cu in the samples from Yaroslavl was below the average by 33.08%.

The average concentration of Zn, Cu, and Pb increased by 1.13, 1.45, and 2.80 times, respectively, between 2003 and 2021. However, the concentration of Fe remained almost the same in 2018–2021, while the concentration of As demonstrated a downward trend in the same period.

The concentration of water-soluble metal forms in the soil had a major effect on the accumulation of Zn, Cu, Fe, and As in feed vegetables grown in the Central Federal District.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Algerian date palm (*Phoenix dactylifera* L.) fruit cultivars: HPLC fingerprinting and antibacterial activity

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Abstract:
The abusive use of antibiotics causes the destruction of intestinal flora and the proliferation of antibiotic-resistant pathogens. Date palm is used in traditional medicine in the Saharan regions due to its biological properties.
The study aimed to identify the phytochemical composition and assess the antibacterial activity of the methanolic extracts of three date cultivars from Algeria. Their total phenolic, flavonoid, and flavonol contents were measured spectrophotometrically. The phytochemical screening was conducted by HPLC fingerprinting using twenty-three pure phenolic compounds as standards. The antibacterial activity against pathogenic bacterial species was assessed using the disk diffusion method.
The colorimetric methods showed that the total phenolic, flavonoid, and flavonol contents ranged from 2.13 ± 0.09 to 2.67 ± 0.02 mg GAE/100 g DW, 1.33 ± 0.21 to 1.55 ± 0.13 mg CEQ/100 g DW, and 0.41 ± 0.23 to 0.47 ± 0.05 mg REQ/100 g DW, respectively. HPLC fingerprinting showed that the extracts of date cultivars served as an excellent source of bioactive compounds (gallic acid, tannic acid, ferulic acid, vanillin, caffeine, quercetin, luteolin, rutin, aspegenin, isorhamnetin, and hesperidin). They also exhibited an antibacterial potential with an inhibition zone diameter ranging from 8.40 to 12.50 mm.
The results clearly demonstrate the antibacterial potency of date palm fruits, which could be attributed to their considerable content of phenolic compounds such as gallic acid, rutin, quercetin, and luteolin.

Keywords: *Phoenix dactylifera* L., high-performance liquid chromatography fingerprinting, phenolic compounds, flavonoids, flavonol, secondary metabolites

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INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is an important fruit crop for the populations of the Middle East and North Africa. This fruit has great nutritional and economic importance [1, 2].

Since antiquity, the date and its pits have been used in traditional medicine in the Saharan regions, more precisely in the oases, where the date palm was cultivated [3]. Dates have high energy values and are rich in reductive and easily assimilated sugars, minerals (selenium, potassium, calcium, magnesium, manganese, and iron) and vitamins (A, B, and C). In addition, this fruit is endowed with numerous health benefits resulting from the mixture of secondary metabolites (polyphenols, anthocyanins, carotenoids, tannins, procyanidins, sterols, flavonols, flavones, anthocyanidins, isoflavones, phytoestrogens, phenolic acids, cinnamic acid derivatives, and volatile compounds) [4]. The date palm has been the subject of phytochemical, pharmacological, and nutritional research [5–8]. Various studies have determined the physicochemical composition of dates but scarce field research has only focused on phenolic components and involved only a few varieties of this fruit. These bioactive compounds are gaining increasing interest, given their important biological properties [9, 10].

This study was part of a program to valorize the Algerian flora through the search for new compounds or active ingredients. We aimed to determine phenolic compounds and to assess the antibacterial effect of methanolic extracts from three cultivars (Hamraya, Figheth, and Tamajort) of the date palm (*P. dactylifera*) fruits growing in the El-Oued region (Algeria).

STUDY OBJECTS AND METHODS

Plant material collection. Three date palm fruit cultivars (*Phoenix dactylifera* L.) were collected from the El-Oued region (South-East of Algeria) at the final stage of fruit ripeness, at the beginning of the 2012 harvest season. They are locally named Hamraya (33'34'30,09N, 6'49'43,11E), Figheth (33'35'15,56N, 6'49'36,83E), and Tamajort (33'33'39,99N, 6'47'27,73E).

Preparation of extracts. The preparation of methanolic extracts was performed according to the research by Biglari *et al.* [11]. In particular, 100 mg of the edible parts of each cultivar were macerated in 300 mL of methanol-water (4:1, v/v) at room temperature under continuous shaking for 5 h. The mixture was filtered and concentrated to obtain crude extracts, which were kept at 4°C until used.

Total phenolic content assay. The total phenolic content was determined by the spectrophotometric method described by Al-Farsi *et al.* [12]. For this, 200 µL of each extract was added to 1.5 mL of the Folin-Ciocalteu reagent. The solutions were mixed and incubated in the dark for 5 min. Then, 1.5 mL of sodium bicarbonate (60 g/L) was added to the reaction medium. After 90 min of incubation at room temperature, the absorbance of all extracts was measured with a UV-visible spectrophotometer at 725 nm against the blank without extract. The phenolic content was expressed as milligrams of gallic acid equivalent per 100 g dry weight (mg GAE/100 g DW) based on a calibration line constructed from the standard solution of gallic acid.

Total flavonoids assay. Total flavonoids were determined by the method described by Biglari *et al.* [11]. For this, 4 mL of distilled water and 1 mL of each extract were added to 0.3 mL of 5% sodium nitrite ($NaNO_2$) and 0.3 mL of 2% aluminum chloride ($AlCl_3$) in methanol. After incubation for 5 min at room temperature, 2 mL of 1% sodium hydroxide (NaOH) in methanol was added. The mixture was diluted to 10 mL with distilled water. The absorbance of the resulting mixture was measured directly with a UV-visible spectrophotometer at 510 nm against the blank. The flavonoid contents were expressed as milligrams of catechin equivalent per 100 g dry weight (mg CEQ/100 g DW) based on a calibration line constructed from the standard solution of catechin.

Total flavonols assay. To determine total flavonols, 500 µL of each methanolic extract was added to 500 µL of 2% aluminum chloride ($AlCl_3$) and 500 µL of 5% sodium acetate (CH_3COONa). The absorbance was determined at 440 nm after incubation for 2.5 h at room temperature. The flavonol contents were expressed as milligrams of rutin equivalent per 100 g dry weight (mg REQ/100 g DW) based on a calibration line constructed from the standard solution of rutin [13].

HPLC analysis of phenolic compounds. To determine phenolic compounds, 10 mg of powdered extracts of *P. dactylifera* were dissolved in 10 mL of methanol to a final concentration of 1 mg/mL. The solution was filtered through a 0.45-µm syringe filter for sterilization. Then, 1 mg of each standard was dissolved individually in 1 mL of methanol and sterile-filtered through a 0.45-µm syringe filter before subjecting to high-performance liquid chromatography (HPLC). The analysis of phenolic compounds in various extracts was carried out using HPLC coupled with a visible UV multi-wavelength detector under the following operating conditions:

– steel column: 25×0.46 cm;
– stationary phase: C18;
– elution solvent: methanol:acetonitrile (30:70 v/v);
– wavelengths: 220, 280, 300, and 365 nm; and
– injection loop capacity: 20 µL.

To identify the compounds, 23 pure phenolic compounds were used as standards, namely rutin, naringin, quercetin, luteolin, isorhamnetin, 2,5-dimethyl hydroxycinnamic acid, 3,4,5-trimethoxybenzoic acid, 3,4,5-trimethoxycinnamic acid, ferulic acid, gallic acid, m-anisic acid, o-anisic acid, syringic acid, transcinnamic acid, 3,4-dimethoxycinnamic acid, 2,5-dihydroxycinnamic acid, apigenin, caffeine, vanillin, tannic acid, naringenin-7-O-glucoside, hesperidine, and caffeic acid. The peaks were identified by comparing the retention time of the standard compounds with that of different peaks obtained in the HPLC analysis of the extracts [13].

Antibacterial activity. The bacterial strains used to evaluate the antibacterial potentials of the date extracts included three Gram-positive (*Staphylococcus aureus* ATCC25223, *Bacillus spizizenii* ATCC6633, and *Listeria monocytogenes* ATCC15313) and three Gram-negative (*Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC8739, and *Salmonella typhimurium* ATCC14028) strains. The bacterial strains were obtained from the Pasteur Institute of Algiers (Algeria). All the bacteria were grown on nutrient agar at 4°C.

The antibacterial potentials of the methanolic date extracts were determined using the agar disc diffusion method [14]. The bacterial strains were cultured in a nutrient broth for 24 h and diluted with sterilized peptone water. Suspensions of the tested microorganisms (0.5 McFarland units) were spread onto the media plates. Sterile filter paper disks of 6 mm in diameter were impregnated with 20 µL of each extract. Methanol was used as a negative control, while pure standard antibiotics (streptomycin and cefazolin) at a concentration of 1 mg/mL were used as a positive control. The plates were incubated at 37°C for 24 h. The zones of inhibition appearing around the disks were measured and recorded in mm. All the tests were performed in triplicate.

Statistical analysis. The results were given as mean ± standard deviation (SD). The analysis of variance (ANOVA) was used to look at the differences in mean values. Turkey's test was used to determine statistically significant differences ($p < 0.05$).

Table 1 Yields and bioactive contents of methanolic extracts of date cultivars (*Phoenix dactylifera* L.)

Cultivars	Yield, % w/w	Total phenolic contents, mg GAE/100 g DW	Flavonoid contents, mg CEQ/100 g DW	Flavonol contents, mg REQ/100 g DW
Hamraya	27.28 ± 0.23[b]	2.13 ± 0.09[a]	1.55 ± 0.13[a]	0.47 ± 0.05[a]
Figheth	33.14 ± 0.04[a]	2.46 ± 0.17[a]	1.49 ± 0.17[b]	0.43 ± 0.32[a]
Tamajort	32.02 ± 1.29[a]	2.67 ± 0.02[a]	1.33 ± 0.21[b]	0.41 ± 0.23[a]

[a–b]: values (mean ± standard deviation, n = 3) in the same column sharing different letters are significantly different ($p < 0.05$)

Table 2 Phenolic compounds detected in methanolic extracts of *Phoenix dactylifera* L. by HPLC at 220 and 280 nm

	220 nm		280 nm	
	Phenolic compounds	Retention time, min	Phenolic compounds	Retention time, min
Hamraya	3,4,5-Trimethoxybenzoic acid	10.721	Gallic acid	3.260
	m-Anisic acid	12.437	Tannic acid	3.270
			Caffeine	6.405
			Naringenin-7-o-glucoside	10.387
			Trans-cinnamic acid	13.821
			2,5-Dimethyl hydroxycinnamic acid	14.823
			Hesperidin	15.070
Figheth	3,4,5-Trimethoxybenzoic acid	11.174	Gallic acid	3.193
	m-Anisic acid	11.936	Tannic acid	3.270
			Caffeine	6.354
			Naringenin-7-o-glucoside	10.338
			Trans-cinnamic acid	13.915
			2,5-Dihydroxycinnamic acid	14.823
			Hesperidin	15.070
Tamajort	not detected	–	Gallic acid	3.182
			Tannic acid	3.399
			Caffeine	6.358
			Naringenin-7-o-glucoside	10.345
			Trans-cinnamic acid	13.900
			2,5-Dimethyl hydroxycinnamic acid	14.834
			Hesperidin	15.158

RESULTS AND DISCUSSION

Bioactive content. The results shown in Table 1 correspond to the yield and bioactive contents (phenolic, flavonoid, and flavonol amounts) of the methanolic extracts of three date cultivars (*Phoenix dactylifera* L.) from Algeria. As we can see, the Figheth and Tamajort cultivars had the highest yields. There was no difference in the total phenolic content ($p > 0.05$) between the extracts of Tamajort, Figheth, and Hamraya. The Hamraya extract had the maximum flavonoid amount, compared to the Figheth and Tamajort cultivars (Table 1).

Finally, we found no significant differences ($p > 0.05$) between the flavonol contents of the Hamraya, Figheth, and Tamajort varieties.

The yield varies according to several parameters: the plant material studied (particle size), the physicochemical characteristics of the solvents used, and the solvents' polarity. It also depends on storage conditions, duration, harvest period, the method, and extraction conditions [13].

To the best of our knowledge, there are no publications on the phenolic and flavonoid compounds of the date cultivars under study analyzed by using HPLC coupled with a visible UV multi-wavelength detector (220, 280, 300, and 365 nm). However, our findings were in accordance with those of Biglari *et al.* who showed that the total polyphenol contents of dates from Iran ranged from 2.89 to 6.64 mg GAE/100 g DW [11]. In addition, the study by Alam *et al.* on the extracts of 26 date varieties from the United Arab Emirates and Pakistan found total phenols ranging from 46 to 397 mg GAE/100 g fresh weight (FW) [15].

Kadum *et al.* tested the ethanolic extracts of five date varieties (Ajwa, Anbara, Piyarom, Rabbi, and Deglet Nour) from Malaysia [16]. They found that the flavonoid amounts varied from 38.63 to 57.07 mg RE/100g DW.

The results in this study were consistent with our previous study, where flavonoids and flavonols of the dates from Algeria varied from 1.06 ± 0.12 to 4.23 ± 0.29 mg CEQ/100 g DW and from 0.44 ± 0.10 to 1.43 ± 0.15 mg REQ/100 g DW, respectively [17].

In the study by Benmeddour *et al.*, the flavonol amounts of date cultivars from Biskra region (Algeria) ranged between 6.73 and 36.64 mg REQ/100 g DW [13].

Several factors can affect the bioactive content of plants. Indeed, studies have shown that these are

Table 3 Phenolic compounds detected in methanolic extracts of *Phoenix dactylifera* L. by HPLC at 300 and 365 nm

	300 nm		365 nm	
	Phenolic compounds	Retention time, min	Phenolic compounds	Retention time, min
Hamraya	Caffeic acid	7.062	Rutin	8.664
	Vanillin	8.604	o-Anisic acid	9.668
	Ferulic acid	9.266	Luteolin	12.776
	3-Hydroxy-4-methoxycinnamic acid	9.677	Quercetin	12.810
	3,4,5-Trimethoxycinnamic acid	12.816	Aspegenin	14.497
	3,4-Dimethoxycinnamic acid	14.882	Isorhamnetin	15.059
Figheth	Caffeic acid	7.080	Rutin	8.779
	Vanillin	8.771	o-Anisic acid	9.740
	Ferulic acid	9.356	Luteolin	12.344
	3-hydroxy-4-methoxycinnamic acid	9.669	Quercetin	12.830
	3,4,5-Trimethoxycinnamic acid	12.902	Aspegenin	14.756
	3,4-Dimethoxycinnamic acid	14.950	Isorhamnetin	15.059
Tamajort	Caffeic acid	7.076	Rutin	8.651
	Vanillin	8.568	o-Anisic acid	9.732
	Ferulic acid	9.356	Luteolin	12.675
	3-Hydroxy-4-methoxycinnamic acid	9.816	Quercetin	12.814
	3,4,5-Trimethoxycinnamic acid	12.898	Aspegenin	14.403
	3,4-Dimethoxycinnamic acid	14.935	Isorhamnetin	14.745

extrinsic factors (such as geographical and climatic factors), genetic factors, and the degree of the plant's maturation [12, 18].

HPLC analysis of phenolic compounds. The phenolic compounds of the date methanolic extracts were detected by HPLC at 220, 280, 300, and 365 nm. The retention times of the standard phenolic compounds were compared with the peaks of the chromatograms of the extracts. At 220 nm, 3,4,5-trimethoxybenzoic acid and m-anisic acid were detected only in the methanolic extracts of Figheth and Hamraya. At 280 nm, gallic acid, tannic acid, caffeine, naringenin-7-o-glucoside, trans-cinnamic acid, 2,5-dimethyl hydroxycinnamic acid, and hesperidin were detected in the methanolic extracts of Hamraya, Figheth, and Tamajort (Table 2).

The chromatographic analyses at 300 nm identified caffeic acid, vanillin, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, 3,4,5-trimethoxycinnamic acid, and 3,4-dimethoxycinnamic acid in all the date extracts. The qualitative analysis at 365 nm revealed the presence of rutin, o-anisic acid, luteolin, quercetin, aspegenin, and isorhamnetin in all the extracts studied (Table 3).

Numerous studies have identified polyphenolic compounds in *P. dactylifera* extracts by HPLC analysis. Indeed, the study by Souli *et al.* identified and quantified trans-ferulic and syringic acids as major phenolic compounds in most Tunisian date cultivars [19].

The chromatographic analyses of the extracts of Al-Qasim cultivated in Saudi Arabia revealed the presence of 3,30-di-O-methyl ellagic acid, 7-methoxy-quercetin-O-hexose isomers, caffeic acid, ferulic acid, isomers of quercentin-rutinoside, kaempferol methylether, p-hydroxybenzoic acid, phytol, punicalagin, and quercetin-3-O-glucoside (isoquercitrin) [20].

Dhaouadi *et al.* quantified phenolic compounds in the aqueous extract of the Deglet-Nour cultivar grown in Tunisia [21]. In their study, the amounts of coumaric, gallic, vanillic, cinnamic, 3,4-dicaffeoylquinic, 5-O-caffeoyl shikimic, caffeoyl-quinic, and caffeic acids were 23.03, 6.79, 2.55, 35.79, 13.86, 17.70, 285, and 9.81 mg/100 g, respectively. Similarly, Kchaou *et al.* established a phenolic profile of the hydroacetonic extracts of three Tunisian date cultivars, including gallic, vanillic, caffeic, syringic, coumaric, ferulic, and sinapic acids, as well as rutin [22]. El Sohaimy *et al.* tested four Omani date varieties by HPLC. The separation showed the presence of gallic, coumaric, caffeic, vanillic, and syringic acids with respective contents of 19.14, 1.67, 1.75, 0.27, and 0.37 mg/100 g [23].

In a study by Shahdadi *et al.*, aqueous and ethanolic extracts from Egyptian date cultivars contained 7.51 and 5.28 μg/g of gallic acid, 2.85 and 1.79 μg/g of tannic acid, and 0.15 and 0.22 μg/g of ferulic acid, respectively [24]. In contrast, the study reported the absence of cinnamic acid. The differences between our results and those reported in literature can be attributed to the geographic origin of the fruits. The phenolic compounds in *P. dactylifera* extracts may also differ because of the extraction solvent [22, 25, 26].

Antibacterial activity. The methanolic extracts of date fruits were screened for their antibacterial activities against six pathogenic bacterial species (Table 4). Theantibacterial activity was recorded when the inhibition zone was greater than 6 mm. The results of antibacterial screening revealed significant antibacterial activity. The inhibition zone diameters ranged from 08.40 ± 0.00 to 12.50 ± 1.00 mm. Streptomycin and cefazolin, which were used as positive experimental

Table 4 Inhibition zone diameters for the methanolic extracts of date cultivars against pathogenic bacterial species

Pathogenic bacterial species	Inhibition zone diameters, mm				
	Cultivars			Antibiotics	
	Hamraya	Figheth	Tamajort	Streptomycin	Cefazolin
Staphylococcus aureus ATCC638P	8.40 ± 0.00	9.30 ± 0.50	10.50 ± 0.50	19.16 ± 0.76	25.40 ± 0.00
Bacillus spizizenii ATCC6633	10.40 ± 0.60	12.50 ± 1.00	9.00 ± 0.50	22.80 ± 1.31	31.50 ± 0.00
Listeria monocytogenes ATCC15313	10.00 ± 1.00	10.50 ± 0.60	n.a.	29.50 ± 1.50	27.40 ± 0.00
Pseudomonas aeruginosa ATCC27853	n.a.	8.00 ± 0.50	n.a.	25.10 ± 2.59	34.20 ± 0.00
Escherichia coli ATCC8739	8.40 ± 0.70	10.30 ± 0.00	n.a.	28.83 ± 1.04	25.60 ± 0.00
Salmonella typhimurium ATCC14028	10.50 ± 0.60	8.500 ± 0.002	10.20 ± 0.35	23.53 ± 1.28	28.80 ± 0.00

* n.a. – no activity revealed; values are presented in mean ± SEM (n = 3)

controls against all bacterial strains assayed, produced an inhibition zone diameter ranging from 19.16 ± 0.76 to 34.20 ± 0.00 mm, while no inhibitory effect could be observed for methanol used as a negative control.

Among the three extracts, Figheth was the most effective against *Bacillus spizizenii* ATCC6633, with the largest zone of inhibition (12.50 ± 1.00 mm), while Hamraya showed the smallest inhibition zone diameter (08.40 ± 0.00 mm) against *Staphylococcus aureus* ATCC638P. Table 4 shows that the methanolic extract of Tamajort had no effect on *Listeria monocytogenes* ATCC15313, *Pseudomonas aeruginosa* ATCC27853, and *Escherichia coli* ATCC8739.

Among natural substances widespread in medicinal plants, flavonoids and organic acids belong to the promising groups of bioactive compounds with strong antibacterial potency [27].

Alshwyeh tested the methanolic extracts of Saudi Arabian native date palm (Ajwa, Khalas Alkharj, and Al-Qasim) cultivars against *Streptococcus pnuemoniae*, *Bacillus subtilis*, *Klebsiella pneumonia*, *S. aureus*, and *E. coli* [20]. They found that the extracts displayed a broad spectrum of inhibitory effects against these pathogenic bacteria.

The inhibitory activity of plant extracts against the growth of microorganisms was attributed to the presence of phenolic compounds [28]. These antibacterial compounds act essentially by enzyme inhibition of DNA gyrase and disturb the function of bacterial cell membranes, retarding the growth and multiplication of bacteria.

Different antibacterial mechanisms of plant flavonoids were reported by Faegheh *et al.* and Górniak *et al.* [29, 30]. In particular, flavonoids inhibit nucleic acid synthesis, cytoplasmic membrane function, energy metabolism, biofilm attachment, and porin on the cell membrane, as well as alter membrane permeability and attenuate pathogenicity.

Abdullah *et al.* tested the antibacterial potential of hot aqueous and methanolic extracts of Ajwa date fruit against Gram-negative bacteria (*Salmonella typhi*, *E. coli*, *Vibrio cholera*, and *Shigella flexneri*) [31]. They found that the methanolic extract showed a higher antibacterial activity than the aqueous extract, suggesting that different extraction methods yield different phytochemicals producing the bactericidal effect.

Flavonoids cause increased permeability of the internal bacterial membrane and disruption of membrane potential. According to Usman Amin *et al.*, the ring of flavonoids may play a role in intercalation or hydrogen binding with nucleic acid base stacks, which may explain their inhibitory action on the synthesis of DNA and mRNA [32].

Flavonoids are known for their powerful antioxidant power. Indeed, they could potentially have an effect on iron chelation, which prevents the intracellular penetration of the co-factor Ca^{2+} into the bacterial cell [33].

CONCLUSION

Our study was designed to determine the phytochemical composition and the antibacterial potential of the methanolic extracts of three cultivars of date palm fruits. HPLC revealed the presence of gallic acid, tannic acid, caffeine, naringenin-7-o-glucoside, trans-cinnamic acid, 2,5-dimethyl hydroxycinnamic acid, hesperidin, caffeic acid, vanillin, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 3,4-dimethoxycinnamic acid, rutin, o-anisic acid, luteolin, quercetin, aspegenin, and isorhamnetin in all the date cultivars. The results showed that the extracts serve as an excellent source of bioactive compounds (polyphenols, flavonoids, and flavonols) and exhibit antibacterial potency with an inhibition zone diameter ranging from 8.40 ± 0.00 to 12.50 ± 1.00 mm. The results clearly demonstrate the antibacterial activity of date palm fruits, which could be attributed to their considerable content of natural compounds. Thus, they can replace the antibiotics which are restricted because of several side effects.

CONTRIBUTION

S. Ali Haimoud and R. Allem conceived and designed the analysis; contributed data and analysis tools;

and performed the analysis. S. Ali Haimoud collected the data and wrote the paper.

CONFLICT OF INTEREST

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

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Embryogenesis induction of carrot (*Daucus carota* L.) in isolated microspore culture

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Abstract:
Haploid technologies are used to create homozygous lines for accelerated breeding. We aimed to optimize the technology for using the isolated microspore culture *in vitro* to obtain doubled haploids of the carrot (*Daucus carota* L.).
We studied two carrot varieties with different responsiveness to embryogenesis, Altajskaya lakomka and Breeding line 17. Carrot microspores were isolated from buds and cultivated in liquid nutrient media supplemented with an antibiotic and activated carbon *in vitro*. They were exposed to different thermal treatments.
The experiment showed the benefits of combining cold pre-treatment of buds (5°C for 1 day) with heat shock of isolated microspores *in vitro* (32°C for 2 days). The induction of embryogenesis on the NLN-13 medium was twice as high as on the MSm-13 medium. The use of 1% activated carbon in 0.5% agarose increased the yield of embryoids by more than 1.5 times. 100 mg/L of ampicillin was found to be the most efficient concentration. After 30 days of cultivation under optimized conditions, the yield was 161.3 and 44.0 embryoids per Petri dish for the cultivar Altajskaya lakomka and Breeding line 17, respectively.
The induction of carrot embryogenesis is determined by the type and duration of thermal stress, the composition of the nutrient medium, the use of activated carbon as a sorbent, the addition of β-lactam antibiotics, and the type of explant exposed to thermal treatment. Our technology enabled us to obtain homozygous doubled haploid lines of carrots during a year, and these lines were included in the breeding process to create F_1 hybrids.

Keywords: *Daucus carota*, culture medium, ampicillin, haploids, carrot, penicillin, cold and heat stress, cefotaxime, embryogenesis

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INTRODUCTION

The carrot (*Daucus carota* L.) is an economically important root vegetable of the *Apiaceae* Lindley (*Umbelliferae* Juss.) family. It is rich in carotene (provitamin A) and contains an essential oil. Since the carrot is a cross-pollinated biennial crop, it takes a lot of time and effort to obtain inbred lines and select parental pairs for crossing. Moreover, it is difficult to achieve a high level of homozygosity by using traditional breeding methods.

Over the last 100 years, scientists have looked for ways to create genetic diversity by using doubled haploids. So far, they have described protocols for obtaining doubled haploids for 384 species, including the culture of isolated microspores *in vitro* for *Apiaceae* vegetables. This technology is based on the ability of microspores to switch from gametophytic to sporophytic development under conditions *in vitro*. Exposed to various stress factors, they form embryoids from which doubled haploids are obtained.

This approach speeds up the creation of initial homozygous material (doubled haploid lines) for selection and expands the range of genetic recombinant forms, including those with recessive traits, thus facilitating the search for rare genotypes [5].

Embryogenesis induction protocols have been developed to obtain haploid and doubled haploid plants of the *Apiaceae* family (cumin, carrot, celery, fennel, and dill) using the cultures of unpollinated ovules, anther, and isolated microspores [6–10].

However, the practical application of unpollinated ovules and anther cultures is limited by the low efficiency of embryogenesis [11]. Moreover, there is a risk of obtaining callus and embryoids from somatic tissues during the cultivation of anthers and unpollinated ovules [12]. Therefore, isozyme or DNA analysis is required to confirm homozygosity [7]. Yet, the culture of isolated microspores excludes the interference of somatic tissues during embryogenesis.

The callus culture obtained from isolated carrot microspores was first described by Matsubara *et al.* in 1995, followed by studies on the regeneration of doubled haploids in the carrot [10, 13, 14]. Federal State Budgetary Scientific Institution Federal Scientific Vegetable Center (Russia) studied the process of embryogenesis and developed a method for obtaining doubled haploid plants from the culture of isolated carrot microspores [15, 16].

Many factors affect the efficiency of embryogenesis in the microspore culture *in vitro*, including genotype, microspore development stage, donor plant growth conditions, medium composition, and cultivation conditions [5].

Stress factors such as cold and heat treatment have a direct influence on microspores and can stimulate the induction of embryogenesis. Nitsch and Norreel first described the use of cold pre-treatment for *Datura innoxia* L. anthers [17]. Its positive effect was presumably associated with slowing down degradation in anther tissues. Such treatment protects microspores from toxic compounds released by degrading anthers and ensures the survival of most embryogenic microspores. Cold pre-treatment of buds has also been shown to increase the frequency of endoreduplication. This stimulates the spontaneous doubling of haploid plants and significantly enhances the efficiency of doubled haploid production. Lowering the temperature by 10°C prior to the isolation of microspores from the donor plants of the *Brassica* species slows down the plant's growth and allows for a longer time when buds can be selected at a certain stage of development. Cold treatment of donor plants improves the embryogenic responsiveness of microspores. A similar phenomenon was observed in plants of the *Brassica* family [18].

Cold pre-treatment is mainly used for buds, while heat treatment (33–37°C) is often used to induce microspores in the culture *in vitro*. In anther culture, cold (4°C) and heat (32°C) pre-treatments had a positive effect on callus formation and embryogenesis of some carrot genotypes [11]. Various cold treatment schemes (1–3 days at 4°C) had a negative effect on most carrot varieties, increasing the induction of embryogenesis in only some of them [14, 15].

The composition of the nutrient medium affects embryogenesis efficiency. Such media as ½ NLN-13, NLN-13, MSm-13, ½ MS, and B5 were used to obtain embryoids from carrot microspores. Matsubara *et al.* supplemented ½ MS with 2,4-D (1 mg/L), BAP (1 mg/L), sucrose (100 g/L), and NLN-13 [13]. Gorecka *et al.* and Kiszczak *et al.* used the nutrient medium B5 supplemented with L-glutamine (500 mg/L), L-serine (100 mg/L), 2,4-D (0.1 mg/L), NAA (0.1 mg/L), and sucrose (100 g/L) [10, 19]. Li *et al.* obtained carrot plants on the NLN-13 medium containing 2,4-D (0.1 mg/L), NAA (0.1 mg/L), and activated carbon [14][1].

High concentrations of ethylene released during microspore cultivation can have a toxic effect on the normal development of embryoids. According to literature, activated carbon is used as an adsorbing agent for ethylene [20]. In addition, it can absorb some phenols produced by damaged tissues. Shumilina *et al.* reported a positive effect of activated carbon on the development of somatic embryoids of the *Brassica* family [18].

Endogenous and exogenous microbial contamination of the culture is one of the main problems that can occur during androgenesis, which may result in the loss of valuable and rare specimens *in vitro*. *β*-lactam antibiotics are most commonly used to prevent that. They inhibit the synthesis of peptidoglycan, the main component of the bacterial cell wall, thereby exerting a bactericidal effect. Some antibiotics can have a regenerative effect, like plant growth regulators. This ability is associated with the similarity of their chemical structure with that of some phytohormones [21]. *β*-lactam antibiotics are similar to auxins, since their molecular structures contain four-membered (*β*-lactam ring) and five-membered (pyrrole) nitrogenous heterocycles [22, 23].

The microspore culture *in vitro* is an advanced technology that speeds up the selection process by rapidly generating homozygous doubled haploid lines. However, it requires further research to ensure a high level of efficiency. We aimed to study the effect of various factors on the yield of carrot (*D. carota* L.) embryoids in the microspore culture *in vitro*. The factors included cold and heat treatment of explants and isolated microspores, the composition of nutrient media, as well as the presence of an antibiotic and activated carbon in the medium.

[1] MSm – modified Murashige and Skoog medium [1], NLN – Nitsch, Lichter and Nitsch medium [2], MS – Murashige and Skoog medium [3], B5 – Gamborg medium [4], 2,4-D – 2,4-dichlorophenoxyacetic acid, BAP – 6-benzylaminopurine, NAA – 1-naphthaleneacetic acid.

STUDY OBJECTS AND METHODS

Plant material and growth conditions. Experimental samples of the carrot (*Daucus carota* L.) included those of the cultivar Altajskaya lakomka and Breeding line 17.

The roots were vernalized at 4°C for 4 months and then planted in a climatic chamber with 9000 lux illumination, a 16/8 h (day/night) photoperiod, and a permanent temperature of 19°C.

Umbels were collected from each sample at the beginning of flowering at an optimal stage of development according to their morphology (flat). To determine the stage of microspore development, buds from the two outer rows of umbels were placed in a mixture of 96% ethanol and glacial acetic acid (3:1 by volume) for 1–2 h and then washed with 70% ethanol. The fixed anthers were crushed in a drop of 2% acetocarmine on a glass slide and quickly heated over a burner flame (not boiled). When all the tissues were removed, they were covered with a glass slide and examined under an Axio Imager A2 light microscope (Carl Zeiss Microscopy GmbH, Germany). The stage of development was determined by the arrangement of vacuoles and nuclei. For cultivation, buds were selected with microspores mainly at the late vacuolized mononuclear stage and pollen grains at the early two-celled stage (a moderate amount) [15].

Microspore culture. Carrot buds from the two outer rows of umbels were sterilized in 96% ethanol (30 s) and then in a 50% aqueous solution of the Belizna preparation (containing 5% sodium hypochlorite) with 2–3 drops of Tween-20 (5 min). After that, they were washed three times in sterile distilled water. Microspores were isolated and cultivated according to an optimized method developed for the *Brassicaceae* family [24]. The microspore suspension density was determined using a Goryaev counting chamber (MiniMed, Bryansk, Russia). The suspension with a density of 2×10^4 was used for incubation.

The isolated microspores were cultivated in 6-cm disposable Petri dishes (filled with 3–4 drops of agarose with activated carbon) on a 5-cm^3 liquid nutrient medium, NLN-13 or MSm-13 (pH = 5.8), containing 13% sucrose [1, 2]. The medium was supplemented with ampicillin (100 mg/L) and plant growth regulators, 1-naphthaleneacetic acid and 2,4-D (1 mg/L). The cultivation took place in the dark in a Binder BF 260 incubator (Tuttlingen, Germany) at a temperature according to the experimental plan.

We counted the number of embryoids (per Petri dish) formed on days 28–32 of cultivation.

Effect of thermal treatment on isolated microspores. The culture of isolated microspores *in vitro* on NLN-13 was exposed to cold treatment at 5°C for 1–3 days, heat treatment at 32°C for 1–2 days, and joint treatment with cold at 5°C for 2 days and heat at 32°C for 2 days [2]. The experiment included seven samples and was performed under different conditions in three repetitions:

1. 25°C throughout the entire period (control);
2. 5°C for 1 day, then 25°C;
3. 5°C for 2 days, then 25°C;
4. 5°C for 3 days, then 25°C;
5. 32°C for 1 day, then 25°C;
6. 32°C for 2 days, then 25°C; and
7. 5°C for 2 days, 32°C for 2 days, then 25°C.

Effect of thermal treatment on explants and isolated microspores. Two types of explants were obtained from four umbels of each carrot sample – buds separated from inflorescences and whole inflorescences. They were pre-treated with cold at 5°C for 1–2 days. Then, the culture of isolated microspores *in vitro* on NLN-13 was exposed to heat shock at 32°C for 2 days [2].

The experiment was performed in seven repetitions and three replicates, namely:

1. 32°C for 2 days, then 25°C (control);
2. 5°C for 1 day, 32°C for 2 days, then 25°C; and
3. 5°C for 2 days, 32°C for 2 days, then 25°C.

Effect of the nutrient medium composition. Two liquid nutrient media were used for the experiments, NLN-13 and MSm-13 (pH = 5.8), containing 13% sucrose and supplemented with ampicillin (100 mg/L) and plant growth regulators NAA and 2.4-D (1 mg/L) [1, 2].

The experiment was performed in seven repetitions.

Effect of activated carbon. 0.5 g of agarose and 1 g of activated carbon were dissolved in 100 cm^3 of distilled water. The preparation was autoclaved and dropped into Petri dishes, 3–4 drops each. Carrot buds were pre-treated with cold at 5°C for 1 day. Then the culture of isolated microspores *in vitro* on NLN-13 was subjected to heat treatment at 32°C (2 days) and then at 25°C [2].

The experiment was performed in two biological replicates: without activated carbon (control) and with activated carbon.

Three analytical replicates were performed for each experimental variant.

Effect of antibiotics. Ampicillin (Biosintez, Russia), cefotaxime (Sintez, Russia), and penicillin (Biosintez, Russia) were used in a lyophilized injection form in sterile 1-g vials. To prepare an antibiotic solution, sterile water (10 cm^3) was added to a sterile antibiotic (1 g). Then, the solution was aliquoted by 1 cm^3 and stored at –20°C. Carrot buds were pre-treated with cold at 5°C for 1 day. The culture of isolated microspores *in vitro* on NLN-13 was subjected to heat treatment at 32°C (2 days) followed by 25°C [2]. The antibiotics were added to the nutrient medium according to the experimental plan.

The phytotoxicity of antibiotics was determined in four replicates, namely: without antibiotic (control), cefotaxime (100 mg/L), ampicillin (100 mg/L), and penicillin (100 mg/L).

The optimal concentration of ampicillin was determined in five replicates, namely: without antibiotic (control), ampicillin (50 mg/L), ampicillin (100 mg/L), ampicillin (200 mg/L), and ampicillin (1000 mg/L);

Table 1 Carrot embryoids per Petri dish at various heat treatments of isolated microspore culture *in vitro*

Sample	Thermal treatment							LSD_{05}*
	a	b			c		d	
		1 day	2 days	3 days	1 day	2 days		
Breeding line 17	0	2.0 ± 0.6	2.0 ± 0.6	1.0 ± 0.6	0.7 ± 0.7	0.7 ± 0.7	0	F < Ft
Altajskaya lakomka	15.0 ± 1.2[b]	22.0 ± 1.7[c]	24.7 ± 0.9[c]	6.7 ± 1.5[a]	7.0 ± 1.2[a]	16.7 ± 0.7[b]	15.0 ± 2.0[b]	4.0

a – 25°C (control); b – 5°C; c – 32°C; d – 5°C for 2 days, then 32°C for 2 days

values marked with different letters are significantly different at $p \leq 0.05$

*least significant difference ($p < 0.05$)

Table 2 Carrot embryoids per Petri dish during cold treatment of explants and heat treatment of isolated microspore culture *in vitro*

Sample	Thermal treatment			LSD_{05}*
	a	b	c	
Breeding line 17	0.5 ± 0.1[a]	28.9 ± 6.5[b]	0[a]	10.6
Altajskaya lakomka	38.9 ± 4.7[a]	58.2 ± 5.2[b]	42.2 ± 2.7[a]	12.2

a – 32°C for 2 days (control); b – 5°C for 1 day, then 32°C for 2 days; c – 5°C for 2 days, then 32°C for 2 days

values marked with different letters are significantly different at $p \leq 0.05$

*least significant difference ($p < 0.05$)

Three analytical replicates were performed for each experimental variant.

All the reagents, except for the antibiotics, were produced by Sigma-ALDRICH (USA) and were labeled as "suitable for the plant cell culture".

Statistical data analysis. Experimental data were processed in Microsoft Excel 2010. One-way and two-way analysis of variance (ANOVA) was performed on the basis of the Fisher test. The least significant difference (LSD) was evaluated using Student's t-test with a probability of 0.95. Experimental data are given as means ± error of the mean.

RESULTS AND DISCUSSION

Effect of cold and heat treatment of isolated carrot microspores and explants on the induction of embryogenesis in culture *in vitro*. The process of primary and secondary embryogenesis in the microspore culture of the carrot (*Daucus carota* L.) was described earlier by Shmykova *et al.* [15]. The technology for obtaining doubled haploids in the microspore culture *in vitro* has been developed for individual carrot genotypes [5, 14]. However, due to a large number of factors that influence embryogenesis and regeneration, these processes need to be continuously reviewed, taking into account innovations and genotypes.

In 2020–2021, we conducted an experiment in two stages to assess carrot microspore responsiveness to embryogenesis at different temperatures. At the first stage (2020), isolated microspores in the culture *in vitro* were subjected to heat treatment (Table 1). At the second stage (2021), explants were pre-treated with cold and then isolated microspores in the culture *in vitro* were exposed to heat (Table 2).

At the first stage of the experiment in 2020, we studied the effect of cold and heat treatments, as well as their combinations, on the embryogenic induction of microspores by the carrot samples (Table 1). We found that cold treatment at 5°C stimulated the formation of carrot embryoids. The best results were obtained by cold treatment during 1 and 2 days (22.0 and 24.7 embryoids per Petri dish, respectively). However, longer cold treatment (up to 3 days) reduced the induction of embryogenesis to 6.7 embryoids per Petri dish, which was half the control number of 15.0 embryoids per Petri dish.

The heat stress at 32°C did not have a positive effect on the induction of embryogenesis in the microspore culture *in vitro*. In particular, 1 and 2 days of heat treatment produced only 7.0 and 16.7 embryoids per Petri dish, respectively, which corresponded to the control level. A combination of cold (5°C) and heat (32°C) treatments during 2 days had no significant effect either, with the number of embryoids reaching the control level (15.0 per Petri dish). According to Table 1, the Breeding line 17 samples showed little responsiveness to the experimental conditions (0–2.0 embryoids per Petri dish) and produced results with no statistically significant differences (F < Ft). The Altajskaya lakomka samples were much more responsive, with 6.7–24.7 embryoids per Petri dish. In particular, we found this cultivar responsive to cold treatment at 5°C for 1–2 days. Therefore, this treatment regime was used at the next stage of the experiment the following year.

At the second stage of the experiment in 2021, we combined cold pre-treatment of explants (5°C for 1 and 2 days) with heat treatment of isolated microspores (32°C for 2 days). This combination increased embryogenic induction to 28.9 and 58.2 embryoids per Petri dish for the Breeding line 17 and cultivars Altajskaya lakomka, respectively. A longer cold pre-treatment of explants (2 days) resulted in the embryogenic induction at the control level (Table 2).

The cultivar Altajskaya lakomka showed high responsiveness to the experimental conditions at the second stage, while the Breeding line 17 samples formed

Table 3 Carrot embryoids per Petri dish depending on nutrient medium composition

Sample	Nutrient medium		LSD_{05}*
	MSm	NLN	
Breeding line 17	5.8 ± 2.0	13.8 ± 4.8	F < Ft
Altajskaya lakomka	28.2 ± 2.0[a]	64.6 ± 2.9[b]	7.0

values marked with different letters are statistically significant at $p \leq 0.05$

* least significant difference ($p < 0.05$)

Table 4 Carrot embryoids per Petri dish at cold treatment of various types of explants

Sample	Explants		LSD_{05}***
	Buds*	Inflorescences**	
Breeding line 17	0.2 ± 0.1[a]	19.4 ± 4.8[b]	9.5
Altajskaya lakomka	50.7 ± 3.7	42.1 ± 3.7	F < Ft

* buds cut from carrot inflorescences;

** buds left on carrot inflorescences;

*** least significant difference ($p < 0.05$)

values marked with different letters are statistically significant at $p \leq 0.05$

Table 5 Carrot embryoids per Petri dish in the isolated microspore culture *in vitro* on the media with and without activated carbon

Sample	Samples		LSD_{05}*
	Without activated carbon (control)	With activated carbon	
Breeding line 17	12.0 ± 1.2[a]	18.6 ± 1.5[b]	4.8
Altajskaya lakomka	51.3 ± 5.4[a]	93.3 ± 12.8[b]	35.8

values marked with different letters are statistically significant at $p \leq 0.05$

* least significant difference ($p < 0.05$)

embryoids only after a 1-day cold pre-treatment of explants. Yet, even with cold pre-treatment, Breeding line 17's embryogenic activity was twice as low as that of the Altajskaya lakomka (28.9 and 58.2 embryoids per Petri dish, respectively). Moreover, a 2-day cold pre-treatment of Breeding line 17 explants at 5°C was totally ineffective, producing 0 embryoids per Petri dish.

Cold stress can stimulate embryogenic induction. In our experiments, however, its positive effect at 5°C was short-lived. In particular, only one day of cold treatment increased the yield of embryoids, with longer treatments leading to decreased embryogenic induction. This is probably associated with the fact that microspores quickly reached a stage of development optimal for embryogenesis. Our results were consistent with those from studies on carrot anthers [11, 14].

Responsiveness to microspore embryogenesis greatly depends on the genotype of a donor plant. Differences in embryogenic induction are also determined by the year of the study [14]. In our experiment, the cultivar Altajskaya lakomka proved highly responsive to embryogenesis, while Breeding line 17 revealed low embryogenic potential. We believe it was due to the fact that Breeding line 17 microspores were at an unsuitable stage of development at the time of the experiment. It may also be that the 24-h duration of the cold stress (5°C) was too long for the carrot buds and it needs to be shortened.

In addition, the genotype of a donor plant affected embryogenic induction. In particular, a combination of cold treatment of the Altajskaya lakomka buds at 5°C for 2 days and the subsequent heat treatment at 32°C for 2 days produced different numbers of embryoids in 2020 and 2021 (15.0 and 42.2 per Petri dish, respectively) (Tables 1 and 2). This could be because the experiments were carried out during mass flowering and inflorescences were collected from different donor plants.

Effect of the nutrient medium composition. We found that embryogenetic induction differed depending on the composition of the nutrient medium. Both carrot varieties, Breeding line 17 and Altajskaya lakomka (Table 3), showed a twice higher induction potential on the NLN-13 medium (13.8 and 64.6 embryoids per Petri dish, respectively) than on the MSm-13 medium (5.8 and 28.2 embryoids per Petri dish, respectively).

The differences in the composition of the nutrient medium for Breeding line 17 samples were statistically insignificant.

The choice of the nutrient medium has a significant effect on the efficiency of embryogenesis. Matsubara *et al.* were the first to observe the division of carrot microspores on a liquid nutrient medium ½ MS supplemented with 2,4-D (1 mg/L) and BAP (1 mg/L) [3, 13]. However, no embryoids were obtained in that study. Gorecka *et al.* successfully used the B5 medium supplemented with glutamine (500 mg/L), L-serine (100 mg/L), 2,4-D (0.1 mg/L), and NAA (0.1 mg/L) for the microspore culture [10]. Li *et al.* induced carrot microspores on the NLN-13 medium supplemented with 2,4-D (0.1 mg/L) and NAA (0.1 mg/L) [2, 4, 14]. Vjurtts *et al.* reported a successful formation of carrot embryoids on the ½ NLN-13 and MS-13 media with various additives [2, 3, 16]. In later studies, Shmykova *et al.* observed the induction of embryogenesis in carrots on the ½ NLN-13 medium supplemented with 2,4-D (0.2 mg/L) and kinetin (0.2 mg/L) [2, 15].

In our study, the NLN-13 medium yielded twice as many embryoids per Petri dish as the MSm-13 medium. This pattern was observed for both varieties. In earlier experiments conducted on other carrot genotypes, the NLN-13 medium also showed the highest induction of embryogenesis [16]. These results are due to the fact that NLN-13 was developed directly for cultivating rapeseed microspores, with stress effects taken into account. For this, the medium contained reduced glutathione and an increased content of boron ions, which triggered cell division. The MSm-13 medium, however, was used to

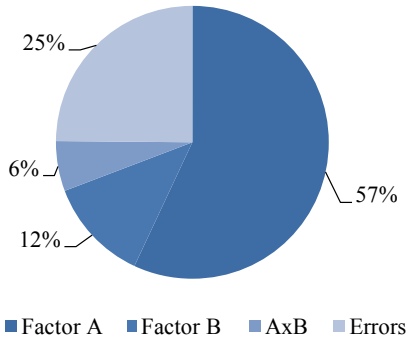


Figure 1 Two-way analysis of variance with repetitions: effects of nutrient medium (Factor A) and thermal treatment (Factor B) on the efficiency of carrot embryogenesis in the isolated microspore culture *in vitro* for the cultivar Altajskaya lakomka

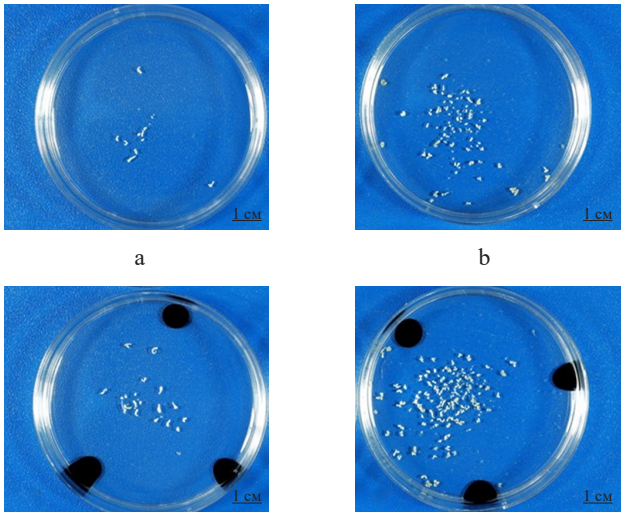


Figure 3 Formation of embryoids in carrot samples of the varieties Breeding line 17 (a and c) and Altajskaya lakomka (b and d) on the medium with and without activated carbon (6 cm Petri dishes)

induce gynogenesis and obtain carrot embryoids from callus cells [1, 12]. Therefore, it is better suited for further cultivation of the embryoids obtained on NLN-13 and for growing seedlings of carrot plants [2].

Effect of the explant type. We used two types of explants: buds separated from inflorescences and whole inflorescences cut and placed in a glass of water (Table 4). The explants were pre-treated with cold, after which microspores were isolated and introduced into the culture *in vitro*.

The effect of cold pretreatment on the induction of embryogenesis depended on the carrot genotype. Particularly, the Altajskaya lakomka samples showed the best results when microspores were isolated from carrot buds (50.7 embryoids per Petri dish). Yet, the influence of the explant type was insignificant for this cultivar. As for the Breeding

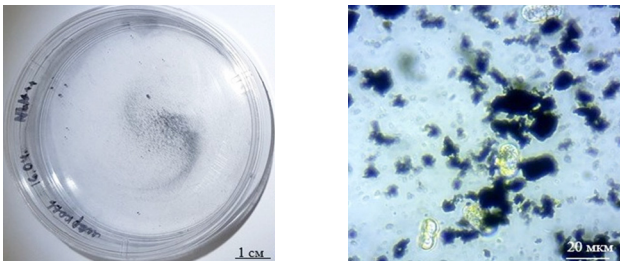


Figure 2 Adhesion of particles of powdered activated carbon to microspores of carrot: (a) general view of Petri dishes with activated carbon; (b) carrot microspores with adhering particles of activated carbon

line 17 samples, the largest number of embryoids (19.4 per Petri dish) was produced by the microspores isolated from inflorescences. The differences between the two types of explants were significant for this cultivar.

According to the two-way analysis of variance with repetitions, the composition of the nutrient medium had the biggest effect on the efficiency of carrot embryogenesis in the microspore culture *in vitro*, accounting for 57% (Fig. 1). The effect of thermal treatment amounted to 12%, while the combined effect of these two factors was only 6%.

The influence of these factors on the efficiency of embryoid formation was not unambiguous, which may be due to differences in the carrot genotypes. However, the one-way analysis of variance revealed no significant differences. A larger number of samples and factors to be studied might give a better understanding of how different types of explants affect the induction of embryogenesis.

Effect of activated carbon. Adding activated carbon powder to a liquid nutrient medium causes carrot microspores to stick together and impairs their further development (Fig. 2). Therefore, we used a preparation of activated carbon with agarose.

The use of activated carbon as an adsorbing agent in the lower layer of the liquid nutrient medium increased the formation of embryoids in the microspore culture *in vitro* 1.5–1.8 times, compared to the carbon-free control (Table 5, Fig. 3). The cultivar Altajskaya lakomka proved more responsive and yielded 4–5 times more embryoids that Breeding line 17.

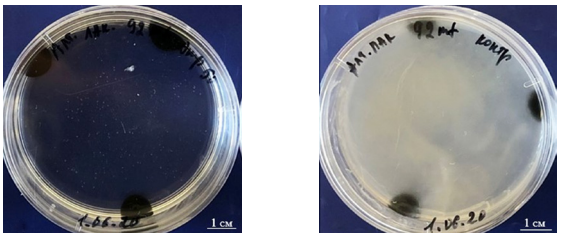
The one-way analysis of variance showed that the addition of activated carbon to the nutrient medium was a significant factor, the data belonging to different groups.

Earlier studies reported that adding activated carbon to the bottom layer of a two-layer nutrient medium increased the rate of embryoid formation in *Brassica napus*. Gland *et al.* suggested that activated carbon removes toxic substances released by inactive microspores, stimulating embryoids to develop [25]. In addition, it may adsorb phenolic compounds that are

Table 6 Influence of ampicillin concentration on embryogenic activity in the isolated carrot microspore culture *in vitro*

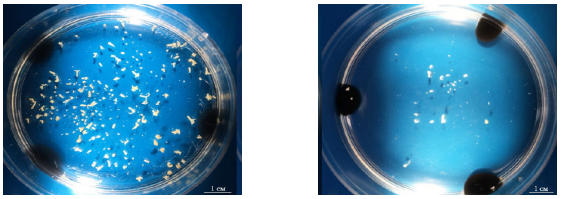
Sample	Ampicillin concentration, mg/L				
	0 (control)	50	100	200	1000
Altajskaya lakomka	0[a]	16.0 ± 1.6[a]	130.5 ± 22.4[b]	11.8 ± 1.9[a]	0[a]

Values marked with different letters are statistically significant at $p \leq 0.05$, $LSD_{05} = 31.1$



a b

Figure 4 Carrot microspore culture on day 3. Cultivation: (a) uninfected medium with ampicillin; (b) microbial infection in the medium without ampicillin



a b

Figure 6 Petri dishes with carrot embryoids visible to the naked eye after 30 days of cultivation: (a) Altajskaya lakomka; (b) Breeding line 17

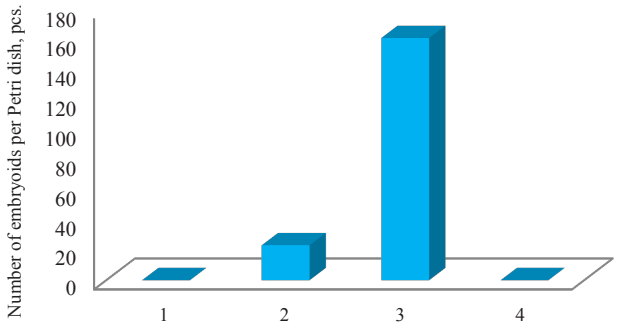


Figure 5 Effect of antibiotics (100 mg/L) on carrot embryogenesis (cultivar Altajskaya lakomka) in the microspore culture *in vitro* (values marked with different letters are statistically significant at $p \leq 0.05$, $LSD_{05} = 27.8$): 1 – control (without antibiotic); 2 – cefotaxime (23.3 ± 4.4[a]); 3 – ampicillin (161.3 ± 17.5[b]); 4 – penicillin (0[a])

released from damaged tissues during cultivation. In a study on embryogenesis and regeneration of wheat seedlings (*Triticum aestivum* L.), activated carbon increased the percentage of morphogenic callus and the number of green meristematic foci. The largest number of green shoots was obtained on the medium containing activated carbon (0.5 g/L) [26].

We found that supplementing the nutrient medium with a 1% suspension of activated carbon in 0.5% agarose intensified embryogenesis in the carrot microspore culture *in vitro*. Guo and Pulli showed a positive effect of adding activated carbon in agarose [27]. In a study by Prem *et al.*, activated carbon was added without agarose and had a negative effect [28]. The authors suggested that carbon particles stuck to the microspores and embryoids, thereby preventing their growth and development, which was confirmed by our experiments.

According to our results, using activated carbon in the microspore culture of more responsive genotypes significantly increases the formation of embryoids. Even the less responsive Breeding line 17 showed a slight increase in embryoids compared to the control (without activated carbon).

Effect of *β*-lactam antibiotics. When using the standard protocol for the cultivation of microspores, we found a serious loss of starting material in some samples due to endogenous microbial contamination *in vitro*. In the experiments without antibiotics, microbial contamination and a total loss of starting material in some samples were observed on the third day. Yet, adding ampicillin to the nutrient medium, even at the lowest concentration of 50 mg/L, prevented microbial contamination (Fig. 4).

The phytotoxicity of various antibiotics (cefotaxime, ampicillin, penicillin) was determined in the microspore culture *in vitro* of the highly responsive cultivar Altajskaya lakomka according to previous studies on cabbage crops [23]. The sample without antibiotics (control) had bacterial contamination (no embrioids), which led to complete inhibition of the microspore culture. Ampicillin provided the largest number of carrot embryoids (161.3 per Petri dish), while cefotaxime reduced this number 7 times (23.3 embryoids per Petri dish). The addition of penicillin suppressed the division of microspores and the formation of embryoids (Fig. 5).

To study the induction effect of *β*-lactam antibiotics on the embryoid yield, we carried out an experiment with various concentrations of ampicillin as the least phytotoxic antibiotic. We found the largest number of carrot embryoids (130.5 per Petri dish) on the nutrient medium with 100 mg/L of ampicillin (Table 6). The minimum concentration of 50 mg/L did not stimulate the embryogenesis significantly (16.0 embryoids per Petri dish). Yet, concentra-

tions exceeding 100 mg/L suppressed the development of carrot embryoids (11.8 per Petri dish).

The *β*-lactam antibiotics proved effective in the culture of isolated carrot microspores *in vitro*. Except for their high antibacterial activity, they should not have a negative effect on the induction and development of embryoids. Earlier studies showed that antibiotics can induce regeneration, but at high concentrations they inhibit the development of explants due to their toxicity [22, 23, 29].

Grzebelus and Skop, who studied the effect of *β*-lactam antibiotics on regeneration in the carrot protoplast culture, found that cefotaxime had a stimulating effect at a concentration of 400–500 mg/L [21]. According to Meng *et al*., ampicillin and cefotaxime had a selective effect on the frequency of regeneration of Chinese cabbage somatic embryoids [22]. In particular, cefotaxime inhibited re-differentiation of shoots and roots, while ampicillin stimulated their differentiation. In a study by Gerszberg and Grzegorczyk-Karolak, ampicillin at concentrations of 100–400 mg/L not only had an antimicrobial effect, but also regenerated tomato buds, while cefotaxime at a concentration of over 200 mg/L had a negative effect [29].

Despite the widespread application of antibiotics in various methods, their use in the microspore culture *in vitro* is an innovation. Therefore, it is not included in the standard protocol for producing doubled haploid plants by androgenesis. Yet, we have found a few studies on wheat, triticale, and rapeseed. Asif *et al.* found that cefotaxime at a concentration of 100 mg/L had a stimulating effect on the embryogenesis of wheat and triticale in the microspore culture *in vitro* [30]. Other authors reported its inducing effect on the embryogenesis and regeneration of rapeseed at certain concentrations and treatment times [31].

According to our results, ampicillin at a concentration of 100 mg/L was the most effective of all the *β*-lactam antibiotics under study in inducing carrot embryogenesis in the microspore culture *in vitro*. It not only controlled unwanted microbial infection, but also significantly increased embryoid yield. Similar results were obtained earlier for cabbage crops [24].

CONCLUSION

The induction of embryogenesis in the culture of isolated microspores *in vitro* is determined by many factors. Although there is a protocol for obtaining haploid and doubled haploid carrot plants, this process is highly dependent on the sample and microspore cultivation conditions. Therefore, this technology needs to be improved for specific genotypes.

In our study, the following factors determined the efficiency of carrot embryogenic induction: a responsive donor plant genotype (Altajskaya lakomka), cold treatment of plant buds at 5°C for 1 day and heat treatment of isolated microspores at 32°C for 2 days, the NLN-13 nutrient medium with activated carbon as an adsorbent, and ampicillin at a concentration of 100 mg/L. We optimized the technology to increase the induction of embryogenesis not only in the highly responsive sample Altajskaya lakomka, but also Breeding line 17 with low responsiveness (157.3 and 44.0 embryoids per Petri dish, respectively, after 30 days of cultivation) (Fig. 6).

CONTRIBUTION

Concept development: E.A. Domblides and O.V. Romanova; methodology: E.A. Domblides; software: V.A. Akhramenko; data validation: E.A. Domblides, O.V. Romanova, and A.I. Mineikina; formal analysis: E.A. Domblides, O.V. Romanova, and A.I. Mineikina; research: T.S. Vjurtts, A.I. Mineykina, O.V. Romanova, Ya.P. Tukuser, and Yu.V. Kulakov; resources: A.V. Soldatenko; data curation: T.S. Vjurtts, A.I. Mineykina, and O.V. Romanova; the initial draft: E.A. Domblides, O.V. Romanova, and A.I. Mineykina; reviewing and editing: E.A. Domblides, O.V. Romanova, and A.I. Mineykina; visualization: Yu.V. Kulakov; project management and control: A.V. Soldatenko.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Grain bran hydrolysates in the production of fruit distillates

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Abstract:
Currently, there is an urgent need for domestic fermentation activators based on low-cost secondary raw materials. We aimed to study the effect of microbial enzyme preparations with different action on the hydrolysis of proteins and phytin of grain bran to obtain fermentation activators that could become an alternative to imported ones.
We studied wheat and rye brans; microbial enzyme preparations with cytolytic, proteolytic, and phytase action; multi-enzyme compositions; and grain bran hydrolysates. Firstly, we determined the kinetic characteristics of enzyme preparations. Secondly, we evaluated their effectiveness in the hydrolysis of the brans. Thirdly, we developed multi-enzyme compositions. Finally, we determined the concentration of soluble forms of phosphorus and free amino acids in the hydrolysates.
We determined optimal temperature and pH values for the enzyme preparations. The multi-enzyme compositions contributed to a high accumulation of reducing substances, water-soluble protein, and phosphorus. The concentration of free amino acids in the hydrolysates obtained under the action of the bran's own enzymes was about 20% higher in the wheat samples, compared to the rye samples. However, when using multi-enzyme compositions in addition to the bran's own enzymes, the concentration of free amino acids was 1.5 times higher in the rye hydrolysates, compared to the wheat hydrolysates.
The use of multi-enzyme compositions under optimal conditions can double the content of phosphorus and free amino acids available for yeast, compared to the control. Our results can be used for further research into using grain bran hydrolysates as an alternative source of nitrogen and phosphorus nutrition for yeast at the fermentation stage of fruit distillate production

Keywords: Grain bran, microbial enzyme preparations, multi-enzyme compositions, hydrolysates, free amino acids, soluble forms of phosphorus

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INTRODUCTION

Enzyme preparations of microbial origin with different action are widely used in various branches of the food industry [1–7]. Enzymatic modification of the main biopolymers of raw materials used in fermentation and winemaking facilitates the conversion of material components into a soluble state. This is due to the release of some substances from the bound state, as well as the hydrolysis of the main high-molecular polymers to soluble forms. Enzymatic hydrolysis intensifies certain stages of production, increases the yield of the end product, and improves its quality. Grain bran is a by-product of flour milling used as a substrate for enzymes [8–11].

In Russia, grain (wheat and rye) bran averages 21.3% of flour production. It is classified as a low-cost renewable raw material [7, 12, 13]. Hydrolysates

Table 1 Enzyme preparations based on standard substrates

Enzyme preparation	Producer	Standard activity	Optimal pH	Optimal temperature, °C
Cellulolytic enzyme preparations				
Agroksil Premium	Agroferment, Russia	3000 units of carboxymethyl cellulase activity 4000 units of xylase activity 2500 units of *β*-xylase activity	5.0	50
Phytase				
Agrofit	Agroferment, Russia	5000 units of phytase activity	5.0	40
Proteolytic enzyme preparations				
Penicillopepsin + Orizin (1:1)	Agroferment, Russia	serine proteinase, 6500 units of proteolytic activity	10.5	40
		acid proteinase, 300 units of proteolytic activity	4.7	30
Neutrase 0.8 L	Novozymes, Denmark	800 units of proteolytic activity	5.5–7.5	45–55

Table 2 Grain bran samples

Type of bran	Content, %			
	Moisture	Total protein (N×6.25)	Ash	Phosphorus
Wheat	13.78	13.38	4.86	0.48
Rye	10.44	13.82	5.17	0.55

obtained through the enzymatic modification of grain bran are used to produce distillates for fruit vodkas.

Fermentation is the key stage in the preparation of raw materials for distillation. Ethanol and secondary fermentation products result from complex biochemical processes under the action of the enzymatic system of *Saccharomyces* yeast.

Fruit materials used in the production of fruit vodkas have a reduced content of nitrogenous components and organic phosphorus [14–18]. Moreover, when processing highly acidic fruit materials, pulp or juice is traditionally diluted with softened water, which further reduces the concentration of components necessary for the normal functioning of the yeast population.

The lack of nitrogen and phosphorus in the fermented medium slows down the reproduction of yeast, increasing the process time and the risk of foreign microflora contamination [19]. Finally, the unbalanced biochemical composition changes yeast metabolism, leading to the accumulation of undesirable volatile substances such as acetaldehyde, acetic acid, propionic acid, and others [20–22].

The fermentation of fruit materials can be controlled by using foreign-produced fermentation activators based on sedimentary yeast autolysates [23–25]. Russia does not produce such preparations on a commercial scale.

Alternatively, grain bran could be used as a low-cost material with a high content of nitrogen and phosphorus compounds. Currently, grain bran is widely used as a source of dietary fiber in the production of bread and functional products. Previous studies have shown that grain bran can be an additional source of nitrogen nutrition for yeast in distillate production [27]. In our former study [12], we found that bran, primarily rye bran, has a high content of insoluble phosphorus compounds (phytin) which can be converted into a soluble form through enzymatic hydrolysis. The enzymatic hydrolysis of phytin during the fermentation of rye must is described by Polyakov *et al.* [28].

We aimed to determine process conditions for obtaining hydrolysates for fruit distillates from wheat and rye bran through the directed enzymatic destruction of proteins and phytin.

STUDY OBJECTS AND METHODS

Wheat and rye bran samples were obtained from two grain-processing enterprises in the Moscow region, Zernoprodukt (Noginsk) and Istra-khleboprodukt (Istra).

We used enzyme preparations of domestic and foreign production (Table 1).

The effectiveness of cellulolytic, proteolytic, and phytase enzyme preparations was determined by how well they accumulated reducing substances, soluble protein, and PO_4^{3-} ions, respectively. PO_4^{3-} ions were quantified by the colorimetric method using a calibration curve [29].

We created two multi-enzyme compositions (MEC) with cellulolytic, proteolytic, and phytase action, namely MEC 1 (Agroksil Premium + Agrofit + Penicillopepsin + Orizin) and MEC 2 (Agroksil Premium + Agrofit + Neutrase 0.8 L).

Wheat and rye bran samples are characterized in Table 2.

The enzyme preparations were introduced with an activity of 0.5 to 1.5 units/g of bran. The substrate's concentration varied from 20 to 120 mg/mL. Optimal temperature and pH were determined by studying enzyme activity at 30–70°C and 3.0–8.0, respectively.

The incubation mixture was composed of ground bran and water (1:10), citrate or phosphate-citrate buffer 0.1 M (20% of volume) with an appropriate pH value, and an enzyme preparation with an activity of

0.5 to 1.5 units/g of bran. Hydrolysis was carried out for 30 min, which corresponded to the zero order of the enzymatic reaction.

We used four control samples hydrolyzed under the action of bran's own enzymes, namely:

– wheat bran:water (1:10) hydrolyzed at pH 4.5, for 4 h at 40°C (Control 1);
– rye bran:water (1:10) hydrolyzed at pH 4.5, for 4 h at 40°C (Control 2);
– wheat bran:water (1:10) hydrolyzed at pH 5.5, for 4 h at 50°C (Control 3); and
– rye bran:water (1:10) hydrolyzed at pH 5.5, for 4 h at 50°C (Control 4).

Our experimental samples were prepared as follows:

– wheat bran + MEC 1 hydrolyzed at 40°C, pH 4.5 (Experiment 1);
– rye bran + MEC 1 hydrolyzed at 40°C, pH 4.5 (Experiment 2);
– wheat bran + MEC 2 hydrolyzed at 50°C, pH 5.5 (Experiment 3); and
– rye bran + MEC 2 hydrolyzed at 50°C, pH 5.5 (Experiment 4).

The wheat and rye brans were modified with the multi-enzyme compositions. The incubation mixture consisted of 10 g of ground bran, 100 mL of distilled water (20% of volume), and a buffer. The enzyme and substrate mixtures were preincubated at 40 or 50°C for 10 min. Enzymatic modification was carried out in two stages, 2 h each. First, we introduced the Agroksil Premium + Agrofit enzyme preparation and then, the Penicillopepsin + Orizin or Neutrase 0.8 L enzyme preparation in optimal amounts. The enzymes were inactivated by rapidly heating the incubation mixture to 85°C for 5 min. The supernatant was separated by centrifugation at 6000 rpm to use the resulting hydrolysate for further studies.

The contents of total protein, soluble protein, and reducing sugars were determined by the Kjeldahl method (N×6.25), the Lowry method, and the Bertrand method, respectively. Ash was measured by burning flour to determine the mass of the residue (State Standard 27494-2016). Moisture was determined according to State Standard 9404-88.

The content of phosphorus in the bran samples was determined in accordance with State Standard 30615-99. For this, we dry-mineralized the sample, dissolved ash, carried out a color reaction with a molybdenum-vanadium reagent, and measured the intensity of the yellow color at 440 nm in 10-mm cuvettes on an SF-2000 spectrophotometer (LOMO, Russia). The concentration of phosphorus in the liquid phase (water extracts from bran and experimental hydrolysates) was determined in accordance with State Standard R 51430-99. For this, we carried out a reaction of phosphate with molybdate in an acidic medium, resulting in a molybdate-phosphorus complex. Then, the complex was selectively reduced to molybdenum blue in the presence of ascorbic acid. Finally, we measured the optical density of the colored solution, which was directly proportional to the phosphorus content in the sample, at 720 nm in 10-mm cuvettes on the SF-2000 spectrophotometer.

Amino acids were separated by high-performance liquid chromatography on an Agilent Technologies 1200 Series instrument (Agilent, USA) with a Luna 5u C18(2) 150×4.6 mm 5 µ chromatographic column (Phenomenex, USA) with a pre-column in accordance with State Standard 34230-2017. The eluent (acetonitrile/acetate buffer solution) flow rate was 1.0 cm^3/min. The gradients for 0–28, 29–40, 41–50, 51–55, 56–60, and 61–63 min were 10/90, 28/72, 25/75, 50/50, 90/10, and 10/90%, respectively.

The results were processed by the methods of mathematical statistics in Microsoft Excel (Excel 19.0, 2018, Microsoft, USA) [29]. In particular, mean values, standard deviations, and confidence intervals were determined from three to five measurements for each sample.

RESULTS AND DISCUSSION

Firstly, we studied the main kinetic characteristics of the enzyme preparations used to modify wheat and rye bran. Figures 1 and 2 show the effect of temperature and pH on the activity of various enzyme preparations used with wheat bran.

As we can see, in the hydrolysis of non-starch polysaccharides of wheat and rye brans with Agroxil Premium (Figs. 1 and 2), the optimal pH was 4.5–5.0 (with a slight shift to the acidic region) and the optimal temperature was 40°C (with 80% of maximum activity retained at 50°C). In the hydrolysis of proteins with Penicillopepsin + Orizin, the optimal pH was 5.0 (with no activity from the alkaline serine proteinase included in the enzyme preparation), while the optimal temperature was 40°C. In the hydrolysis of proteins with Neutrase 0.8 L, the optimal pH ranges were 5.0–5.5 and 5.5–6.0, whereas the optimal temperatures were 45–50 and 50–55°C for wheat and rye brans, respectively. In the hydrolysis of phytin (inositol hexa phosphoric acid) with the Agrofit enzyme preparation, the optimal pH was 5.0 and the optimal temperature was 40°C for both brans.

The optimal amounts of the enzyme preparations at saturating concentrations of the substrate were 1.2, 1.0, 0.7, and 0.5 units of activity/g of bran for Agroksil Premium, Penicillopepsin + Orizin, Neutrase, and Agrofit, respectively.

Secondly, we studied the effectiveness of the enzyme preparations in their action on wheat and rye brans. It was determined by the accumulation of reducing substances, soluble protein, and PO_4^{3-} ions (Fig. 3). As we can see, all the enzyme preparations hydrolyzed the substrates quite actively. However, the Neutrase 0.8 L preparation was significantly more effective than the Penicillopepsin + Orizin complex under the given conditions of the enzymatic reaction. In addition, the activity of phytase was 30% higher when used on rye bran compared to wheat bran, which is

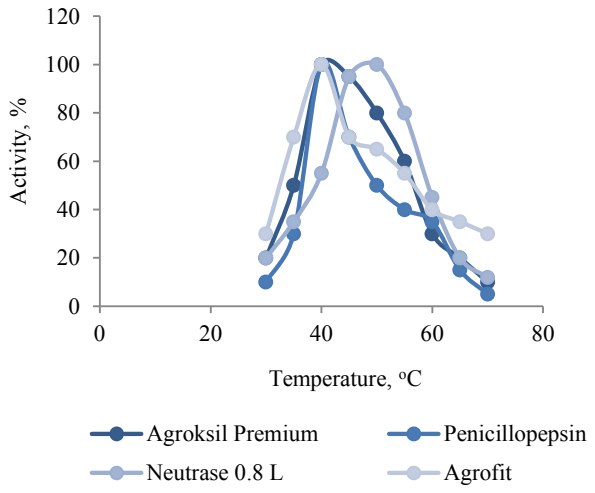

Figure 1 Effect of temperature on the activity of enzyme preparations modifying wheat bran

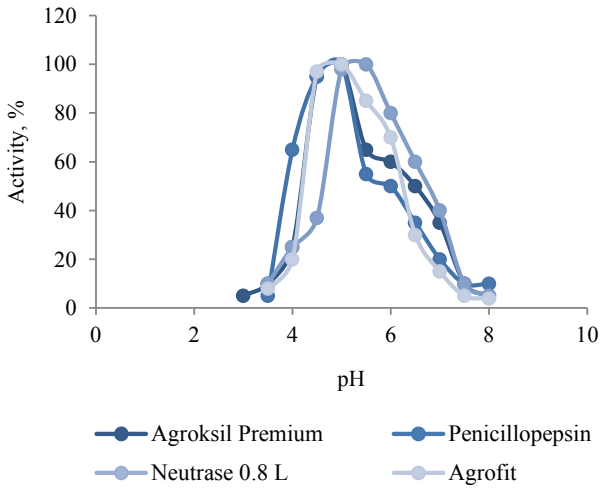

Figure 2 Effect of pH on the activity of enzyme preparations modifying wheat bran

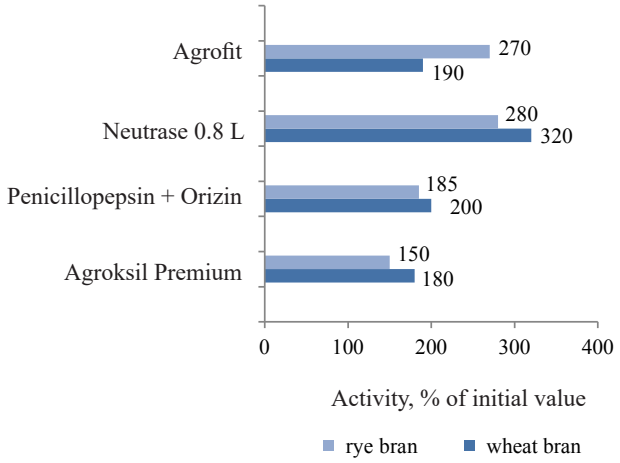

Figure 3 Effectiveness of enzyme preparations in their action on wheat and rye bran

obviously associated with the high activity of rye's own phytase [30, 31].

Thirdly, based on the kinetics of enzymatic reactions and the effectiveness of the enzyme preparations in their action on wheat and rye brans, we designed two multi-enzyme compositions (MEC) with cellulolytic and proteolytic action, as well as phytase, namely MEC 1 (Agroksil Premium + Agrofit + Penicillopepsin + Orizin) and MEC 2 (Agroksil Premium + Agrofit + Neutrase 0.8 L).

When using microbial enzyme preparations with different specificity of action, we should take into account endogenous enzyme systems, mainly acidic proteinase and grain phytase concentrated in the peripheral parts of the grain [5, 7, 30]. We used four control samples with wheat and rye brans differing in hydrolysis pH and temperature. The choice of pH was determined by the optimal values for the substrate's

Table 3 Effectiveness of the multi-enzyme compositions in their action on wheat and rye brans

Sample	Reducing substances, %	Protein (Lowry method), mg/mL	PO_4^{3-}, g/L or mg/mL
Wheat bran			
Control 1 (pH 4.5, 40°C)	0.18	0.070	0.017
Experiment 1 (with MEC 1)	0.34	0.146	0.035
Control 3 (pH 5.5, 50°C)	0.20	0.078	0.017
Experiment 3 (with MEC 2)	0.32	0.158	0.039
Rye bran			
Control 2 (pH 4.5, 40°C)	0.22	0.094	0.020
Experiment 2 (with MEC 1)	0.33	0.180	0.055
Control 4 (pH 5.5, 50°C)	0.22	0.110	0.020
Experiment 4 (with MEC 2)	0.30	0.218	0.060

MEC – multi-enzyme composition

own acidic proteinases and phytases, as well as those of the studied enzyme preparations. The results of the experiments are presented in Table 3.

The results (Table 3) indicate that MEC 1 and 2 contributed to an active accumulation of reducing substances, water-soluble protein, and PO_4^{3-} ions when used with both wheat and rye brans. Their content increased by an average of 1.3–2.3 times, with higher values for wheat bran compared to rye bran. In particular, wheat bran exposed to MEC 1 and MEC 2 had increases (compared to the controls) in reducing substances, water-soluble protein, and PO_4^{3-}

Table 4 Phosphorus concentrations in experimental and control samples of wheat and rye bran hydrolysates

Sample	Dissolved phosphorus concentration, mg%	Dissolved phosphorus, % of initial phosphorus in bran
Wheat bran		
Initial sample	26.9	5.6
Control 1 (pH 4.5, 40°C)	79.1	16.6
Experiment 1 (with MEC 1)	151.2	31.5
Control 3 (pH 5.5, 50°C)	88.5	18.4
Experiment 3 (with MEC 2)	177.6	34.7
Rye bran		
Initial sample	33.4	6.1
Control 2 (pH 4.5, 40°C)	94.9	17.4
Experiment 2 (with MEC 1)	165.4	30.3
Control 4 (pH 5.5, 50°C)	110.3	20.2
Experiment 4 (with MEC 2)	199.7	36.6

MEC – multi-enzyme composition

ions of 88 and 60%, 87 and 102%, as well as 105 and 129%, respectively. For rye bran, these indicators were somewhat lower: the increases in reducing substances, water-soluble protein, and PO_4^{3-} ions amounted to 50 and 36%, 91 and 98%, as well as 175 and 200%, when used with MEC 1 and MEC 2, respectively.

However, the absolute concentration of phosphorus in all the rye bran hydrolysates was higher than in the wheat bran hydrolysates, and the efficiency of phytase in the multi-enzyme compositions was higher at pH 5.5 (Table 4). Thus, we found that using Agrofit within a multi-enzyme composition more than doubled the amount of soluble phosphorus available for yeast, compared to the control.

Finally, we determined the composition and concentration of free amino acids in the wheat and rye bran hydrolysates obtained using two multi-enzyme compositions.

Based on their effect on yeast growth and reproduction, amino acids can be divided into easily-assimilated (aspartic acid, arginine, valine, histidine, isoleucine, and tryptophan) and poorly-assimilated (leucine, methionine, tyrosine, threonine, serine, and lysine). It has been proven that the yeast cell can assimilate most amino acids (except for proline), di- and tripeptides, as well as ammonia nitrogen. Noteworthily, the enzymatic activity of yeast cells can significantly increase in natural nutrient media with a cultivar of amino acids, such as aqueous bran extracts. In this case, yeast can directly assimilate amino acids during reproduction [32, 33]. The content of assimilable nitrogen in the medium determines the rates of yeast growth, sugar utilization, and fermentation, as well as biomass yield. The assimilation of amino acids as a result of enzymatic deamination produces various volatile components which contribute to the aroma and taste of alcoholic beverages based on distillates.

Regardless of raw materials (starch-, inulin-, sugar-containing or fruit), distillates contain predominantly higher alcohols, especially propyl, isobutyl, and isoamyl. It is known that these volatile compounds can be synthesized from the corresponding amino acids, namely threonine, valine, and leucine, respectively. Their content in bran is 8–10% of total free amino acids [27]. We found aspartic and glutamic acids, as well as asparagine to dominate in the control and experimental samples of wheat and rye bran hydrolysates (Tables 5 and 6).

We found that the concentration of free amino acids in the control wheat bran hydrolysates (obtained under the action of bran's own enzymes) was on average 20% higher than in the rye hydrolysates. However, the total concentration of amino acids in the rye hydrolysates was more than 1.3 times higher than in the wheat hydrolysates.

As can be seen in Tables 5 and 6, the multi-enzyme compositions (MEC 1 and MEC 2) increased the total concentration of free amino acids in the experimental hydrolysates by an average of 1.5–2.0 times compared to the controls. Noteworthily, the type of bran or multi-enzyme composition had almost no effect on the increase in free amino acids in the hydrolysates compared to their initial content in the raw material. This increase ranged from 192 to 205%.

We also found that the multi-enzyme compositions increased the content of the most valuable amino acids for nitrogen nutrition of yeast in the experimental bran hydrolysates [34]. Particularly, the concentrations of aspartic acid in the wheat and rye bran hydrolysates increased 1.8 and 2.5 times, respectively. The content of valine in the experimental hydrolysates increased 4–5 times compared to the initial sample.

Our analysis of the experimental samples versus the controls showed changes not only in the total concentration of free amino acids, but also in their ratios. Importantly, the total content of threonine, valine, and leucine – essential amino acids for the production of distillates – almost doubled (15–20%) compared to the initial bran samples (8–10%). Such findings have never been reported before.

CONCLUSION

We managed to scientifically substantiate the use of enzyme preparations with cellulolytic, proteolytic, and phytase action to produce wheat and rye bran hydrolysates with a high content of free amino acids and soluble phosphorus compounds as an alternative to fermentation activators based on sedimentary yeast autolysates.

We studied the kinetic characteristics of the enzyme preparations included in the multi-enzyme compositions. Also, we determined the optimal conditions for enzymatic reactions with wheat and rye brans used as a substrate, namely the initial rate of enzymatic reaction,

Table 5 Free amino acids in wheat bran hydrolysates

Amino acid	Concentration, mg/L				
	Initial sample	Control 1 (pH 4.5, 40°C)	Experiment 1 (with MEC 1)	Control 3 (pH 5.5, 50°C)	Experiment 3 (with MEC 3)
Aspartic acid	63.0	64.5	115.5	69.5	115.5
Glutamic acid	46.0	49.5	90.0	76.5	97.5
Asparagine	209.0	219.0	322.5	217.5	321.0
Histidine	21.0	34.5	49.5	36.0	48.0
Serene	12.0	18.0	28.5	21.0	24.0
Glutamine	9.0	25.5	37.5	24.0	39.0
Arginine	25.5	31.5	36.0	36.0	34.5
Glycine	66.0	105.5	162.0	97.5	157.5
Threonine	61.5	88.5	145.5	87.0	156.0
Alanine	4.5	24.0	39.0	15.0	39.0
Tyrosine	6.0	16.5	54.0	12.0	36.0
Valine	22.5	55.5	94.5	52.5	91.5
Methionine	4.5	30.0	42.0	21.0	40.5
Tryptophan	182.5	192.0	282.0	204.0	280.5
Isoleucine	15.0	37.5	60.0	34.5	58.5
Phenylalanine	13.5	63.0	100.5	43.5	97.5
Leucine	15.0	90.0	141.0	64.5	136.5
Lysine	24.0	81.0	126.0	70.5	121.5
Total	800.5	1226.0	1926.0	1182.5	1894.5
% vs. initial sample	–	130.0	205.0	125.0	202.0
% of protein nitrogen in material	5.9	8.8	13.7	8.4	13.5

Table 6 Free amino acids in rye bran hydrolysates

Amino acid	Concentration, mg/L				
	Initial sample	Control 2 (pH 4.5, 40°C)	Experiment 2 (with MEC 2)	Control 4 (pH 5.5, 50°C)	Experiment 4 (with MEC 4)
Aspartic acid	106.5	127.5	261.0	117.0	268.5
Glutamic acid	70.5	76.5	141.0	169.5	183.5
Asparagine	467.5	474.0	825.0	544.5	852.0
Histidine	18.0	42.0	72.0	40.5	73.5
Serene	13.5	22.5	34.5	24.0	33.0
Glutamine	37.5	57.0	93.0	48.0	96.0
Arginine	19.5	36.0	45.0	43.5	45.0
Glycine	58.5	114.0	175.5	88.5	174.0
Threonine	52.5	93.0	177.0	102.0	189.0
Alanine	22.5	40.5	78.0	33.0	75.0
Tyrosine	4.5	73.5	18.0	37.5	13.5
Valine	30.0	76.5	157.5	61.5	157.5
Methionine	4.5	36.0	60.0	22.5	60.0
Tryptophan	52.5	58.5	85.5	57.0	97.5
Isoleucine	13.5	48.0	91.5	37.5	99.0
Phenylalanine	15.0	75.0	138.0	46.5	148.5
Leucine	12.0	112.5	198.0	66.0	207.0
Lysine	21.0	85.5	141.0	64.5	139.5
Total	1019.5	1648.5	2791.5	1603.5	2912.0
% vs. initial sample	–	114.0	192.0	111.0	201.0
% of protein nitrogen in material	7.7	11.9	19.4	13.3	20.9

temperature, pH, enzyme concentration, and saturating substrate concentration.

We developed two multi-enzyme compositions that contained enzyme preparations with cytolytic, proteolytic, and phytase action and studied their effectiveness. According to our results, the multienzyme compositions contributed to an active accumulation of reducing substances, water-soluble protein, and soluble

phosphorus available for yeast. Their action on wheat and rye brans more than doubled the concentration of water-soluble phosphorus.

Our study showed that the concentration of free amino acids in the hydrolysates obtained under the action of bran's own enzymes was 20% higher in the wheat samples, although the absolute value of this indicator was higher in the rye samples.

The multi-enzyme compositions increased the total concentration of free amino acids in the experimental hydrolysates by an average of 1.5–2.0 times, including the most valuable amino acids for nitrogen nutrition of yeast – aspartic acid (2.5 times) and valine (4–5 times), compared to the hydrolysates obtained under the action of bran's own enzymes.

Our results can be used for further research into grain bran hydrolysates as an alternative source of nitrogen and phosphorus nutrition for yeast at the fermentation stage of fruit distillate production.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Assessment of trace elements in canned fish and health risk appraisal

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Abstract:
Trace elements are dangerous to human health and there is a rising concern about the quality of processed foods in some parts of the world, especially in Iraq.
The chemical composition (total sold, moisture, and ash) and concentrations of trace elements in canned fish (Skipjack tuna, Sardines, Tuna fish, Sardines, and Mackerel) from the Kalar market, Iraq were determined by using an inductively coupled plasma-optical emission spectrometer.
The ranges obtained for the elements in mg/kg were as follows: Se (0.025–0.77), As (0.02–1.07), B (0.05–0.7), Ag (0.04–0.83), Ba (0.05–0.975), Mg (29.8–37.5), Mn (0.97–2.09), Cu (0.91–3.09), and Zn (5.12–11.7). The studied canned fishes pose no risk with respect to the estimated daily intake of Se, As, B, Ag, Ba, Mg, Mn, Cu, and Zn. The total target hazard quotients for the studied metals from individual fish species (except Fme, Fma, and Fsh) were more than one, which was responsible for non-carcinogenic risks. The target carcinogenic risk value for arsenic was also higher than the standard (10^{-4}) set by the United States Environmental Protection Agency.
It revealed that the consumption of canned fish causes a chronic cancer risk to humans.

Keywords: Proximate chemical composition, trace elements, toxic elements, health risk, canned fish, Iraq

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INTRODUCTION

Popular demand for healthy food supply has expanded globally in recent decades [1]. With a rise in environmental pollution, food contamination by various pollutants is now considered a major issue in industrialized, emerging, and developing countries [2–4]. In recent years, the problems of environmental contamination, particularly food contamination by a cultivar of chemical pollutants including potentially toxic elements, are receiving a lot of attention from scientists around the world [5, 6]. The environment may be exposed to trace elements from various sources, particularly processed canned food, where they pose a serious threat due to their toxicity and bioaccumulation in the food chain.

Potentially toxic elements are important due to their toxicity as well as essentiality. Potentially toxic elements are classed as potentially unhealthy (arsenic, cadmium, lead, mercury, and nickel, among others), probably toxic (vanadium and cobalt), and necessary (copper, zinc, iron, manganese, and selenium) [7, 8]. When consumed in large quantities, potentially toxic elements can be extremely hazardous even at low concentrations. When metal consumption is overly high, essential and possibly essential metals might generate hazardous effects [9]. As a result of increased concern about the health benefits and hazards of food intake, substantial attention has been dedicated in recent decades to the research of essential and harmful element content in foodstuffs, particularly canned foods. However, contamination of the food chain is a key mechanism for these potentially toxic elements to enter the human body [6, 10]. In Iraq, assessing the risks and benefits of canned fish consumption is critical because canned fish meets 60–80% of the country's animal protein needs. It is also a key source of essential minerals, vitamins, and fatty acids, all of which are important in child development and adult health [9]. Furthermore, it is critical to monitor

the level of potentially toxic elements in canned fish to ensure the safety of fish protein supplied to consumers and to comprehend the adverse impacts of canned fish consumption among individuals and populations.

Canned fish is widely consumed because it contains protein, omega-3 fat acids, liposoluble vitamins, as well we micro- and macroelements [11]. Fish is a significant source of nutrition for many people since it supplies animal protein that is not available in cereal-based diets [12, 13]. Recently, the global consumption of fish has expanded in tandem with growing awareness of its nutritional and therapeutic benefits. In addition to being a good source of protein, fish is also rich in critical minerals, vitamins, and unsaturated fatty acids [14]. Although seafood is considered to be the primary source of high biological protein, polyunsaturated oil, and minerals such as calcium, potassium, and zinc, since they are at the top of the food pyramid, fish can potentially make for trace metals bio-magnification and act as a potential means of transmission to humans [15]. Fish has a high content of amino acids, which is very well suited for human dietary needs. The nutritional value of its protein compares favorably to that of egg, milk, and meat [16].

Minerals are important for certain functions of the body. Some metals, such as zinc, copper, and iron, are basic to life and play a significant role in the functioning of essential enzyme systems [17]. Fish is a healthy source of calcium, potassium, phosphorus, copper, iodine, cobalt, manganese, and other trace minerals that are vital for preserving healthy teeth and bones [18].

Canning increases the shelf life of a canned product for many years. Yet, producers, nutritionists, cooks, and customers are specifically interested in the composition of fish as they want to know its nutritional contribution to a healthy diet [20]. Canned fish is very popular in Iraq since it is convenient and inexpensive for most working families. Some studies have determined potentially toxic elements in canned fish, including the ones in Turkey, Iran, Egypt, USA, Italy, Spain, Lebanon, Austria, Czech Republic, and Poland [11, 17, 21–35]. However, information regarding potentially toxic elements concentrations in canned fish marketed in Iraq is scarce, although canned skipjack tuna, sardines, tuna fish, sardines, and mackerel are extensively consumed.

Until now, there has been no detailed scientific research in Iraq concerning potentially toxic elements contamination in canned or tinned fish and their probable risk to human health. Therefore, we aimed to determine the proximate chemical composition of canned fish (total solids, moisture, and ash content), to evaluate the concentration of potentially toxic elements (Se, As, B, Ag, Ba, Mg, Mn, Cu, and Zn) in an edible portion of some of the commercially imported canned fish species on the market in Kalar City, northern Iraq, and to estimate the risks of these trace elements in everyday intake.

STUDY OBJECTS AND METHODS

Samples collection and preparation. Canned fish samples (one brand of skipjack tuna, seven brands of sardines, one brand of tuna, and one brand of mackerel) were purchased from various supermarkets in Kalar City, northern Iraq (Fig. 1). A minimum of five canned fish samples per brand were obtained at random from various retail stores. After collection, combinations of at least five samples of each fish species were prepared and homogenized in stainless-steel blender cups. Their 100 g test portions were stored at −20°C in the central laboratory of College of Education, University of Garmian (Kalar City, Iraq). Then, all the samples were freeze-dried for 48 h until constant weight was attained and sealed in airtight plastic bags. For chemical analysis, each can's material was thoroughly homogenized in a food blender using stainless steel cutters [19].

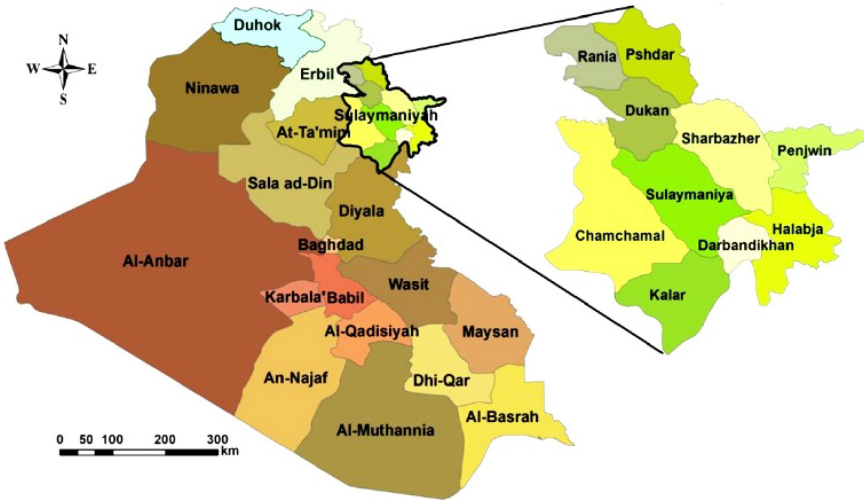

Figure 1 Map of the studied area of Kalar City, Iraq for canned fish sampling

Table 1 Fish samples collected from various supermarkets in Kalar City, Iraq

No.	Samples	Fish weight, g	Country of origin	Product local name	Scientific name	Storage media
1	Fru	130	Indonesia	Rubal	Skipjack tuna	Soya oil
2	Fsh	125	Iraq	Shabab	Sardines	Vegetable oil
3	Fsa	90	AL Maghreb	Sarden	Tuna fish	Vegetable oil
4	Fkl	120	Thailand	Klode	Sardines	Vegetable oil
5	Fal	52	Turkey	Altunsa	Sardines	Vegetable oil
6	Fze	104	Turkey	ZER	Sardines	Vegetable oil
7	Fsi	130	Vietnam	Siblue	Mackerel	Soya oil
8	Fme	112	Indonesia	Melo	Sardines	Sunflower oil
9	Fma	90	AL Maghreb	maria	Sardines	Vegetable oil
10	Fbe	120	Iran	berkeh	Sardines	Vegetable oil

Table 2 Levels of metals in DORM-4 Fish protein certified reference material (mg/kg) for the validation of analytical method

Elements	Certified value	Measured value (n = 3)	Recovery, %
Se	3.45 ± 0.40	3.37 ± 0.21	98
As	6.87 ± 0.44	6.85 ± 0.78	100
B	n.a.	n.a.	n.a.
Ag	0.0252 ± 0.0050	0.0248 ± 0.0020	98
Ba	n.a.	n.a.	n.a.
Mg	910 ± 80	889 ± 39	98
Mn	3.17 ± 0.26	3.15 ± 0.21	99
Cu	15.70 ± 0.46	15.30 ± 0.22	97
Zn	51.6 ± 2.8	51.1 ± 1.3	99

n.a. – not available

Table 1 shows the fish types, brands, volumes, and other information used by canned fish makers, such as scientific names and storage media.

Digestion and elemental analyses. For each canned fish sample, 4 g of fish muscle (wet weight) was weighed and placed in a Teflon digestion vessel with 15 mL of pure nitric acid. The samples were then microwaved as follows: Step 1: 25°C for 10 min at 1000 W; Step 2: 96°C for 30 min; Step 3: 180°C for 10 min at 1000 W; 180°C for 10 min before cooling to room temperature; Step 4: 2 mL of 30% hydrogen peroxide was added, and the mixture was exposed to Step 3 again.

In the final step, hydrogen peroxide was used to break down organic materials that may have remained undissolved throughout the concentrated nitric acid digestion (Steps 1–3). Finally, the digests were prepared in acid-washed standard flasks to 25 mL with deionized water and placed in acid-washed 50-mL polyethylene bottles. The chemicals used to dissolve the samples were of analytical quality. Ultra-pure water was used in the study [36]. The moisture and ash contents were calculated using the AOAC method [37]. All metal concentrations were determined using an inductively coupled plasma–optical emission spectrometer in three replications [38].

Instrument analysis and quality assurance. The instrument conditions were as follows: a Scott spray chamber; nebulizer: cross flow; RF power: 1400 W; pump speed: 30 RPM; coolant flow: 14 L/min; auxiliary flow: 0.9 L/min; nebulizer gas flow: 0.8 L/min; preflush: 40 s; measure time: 28 s; replicate measurement: 3; multielement stock solutions containing 1000 mg/L obtained from Bernd Kraft (Duisburg, Germany).

Standard solutions were generated from stock solutions (Merck, multi-element standard). All of the instrumental conditions used for concentration determinations were defined [39]. The analytical procedure was confirmed using DORM-4 Fish protein-certified reference material for trace metals. The National Research Council of Canada prepared and supplied these fish samples. The results showed that the certified and observed values were in good agreement. Table 2 shows that the percentage recoveries of the analyzed potentially toxic elements ranged from 97 to 100 percent.

Calculation for health risk due to metal contamination. ***Estimated daily intakes.*** Estimated daily intakes (EDI) for potentially toxic elements were calculated by multiplying the respective average concentration in fish samples by the weight of food item consumed by a person (body weight of 60 kg for an adult in Iraq), as obtained from the household income and expenditure survey, and then using the following formula [40]:

$$EDI = (FIR \times C)/BWT \quad (1)$$

where EDI is the estimated daily intakes; FIR is the food ingestion rate, g/person/day; C is the metal content in fish samples, mg/kg; BWT is the body weight for adult residents of 60 kg [41]. On a wet weight basis, the daily consumption of fish is 10.96 g [9].

Non-carcinogenic risk. The procedure for assessing non-carcinogenic risks was based on the risk-based concentration table published by the United States Environmental Protection Agency (USEPA) Region III [42]. The target hazard quotient was used to quantify the non-carcinogenic danger of each specific metal from fish consumption (USEPA, Risk-Based Concentration Table). It is "the ratio of a single substance exposure level over a specified time period

Table 3 Approximate chemical configuration of the fish flesh samples collected from various supermarkets in Kalar City, Iraq (n = 3)

Fish samples	Total solid, %	Moisture, %	Ash, %
Fme	36.30 ± 1.80	64.00 ± 0.98	4.40 ± 1.10
Fsi	37.90 ± 2.01	62.10 ± 1.10	4.20 ± 0.03
Fma	32.80 ± 3.00	67.20 ± 2.10	4.70 ± 0.70
Fbe	39.70 ± 2.23	60.30 ± 1.20	4.30 ± 0.80
Fal	34.60 ± 1.87	65.40 ± 2.00	2.19 ± 0.39
Fru	31.30 ± 0.99	68.70 ± 0.90	1.22 ± 0.50
Fsh	32.40 ± 1.20	67.60 ± 1.07	3.20 ± 0.50
Fkl	29.80 ± 2.10	70.20 ± 1.01	1.50 ± 0.35
Fze	37.50 ± 1.40	62.50 ± 2.01	3.10 ± 0.70
Fsa	30.21 ± 2.50	69.79 ± 0.80	1.90 ± 0.80

(e.g., sub-chronic) to a reference dose (Rf.D.) for that substance derived from a similar exposure period". The following equation was used to calculate the target hazard quotient:

$$THQ = [(EFr \times ED \times FIR \times C)/(Rf.D. \times \times BWT \times AT)] \times 10^{-3} \quad (2)$$

$$\text{Total THQ (THQ)} = THQ_{\text{toxicant 1}} + + THQ_{\text{toxicant 2}} + \ldots\ldots\ldots + THQ_{\text{toxicant n}} \quad (3)$$

where THQ is the target hazard quotient; EFr is the exposure frequency (365 days/year); ED is the exposure duration (30 years); FIR is the food ingestion rate (59.91 g/person/day); C is the metal concentration in foods, mg/kg; Rf.D. is the oral reference dose, mg/kg/day; AT is the averaging time for non-carcinogens (365 days/year×number of exposure years, assuming 30 years). The oral reference doses were based on 0.005, 0.0003, 0.2, 0.005, 0.07, 0.14, 0.04, and 0.3 mg/kg/day for Se, As, B, Ag, Ba, Mn, Cu, and Zn, respectively [42]. If the target hazard quotient is equal to or higher than 1, there is a potential health risk and related interventions and protective measures should be taken [43, 44].

Carcinogenic risks. The risk was calculated for carcinogens as an increasing probability of a person developing cancer over a lifetime of exposure to that probable carcinogen [42]. The target carcinogenic hazards associated with As consumption was computed using the calculation provided in the USEPA Region III Risk-Based Concentration Table:

$$TR = [(EFr \times ED \times FIR \times C \times \times CSFo)/(BWT \times AT)] \times 10^{-3} \quad (4)$$

where EFr is the exposure frequency (365 days/year); ED is the exposure duration (70 years) (USEPA, Regional Screening Level Summary Table: November 2011); AT is the averaging time for carcinogens (365 days/year×70 years); CSFo is the oral carcinogenic slope factor. For As, CSFo was 1.5 mg/kg/day from the Integrated Risk Information System (Risk-Based Concentration Table). Total As in marine food (fish) is mostly organic and a little amount may remain as inorganic. In this study, we assumed the conversion coefficient of total As to inorganic As by 0.05 in fish to produce a carcinogenic risk.

Statistical analysis. Each experimental analysis was done in triplicate. All statistical analysis was performed using statistical package SPSS version 20.0 for Windows (IBM Corp., Armonk, NY, USA). Significance was accepted at 0.05 level of probability ($p < 0.05$). Difference in metal concentration in fish was detected using One Way Analysis of Variance (ANOVA). The tables were plotted with OriginPro 6.1 (Origin Lab Corporation, Northampton, Massachusetts, USA). The results were described as mean ± SD. The statistical differences between fishes were analyzed using mean concentrations of metals [45].

Ethical approval: The research received ethical approval because it did not involve human or animal use.

RESULTS AND DISCUSSION

Concentration of basic components in canned fish. Proteins, lipids (fat or oil), and ash (minerals) are the main constituents in the edible portion of fish. The analysis of these basic components is called proximate analysis. The estimated chemical composition of the fish examined is shown in Table 4. In the fish samples, the moisture content ranged from 70.2% (Fkl) to 60.3% (Fbe). The ash content in the fish flesh ranged between 1.22% in Fru and 4.7% in Fma fish samples. The opposite relationship between moisture and overall solid content could be seen from the results, as all fish processing technologists were well aware of it. We also found that only three of the fishes had an ash content below 2% – Fsa, Fkl, and Fru, so they were weak in minerals. The overall solid content was generally high in the same way, reaching from 37.9 to 39.7%, and crude ash ranged from 4.4 to 1.22%, as seen in Table 4. All the fish species studied were high in moisture and ash and they complied with the human dietary requirements [46]. Our data were in line with the study conducted by Tawfik [47]. The total solid concentrations of the necessary substrates provide valuable information on the expected biogas yield and efficiency of the process [48]. The highest moisture content was observed in sample Fkl (70.2%) and the lowest moisture was in Fsi Mackerel fish type (62.1%), but not significant with Fze samples (62.5%). In our study, the variations in the minerals of the studied canned fish types can be due to their water concentration, the biological state of the fish and/or their capability to consume the elements from their diet, as well their ability to absorb water [49].

Element concentration in fish species. Although researchers have presented the elemental concentrations in various tissues of fish, such as liver, kidneys, gills, gonads, and muscles, we evaluated only the

Table 4 Elements concentration (mg/kg) in studied fish flesh samples collected from various supermarkets in Kalar City, Iraq

Fish samples	Se	As	B	Ag	Ba	Mg	Mn	Cu	Zn
Fme	0.170 ± 0.002	0.02 ± 0.01	0.052 ± 0.001	0.04 ± 0.01	0.135 ± 0.010	37.50 ± 1.21	2.010 ± 0.029	1.23 ± 0.21	11.7 ± 0.5
Fsi	0.26 ± 0.10	0.300 ± 0.051	0.275 ± 0.010	0.057 ± 0.09	0.80 ± 0.02	32.30 ± 2.01	0.98 ± 0.04	2.46 ± 0.15	6.65 ± 0.71
Fma	0.170 ± 0.001	0.020 ± 0.001	0.052 ± 0.030	0.040 ± 0.005	0.690 ± 0.012	37.50 ± 2.91	1.21 ± 0.25	1.87 ± 0.31	5.55 ± 0.12
Fbe	0.4400 ± 0.0021	0.70 ± 0.06	0.425 ± 0.050	0.225 ± 0.004	0.90 ± 0.01	33.01 ± 1.01	1.12 ± 0.32	2.10 ± 0.01	7.430 ± 0.001
Fal	0.10 ± 0.01	0.250 ± 0.012	0.50 ± 0.07	0.210 ± 0.001	0.3750 ± 0.0015	29.8 ± 1.9	1.09 ± 0.32	3.11 ± 0.50	6.228 ± 0.210
Fru	0.73 ± 0.01	1.07 ± 0.08	0.53 ± 0.03	0.320 ± 0.001	0.1500 ± 0.0012	30.4 ± 2.1	0.97 ± 0.04	2.650 ± 0.116	6.625 ± 1.270
Fsh	0.175 ± 0.015	0.10 ± 0.05	0.25 ± 0.01	0.50 ± 0.00	0.85 ± 0.00	30.90 ± 0.98	1.73 ± 0.30	2.90 ± 0.51	9.05 ± 1.05
Fkl	0.275 ± 0.010	0.25 ± 0.02	0.05 ± 0.00	0.83 ± 0.05	0.70 ± 0.04	34.30 ± 1.08	0.99 ± 0.03	3.09 ± 0.32	5.12 ± 0.90
Fze	0.025 ± 0.001	0.30 ± 0.04	0.70 ± 0.03	0.320 ± 0.001	0.975 ± 0.090	35.1 ± 2.0	1.85 ± 0.06	0.910 ± 0.001	7.425 ± 0.980
Fsa	0.250 ± 0.001	0.65 ± 0.03	0.50 ± 0.07	0.220 ± 0.012	0.050 ± 0.013	36.5 ± 2.4	2.090 ± 0.053	2.10 ± 0.04	9.875 ± 1.070
Mean ± SD	0.26 ± 0.20	0.37 ± 0.34	0.33 ± 0.23	0.28 ± 0.24	0.56 ± 0.35	33.70 ± 2.89	1.40 ± 0.46	2.24 ± 0.75	7.56 ± 2.06
LOD	0.002	0.001	0.05	0.005	0.006	0.001	0.004	0.004	0.005

LOD – Limit of detection

edible portion of canned fish for elemental concentrations [50]. Table 5 shows nine potentially toxic elements (Se, As, B, Ag, Ba, Mg, Mn, Cu, and Zn) in ten imported canned fish species collected from various supermarkets in Kalar City, Iraq. The ranking of the elements' mean concentrations in the canned fish samples were Mg (33.7) > Zn (7.56) > Cu (2.24) > Mn (1.40) > Ba (0.56) > As (0.37) > B (0.33) > Ag (0.28) > Se (0.26) (mg/kg), respectively. The concentrations of different elements varied considerably among the canned fish species.

Selenium, copper, magnesium, manganese, and zinc are potentially toxic elements required for regular physiological function, but in high amounts they can be hazardous. The ranges of Se, As, B, Ag, Ba, Mg, Mn, Cu, and Zn in the canned fish items were 0.025–0.73, 0.02–1.07, 0.05–0.7, 0.04–0.83, 0.05–0.975, 29.8–37.5, 0.97–2.09, 0.91–3.11, and 5.12–11.7 mg/kg, respectively. A study conducted by Ashraf *et al.* found that mean concentrations of Cu and Zn in canned tuna were 2.94, and 10.4 mg/kg, respectively, which were identical to our study [51].

The lowest and highest selenium levels in the canned fish species were found as 0.025 mg/kg in Fze (Turkish sardines) and 0.73 mg/kg in Fru (Indonesian Skipjack tuna). Selenium contents in fish have been reported in literature to be in the range of 0.234–0.389 mg/kg from Puerto Rico, 0.041–1.13 mg/kg in fish and seafood, 1.1–3.0 mg/kg in edible fish muscle from Portugal [52–54]. For adults, the maximum daily dietary intake of selenium is 0.006 mg/kg-bwt/day [55].

Arsenic is a major heavy metal (metalloids) that is both hazardous and cancer-causing. All the analyses in this study were performed for total (including organic and inorganic) arsenic, despite the fact that the majority of arsenic detected in fish and seafood is in organic form, which is less harmful [56]. There was no standard limit for As in fresh water fish, as well as canned fish, but the Serbian Regulation [57]. Total arsenic levels have already been set at 3 mg/kg for freshwater and saltwater fish, 3 mg/kg for marine fish products, and 12 mg/kg for tuna fish products. In our study, As concentrations in all the examined samples were less than this level. Arsenic was found in all the fish samples with a mean (range) concentration of 0.37 (0.02–1.07 mg/kg) (Table 5). Andayesh *et al.* found arsenic values ranging from 0.257 to 1.452 mg/kg in canned tuna obtained from Tehran's seafood market, Iran, which was slightly higher than in our study [22]. Also, Ikem and Egiebor reported lower As levels with a maximum concentration of 1.72 mg/kg in canned tuna and 1.12 mg/kg in canned sardines collected from the market in Georgia and Alabama, USA [11]. Morgano *et al.* identified As in tuna to be in the range of 0.187–3.677 mg/kg, which is consistent with the findings of our study [58].

The adverse effects due to chronic exposure of humans to silver are a permanent bluish-gray discoloration of the skin (argyria) or eyes (argyrosis). The lowest and highest silver levels in the canned fish species were found as 0.04 mg/kg in Fme and Fma and 0.83 mg/kg in Fkl (Table 4). Silver contents in literature have been reported in the range of 0.00–0.20 mg/kg in canned fish from Georgia and Alabama, USA; 0.00–2.28 mg/kg in dietary fish in France, 0.021–0.580 mg/kg in marine fish from Southeast Asia, and 0.14–0.43 mg/kg in two marine fishes in China's Fujian province [11, 59–61]. The estimated daily intake of silver from the fish diet was reported to be 0.011 mg kg-bw/d. The USEPA has set a daily intake limit of 0.0132 mg/kg-bwt for all types of silver (Risk-Based Concentration Table, April, 2005). As a result, our daily silver consumption was substantially below the safe threshold.

Copper levels were found to be the lowest and highest in the canned fish species as 0.91 mg/kg in Fze and 3.11 mg/kg in Fal (Table 5). Copper levels have been reported in literature to be in the range of

Table 5 Comparison between the dietary intakes of trace elements from composite fish flesh samples collected from various supermarkets in Kalar City, Iraq and the corresponding maximum tolerable daily intake (MTDI)

Fish samples	Estimated daily intake of trace elements, mg								
	Se	As	B	Ag	Ba	Mg	Mn	Cu	Zn
Fme	0.010	0.001	0.003	0.002	0.008	2.247	0.120	0.074	0.701
Fsi	0.016	0.018	0.016	0.003	0.048	1.935	0.059	0.147	0.398
Fma	0.010	0.001	0.003	0.002	0.041	2.247	0.072	0.112	0.333
Fbe	0.026	0.042	0.025	0.013	0.054	1.978	0.067	0.126	0.445
Fal	0.006	0.015	0.030	0.013	0.022	1.785	0.065	0.186	0.373
Fru	0.043	0.064	0.032	0.019	0.009	1.821	0.058	0.159	0.397
Fsh	0.010	0.006	0.015	0.030	0.051	1.851	0.104	0.174	0.542
Fkl	0.016	0.015	0.003	0.050	0.042	2.055	0.059	0.185	0.307
Fze	0.001	0.018	0.042	0.019	0.058	2.103	0.111	0.055	0.445
Fsa	0.015	0.039	0.030	0.013	0.003	2.187	0.125	0.126	0.592
Total estimated daily intake	0.155	0.220	0.200	0.165	0.337	20.21	0.841	1.343	4.532
Maximum tolerable daily intake	0.3[a]	0.126[b]	0.50[a]	0.25[c]	12[d]	60[b]	2.0–5.0[e]	30[b]	60[b]

[a](EFSA, 2006), [b](FAO, 2006); [c](USEPA, 2005); [d](SCHER, 2012); [e](NRC, 1989)

Table 6 Relative contribution of estimated daily intakes of trace elements from consumption of canned fish species collected from various supermarkets in Kalar City, Iraq

Fish samples	% contribution of estimated daily intake of trace elements								
	Se	As	B	Ag	Ba	Mg	Mn	Cu	Zn
Fme	3.39	0.95	0.62	0.96	0.07	3.74	6.02	0.25	1.17
Fsi	5.19	14.26	3.30	1.37	0.40	3.23	2.94	0.49	0.66
Fma	3.39	0.95	0.62	0.96	0.34	3.74	3.62	0.37	0.55
Fbe	8.79	33.28	5.09	5.39	0.45	3.30	3.35	0.42	0.74
Fal	2.00	11.89	5.99	5.03	0.19	2.98	3.27	0.62	0.62
Fru	14.48	51.11	6.35	7.67	0.07	3.04	2.91	0.53	0.66
Fsh	3.49	4.75	3.00	11.98	0.42	3.09	5.18	0.58	0.90
Fkl	5.49	11.89	0.60	19.89	0.35	3.42	2.97	0.62	0.51
Fze	0.50	14.26	8.39	7.67	0.49	3.50	5.54	0.18	0.74
Fsa	4.99	30.91	5.99	5.27	0.02	3.64	6.26	0.42	0.99

0.30–4.87 mg/kg in canned fish from Serbia; 0.01–5.33 mg/kg in canned fish from Georgia and Alabama, USA; 5.17–9.45 mg/kg in fish species from Bangladesh, 0.06–0.35 mg/kg from the Pearl River Delta, South China; and 0.15–0.27 mg/kg in fish from Puerto Rico [11, 52, 62–64]. The maximum copper level that can be consumed through food is 0.50 mg/kg-bwt/day [65]. In our study, copper's estimated daily intake value was within acceptable limits.

Zinc deficiency has been linked to lack of appetite, growth retardation, skin changes, and immunological problems in humans [17]. The lowest and highest zinc concentrations were observed as 5.12 mg/kg in Fkl (sardines, Thailand) and 11.2 mg/kg in Fme (sardines, Indonesia). Zinc contents in literature have been reported in the range of 1.35–44.50 mg/kg in canned fish from Serbia; 42.83–418.00 mg/kg in some eatable fishes from Bangladesh; 38.8–93.4 mg/kg in commercial fish species from the Black Sea, Turkey; and 14.0–97.8 mg/kg in canned fish from Georgia and Alabama, USA [11, 17, 62, 66]. As shown in Table 8, the Zn content in our study was in a good ratio compared to other fish flesh.

The lowest and highest levels of barium were 0.05 mg/kg in tuna fish (Fsa) and 0.975 mg/kg in sardines (Fze). There is little information regarding Ba content in fish and fish products. However, literature has reported Ba contents in the range of 0.0001–0.9450 mg/kg in dietary fish of France; 3.44–6.96 mg/kg in different fish species from Turkey; and 0.003–0.208 mg/kg in edible marine fish from Rio de Janeiro, Brazil [14, 59, 67]. As presented in Table 6, Brazilian fish had a mean Ba concentration of 0.67 ± 0.07 in a recent study. Yet, other countries had higher mean Ba concentrations.

The amounts of trace element contamination in various canned fish species may vary depending on factors such as contamination gradient, aquatic physicochemical parameters, sex, species, metabolism, age, and diet [68]. Trace element contamination in canned fish products imported to Iraq may occur due

Table 7 Target hazard quotient and target carcinogenic risk of toxic elements due to consumption of canned fish collected from various supermarkets in Kalar City, Iraq

Fish samples	Target hazard quotient									Carcinogenic risk
	Se	As	B	Ag	Ba	Mn	Cu	Zn	Total	As*
Fme	3.4E-02	6.7E-02	2.6E-04	8.0E-03	1.9E-03	1.4E-02	3.1E-02	3.9E-02	1.9E-01	1.5E-02
Fsi	5.2E-02	1.0E+00	1.4E-03	1.1E-02	1.1E-02	7.0E-03	6.1E-02	2.2E-02	1.2E+00	2.2E-01
Fma	3.4E-02	6.7E-02	2.6E-04	8.0E-03	9.8E-03	8.6E-03	4.7E-02	1.8E-02	1.9E-01	1.5E-02
Fbe	8.8E-02	2.3E+00	2.1E-03	4.5E-02	1.3E-02	8.0E-03	5.2E-02	2.5E-02	2.6E+00	5.2E-01
Fal	2.0E-02	8.3E-01	2.5E-03	4.2E-02	5.3E-03	7.8E-03	7.8E-02	2.1E-02	1.0E+00	1.9E-01
Fru	1.4E-01	3.6E+00	2.6E-03	6.4E-02	2.1E-03	6.9E-03	6.6E-02	2.2E-02	3.9E+00	8.1E-01
Fsh	3.5E-02	3.3E-01	1.2E-03	1.0E-01	1.2E-02	1.2E-02	7.2E-02	3.0E-02	6.0E-01	7.5E-02
Fkl	5.5E-02	8.3E-01	2.5E-04	1.7E-01	1.0E-02	7.1E-03	7.7E-02	1.7E-02	1.2E+00	1.9E-01
Fze	5.0E-03	1.0E+00	3.5E-03	6.4E-02	1.4E-02	1.3E-02	2.3E-02	2.5E-02	1.1E+00	2.2E-01
Fsa	5.0E-02	2.2E+00	2.5E-03	4.4E-02	7.1E-04	1.5E-02	5.2E-02	3.3E-02	2.4E+00	4.9E-01
Total	5.2E-01	1.2E+01	1.7E-02	5.5E-01	8.0E-02	1.0E-01	5.6E-01	2.5E-01	1.4E+01	2.7E+00

*Assuming 10% inorganic As present in fish to produce carcinogenic risk (Saha and Zaman, 2013)
Bold indicates target hazard quotient value > 1

to transportation, production handling, canning process, and storage conditions [11].

Health risk assessment. ***Estimated daily intake.*** Table 6 shows the estimated daily intake for potentially toxic elements (mg/kg-bwt/day) from canned fish in our study. The dietary exposure to potentially toxic elements from eating canned fish as part of the daily diet of adults in the study area is determined by the estimated daily intake. The mean concentration of individual potentially toxic elements and the individual consumption rate can be used to estimate the daily intake [69].

We received information regarding potentially toxic element intake on a daily basis from the estimated daily intake from canned fish consumption. We found that the total daily potentially toxic element intake was lower than the maximum tolerated intake value (Table 5). Although the total estimated daily intake was low due to low canned fish consumption, a long-term intake of contaminated canned fish from the research area could have major health consequences for Iraq's people [44, 70]. Due to Iraqis' low consumption of canned fish, the estimated daily intake was lower than the maximum tolerated intake value. The total estimated daily intakes for Se, As, B, Ag, Ba, Mg, Mn, Cu, and Zn were 0.155, 0.220, 0.200, 0.165, 0.337, 20.21, 0.841, 1.343, and 4.532 mg/kg-bwt/day, respectively (Table 5).

Table 6 shows the estimated daily intake of potentially toxic elements [estimated daily intake (percent contribution)] from canned fish species compared to the recommended daily dietary requirements. For estimated daily intake, the contribution percentage of As ranged from 0.95 to 51.11. The largest percentage of As contribution was found in Fru (51.11%), while the lowest was reported in Fme and Fma (0.95 percent). The percentage of contribution of Se for estimated daily intake ranged from 0.50–14.48. The highest Ag percentage was observed in Fkl (19.89%). Manganese contribution in estimated daily intake was estimated to be between 2.9 and 6.26%. The maximum Mn percentage for estimated daily intake contribution was found in Fsa (6.26%), while the lowest was found in Fru (2.9%). From the contribution of heavy metals, we concluded that eating these fish species taken from several shops in Kalar, Iraq, was safe and that the health risks associated with consuming these canned fishes were minimal.

Non-carcinogenic and carcinogenic risk. Table 5 shows the non-carcinogenic (target hazard quotient) and carcinogenic risks of heavy metals (Se, As, B, Ag, Ba, Mn, Cu, and Zn) in canned fish contaminated with potentially toxic elements. In decreasing sequence, the target hazard quotients for four heavy metals were calculated as As > B > Ba > Mn > Zn > Se > Ag > Cu. The total THQ values for Se, As, B, Ag, Ba, Mn, Cu, and Zn were 5.2E-01, 1.2E+01, 1.7E-02, 5.5E-01, 8.0E-02, 1.0E-01, 5.6E-01, and 2.5E-01, respectively.

Considering individual elements from individual fish species, the target hazard quotient value for As exceeded the standard value (> 1) for some of the fish species, which was a serious concern for the consumption of these canned fishes. Humans are exposed to non-carcinogenic Arsenic risks by eating these canned fishes. The target hazard quotient values of As for all the canned fish species were (in the decreasing order) Fru > Fbe > Fsa > Fze > Fsi > Fkl > Fal > Fsh > Fme > Fma (Table 7). However, Fru presented the maximum target hazard quotient for As (3.6E+00) followed by Fbe (2.3E+00) and Fsa (2.2E+00). The lowest target hazard quotient for arsenic as a single metal was in Fma and Fme (6.7E-02).

Our analysis revealed that the total target hazard quotient from different fish species was quite high (except Fma, Fme, and Fsh), with potentially toxic elements investigated having an ability to cause non-carcinogenic hazards (target hazard quotient > 1). (Table 7). In Kalar City, Iraq, excessive and continuous

Table 8 Comparison between present study's results and those of various other studies

Country	Se	As	B	Ag	Ba	Mg	Mn	Cu	Zn	References
Present study	0.26 ± 0.20	0.37 ± 0.34	0.33 ± 0.23	0.28 ± 0.24	0.56 ± 0.35	33.70 ± 2.89	1.40 ± 0.46	2.24 ± 0.75	7.56 ± 2.06	[75]
Nigeria	0.27 ± 0.01	0.47 ± 0.07	0.24 ± 0.08	0.29 ± 0.35	0.51 ± 0.21	35.2 ± 2.0	0.9 ± 0.8	2.11 ± 0.60	3.4 ± 0.5	[66]
China	0.41 ± 0.31	0.57 ± 0.20	0.27 ± 0.04	0.24 ± 0.21	0.59 ± 0.10	25.7 ± 0.9	1.3 ± 0.5	n.f.	13.1 ± 2.6	Risk-Based Concentration Table, April, 2005
Macedonia	n.f.	0.61 ± 0.31	0.46 ± 0.05	0.31 ± 0.04	0.55 ± 0.13	n.f.	1.1 ± 0.4	n.f.	n.f.	[24]
India	0.21 ± 0.04	0.45 ± 0.14	n.f.	n.f.	0.50 ± 0.32	29.8 ± 0.7	0.90 ± 0.09	n.f.	10.6 ± 1.7	[19]
Brazil	0.20 ± 0.03	n.f.*	0.29 ± 0.9	0.28 ± 0.08	0.61 ± 0.07	40.1 ± 0.5	1.5 ± 0.3	1.98 ± 0.40	8.20 ± 10.98	[16]
Bangladesh	0.300 ± 0.025	0.33 ± 0.50	0.34 ± 0.06	0.29 ± 0.41	0.48 ± 0.05	42.1 ± 2.6	1.60 ± 0.12	1.50 ± 0.17	n.f.	[32]
Iran	n.f.	0.23 ± 0.21	0.31 ± 0.21	0.19 ± 0.09	n.f.	38.8 ± 2.6	1.38 ± 0.07	2.02 ± 0.9	9.3 ± 0.3	[39]

*n.f. – not found

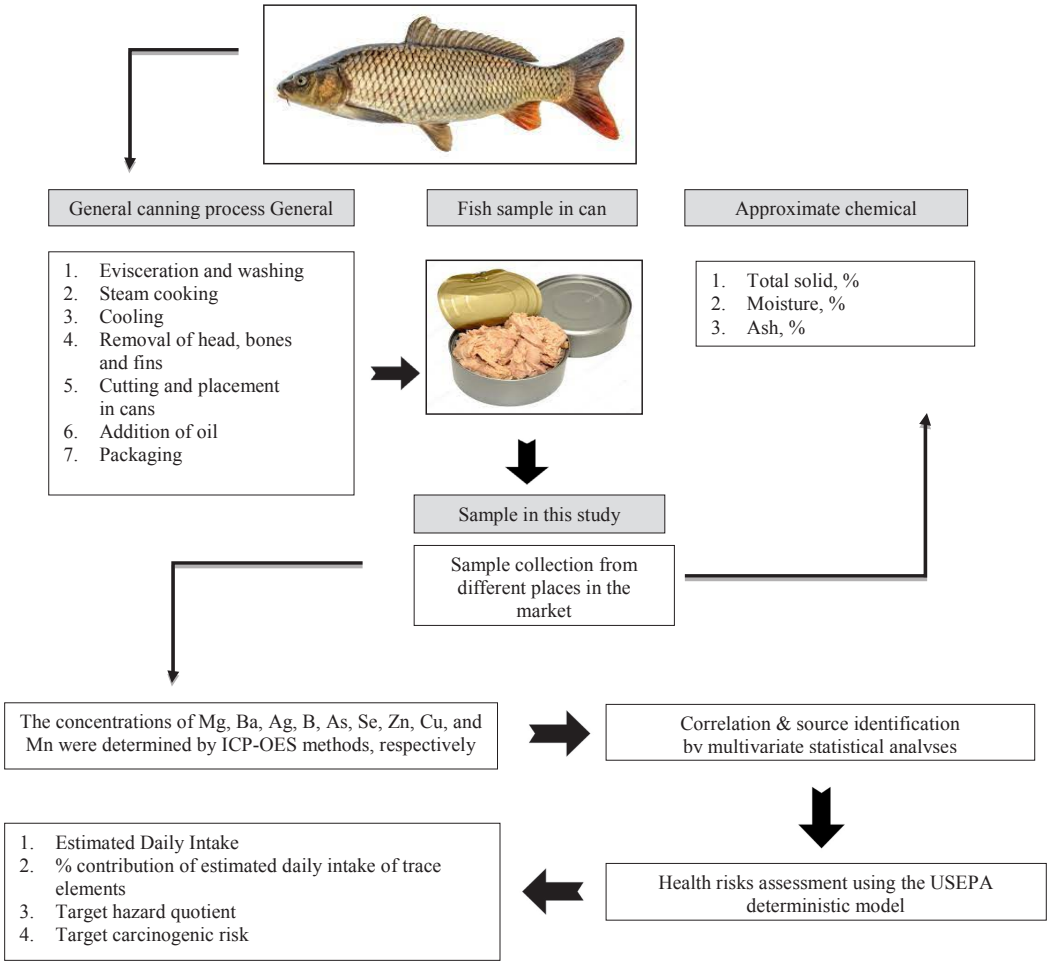

Figure 2 A flow diagram of the research process

eating of the examined fish species could pose serious non-carcinogenic dangers.

The amount of As in various canned fishes was used to determine the carcinogenic risk. Based on the amount, arsenic can have a non-carcinogenic or carcinogenic effect. Table 8 shows the cancer risks for the participants in the study area who ate various canned fishes. Arsenic had a cancer risk value ranging from 1.5E-03 to 8.1E-02. Arsenic is a potentially harmful element found in fish mostly as a result of its presence in the aquatic environment. It is classified as a carcinogen (USEPA group A) and enters aquatic habitats

through the weathering of bedrock, but more frequently through anthropogenic sources [71]. Several health problems induced by chronic exposure to inorganic As have been described in the human body. They affect the gastrointestinal and respiratory tracts, skin, liver, neurological, cardiovascular, and hematological systems [72]. The cancer risk reference value ranges from 10^{-6} to 10^{-4}. The risk of cancer is insignificant if the target carcinogenic risk value is less than 10^{-6}, whereas target carcinogenic risk values greater than 10^{-4} are not safe for humans and may induce cancer [42]. In our study, the cancer risk for As was slightly higher than the reference value. Our results showed that customers in Kalar City, Iraq are exposed to As through canned fish eating and have an increased lifetime chance of developing cancer.

CONCLUSION

According to our results for trace elements (essential and harmful), the analyzed canned fish species, except for Indonesian canned fish, were nutritionally and toxically safe for human consumption. Skipjack tuna had a high arsenic level. The levels of magnesium, selenium, and ash were high in Indonesian sardines. We concluded that the concentrations of trace elements in the muscles of the commercial canned fish studied fall within the limits of international law and are appropriate for human consumption. The target hazard quotient was higher than one in As for most of the fish items and as a single element. In the study area, arsenic may pose a non-carcinogenic health risk. There was a high risk of cancer from consuming hazardous element-contaminated canned salmon. Those who consume canned fish polluted with arsenic on a regular basis have a lifetime risk of cancer.

We evaluated canned fish consumption, which accounts for only 5% of daily calorie intake per capita in Iraq. Other food sources include rice, vegetables, fruits, cereals, seafood, and non-piscine protein sources. They may need to be investigated to determine the exact health hazards associated with trace element intake from such food products. Education and public awareness of the appropriate levels of potentially toxic elements in commercially imported fish are critical, and such information must be made available to the public to ensure that nature and human health coexist in peace.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

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

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White pitahaya as a natural additive: potential usage in cosmetic industry

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Abstract:
The awareness of some harmful side effects of the chemicals contained in synthetic cosmetics has increased the demand for herbal-based cosmetic products today.
White pitahaya fruit and peel methanol extracts were prepared to determine their usage potential in the cosmetic industry. Firstly, we investigated their antimicrobial activity against some test microorganisms using the disc diffusion assay. We also determined their minimal inhibition and minimal bactericidal or fungicidal concentrations. Then, we assayed the antimicrobial activity of a commercial cream containing white pitahaya extracts and the probiotic *Lactobacillus fermentum* MA-7 strain against the test microorganisms. Finally, we measured the sun protection factors of the white pitahaya fruit and peel extracts and the cream with the extracts.
The white pitahaya fruit and peel extracts exhibited antimicrobial activity against the test microorganisms. The cream formulation containing a pitahaya fruit extract had the highest inhibition zone diameter of 11.25 mm against *Escherichia coli* O157:H7. The highest sun protection value among the extracts and cream with extracts was determined for peel extract as 6.66 and 23.34, respectively.
The results indicate that pitahaya fruit and peel extracts have effective antibacterial and antifungal properties, as well as high sun protection factors, and therefore they could be used as natural preservatives in the cosmetic industry.

Keywords: Extract, antimicrobials, probiotics, cream formulation, solar protection

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INTRODUCTION

Pitahaya is a tropical fruit belonging to the *Cactaceae* family which attracts the attention of researchers and producers due to its appearance and color [1]. It was first used as an ornamental plant, and then its fresh fruit began to be consumed [2]. Pitahaya fruit is rich in vitamins and fiber, which helps the digestive system, blocks diabetes, removes toxic substances such as heavy metals, as well as controls cholesterol and blood pressure levels [2]. Pitahaya is known to have a high content of antioxidants which help fight many diseases such as arthritis, atherosclerosis, cancer, heart disease, inflammation, and brain dysfunction [3].

Food-borne and clinical pathogens pose a significant threat to human health. In developed countries, these pathogens cause millions of infectious gastrointestinal diseases each year [4]. The influence of fungal microorganisms on human health has been increasing rapidly in recent years, with the global mortality rate from fungal diseases exceeding that from malaria or breast cancer [5]. Chemical-containing substances can be used against these pathogens, but they have harmful effects on human health, such as resistance to antimicrobials or toxicity.

Therefore, there is a growing interest in finding safer and more effective natural preservatives. Plants are natural sources of antimicrobials that can be used against various infectious pathogens, such as bacteria and fungi. More than 1340 plants with antimicrobial activity have been identified and more than 30 000 natural antimicrobial compounds have been isolated [6].

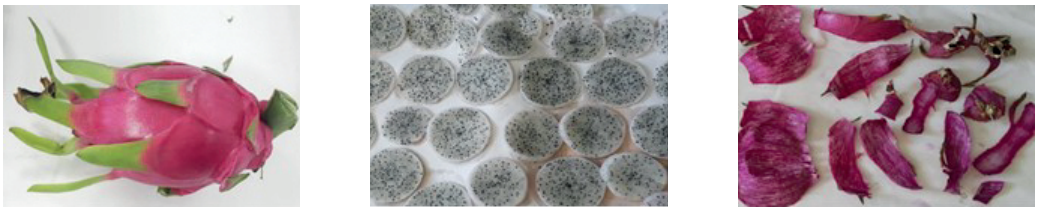

Figure 1 White pitahaya fruit and peel

Formulations containing probiotics are increasingly in demand for skin care, prevention and treatment of skin aging and skin diseases. Thus, the use of probiotics in the cosmetic industry represents an emerging area for skin health [7]. Roudsari *et al.* reported that probiotics in cosmetic formulations have protective effects against atopic dermatitis, acne, eczema, allergic inflammation, or hypersensitivity of the skin to ultraviolet-induced damage [8]. The skin, one of the important organs of the human body, acts as a protective wall that prevents disease-causing pathogenic microorganisms from entering the internal system of the body.

It has been reported in many studies, e.g. by Nichols and Katiyar, that prolonged exposure to sunlight causes various skin conditions [9]. Solar UV rays are divided into three regions: UV-A (320–400 nm), UV-B (290–320 nm), and UV-C (200–290 nm). UV-A rays cause serious problems such as suppression of the immune system, premature skin aging, and endothelial cell necrosis that damages the dermal blood vessels [10]. UV-B rays cause sunburn, suppression of the immune system, photo carcinogenesis, and pigmentation [11]. UV-C rays react with oxygen atoms in the atmosphere and convert oxygen into ozone, which is absorbed by the atmosphere before reaching the Earth [12]. Sunscreen creams are used to protect the skin against the damage of these rays. However, chemical sunscreen creams can have some harmful effects on people with sensitive skin. Recent years have seen a trend towards using natural products to reduce or eliminate these harmful effects. In particular, researchers have focused on the development of herbal sunscreens that have no side effects by including naturally produced bioactive substances into sunscreens [10].

Fruit extracts are known to have ultraviolet (UV) protection, as well as antimicrobial and antioxidant activities due to the compounds they contain [13, 14]. Therefore, plant extracts have the potential to be used as bioactive natural products in food preservation, pharmaceutical industry, healthcare, and cosmetics [15].

We aimed to 1) evaluate the antimicrobial activity of pitahaya extracts; 2) develop a cream formulation containing pitahaya as a natural ingredient; 3) measure the sun protection factors of the pitahaya extracts to determine their potential use in the cosmetic industry as an additive in natural sunscreens; and 4) investigate the solar protection activity of the commercial cream with pitahaya extracts.

STUDY OBJECTS AND METHODS

Plant material. The white pitahaya fruit was purchased in Antalya (Turkey) in September 2021.

Preparation of extracts. The pitahaya fruit samples were washed with distilled water. Then, the fruits and peels were air-dried separately at room temperature (Fig. 1). The dried fruit samples were ground with a Waring blender. For extraction, 10 g of the plant material was mixed with 30 mL of methanol (96%). The fruit samples were extracted every day for 6 h (3 days) in a hot water bath. The extracts were concentrated by using a rotary evaporator. The white pitahaya fruit and peel extracts were dissolved in dimethyl sulfoxide (DMSO) and then sterilized with a 0.45-µm filter. The extracts were stored at 4°C until used.

Determination of antimicrobial activity. ***Disc diffusion assay.*** The disc diffusion method was used to determine the antimicrobial activity of the methanol extracts from white pitahaya fruit and peel against test microorganisms, including food-borne and clinical bacteria (*Salmonella enteritidis* RSKK 171, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* O157:H7) and two yeasts (*Candida albicans* ATCC 10231 and *Candida glabrata* RSKK 04019).

The food-borne and clinical pathogens were cultured at 37°C in Tryptic Soy Broth and Nutrient Broth media, while the yeasts were cultured at 30°C in Yeast Peptone Dextrose media for 24 h. The test microorganisms were washed twice with a saline solution and their concentration was adjusted to 0.5 McFarland. 100 µL of the prepared microbial suspension (0.5 McFarland) was spread onto agar media. Sterile discs (6 mm in diameter) were placed on the solid agar and 20 µL (4000 µg/disc) of the white pitahaya fruit and peel extracts were then dropped onto the discs in triplicates. The petri dishes were incubated at appropriate temperatures for 24 h. After incubation, the inhibition zones around the discs were measured with a caliper and recorded.

Micro-dilution assay. The minimum inhibition concentration and minimum bactericidal concentration or minimum fungicidal concentration of the extracts were determined using the micro-dilution assay. Test microorganisms adjusted to 0.5 McFarland were added to each tube containing the extract and the media. The mixtures in the tubes were incubated under the same conditions as in the disc diffusion assay for each

microorganism. After incubation, the concentration of the extract in the non-growth tube was recorded as minimum inhibition concentration values. Then, the samples from the tubes were inoculated onto solid media using the spot-dropping method. The petri dishes were incubated for 24 h at the same temperature for each microorganism which is indicated in the minimum inhibition concentration determination assay. At the end of the incubation, the extract concentrations that prevented bacterial growth on the solid media were evaluated as minimum bactericidal concentration or minimum fungicidal concentration values.

Antimicrobial activity of the cream with pitahaya extract and probiotic. A new method was designed by modifying the protocols of Handali *et al.* and Chen *et al.* [16, 17]. To formulate an antimicrobial cream, we used a commercial cream, white pitahaya fruit extracts, and the *Lactobacillus fermentum* MA-7 probiotic strain isolated from human milk [18].

In this study, three different experimental samples were assayed. The control was the cream (10% w/v) adjusted with distilled water to the final volume. The experimental samples contained: a) the cream (10% w/v) with the *L. fermentum* MA-7 probiotic strain; b) the cream (10% w/v) with the white pitahaya extract (20% w/v); and c) the cream (10% w/v) with the white pitahaya extract (20% w/v) and the *L. fermentum* MA-7 strain.

After vortexing, all the groups were sonicated for 15 min and then sterilized with a 0.45-μm filter. The antimicrobial activity of the prepared mixtures was determined by the well diffusion method. The test microorganisms used in this study included *S. enteritidis* RSKK 171, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *E. coli* O157:H7, *L. monocytogenes* ATCC 7644, *C. albicans* ATCC 10231, and *C. glabrata* RSKK 04019. They were washed twice with a saline solution and their concentrations were adjusted to 0.5 McFarland. The microorganism suspensions (100 μL) were spread onto solid media. 100 μL of the mixture was added to each well (6 mm in diameter). The volume of the wells corresponded to 0.1. The experiment was carried out in three repetitions. The petri dishes were incubated for 24 h at 37°C for the food-borne and clinical pathogens and at 30°C for the yeasts. The inhibition zones around the wells were then measured with a caliper and recorded.

Sun protection factor of pitahaya extract. The sun protection factor (SPF) of the white pitahaya fruit and peel extracts was determined spectrophotometrically. The extract (0.006 g) was mixed with 3 mL of ethanol (96%) and homogenized. The homogeneous mixture was measured using a spectrophotometer (Beckman Coulter, USA) at 5-nm intervals in the wavelength range of 290–320 nm. The recorded values were calculated using the Mansur equation [19]:

$$\mathrm{SPF} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}(\lambda) \times \mathrm{I}(\lambda) \times \mathrm{Abs}(\lambda)$$

where CF is the correction factor (= 10); EE(λ) is the erythemetogenic effect radiation wavelength (λ); I(λ) is the intensity of sunlight at wavelength (λ); Abs(λ) is the absorbance of extracts at wavelength (λ).

Sun protection factor of the pitahaya extract and cream samples. The sun protection factor of the pitahaya extract and commercial cream samples was determined using a modified method of Bambal *et al.* and Imam *et al.* [20, 21]. For this, 1 g of cream and 0.5 g of white pitahaya (fruit and peel) extracts were mixed, and 8.5 g of distilled water was added to the mixture. The mixture was stirred until it became homogeneous. 0.1 g of the homogeneous mixture was taken into another tube and completed to 10 mL volume with ethanol (40%). After sonication (30 kHz, 100% amplitude) for 5 min, the mixture was filtered through Whatman No. 1 filter paper. The mixture (0.5 mL) was adjusted to 5.0 mL with ethanol (40%) in another tube. Then, 0.5 mL of this mixture was taken, and the volume was completed with ethanol (40%) to 2.5 mL. The mixtures adjusted to 2.5, 5.0, and 10.0 mL were measured in three repetitions using the spectrophotometer (Beckman Coulter, USA) at 5-nm intervals in the wavelength range of 290–320 nm. The sun protection factor values of the control and experimental samples were calculated using the Mansur equation presented above [19].

RESULTS AND DISCUSSION

We investigated the antimicrobial and antifungal activities of the white pitahaya fruit and peel extracts on food-borne and clinical test microorganisms. Disc diffusion assay results are given in Table 1. As we can see, the highest inhibition zone diameter (11.57 mm) was found against *Pseudomonas aeruginosa* ATCC 2785 in the peel extract, while the lowest inhibition zone diameter (6.17 mm) was found against *Escherichia coli* O157:H7 in the fruit extract. As for the antifungal activity, the fruit and peel extracts had inhibition zone diameters of 11.66 and 13.15 mm, respectively, against *Candida albicans* ATCC 10231 and 12.21 and 12.93 mm, respectively, against *Candida glabrata* RSKK 04019.

Nurmahani *et al.* stated that ethanol, hexane, and chloroform extracts of white pitahaya peel showed an inhibition zone diameter of 7–9 mm against *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 14506, *Salmonella typhimurium* ATCC 13311, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Yersinia enterocolitica* ATCC 23715, and *Campylobacter jejuni* ATCC 29428 [22]. In our study, the inhibition zone diameters were larger compared to their results. In another study by Mahdi *et al.*, who used the well diffusion method, the inhibition zone diameters of 7–11 mm were obtained for white pitahaya aqueous extracts against *E. coli*, *Klebsiella* spp., *Staphylococcus epidermidis*, *S. aureus*, and *C. albicans* [23]. The

Table 1 Antimicrobial activity of white pitahaya methanol extracts

Test microorganisms	Inhibition zone diameter, mm				
	Extracts		Antibiotics		
	Fruit extract	Peel extract	Ampicillin	Kanamycin	Fluconazole
Salmonella enteritidis RSKK 171	7.98 ± 0.21	9.28 ± 0.12	14.02 ± 0.30	15.48 ± 1.40	–
Pseudomonas aeruginosa ATCC 27853	9.87 ± 0.49	11.57 ± 0.79	23.53 ± 0.60	20.37 ± 0.20	–
*Listeria monocytogene*s ATCC 7644	6.30 ± 0.14	6.35 ± 0.38	29.57 ± 0.10	25.40 ± 1.30	–
Escherichia coli O157:H7	6.33 ± 0.09	7.06 ± 0.02	17.76 ± 0.00	19.33 ± 0.40	–
Enterococcus faecalis ATCC 29212	6.17 ± 0.22	6.82 ± 0.28	24.02 ± 0.30	13.48 ± 1.40	–
Candida albicans ATCC 10231	11.66 ± 0.07	13.15 ± 0.83	–	–	21.85 ± 1.76
Candida glabrata RSKK 04019	12.21 ± 1.44	12.93 ± 0.64	–	–	20.35 ± 0.10

Table 2 Minimum inhibition and bactericidal or fungicidal concentrations of white pitahaya methanol extracts

Test microorganisms	Minimum inhibition concentration, mg/mL		Minimum bactericidal concentration or minimum fungicidal concentration, mg/mL	
	Fruit extract	Peel extract	Fruit extract	Peel extract
Salmonella enteritidis RSKK 171	40	80	40	80
Pseudomonas aeruginosa ATCC 27853	20	40	20	80
Listeria monocytogenes ATCC 7644	40	80	> 80	80
Escherichia coli O157:H7	80	80	> 80	80
Enterococcus faecalis ATCC 29212	80	40	> 80	> 80
Candida albicans ATCC 10231	80	40	80	40
Candida glabrata RSKK 04019	40	20	40	20

Table 3 Minimum bactericidal concentration/minimum inhibition concentration or minimum fungicidal concentration/ minimum inhibition concentration ratios of white pitahaya methanol extracts

Test microorganisms	Minimum bactericidal/inhibition concentration or minimum fungicidal/inhibition concentration	
	Fruit extract	Peel exctract
Salmonella enteritidis RSKK 171	1	1
Pseudomonas aeruginosa ATCC 27853	1	2
Listeria monocytogenes ATCC 7644	n.d.	1
Escherichia coli O157:H7	n.d.	1
Enterococcus faecalis ATCC 29212	n.d.	n.d.
Candida albicans ATCC 10231	1	1
Candida glabrata RSKK 04019	1	1

n.d. – not determined

differences in the antimicrobial activity assay results may be due to the differences in the origin of the pitahaya fruit samples, the solvents, as well as extraction and test methods used in the studies.

The disc diffusion assay results indicated the white pitahaya extracts' antimicrobial or antifungal activity against all the tested microorganisms. The minimum inhibition concentration, minimum bactericidal concentration or minimum fungicidal concentration values are shown in Table 2.

Among the tested white pitahaya extracts, the lowest minimum bactericidal concentration value was found to be 20 mg/mL for the fruit extract against *P. aeruginosa* ATCC 27853. The minimum bactericidal concentration values of the fruit extracts varied from 20 to ≥ 80 mg/mL. The minimum bactericidal concentration values for the peel extracts were found to be 80 or ≥ 80 mg/mL against the tested bacterial strains. The lowest minimum fungicidal concentration value was 20 mg/mL for the peel extract against *C. glabrata* RSKK 04019.

The tested extract was considered bactericidal if the minimum bactericidal/inhibition concentration ratio was ≤ 4 and bacteriostatic if the minimum bactericidal/inhibition concentration ratio was > 4 [24, 25]. The minimum bactericidal/inhibition concentration or minimum fungicidal/inhibition concentration ratios of some white pitahaya fruit and peel extracts were found to be 1 and 2. We established that those extracts had

Table 4 Antimicrobial activity of cream samples

Test Groups / Test Microorganisms	Inhibition zone diameter, mm			
	Cream (control)	Cream with *Lactobacillus fermentum* MA-7	Cream with extract	Cream with extract and *Lactobacillus fermentum* MA-7
Salmonella enteritidis RSKK 171	–	1.62 ± 0.82	–	9.98 ± 5.57
Enterococcus faecalis ATCC 29212	–	–	–	2.72 ± 0.61
Pseudomonas aeruginosa ATCC 27853	6.44 ± 0.85	3.28 ± 0.30	3.76 ± 0.94	5.06 ± 0.58
Escherichia coli O157:H7	–	6.40 ± 0.24	–	11.25 ± 1.10
Listeria monocytogenes ATCC 7644	–	–	–	8.54 ± 0.38
Candida albicans ATCC 10231	–	6.76 ± 0.60	–	6.39 ± 0.11
Candida glabrata RSKK 04019	2.76 ± 0.53	8.73 ± 0.62	4.23 ± 1.53	3.54 ± 0.89

"– " is no inhibition zone

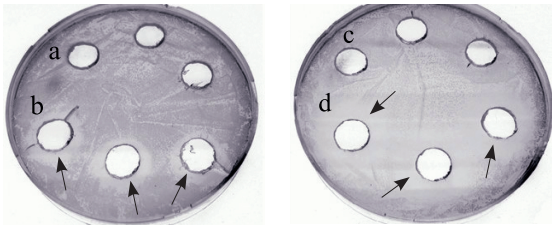

Figure 2 Antimicrobial activity of cream samples against *Escherichia coli* O157:H7: a – cream (control); b – cream with *Lactobacillus fermentum* MA-7; c – cream with extract; d – cream with extract and *Lactobacillus fermentum* MA-7

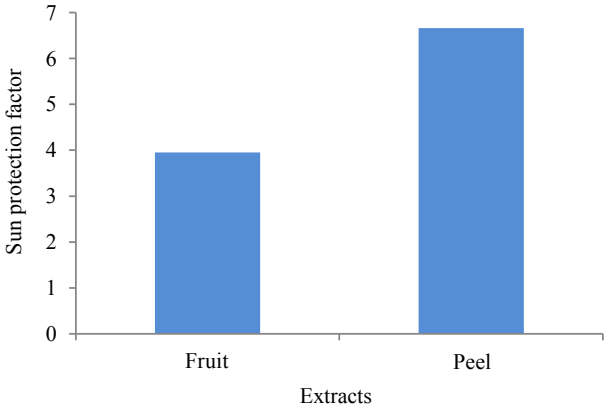

Figure 3 Sun protection factor of white pitahaya methanol extracts

antibacterial and antifungal activities against the tested microorganisms, except for the not determined group (Table 3).

The antimicrobial activity assay results of the cream samples are given in Table 4. As we can see, the cream with the extract and *Lactobacillus fermentum* formed an inhibition zone against all the tested microorganisms. The highest inhibition zone diameter (11.25 mm) among the tested microorganisms was obtained against *E. coli* O157:H7 in the cream with the extract and *L. fermentum*. The control cream and the cream with the extract did not show an inhibition zone against *E. coli* O157:H7, while the cream with *L. fermentum* mixture had a diameter of 6.4 mm.

According to Fig. 2, the antimicrobial activity or increased inhibition zone diameters were observed in the cream with the extract and *L. fermentum*, compared to the control sample. The results for the control and all the test groups showed that the pitahaya extract and the probiotic candidate strain increased the antimicrobial activity of the commercial cream by creating a synergetic effect.

A recent study by Moysidis *et al.* indicated that the cream sample with *L. fermentum* MA-7 increased the rate of healing the wound [26]. Another recent study showed inhibition zone diameters of 2–10 mm in cream with *Vernonia ambigua* leave extracts against *C. albicans*, *S. aureus*, *E. coli*, and *P. aeruginosa* [27].

Sunscreens, which are widely used today to protect the skin from sun rays, constitute a large part of the cosmetic industry. In our study, the white pitahaya fruit and peel extracts had a sun protection factor of 3.95 and 6.66, respectively (Fig. 3). According to the UV blocking rates reported by Imam *et al.*, the white pitahaya fruit and peel extracts had a UV blocking capacity of 75 and 80%, respectively [21].

The sun protection factor values of the cream samples containing white pitahaya fruit and peel extracts are given in Fig. 4. The highest sun protection factor values of 9.26 and 23.34 were observed in the fruit and peel extracts at 10-mL concentrations, respectively. The highest sun protection factor value was observed at the 10-mL concentration for the samples with the extract as 9.26 and 23.34, while that for the commercial cream (control) was found to be 4.94 (Fig. 4). According to Imam *et al.*, the cream samples with pitahaya fruit and peel extracts had UV blocking rates of 90 and 95%, respectively [21]. Compared to the control, the fruit and peel extracts increased the sun protection factor values and the UV blocking rate of the cream.

Higher concentrations of the extract added to the commercial cream increased the sun protection factor value of the cream. The sun protective properties of plant extracts are due to the phenolic compounds and flavonoid substances they contain. Phenolic and

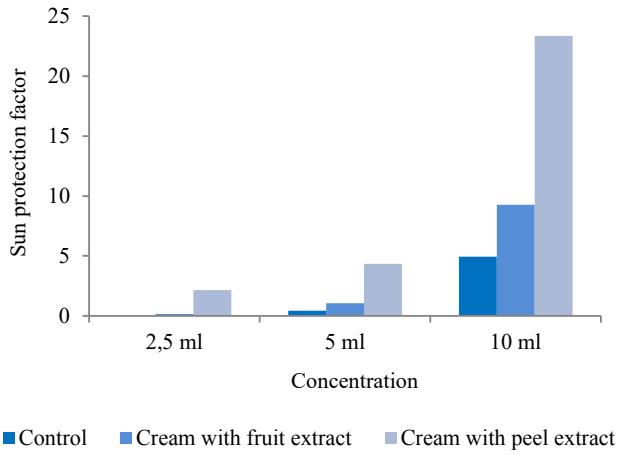

Figure 4 Sun protection factor of commercial cream (control) and cream with white pitahaya methanol extracts

flavonoid compounds found in pitahaya have been reported to contribute to a high sun protection factor value and broad-spectrum UV-A and UV-B light protection [28]. The use of natural additives obtained from plants in various industries enables low-cost production in high quantities [29, 30].

CONCLUSION

We studied the biological activities of pitahaya fruit and peel extracts to determine their usage potential in the cosmetic industry. The extracts exhibited good antibacterial and antifungal activities against the tested microorganisms. We also tested the cream samples containing the pitahaya extracts to determine their antimicrobial activity. The cream with the pitahaya fruit extract and *Lactobacillus fermentum* MA-7 with high antimicrobial activity could become an alternative to chemical preservatives. Additionally, the pitahaya fruit extracts and the cream with the pitahaya extracts exhibited high sun protection values. The results showed that pitahaya fruit and peel extracts could be suitable as natural preservatives for the cosmetic industry.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Preparation of sodium alginate films incorporated with hydroalcoholic extract of *Macrocystis pyrifera* L.

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Abstract:
Agroindustry needs novel materials to replace synthetic plastics. This article introduces sodium alginate films with antioxidant properties. The films, which were incorporated with hydroalcoholic extract of *Macrocystis pyrifera* L., were tested on sliced Hass avocados.

The research featured sodium alginate films incorporated with hydroalcoholic extracts of *M. pyrifera*. Uncoated avocado halves served as control, while the experimental samples were covered with polymer film with or without hydroalcoholic extract. A set of experiments made it possible to evaluate the effect of the extracts on polymeric matrices, release kinetics, and sensory profile of halved Hass avocados.

A greater concentration of hydroalcoholic extracts increased the content of phenolic compounds and their antioxidant activity. As a result, the bands in the carboxylate groups of sodium alginate became more intense. Crystallinity decreased, whereas opacity and mass loss percentage increased, and conglomerates appeared on the surface of the films. These processes fit the Korsmeyer-Peppas kinetic model because they resulted from a combination of diffusion and swelling mechanisms in the films.

The films incorporated with hydroalcoholic extract of *M. pyrifera* proved to be an effective alternative to traditional fruit wrapping materials.

Keywords: Sodium alginate, films, hydroalcoholic extracts, *Macrocystis pyrifera*, coating, storage, avocado

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INTRODUCTION

Polymers, popularly known as plastics, are usually synthetic or semi-synthetic organic compounds with a high molecular weight. These materials are used in almost all industrial sectors. As a result, the total amount of plastics manufactured in the world since 1950 exceeds 8000 Mt [1]. However, its production generates pollutants and greenhouse gases, e.g., carbon dioxide (CO_2), which contribute to environmental pollution and global warming [2].

However, conventional polymers can be replaced by biodegradable materials made of fats, vegetable oils, gluten, proteins, and polysaccharides [3].

Bioplastics are defined as materials produced by living organisms. They are biobased, biodegradable, or both and are used in many sectors, including food processing, agriculture, compost bags, etc. [4].

Red (*Rhodophyta*), green (*Chlorophyta*), and brown (*Phaeophyta*) macroalgae possess a great chemical diversity of primary and secondary metabolites with numerous beneficial properties and a good application potential. They have increasingly attracted attention of many industrial branches, including plastics production [5].

Brown macroalgae contain secondary metabolites with antioxidant, anti-inflammatory, and anti-

microbial [6, 7] properties. *Macrocystis pyrifera* is one of the most popular representatives of this group [8].

M. pyrifera L. is a low-calorie product with a high concentration of mineral ions (Mg^{2+}, Ca^{2+}, P^{5+}, K^{+}, I^{-}), vitamins, low lipid content, alginates, and polyphenols [9].

Polyphenols are antioxidant compounds that delay or prevent the oxidation of oxidizable molecules [10]. As a result, they reduce food deterioration when they are incorporated directly into food itself or its packaging [11].

Alginate is a polysaccharide that consists of *β*-D-mannuronic and *α*-L-guluronic acids [12]. It is biocompatible, biodegradable, low-toxic, and easily available, which causes a great commercial interest [13]. Alginate-based films and coatings are flexible and glossy; they possess excellent water solubility and emulsification capacity, as well as low oil and oxygen permeability [14].

In the food industry, alginate-based films provide temporary protection against water loss. Such films prolong the shelf life of fruits and vegetables by inhibiting post-harvest metabolic processes, i.e., aging and rotting [15]. Alginate-based films are prospective vehicles for polyphenolic compounds, which migrate, partially or totally, from the film onto the food surface [16, 17].

The extract leaves the polymeric matrix by diffusion throughout or swelling of the matrix. Eventually, the release rate decreases because the material swells, and the active agent has to cover a greater distance to exit the system. This diffusion process is governed by Fick's law, in which the concentration is proportional to the diffusion flux density.

However, some swelling-produced systems generate a slow migration, which results in a balance between the internal and external environments. Considering these processes, Higuchi proposed that release occurs as a function of the square root of time (Eq. (1)), while Korsmeyer *et al.* considered that the release depends on material dissolution or structural effects (Eq. (2)) [18, 19]:

$$\frac{M_t}{M_\infty} = K \times t^{1/2} \quad (1)$$

where M_t/M_∞ is the fraction of solute that has been released at time t, and K is the release rate constant;

$$\frac{M_t}{M_\infty} = K \times t^{n} \quad (2)$$

where K is a constant that incorporates structural and geometric characteristics of the release system, and n is the exponent that indicates the release mechanism.

This research focused on the antioxidant effect of sodium alginate films with hydroalcoholic extract of *M. pyrifera* on Hass avocado.

STUDY OBJECTS AND METHODS

Materials. All solvents and reagents were of analytical grade. Medium viscosity brown algae sodium alginate, glycerol 99%, average weight poly (ethylene) glycol Mn 400, sodium carbonate, the Folin-Ciocalteau reagent 2N, gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Ethanol and methanol were produced by Merck. The hydroalcoholic extract of brown macroalgae was prepared from *Macrocystis pyrifera* L., collected in the district of Paracas (Ica, Peru). The Hass avocado fruits were collected from the Province of Chincha (Ica, Peru) and stored at 8°C until the application of the films.

Extraction of brown macroalgae with a hydroalcoholic solution. To produce the extracts, we macerated 10 g of dry and ground brown macroalgae with 100 mL of a hydroalcoholic ethanol:water solution (70:30, v/v). The mix was stirred at 35°C in an amber bottle for 24 h. Then, the mix was filtered, and the solid residue was macerated again with the hydroalcoholic solution. The resulting supernatants were combined and concentrated with a vacuum evaporator to a volume of 100 mL. The resulting product was stored at 10°C.

Determination of the total phenolic content. The total phenolic content of the hydroalcoholic extract was revealed by the Folin-Ciocalteu method, and the results were expressed in gallic acid equivalent per 100 g of brown macroalgae (mg GAE 100 g^{-1}) [20]. According to the standard procedure, 1 mL of the hydroalcoholic *M. pyrifera* extract was mixed with 0.6 mL of the Folin-Ciocalteu reagent. After that, we added 3.2 mL of an aqueous solution of sodium carbonate (Na_2CO_3, 7.5%, w/v). The resulting mix was brought up to 12 mL with ultrapure water and stirred at room temperature in the dark for 60 min. Finally, its absorbance was measured at 765 nm using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer).

Determination of the free radical capture capacity (DPPH method). ***Antioxidant activity.*** The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method [21]. According to the standard procedure, 1 mL of the hydroalcoholic *M. pyrifera* extract was mixed with 1 mL of methanolic solution of DPPH (0.36 mmol L^{-1}) and 2 mL of methanol. The mix was stirred and left at room temperature in the dark for 30 min. Then, its absorbance was measured at 517 nm.

The results were expressed as the inhibition percentage of the DPPH radical according to Eq. (3):

$$\text{Inhibition}(\%) = 100 \times \left(\frac{A_C - A_E}{A_C} \right) \quad (3)$$

where A_C is the control absorbance (DPPH), and A_E is the extract absorbance.

Preparation of the films incorporated with hydroalcoholic extract. We added a mix of plasticizers (ethylene glycol and polyethylene glycol) to 30 mL of sodium alginate polymeric solution 1.5% (w/v) at

a ratio of 9:1 (w/w) under constant stirring at 70°C for 60 min. Subsequently, we added 5 mL of extract solution in a range between 3 and 6 % (w/v). The solution was obtained from the stock solution of *M. pyrifera* extract. The resulting mix was stirred at 70°C for 30 min. Finally, the solutions were molded and dried at 50°C for 24 h.

Description of the films incorporated with hydroalcoholic extract. The opacity was reduced from the transmission values and the film thickness as in Eq. (4). The mean thickness value was registered using a mechanical micrometer (Mitutoyo 103-137) with a precision of 0.01 mm. The transmittance value was obtained by cutting the films into square pieces (20×20 mm). The pieces were placed in the support of solid samples of a Varian Cary® 50 UV-Vis spectrophotometer. The spectra were registered at 300–1000 nm [22].

$$\text{Opacity} = \frac{-Log(T)}{d} \quad (4)$$

where T is the light transmittance of the film at 600 nm, and d is the sample thickness, mm.

The FTIR spectra were obtained by Attenuated Total Reflectance (ATR) using an IRPrestige 21 Shimadzu spectrophotometer at 600–4000 cm^{-1} after acquisition of 20 scans at a resolution of 4 cm^{-1} for each spectrum. Thermogravimetric curves were gathered in an SDT Q600 simultaneous TG/DTA modulus managed by the Thermal Advantage for Q Series software (v. 5.5.24), both from TA Instruments.

The measurements were performed using sample amounts of 5.0 ± 0.1 mg in a dynamic N_2 atmosphere flowing at 50 mL min^{-1}. The temperature range was selected as 25–800°C with a heating rate of 10°C min^{-1}. The XRD diffractograms were obtained in a range of 2 θ from 5 to 100° in a D8 Advance diffractometer (Brüker) equipped with a Cu source (Kα = 1.5418 Å) and a LynxEye model PSD type detector. The diffractometer operated at a voltage of 40 kV and 40 mA (1600 W). The SEM images were obtained with an LEO 440 microscope (Cambridge) equipped with a 7060 detector (Oxford) at resolutions of 10 and 1 µm with a magnification of 1000× and 5000×, respectively.

Table 1 Antioxidant activity of *Macrocystis pyrifera* extract at different concentrations

Extract %, w/v	Total phenolic content, mg GAE 100 g^{-1}	DPPH radical scavenging, %
3.0	25.4 ± 0.2	22.2 ± 0.2
6.0	48.5 ± 0.3	41.2 ± 0.4
10	74.2 ± 0.3	61.0 ± 0.1

The samples were gold-plated in an MED 020 (Bal-Tec) high vacuum metallizer.

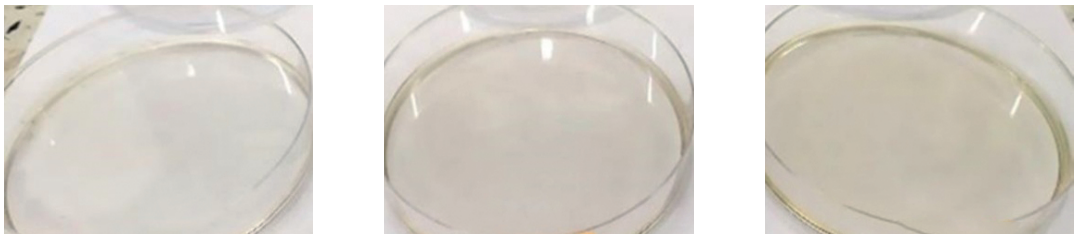
Release test of hydroalcoholic extract. The alginate films incorporated with hydroalcoholic extract of *M. pyrifera* were immersed in 25 mL of a 70% ethanolic solution at 10°C and stirred at 100 rpm. For measurement purposes, 2 mL of release medium were withdrawn at predetermined times. Its absorbance was determined at 271 nm using a UV-1800 UV-Vis spectrophotometer (Shimadzu). This aliquot was returned after reading, and the system was kept under stirring until the next reading [23].

Food protection test. The antioxidant activity of the films with hydroalcoholic extracts was tested on halved Hass avocados. The cut face was covered with simple films and those incorporated with hydroalcoholic extract. The research involved an additional Hass avocado test without coating, which was marked as control sample C. The tests were carried out at 8°C and 50–60% relative humidity on storage day 21.

RESULTS AND DISCUSSION

Total phenolic content and antioxidant activity of extracts. A 10-g sample of *Macrocystis pyrifera* L. yielded 6.86% in the extraction of the hydroalcoholic extract. Its concentration was 10% w/v, the TFC was 74.2 mg GAE 100 g^{-1}, and the percentage inhibition was 61.0%.

After that, 3 and 6% solutions were prepared from diluted stock solution. They were presented as a total polyphenol content of 25.4 and 48.5 mg GAE 100 g^{-1}, with inhibition percentages of 22.2 and 41.2%, respectively. The obtained results were consistent with available scientific publications on *M. pyrifera*. Table 1 shows the total phenolic content values for each solution.



a b c

Figure 1 Films obtained from sodium alginate: (a) base film (no extract); (b) film with 3% hydroalcoholic *Macrocystis pyrifera* extract; and (c) film with 6% extract

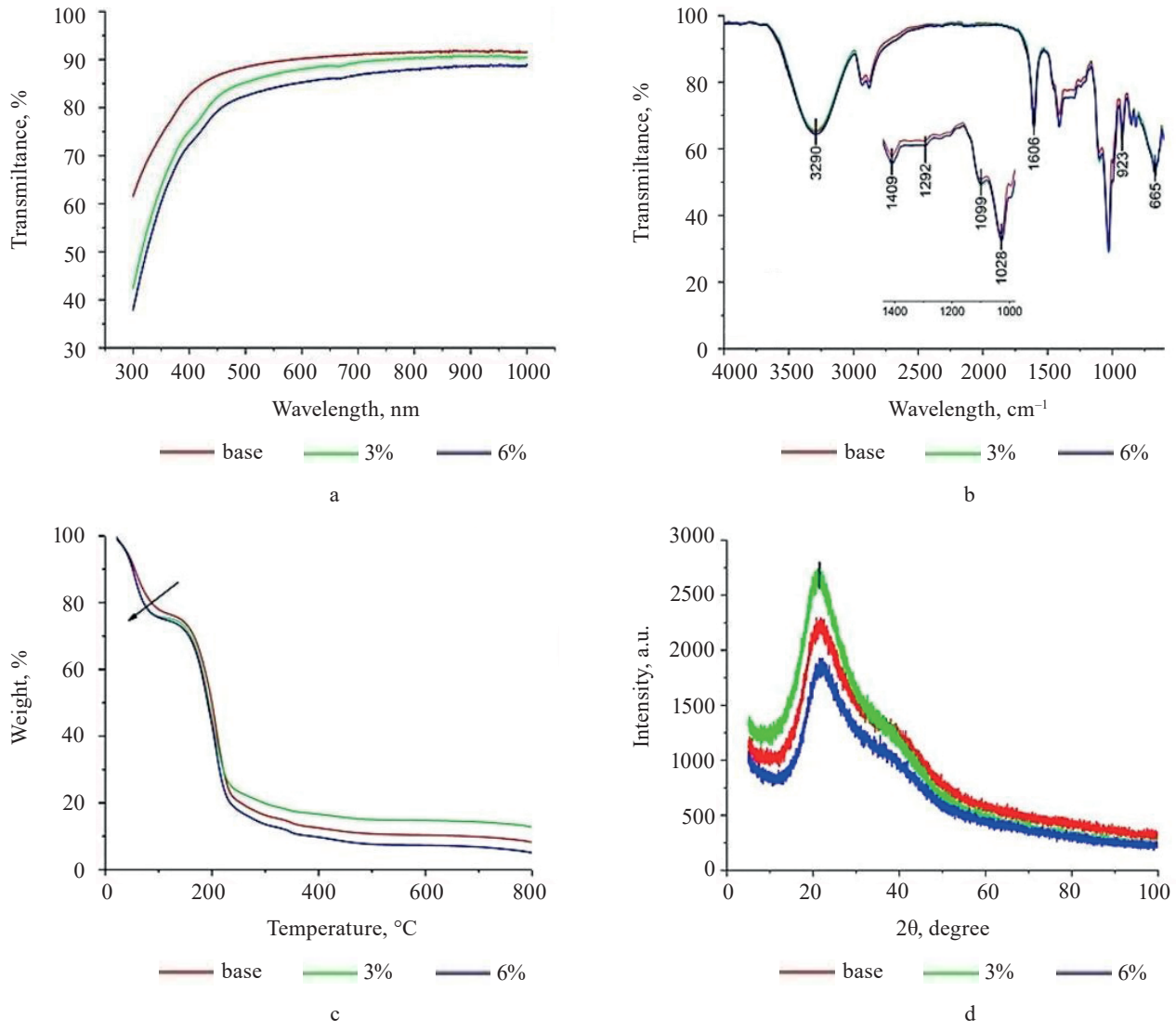


Figure 2 Light transmittance (a), FTIR spectrum (b), TG curves (c), and XRD diffractograms (d) of films from sodium alginate (base) and sodium alginate with 3 and 6% of *Macrocystis pyrifera* extract

Table 2 Transmittance and opacity values of films in the visible spectrum

Film	Thickness, mm	T_{600}, %[a]	Opacity, a.u. (nm/mm)
Base (no extract)	0.185	90.2059	0.24
With 3% *Macrocystis pyrifera* extract	0.173	87.9631	0.32
With 6% *Macrocystis pyrifera* extract	0.178	85.2594	0.39

[a] T_{600} % is the transmittance percent of each film at 600 nm

Sodium alginate films. We prepared three alginate films, namely the base film, which contained no hydroalcoholic extract, and films contained 3 and 6% of hydroalcoholic extract. Figure 1 depicts the obtained films: the hue darkened as the concentration of the hydroalcoholic extract increased.

Optical properties of films. Average thickness and UV-Vis spectrum described the opacity of the films (Fig. 2a). The obtained transmittance percentage was ≥ 80% in the region of 500–800 nm. The transmittance decreased as the hydroalcoholic extract concentration increased because it had a photoprotective effect [24]. The opacity also increased, which means that the extracts were not homogeneously distributed in the polymeric matrix of the films.

Table 2 shows the transmittance value of the films at 600 nm (visible light), as well as their corresponding opacity. The transmittance percentage of the base film was 90.21%. As the concentration of the extract increased, the value fell down to 87.96 and 85.26% in the films with 3 and 6% of the extract, respectively. The opacity was calculated at a wavelength of 600 nm. It demonstrated a slight increase as the concentration of the *M. pyrifera* extract increased.

Table 3 Mass loss values: thermogravimetric analysis of films obtained from sodium alginate with/without *Macrocystis pyrifera* extract

Film	Weight, mg	Thermal event	ΔT, °C	Mass loss, %
Base (no extract)	5.176	Dehydration	25.0–119.8	22.8
		Film degradation	119.8–320.8	61.1
		Polymer degradation	320.8–672.5	5.38
		Carbonization	672.5–800.0	5.14
With 3% *Macrocystis pyrifera* extract	5.142	Dehydration	25.0–109.7	23.9
		Film degradation	109.7–325.5	57.0
		Polymer degradation	325.5–638.2	4.00
		Carbonization	638.2–800.0	7.87
With 6% *Macrocystis pyrifera* extract	5.105	Dehydration	25.0–113.6	24.8
		Film degradation	113.6–314.1	61.9
		Polymer degradation	314.1–617.3	5.68
		Carbonization	617.3–800.0	9.86

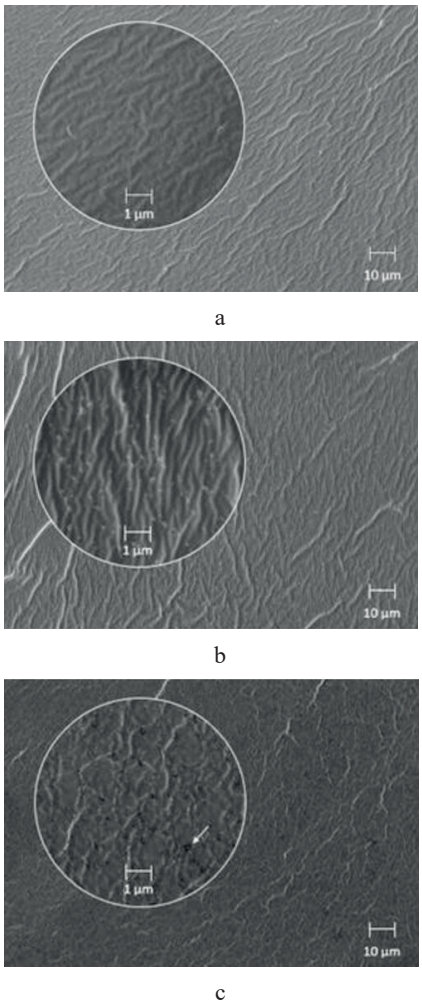


Figure 3 Scanning electron microscopy images of films: (a) base film; (b) film with 3% extract; and (c) film with 6% extract

FTIR of the films. Figure 2b illustrates the FTIR spectrum of the films under study. The increase in the concentration of the extracts intensified the bands corresponding to the symmetric stretching COO^- at 1409 cm^{-1}, torsional vibrations and swinging of $–CH_2$ at 1350–1150 cm^{-1}, C-O stretching of the pyranose ring at 1099 cm^{-1}, and C-O stretching at 1028 cm^{-1} of sodium alginate [25–27]. The obtained results revealed an interaction between the polar groups of the polymeric matrix and the polyphenolic compounds of the extract.

Thermogravimetry. Figure 2c presents the thermogravimetric curves of the films with mass losses that were assigned to the following steps: dehydration, film degradation, polymer degradation, and carbonization [28]. As the extracts became more concentrated, the mass loss in the films increased, especially in the film with 6% of the extract. However, if the concentration of the extract was lower, the mass loss decreased during this step, and a better extract-film interaction was assumed. Table 3 summarizes the thermal events, mass loss, and respective temperature intervals

X-ray diffraction analysis. The X-ray diffraction diffractogram in Fig. 2d demonstrates a broad peak between 20 and 30°. It presents the diffraction pattern of the amorphous structure of the sodium alginate film plasticized with glycerol and PEG 400 (base film). The crystallinity percentage of the base film was 39.3%. However, it reached 39.1% when it was incorporated with 6% of the extract. The low concentration of the extract brought up the crystallinity to 43.1% in the film with 3% of the extract. Therefore, the microstructure of the incorporated film was more homogeneous when the concentration of the extract was lower.

Scanning electron microscopy analysis. The scanning electron microscopy images (Figs. 3a, 3b, and 3c) showed a rough and homogeneous surface (Fig. 3a) with clusters on the surface, which increased the concentration of the *M. pyrifera* extract (Figs. 3b and 3c). The obtained result shows how the secondary metabolites migrated towards the avocado surface.

Kinetic behavior of hydroalcoholic extract release. Figure 4 shows the release curve of the extract at 271 nm for 300 min of testing. Figure 5 indicates the profile of the film with 3 and 6% extract according to the Higuchi model. Figure 6 shows the profile according

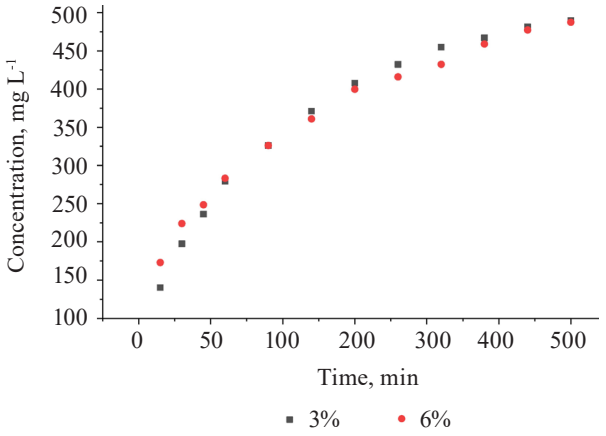


Figure 4 Release profile of *Macrocystis pyrifera* extract in alginate films with 3 and 6% hydroalcoholic *Macrocystis pyrifera* extracts

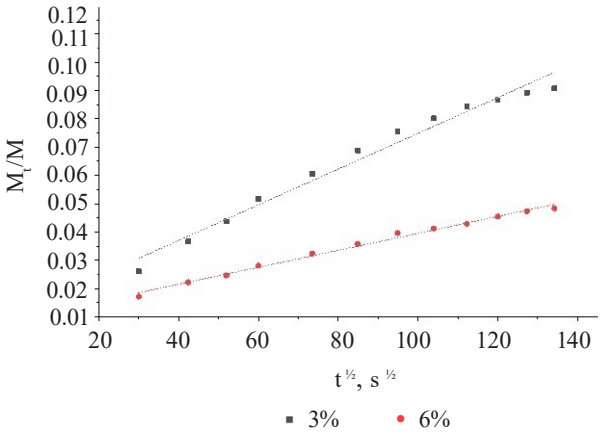


Figure 5 Release profile according to the Higuchi model.

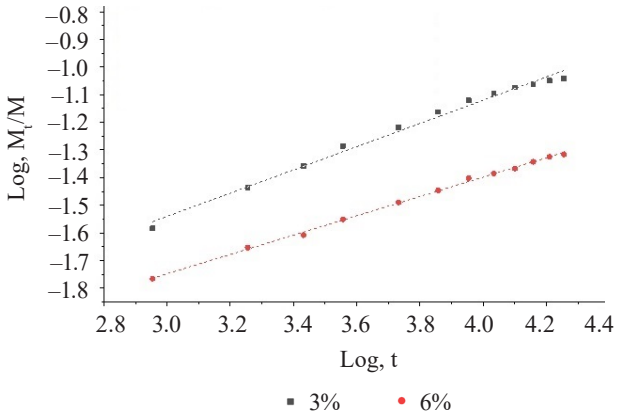


Figure 6 Release profile according to the Korsmeyer and Peppas model.

Table 4 Kinetic parameters of films from sodium alginate with *Macrocystis pyrifera* extracts according to Higuchi and Korsmeyer-Peppas

Film	Higuchi		Korsmeyer-Peppas	
	K_H	R^2	n	R^2
3% extract	6.31×10^{-4}	0.9764	0.42	0.9894
6% extract	2.99×10^{-4}	0.9918	0.35	0.9982

to the Korsmeyer and Peppas model. In both cases, the film with the 3% extract had higher constants R^2 and n. However, the film with the 6% extract could continue to release. The release percentage was not complete because the extract contained polyphenolic compounds: their hydroxyl groups can interact with related groups of alginate or plasticizer [23].

Table 4 shows kinetic parameters K and n calculated according to Eqs. (3) and (4). As $n \leq 0.5$, the swelling and porosity provided a partial diffusion mechanism [29].

Food protection test. In Fig. 7, the endocarp of the control sample (uncoated avocado) is brown around the stone, and so is the sample coated with the base film

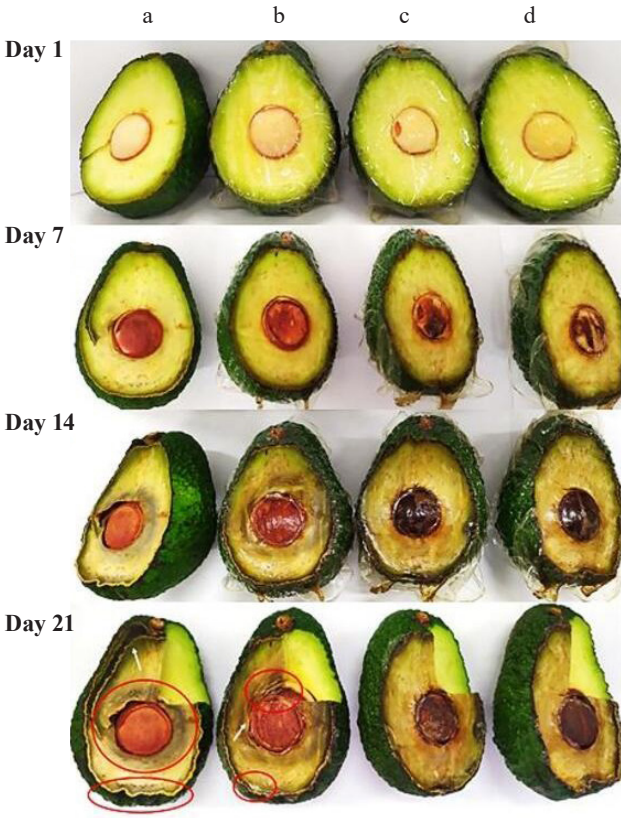


Figure 7 Hass avocado halves on days 1, 7 and 14 of storage. Epicarp, mesocarp, and endocarp of Hass avocado on day 21. (a) Uncoated (control); (b) coated with the base film (no extract); (c) coated with the film from sodium alginate with the 3% *Macrocystis pyrifera* hydroalcoholic extract; and (d) coated with the film from sodium alginate with the 6% extract

(no *M. pyrifera* extract). However, as the concentration of the hydroalcoholic extract increased, the browning intensity around the stone decreased. As a result, the browning in the sample covered with the film with the 6% extract was less intense in color, compared to the avocado covered with the film with the 3% extract. This result proves that the extract migrated from the film onto the fruit surface.

On day 21, none of the samples showed any evidence of browning. Therefore, the interior of the fruit remained intact under the experimental conditions of 8°C and 50–60% relative humidity. However, the epicarp in the control sample and the avocado coated with the base film had a little mold caused by humidity, which was absent in the samples coated with the films with the *M. pyrifera* extracts.

CONCLUSION

The hydroalcoholic extracts interacted with the polymeric matrix of sodium alginate. The increase in their concentration affected the surface and the microstructure of the films, resulting in a greater mass loss during degradation, a more intense opacity, and a lower crystallinity percentage. However, when the concentration was lower, it facilitated the distribution within the polymeric matrix. Alginate films proved to be good vehicles for the administration and release of *Macrocystis pyrifera* extracts. If used as fruit coating, this film can reduce browning.

CONTRIBUTION

The authors were equally involved in the written and experimental part of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

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

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Sesame seed protein: Amino acid, functional, and physicochemical profiles

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Abstract:
Sesame (*Sesamum indicum* L.) is an erect herbaceous annual plant with flat seeds. It is one of the oldest cultivated oilseed plants in the world, especially popular in Africa and Asia.
The present research objective was to describe a sesame protein isolate, i.e., its amino acid profile, functional and physicochemical properties, zeta potential, and hydrodynamic diameter. The surface charge and hydrodynamic diameter in aqueous solutions were obtained for standard sesame seeds, defatted sesame seeds, and the sesame protein isolate.
Defatted sesame seeds yielded the following optimal parameters: salt concentration – 0.6 M, pH – 7, iso-electric point (pI) – 4. The sesame protein isolate was rich in methionine content, which is rare in other plant proteins, but its lysine content was lower than in other isolates. The sesame protein isolate displayed almost identical zeta potential profiles with its pH. The decreasing pH increased the zeta values gradually from the lowest negative value to the highest positive value. The zeta potentials of standard and defatted sesame seeds at pH 7 were –23.53 and –17.30, respectively. The hydrodynamic diameter of the sesame protein isolate (0.33 μm) was smaller than that of sesame seeds (2.64 μm) and defatted sesame seeds (3.02 μm). The sesame protein isolate had a water holding capacity of 1.26 g/g and an oil holding capacity of 3.40 g/g. Its emulsifying properties looked as follows: emulsion capacity – 51.32%, emulsion stability – 49.50%, emulsion activity index – 12.86 m^2/g, and emulsion stability index – 44.96 min, respectively. These values are suitable for the sesame protein isolate and are consistent with the literature.
The sesame protein isolate was a good source of protein (88.98%). Using sesame proteins as functional components can be an important basis for better knowledge of the relationship between electrical charge interactions in food matrices and the structure, stability, shelf life, texture, structural and functional properties of food. Research prospects include the effects of sesame protein isolates on various food systems.

Keywords. Sesame protein isolate, amino acid profile, zeta potential, functional properties, solubility

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INTRODUCTION

Sesame (*Sesamum indicum* L.) is an erect herbaceous annual plant. Its seeds are considered one of the oldest oilseed plants in human history. For instance, peoples of Africa and Asia have cultivated them for many centuries. Nowadays, sesame seeds and oil are used in food production and different industries. Global sesame production was approximately 2.5 million tons in 1997 and 6.5 million tons in 2019 [1].

Sesame seeds are remarkably rich in oil and protein. They contain 45.46–59.28% oil, 21.43–25.77% protein, 2.70–5.10% moisture, 2.89–5.44% ash, 3.20–7.31% fiber, and 4.33–19.33% carbohydrates [2, 3]. The chemical composition of sesame seeds varies according to many factors, e.g., genetics, climate, environment, growth, maturation stage, harvest time, analytical methods, etc. [4].

Sesame proteins have a high potential to be used as an ingredient in the food industry because they increase the nutritional value of foods. One of the main properties of sesame proteins is that they contain such essential amino acids as tryptophan and methionine.

Sesame flour and protein isolates are rich in sulfur-containing amino acids (3.8–5.5%) with methionine prevailing (2.5–4.0%) [5–7]. Most plant proteins, e.g., soy protein, wheat flour, barley grain, ground rice, cornmeal, etc., contain low sulfur amino acids. Therefore, sesame proteins are unique because they contain high amounts of sulfur amino acids [4]. As a result, sesame proteins can serve as a complementary food to legumes and cereals. The polymeric structure of sesame proteins also improves the textural and rheological properties of the food they complement by increasing the acceptability of the final product [8].

Emulsifying capacity and emulsion stability are essential parameters in selecting a protein for particular industrial processes. Proteins prevent coalescence and reduce tension of the water-oil interface. The stabilizing effect of a protein in an emulsion comes from the membrane matrix that surrounds the oil droplet and prevents it from coalescing [5]. Proteins also have a good oil and water holding capacity, foaming stability, and solubility. The importance of these characteristics depends on the type of food product where the protein isolate is used. For example, high foaming and emulsifying protein isolates are preferred in salad dressings and soups.

In contrast, protein isolates with high water and fat holding capacity are preferred in products that contain meat fat [4]. Onsaard *et al.* reported that the emulsifying properties and solubility of sesame protein isolates can be used to replace soy protein isolates [9]. Achouri *et al.* found that sesame protein isolates had lower water retention and oil holding capacities than soy protein isolates [3]. They also proved that the foaming and emulsifying properties of sesame protein isolates are pH-dependent.

Sesame protein isolates or concentrates are easy to obtain by the isoelectric precipitation method. Due to its different functional properties and easy extraction procedures, sesame protein has become a popular commercial protein with a wide range of applications. Sesame protein isolates have a good heat stability and high molecular weight, which makes them suitable for film-forming utilization [10]. However, very few publications feature the zeta potential of sesame protein isolates and the average particle size (hydrodynamic diameter) in an aqueous solution. Most of them focus on the zeta potential and hydrodynamic size measurement of emulsions produced from sesame protein isolates [11, 12].

The industrial role of sesame seeds as a potential source of protein is growing. As a result, composition, physicochemical profile, and functional properties of sesame-isolated proteins are attracting more and more scientific attention. For proper industrial use, isolated proteins need to have a proper effect on specific processed foods. Therefore, food science needs more data on utilization of a specific protein [6]. For instance, very little information is available about how extraction conditions, e.g., salt concentration and pH, affect the extractability of proteins from sesame seeds.

Therefore, the main objectives of this study were as follows: (I) to investigate the extractability of proteins from sesame seeds in water and at different NaCl concentrations (0, 0.3, 0.6, 0.9, and 1.2 M) as a function of pH; (II) to describe the sesame protein isolate and to examine some of its functional properties; (III) to investigate the zeta potential and particle size of the sesame protein isolate in aqueous solution, (IV) to examine the amino acid profile of the sesame protein isolate.

STUDY OBJECTS AND METHODS

Materials and chemicals. The sesame (*Sesamum indicum* L.) seeds were supplied by Pro Gıda, Olam Group Co (Samsun, Turkey). The corn oil was purchased from a local market (Samsun, Turkey). The diethyl ether and sodium hydroxide (NaOH) were obtained from Isolab (Wertheim, Germany). All other chemicals and standards were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (USA). A Milli-Q water purification system (Millipore, Massachusetts, USA) was used to obtain deionized water.

Optimal pH, NaCl concentration, and iso-electric point for protein extractability. Sesame seeds were milled in a kitchen blender (AR1133, Arzum, Turkey) and defatted by constant stirring with diethyl ether (1:4 w/v, sesame/solvent) at room temperature for 3 h. The resulting mix was defatted four more times using the same sesame/solvent proportion. After that, the defatted sesame was filtered using filter paper and dried at 40°C overnight [9]. After drying, the defatted sesame seeds were used for protein extraction.

In order to find the optimal conditions for protein isolation, we determined the optimal salt concentration and pH by modifying the method described by Achouri *et al.* in [3]. Dispersions of defatted sesame seeds (10% w/v) were prepared using different concentrations of sodium chloride (0, 0.3, 0.6, 0.9, and 1.2 M) and shaken at 300 pm at 35°C for 2 h. The dispersions were divided into test tubes by approximately 10 mL. There, their pH was adjusted from 2 to 11 (in 1 unit increments) using 1N HCl or 1N NaOH. The dispersions were continuously stirred at room temperature for 2 h, and their pH was kept at the desired level (2–11). The samples were sonicated for 30 min and centrifuged at 2060×g for 30 min (NF 1200R, Nuve, Ankara, Turkey).

Then, 0.1 mL was taken from the supernatant and added to 3 mL of Bradford's reagent. After 5 min, the absorbance of the mix was registered using a T80+ spectrophotometer (PG Instruments Limited, Leicestershire, UK) at 595 nm wavelength. The pH and salt concentration of the samples with the highest protein content were chosen as optimal conditions to maximize protein isolation.

The iso-electric point (pI) precipitation method was used for protein isolation. The most appropriate pI was determined by the method described by Yuzer *et al.* in [13]. First, a 10% sample dispersion was prepared with the optimal concentration of NaCl solution and adjusted to the optimal pH value for extraction. The dispersion was shaken at 300 rpm for 2 h. After shaking, 10 mL of the liquid part from each sample was poured into six centrifuge tubes. Their pH values were adjusted between 3.5–6.0 with a 0.5-unit change and vortexed for 1 min in a WN-2800 Vortex Mixer (Weightlab Instruments, Istanbul, Turkey). The samples were centrifuged at 3000×g 4°C for 20 min. The protein amount in the supernatants was determined using the Bradford method. The pH value with the lowest protein amount was determined as the iso-electric point of the sample.

Preparation of the sesame protein isolate. The optimal salt concentration and pH values were determined as 0.6 and 7 M, respectively (Fig. 1), to produce a 1:10 (w/v) sample suspension. The suspension was stirred at 35°C at 300 rpm for 2 h. The supernatant portion containing the soluble proteins was obtained by filtration. In order to increase the protein amount and yield, we mixed the remaining solid using the same salt solution and pH and removed the supernatant by filtration.

Afterward, the pH values of the samples were adjusted to the isoelectric point (pH 4, Fig. 1). The proteins were allowed to precipitate and kept at +4°C for 18 h. At the end of the period, the supernatant was removed by centrifugation at 3000×g at 4°C for 30 min to recover the precipitated proteins. The resulting precipitates were washed with distilled water. The pH was then neutralized to 7. The protein isolates were dried with a lyophilizer at –105°C and 100 mbar (ScanVac Coolsafe 110-4 Pro, Labogene, Lynge, Denmark). The resulting sesame protein isolate was stored at –20°C. The protein yield, %, was calculated as follows [3]:

$$\text{Protein yield} = \frac{w_{SPI} \times PA_{SPI}}{w_{DSS} \times PA_{DSS}} \times 100 \quad (1)$$

where SPI is the sesame protein isolate; DSS is the defatted sesame seeds; *w* is the weight, g; PA is the protein amount, %.

Proximate composition and pH analysis. Standard sesame seeds, defatted sesame seeds, and sesame protein isolates were tested for moisture, crude protein (N×6.25), ash, and crude fat according to the AOAC methods [14]. The pH values were determined by a Starter 2100 pH-meter (OHAUS, USA) as follows: 10% (w/v) dispersions of each sample were tested in triplicates.

Zeta potential and hydrodynamic size. The hydrodynamic size (μm) of the samples was determined using a Malvern Zetasizer Nano Zetasizer (Malvern Instruments, Malvern, UK). The device employed the dynamic light scattering principle with refractive indices 1.330 and 1.476 for water and sesame,

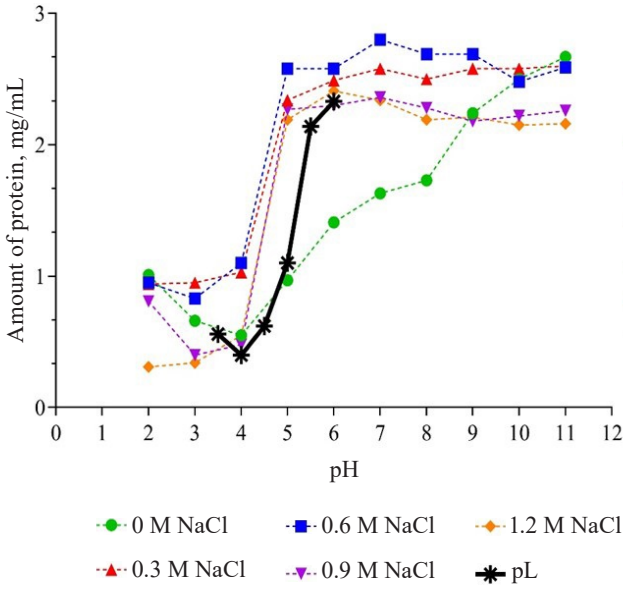

Figure 1 Optimal isoelectric point, salt concentrations, and pH values in sesame protein isolation

respectively. The zeta potential (mV) was measured with the same equipment based on the electrophoretic action of protein solutions. We prepared 0.05% (w/v) protein solutions of the sesame protein isolate with distilled water. Their pH was adjusted from 2 to 11 with 1N HCl or 1N NaOH. For standard sesame seeds and defatted sesame seeds, the sample dispersions contained 0.1% (w/v) deionized water. Their pH was adjusted to 7 with 1N HCl or 1N NaOH. Each sample was homogenized and measured in triplicates.

Amino acid analysis. The amino acid analysis of the sesame protein isolate relied on a liquid chromatography tandem mass spectrometry (LC-MS/MS). The Agilent Infinity 1260 HPLC system consisted of a binary pump, a degasser, and an autosampler coupled with an Agilent 6460 Triple Quad Mass Spectrometer (Agilent Technologies, CA, USA) using the method described by Bilgin *et al.* in [15]. We used a Jasem LC-MS/MS amino acid analysis kit (Sem Laboratories Devices Pazarlama San. ve Tic. Inc., Istanbul, Turkey) with a modified sample preparation procedure to measure amino acid concentrations.

The samples were hydrolyzed as follows. We placed 0.1 g of sample in a screw-cap glass tube, added 4 mL of acidic hydrolysis reagent, and hydrolyzed it at 110°C for 24 h. When the hydrolysate was cooled to room temperature, it was centrifuged at 4000 rpm for 5 min. Then, 100 μL of supernatant was poured into a vial with 900 μL of distilled water. This dilution process was repeated once more to obtain a 4000-fold diluted hydrolysate of the sample. Following the hydrolysis process, the kit sample preparation was applied as follows. We poured 50 μL of diluted hydrolysate into a vial and added 50 μL of the stable isotope-labeled internal standard mix and 700 μL of reagent 1. The mix was vortexed for 5 s. After all the samples underwent

the same procedure, they were injected into the LC-MS/MS system. The calibration curve required for quantifying amino acids was obtained by preparing the five-point calibration set in accordance with the kit sample preparation without hydrolysis and reading in the LC-MS/MS system.

We injected 3 μL of the prepared sample into the Jasem amino acid analytical column set at 30°C in HPLC. The chromatographic separation analysis took 7.5 min with mobile phase A and B gradient programmed for 0.7 mL/min. The tandem electrospray ionization mass spectrometer performed mass spectrometric detection in the positive ionization mode. The mass detector parameters were as follows: gas temperature – 150°C, gas flow – 10 L/min, capillary voltage – +2000 V, and nebulizer pressure – 40 psi.

Scanning electron microscopy (SEM). The surface morphology of the sesame protein was determined by a scanning electron microscope (SEM, JSM-700 LF JEOL, Japan) at a voltage acceleration of 10 kV. In order to increase the electrical conductivity of the samples and obtain a clearer image, the surface of the samples was coated with a 10-nm gold-palladium alloy (Quorum SC7620, UK) before measurement. The scanning electron microscopy images had three different magnifications: 5000×, 10 000×, and 30 000×.

X-ray diffraction (XRD). The X-ray diffraction analysis (Rigaku Smartlab, Germany) was performed to investigate the crystallinity of the samples. The analysis conditions were as follows: room temperature, 2 θ from 5 to 40°, scanning rate – 1°/min, step size – 0.01°, power – 40 kV, Cu Kα radiation – 30 mA [11].

Fourier transform infrared spectroscopy (FTIR). Fourier transformed infrared spectroscopy determined the functional groups of the samples and their interactions. The secondary structure of sesame protein isolates was determined using a Platinum ATR equipped with a FTIR Tensor 27 spectrophotometer (Bruker Optics Inc., USA). The measurements were made at the wavelength range between 400 and 4000 cm^{-1}.

Differential scanning calorimetry (DSC). The thermal properties of the samples were studied by differential scanning calorimetry (DSCQ2000, TA Instruments, USA). Approximately 10 mg of each sample was hermetically sealed in aluminum containers. The samples were then analyzed under the following conditions: nitrogen atmosphere, flow rate – 30 mL/min, heating rate – 10°C/min between 25 and 400°C [16]. An empty aluminum pan served as a reference. The initial temperature (T_0), peak temperature (T_p), and reaction enthalpy (ΔH) of the samples were determined using Universal Analysis 2000, version 4.5A software (TA Instruments, USA).

Thermogravimetric analysis (TGA). The thermal stability of the samples was evaluated using thermogravimetry (Q600 SDT, TA Instruments, USA). Approximately 10 mg of each sample in platinum cups was studied under the following conditions: nitrogen atmosphere, heating rate – 10°C/min between 30 and 600°C, flow rate – 50 mL/min. An empty platinum capsule served as a reference [17]. The results were evaluated using Universal Analysis 2000 software 4.5A (TA Instruments, USA).

Solubility. Solubility percentage was determined as a function of pH (2–11). We prepared 0.5% (w/v) protein dispersions of the sesame protein isolate with deionized water. The pH of the dispersions was adjusted 2 to 11 using 1N HCl or 1N NaOH. The suspensions were stirred in a magnetic stirrer for 1 h at ambient temperature. Then, the suspensions were centrifuged at 3000×g at room temperature for 30 min [18]. After centrifugation, we performed the protein analysis in the supernatant using the Bradford method. The bovine serum albumin standard curve was used to calculate the amount of protein. The solubility, %, was found according to Eq. (2):

$$\text{Solubility} = \frac{\textit{Protein content in supernatant}}{\textit{Total protein content in sample}} \times 100 \qquad (2)$$

Water and oil holding capacities. Approximately 50 mg of the protein isolate was dispersed in a 2-mL centrifuge tube by adding 1.5 mL of distilled water or commercial corn oil and mixed with a vortex at room temperature for 20 s. After mixing, the tubes were capped and held at room temperature for 30 min. The tubes were centrifuged at 14 000 rpm for 20 min. After the supernatant was carefully decanted at a 45° angle, we weighed the centrifuge tube with the sediment. The water or oil retained content was determined by weighing the tubes with the sediment. The water (WHC) and oil (OHC) holding capacities were expressed as 1 g of water or oil absorbed per 1 g of the sesame protein isolate, respectively. They were found according to Eq. (3):

$$\text{WHC or OHC} = \frac{M_2 - M_1}{M_0} \qquad (3)$$

where M_0 is the weight of the sample; M_1 is the weight of the centrifuge tube; M_2 is the weight of the centrifuge tube after the oil or water has been removed [18].

Emulsifying properties. We diluted 0.25 g of the protein isolate with 5 mL of distilled water. The pH value was adjusted to 7.0 using 1N HCl or 1N NaOH. After that, we added 5 mL of corn oil to the protein solution and mixed using a vortex for 1 min. Afterward, the emulsion was centrifuged at 1100×g for 5 min, and the emulsion capacity (EC, %) was determined as in Eq. 4. To define the emulsion stability, the samples were heated in a water bath at 80°C for 30 min and cooled rapidly under running water and ice. The samples were again centrifuged at 2291×g for 5 min, and emulsion stability (ES, %) was calculated as in Eq. (5) [19]:

$$\text{EC} = \frac{\textit{Height of emulsified layer in the tube}}{\textit{Height of the total contents in the tube}} \times 100 \qquad (4)$$

Table 1 Proximate composition, pH, zeta potential, and particle size of standard sesame seeds, defatted sesame seeds, protein isolates, and protein extraction yield (n = 3)

Sample	Moisture, %	Protein, %	Crude fat, %	Ash, %	pH	Zeta potential, mV	Perticle size, μm	Protein extraction yield, %
Sesame seeds	2.68 ± 0.01	25.55 ± 0.14	56.83 ± 0.45	2.96 ± 0.02	6.41 ± 0.03	–23.53 ± 0.45	2.64 ± 0.49	–
Defatted sesame seeds	2.14 ± 0.22	57.37 ± 0.23	6.64 ± 0.18	5.94 ± 0.25	6.23 ± 0.01	–17.30 ± 1.28	3.02 ± 0.98	–
Protein isolate	1.26 ± 0.06	88.98 ± 0.13	0.82 ± 0.25	–	6.98 ± 0.19	–14.20 ± 1.01	0.33 ± 0.05	43.76

Data represent as mean ± SD. Means with different letters in the same column are significantly different ($p < 0.05$)

$$ES = \frac{Height\ of\ emulsified\ layer\ after\ heating}{Height\ of\ the\ emulsified\ layer\ before\ heating} \times 100 \quad (5)$$

The emulsion activity index and emulsion stability index were determined according to the method developed by You *et al.* [20]. We mixed 300 mg of the protein isolate sample with 30 mL of purified water (1% protein dispersion) and then added 10 mL of corn oil. The pH of the mix was adjusted to 7 with 1N HCl or 1N NaOH. The mix went through a homogenizer (Ultra-Turrax T25 digital, IKA, Staufen, Germany) at 20 000 rpm for 1 min. Immediately after the emulsion was formed, 50 μL of emulsion sample from its lower part (liquid phase) was put into a tube and diluted by adding 5 mL of a 0.1% (w/v) sodium dodecyl sulfate (SDS) solution. The absorbance of the resulting mix at 500 nm was measured in the spectrophotometer. The emulsion activity index (EAI, m^2/g) was calculated as in Eq. (6):

$$EAI = \frac{2 \times 2.303 \times A_0 \times N}{c \times \phi \times 10000} \quad (6)$$

where, A_0 is the absorbance at 0 min; N is the dilution factor (100); c is the concentration of the protein dispersion (0.01 g/mL); ϕ is the volumetric fraction of the oil (10/40 = 0.25).

After 10 min, 50 μL of the emulsion was taken from its lower part (liquid phase) and diluted with 5 mL of a 0.1% (w/v) sodium dodecyl sulfate solution. The absorbance registered at 500 nm was used in Eq. (7) to calculate the emulsion stability index (ESI, min):

$$ESI = \frac{A_0 \times t}{A_0 - A_{10}} \quad (7)$$

where, A_{10} is the absorbance at 10 min after the homogenization process; t is the holding time of the emulsion (10 min).

Foaming properties. We diluted 3% (w/v) dispersions of the sample with distilled water and adjusted to pH 7 using 1 N HCl or 1 N NaOH. Then the mix was homogenized at 11 000 rpm for 2 min using an Ultra-Turrax homogenizer. The mix was immediately transferred to a 100-mL graduated cylinder. We recorded the total volumes, liquid volumes, and volumes of the remaining foams after 10 and 30 min of storage at room temperature. The foaming capacity (FC, %) and foaming stability (FS, %) were calculated using Eqs. (8) and (9) [18]:

$$FC = \frac{V_1 - V_0}{V_0} \times 100 \quad (8)$$

$$FS = \frac{V_2}{V_1} \times 100 \quad (9)$$

where V_1 is the volume of foam instantly after homogenization; V_0 is the volume before homogenization; V_2 is the volume of foam remaining after 10 and 30 min at room temperature.

Statistical analysis. We used SPSS Statistics 26.0 (IBM, New York, USA) to evaluate the data and Duncan's multiple comparison test to evaluate significant ($p < 0.05$) differences.

RESULTS AND DISCUSSION

Proximate composition and pH analysis. Table 1 illustrates the proximate composition of standard sesame (*Sesamum indicum* L.) seeds, defatted sesame seeds, sesame protein isolate, and protein extraction yield. Defatted sesame seeds had a greater content of ash and protein. The protein content rose from 25.55% in the standard sesame seeds to 57.37% in the defatted sesame seeds. Probably, the ash content increased as a result of the proportional decrease in the amount of crude fat. The crude fat content of the sesame seeds decreased because it was affected, first, by the solvent during the defatting process and, second, by the further separation of oil during centrifugation. As a result, the crude fat content of the standard sesame seeds, defatted sesame seeds, and sesame protein isolate were 56.83, 6.64, and 0.82%, respectively. The moisture content of the samples varied between 1.26 and 2.68%. These results confirmed those reported in [2, 3, 21]. The protein content of the sesame protein isolate was 88.98%. It was slightly lower than 90.50% achieved by Sharma *et al.*, but higher than 86.33% obtained by Fathi *et al.* and 81.66% obtained by Saatchi *et al.* [6, 10, 12]. The protein extraction yield was determined as 43.76% according to the extraction conditions used in this study.

The pH values of the standard sesame seeds, defatted sesame seeds, and sesame protein isolates were 6.41, 6.23, and 6.98, respectively (Table 1). An insignificant change in pH occurred between standard and defatted

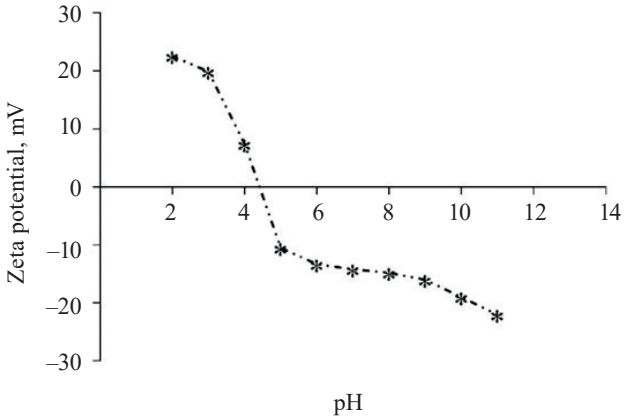

Figure 2 Effect of pH on the zeta potential of the sesame protein isolate

sesame seeds ($p > 0.05$), followed by an increase in the sesame protein isolates as a result of the neutralization in the last stages of isolate production. Unfortunately, very few publications are available on the pH of sesame and its products.

Zeta potential and hydrodynamic size. Food systems include electrically charged particles that interact with the environment and with each other. Electric charge interactions significantly affect food structure, stability, rheological behavior, precipitation, texture, color, shelf life, and flavor. The zeta potential (ζ) is one of the most practical characteristics in investigating electrical relations in food matrices. Functional characteristics of proteins are affected by their foaming and emulsifying capacity, solubility, gelation, surface activity, conformational stability, and interaction with polysaccharides. The zeta potential is important for determining the aggregation and interactions of compounds [22].

Figure 2 shows the zeta potential of the sesame protein isolates at pH 2–11. The aqueous solution of the sesame protein isolate had a neutral charge at pH 4–5. This range included the iso-electric point pH 4 determined for precipitation during the isolation process. The sesame protein isolate displayed almost identical zeta potential profiles with its pH. As the pH increased from 2 to 11, the zeta values gradually changed from the highest (positive) value to the lowest (negative) value. This result was consistent with the fact that the surface charge can change gradually from negative to positive due to the gradual protonation of carboxyl groups and deprotonation of amino groups of proteins [23]. The zeta potential of standard and defatted sesame seeds at pH 7 were –23.53 and –17.30 mV, respectively (Table 1). We found no previous publications on the surface charge in aqueous solutions of standard and defatted sesame seeds, which makes this research relevant. The obtained data revealed how electrical charge interactions in sesame seeds and sesame-containing food components may affect the structure, stability, shelf life, and texture of the finished product.

Table 2 Amino acid composition of the sesame protein isolates

Amino acid	Amount, g/100 g
Essential amino acids	
Phenylalanine	4.47 ± 0.00
Isoleucine	2.32 ± 0.01
Leucine	5.68 ± 0.00
Lysine	2.82 ± 0.01
Methionine	2.78 ± 0.00
Threonine	3.45 ± 0.00
Valine	3.52 ± 0.00
Histidine	2.51 ± 0.01
Non-essential amino acids	
Arginine	11.86 ± 0.01
Alanine	4.33 ± 0.00
Aspartic acid	6.92 ± 0.00
Cysteine	1.57 ± 0.00
Glutamic acid	17.78 ± 0.02
Glycine	2.26 ± 0.00
Proline	3.55 ± 0.00
Serine	4.21 ± 0.00
Tyrosine	2.90 ± 0.00
Total non-essential amino acids	55.38
Total essential amino acids	27.55
Total acidic amino acids[a]	24.70
Total basic amino acids[b]	17.19
Total sulfur amino acids[c]	4.35

Data represent a mean ± SD (n = 2)

[a] Acidic amino acids: aspartic acid and glutamic acid

[b] Basic amino acids: histidine, lysine, and arginine

[c] Sulfur amino acids: methionine and cysteine

Table 1 illustrates the hydrodynamic dimension measurements for the standard sesame seeds, defatted sesame seeds, and sesame protein isolates. The functionality and emulsifying properties of proteins depend on their molecular size [24]. The hydrodynamic diameter of the sesame protein isolates (0.33 μm) was smaller than that of standard sesame seeds (2.64 μm) and defatted sesame seeds (3.02 μm). Ordinary and defatted sesame seeds may have formed larger aggregates through strong hydrophobic interactions in water. These values were similar to those reported in previous scientific publications. For instance, Rahmati *et al.* determined the hydrodynamic dimensions between 100–200 nm at pH 7 in bean protein isolates [24]. Mozafarpour *et al.* reported the hydrodynamic radius of soy protein isolates as 0.32–0.68 μm [25].

Amino acid composition. Table 2 shows the amino acid composition of the sesame protein isolates. Glutamic acid, arginine, and aspartic acid were 17.78, 11.86, and 6.92 g/100 g, respectively. They proved to be the predominant amino acids in the isolate, which confirmed other sesame protein studies [11, 26–29]. Leucine (5.68 g/100 g) appeared to be the most abundant essential amino acid, which approximated the results reported by Yang *et al.* and Lawal *et al.* [27, 28]. The

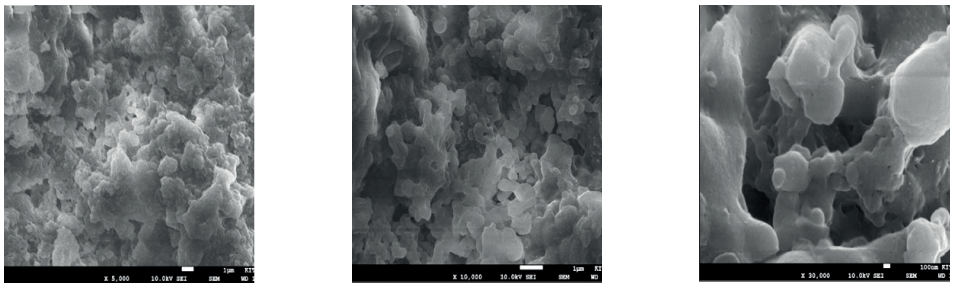

Figure 3 Scanning electron microscopy of the protein isolate: (a) 5000× magnification; (b) 10 000× magnification; (c) 30 000× magnification

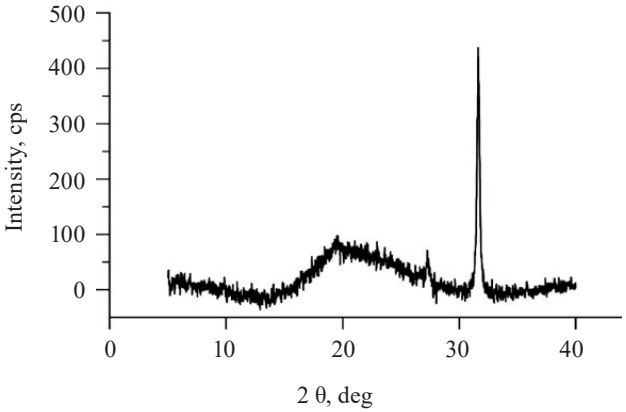

Figure 4 X-ray diffraction pattern of the sesame protein isolate

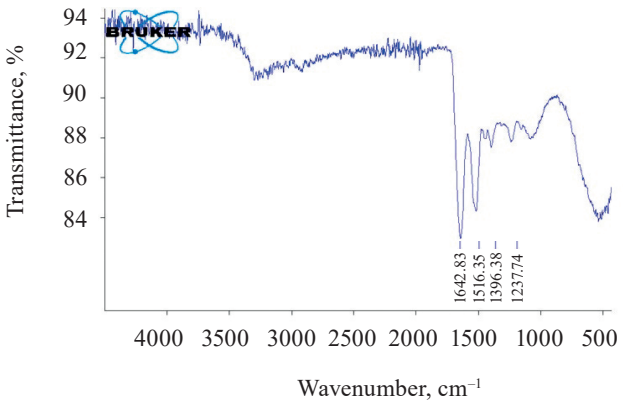

Figure 5 Fourier transform infrared spectroscopy of the sesame protein isolate

sesame protein isolates also contained sulfurous amino acids, primarily methionine, which are rarely found in other plant proteins. However, methionine was more abundant than cysteine, which was also observed by Saatchi *et al.* and Fasuan *et al.* [11, 26].

The sesame protein isolates owed their acidic quality to their high acidic/basic amino acid ratio. The fact that the iso-electric point of the protein is in the acidic pH region can be attributed to the high amount of acidic amino acids, e.g., glutamic and aspartic acids, in its content (Figs. 2 and 5).

The sesame protein isolate was richer in such essential amino acids as phenylalanine, methionine, threonine, valine, and histidine than hemp, soy, and pea proteins reported by Gorissen *et al.* [30]. They also had more leucine and isoleucine than hemp and soy proteins but less than pea protein. However, the sesame protein isolates were insufficient in lysine compared to soy and pea proteins.

Scanning electron microscopy. The structural morphology of the sesame protein isolate was examined with the aid of scanning electron microscopy at three different magnifications (Fig. 3). The particles of the sesame protein isolate powder were irregular in shape and not uniform. The surface was wrinkled, and the particles were agglomerated. Saini *et al.* also reported that the alkali extraction and iso-electric precipitation technique applied to sesame protein isolates transformed the microstructure of the protein into a compact structure with a wrinkled surfacee [7].

X-ray diffraction (XRD). Figure 4 shows the crystal structure of the sesame protein isolate obtained by X-ray diffraction. The image revealed a broad and diffuse background at 19.5°, which means that the major structure of the isolate was amorphous. Other sharp peaks (2θ = 27.3° and 31.6°) could be attributed to minerals or other compounds remaining after protein isolation. These results were consistent with those reported in previous publications [7, 11, 31, 32].

Fourier transform infrared spectroscopy (FTIR). Figure 5 shows the spectra demonstrated by the Fourier transform infrared spectroscopy. The obtained spectra made it possible to determine the functional groups. The sesame protein isolate showed some typical features of the protein spectrum, namely Amide I (1642 cm^{-1}), Amid II (1516 cm^{-1}), and Amid III (1238 and 1396 cm^{-1}) that result from the stretching and bending vibrations, typical of the protein backbone [12, 27, 33]. The characteristic peaks in Amid I and Amid II were predominantly due to N-H stretching vibrations. In Amid III, they were attributed to C-N stretching and N-H bending vibrations [12, 27]. In particular, the density of the Amid I group was stronger than that of Amid II and Amid III. Saatchi *et al.* observed the Amid I, Amid II, and Amid III bands of sesame protein concentrate at 1635, 1518, and 1234 cm^{-1},

Table 3 Differential scanning calorimetry and thermogravimetric analysis results of the sesame protein isolates

	Glass transition temperature (T_g), °C	Denaturation onset temperature (T_0), °C	Peak or denaturation temperature (T_p), °C	Enthalpy of denaturation (ΔH), J/g	Thermal decomposition initial temperature (T_{d1})*, °C	Weight loss (Δ_{W1})*	Thermal decomposition initial temperature (T_{d2})**, °C	Weight loss (Δ_{W2})**	Residue, %
Protein isolate	85.56	88.12	122.56	28.47	31.71	3.80	203.81	62.56	33.43

Data represent as mean ± SD (n = 4)

*first stage of degradation, **second stage of degradation

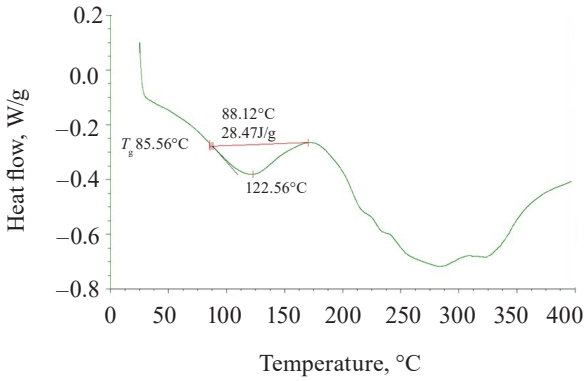

Figure 6 Differential scanning calorimetry of the sesame protein isolate

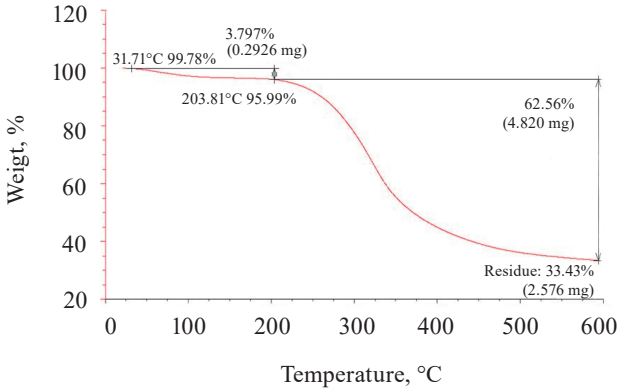

Figure 7 Thermogravimetric analysis of the sesame protein isolate

respectively [12]. These results are very similar to the Fourier transform infrared spectroscopy results obtained for the sesame protein isolate in the current study. Our results were also compatible with other studies [7, 27].

Differential scanning calorimetry (DSC). Differential scanning calorimetry is a convenient method to evaluate protein thermal stability and conformational changes. Figure 6 shows differential scanning calorimetry results for the sesame protein isolate. In our study, the thermograms exhibited endothermic peaks, which are usually attributed to protein denaturation [18].

For the sesame protein isolate, T_0 started at 88.12°C, and peak temperature T_p was at 122.56°C, while the ΔH value was 28.47 J/g. The amorphous fractions of the protein formed a glass transition (T_g), and the T_g value was 85.56°C. Available publications on the thermal stability of sesame proteins give a wide range of results. Sharma and Singh determined the T_g value of the sesame protein isolate as 37°C, T_0 as 98.25°C, and T_p as 207°C [32]. Saini *et al.* reported T_0 as 176.07°C, T_p as 210.78°C, and ΔH as 63.72 J/g [7]. They attributed the high denaturation temperature to higher non-polar residues in the proteins. However, Saatchi *et al.* reported the T_g temperature of the sesame protein concentrates as 100.54°C and the ΔH value as 51.10 J/g [11]. Yang *et al.* determined T_0 as 55.55–61.26°C, T_p as 112.39–113.69°C, and ΔH as 203.4–220.8 J/g [27].

Thermogravimetric analysis (TGA). Thermogravimetry presents the change in mass with progression in temperature and, therefore, can determine the physical and chemical structural changes during the thermal conversion of biomass to food products [27, 34]. Table 3 describes the thermogravimetric analysis while Fig. 7 illustrates the thermogram of the sesame protein isolate. The samples exhibited weight loss in two stages. A slight weight loss was observed in the samples at temperatures below 100°C, probably, due to water removal. The second and significant primary decrease in the mass of the samples was observed in the range of 250–450°C.

For the sesame protein isolate, the first degradation started at 31.71°C with a weight loss of 3.80%, while the second degradation started at 203.81°C with a weight loss of 62.56%. The weight loss below 100°C could be attributed to the water loss and the decomposition of the quaternary structure of proteins. Temperatures above 100°C are known to denature protein subunits and promote protein aggregate formation through electrostatic, hydrophobic, and disulfide exchange binding mechanisms [35]. At 203°C, the weight loss resulted mainly from the cleavage of the covalent bond between the peptide bonds of amino acids. Further heating caused dissociation of *α*-globulins (2S), *β*-globulins (11S), and the secondary structure opening in the subunit.

The sesame protein isolate was rich in sulfur-containing amino acids methionine and cysteine, which decomposed between 220 and 250°C. The weight loss continued at around 300°C, probably, due to the splitting of S–S, O–N, and O–O junctions [7]. Our thermogravimetry results were consistent with those

Table 4 Water and oil holding capacity, as well as foaming and emulsifying properties of the sesame protein isolates

	Water holding capacity, g/g	Oil holding capacity, g/g	Emulsion capacity, %	Emulsion stability, %	Emulsion activity index, m^2/g	Emulsion stability index, min	Foaming capacity, %	Foaming stability after 10 min, %	Foaming stability after 30 min, %
Protein isolate	1.26 ± 0.04	3.40 ± 0.26	51.32 ± 1.86	49.50 ± 0.71	12.86 ± 0.89	44.96 ± 0.01	36.21 ± 2.44	22.79 ± 0.41	20.26 ± 0.36

Data represent as mean ± SD (n = 4)

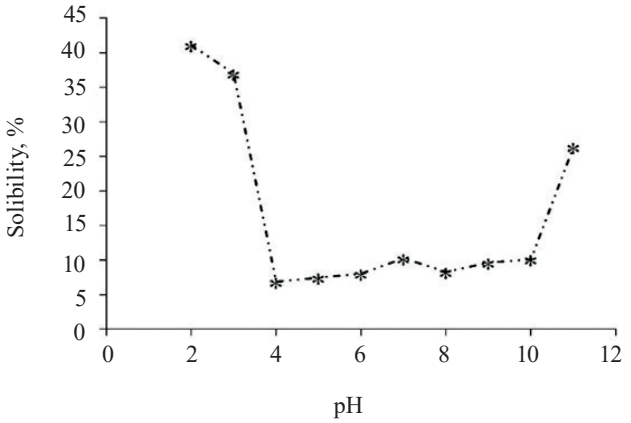

Figure 8 Effect of pH on solubility of the sesame protein isolate

obtained by Saini *et al*., Yang *et al*., and Sharma and Singh [7, 27, 32]. Yang *et al.* reported a two-stage weight loss [27]. They attributed the first weight loss to water evaporation at 59.02°C, while the second weight loss, which occurred at 182.58°C, was probably caused by protein weight loss.

Solubility. Figure 8 shows the protein solubility profile for the sesame protein isolate. The pH-solubility profile was similar for all proteins, and the solubility in water had a typical U-shaped curve. The sesame protein isolate showed minimal solubility in the isoelectric point region (pH 4–5, Fig. 2). The low protein solubility in the region close to the iso-electric point (pI) values could be attributed to the low net charge in this region. The solubility increased as the pH moved away from the isoelectric point. At pH = 2 and 3, the solubility for the sesame protein isolate was at its highest: 41.11 and 36.99%, respectively. The lowest solubility value of 6.87% was registered at pH 4. These results were consistent with those reported by Achouri *et al.* [3]. The solubility remained low at pH 5–10 because high salt concentration (0.6 M NaCl) during protein isolation might contribute to the adsorption of chloride ions by proteins, thus reducing repulsive interactions between protein molecules [3].

Water and oil holding capacities. The water holding capacity of the sesame protein isolate was 1.26 g/g, and the oil holding capacity was 3.40 g/g. The oil holding capacity exceeded the water holding capacity probably because the solubility of the sesame protein isolate was low at neutral pH (10.28%, Fig. 8). Similarly, Gundogan and Can Karaca reported that the water and oil holding capacities of bean protein isolates were between 1.8–2.1 and 4.0–5.4 g/g, respectively [18]. They also reported that protein isolates with a high oil holding capacity could serve as food components to improve such properties as content, consistency, and viscosity in many products.

Emulsifying properties. Table 4 shows such emulsifying properties of sesame protein as emulsion capacity, emulsion stability, emulsion activity index, and emulsion stability index. The emulsion capacity and stability were 51.32 and 49.50%, respectively. Cano-Medina *et al*. reported the highest emulsion capacity values of sesame protein concentrates as 38.0% and the lowest as 19.2% [5]. In the same study, they reported the highest emulsion stability as 50.6% and the lowest as 35.6%. Sharma *et al*. determined the emulsifying activity of sesame protein isolates as 8.70% at pH 9 and 28.96% at pH 12 [6]. Apparently, the differences in the extraction methods and conditions of sesame proteins affect the emulsifying properties.

The emulsion activity index measures the ability of the protein to provide a rapid and adequate coating of the interface area to prevent immediate association and aid in the dispersion of the fat phase [9]. In our study, the emulsion activity index of the sesame protein isolates was 12.86 m^2/g. Onsaard *et al.* reported a similar emulsion activity index (14.95 m^2/g) for sesame protein concentrates extracted at pH 9 [9]. However, they had a higher emulsion activity index for the samples extracted at pH 11 and in salt conditions: 96.66 and 49.70 m^2/g, respectively. Still, our results were consistent with those reported in other studies [3, 26].

Emulsion stability is a time-related property. Interactions of proteins in the oil and aqueous phase affect the stability of the protein film formed at the emulsion interface [9]. Emulsion stability index was 44.96 min, which coincided with the results reported in [9, 26].

However, sesame protein had lower emulsion activity and stability indices compared to other protein isolates, e.g., beans and soy [9, 18]. This result can be explained by the relatively low solubility of sesame protein, compared to that of proteins from other sources.

Foaming properties. Table 4 shows the foaming capacity and stability of the sesame protein isolates at pH 7. The foaming capacity and stability after 10 min were 36.21 and 22.79%, respectively. The foaming

stability after 30 min was 20.26%. Good foamability can be associated with flexible protein molecules that reduce surface tension. In contrast, low foaming may be associated with highly regular globular proteins that resist surface denaturation [9]. Sesame flour has the following protein content: 67.3% globulin, 8.6% albumin, 6.9% glutelin, and 1.4% prolamine. The alkaline protein isolate extracted in water at pH 10 and precipitated at pH 4.0 was reported to contain 41.3% albumin, 41.0% glutelin, 14.8% globulin, and 0.8% prolamin [4]. However, an excess of globular proteins can be cited among the reasons for low foaming capacity and stability of the sesame protein isolates. Previous studies also reported relatively low foaming capacity and stability values of sesame protein isolates and concentrates [6, 9, 26].

CONCLUSION

This study featured sesame (*Sesamum indicum* L.) protein isolates produced from defatted sesame seeds, as well as various properties of standard sesame seeds, defatted sesame seeds, and sesame protein isolates. The extractability of sesame proteins proved sensitive to pH more than to pH 4 and correlated with the increasing NaCl content. The sesame protein isolate appeared to be a good source of protein (88.98%). The sesame protein isolates contained sulfurous amino acids, e.g., methionine (2.78 g/100 g), which is rarely found in plant proteins.

The study is novel in that it reports the zeta potential and hydrodynamic diameter of the sesame protein isolate. The solubility was found to correlate with the zeta potential. Thus, sesame proteins can serve as prospective functional components. They can cast more light on the relationship between the electrical charge interactions in food matrices and the structure, stability, shelf life, texture, and functions of food.

More data on how these proteins interact with each other and behave in foods can help develop appropriate methods that could detect the effects of sesame protein isolates in foods. In this regard, the behavior of sesame protein isolates in various food systems seems a promising research direction. Our next step will be to study the sesame protein isolate we produced and described in this article in various food systems. We also plan to concentrate on the interactions between sesame paste and sesame isolates, salt concentration, and pH conditions.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism. All authors of this study have read the manuscript and agreed to publish it.

CONFLICT OF INTEREST

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

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Antioxidant and antithrombotic properties of fruit, leaf, and seed extracts of the Halhalı olive (*Olea europaea* L.) native to the Hatay region in Turkey

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Abstract:
The olive (*Olea europaea* L.) is one of the most important plants grown in many Mediterranean countries that has a high economic value. Olives, which are specific to each region, have different bioactive components. In this study, we investigated the phenolic/flavonoid contents, as well as antioxidant, antimicrobial, and antithrombotic activities of the fruit, leaf, and seed extracts obtained from the Halhalı olive grown in Arsuz district of Hatay, Turkey.

Antioxidant activities of the phenolic compounds found in the olive fruit, seed, and leaf extracts were determined by employing established in vitro systems. Total phenolics were determined as gallic acid equivalents, while total flavonoids were determined as quercetin equivalents. Also, we evaluated a possible interaction between oleuropein and aggregation-related glycoproteins of the platelet surface via docking studies.

The extracts showed effective antioxidant activity. The seed extract had the highest phenolic content of 317.24 μg GAE, while the fruit extract had the highest flavonoid content of 4.43 μg. The highest potential for metal chelating activity was found in the leaf extract, with an IC_{50} value of 13.33 mg/mL. Also, the leaf extract showed higher levels of antioxidant, antithrombotic, and antimicrobial activity, compared to the fruit and seed extracts. The docking scores of oleuropein against the target molecules GPVI, $\alpha 2\beta 1$, and GPIbα were calculated as –3.798, –4.315, and –6.464 kcal/mol, respectively.

The olive fruit, leaf, and seed extracts used as experimental material in our study have remarkable antioxidant, antimicrobial, and antithrombotic potential.

Keywords: *Olea europaea* L., antioxidant, antithrombotic activity, antimicrobial activity, molecular docking

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INTRODUCTION

The olive (*Olea europeae* L.), the oldest fruit tree known to be cultivated in the world, belongs to the Olea genus of the *Oleaceae* family and takes its name from the Greek “elaia” and the Latin “olea”. People consume its fruit and other products for both nutritional and health benefits [1–3].

Like most plants, the olive tree is constantly exposed to environmental stresses such as high temperatures and UV radiation. To protect itself in this situation, the plant produces chemical components with antioxidant properties defined as phenolic compounds (phenolic acids, phenolic alcohols, secoiridoids, and flavonoids). These components vary qualitatively and quantitatively depending on certain factors such as the olive species, the degree of its fruit/leaf’s ripening, the climate, and the geographical location [4–8]. Phenolic compounds are found in the olive’s fruit, leaves, and seeds. They contribute to the color and flavor of the fruit depending on their concentration, which differs considerably between various tissues [9, 10].

Olive groves, which cover 8 million hectares of Mediterranean countries, have a very important social

and economic value. Olive oil is the main product produced by these countries on a large scale. However, the by-products of such production are used beneficially, too. They include olive cake (olive seed and pomace, the fleshy part), black water, twigs, and leaves.

There is a lot of research on olive leaves and seeds, which have been used in folk medicine for a long time but have become more popular recently. Olive leaves are commercially produced in the form of herbal tea and olive leaf extract is offered in the form of tablets, both considered as food support products. In cosmetics, products containing olive leaf extract are used in skin care due to their antioxidant and antiaging effects.

Recently, some medicines made from olive leaf extract have started to be used for human and animal health [11, 12]. These medicines have natural antibiotic and antiparasitic effects [13]. Olive leaves contain polyphenols (oleuropein, hydroxytyrosol, and luteolin) that can inhibit platelet aggregation and eicosanoid production [14]. Olive leaf extract has an antimicrobial effect against all kinds of bacteria, viruses, yeasts, and fungi. It is used in the treatment of common bacterial infections such as bronchitis and tonsillitis, fungal infections in the vaginal areas of women, and viral infections such as herpes [15–19].

The olive seed, which is the main waste of olive oil and table olive production, is a lignocellulosic structure made up of hemicellulose, cellulose, and lignin. It contains oil that is rich in polyunsaturated fatty acids. The amount of protein in the seed is higher than in the remaining part of the olive [20]. In addition, it contains different polyphenols such as tyrosol, hydroxytyrosol, oleuropein, and 3,4-DHFEA-EDA. The olive seed is also used for therapeutic purposes in folk medicine practices. In particular, to treat rheumatic pain or accelerate wound healing, the olive is crushed with its seed and applied onto the lesion. It can also be used for gastritis and stomach ulcers.

According to recent research, oleuropein is the most active polyphenolic antioxidant in the olive's fruit, seed, and leaves [21, 22]. This compound was discovered as early as 1908 by Bourquelot and Vintilesco in a study on the olive's fruit, but its structure was only identified in 1960 [23]. Most researchers agree that the therapeutic properties of olives and their by-products come from oleuropein, the most abundant phenolic compound in their composition [24–26].

The value of the olive tree lies not only in its fruit, but also in its oil, seeds, branches, roots, and leaves. Therefore, in this study, we aimed to determine phenolic components and antioxidant, antimicrobial, and antithrombotic activities of the fruit, leaf, and seed extract of the olive tree (*O. europaea* L.).

In addition, we evaluated a possible interaction between oleuropein and aggregation-related glycoproteins of the platelet surface via a docking study. The fruit, leaves, and seeds were collected from the Halhalı olive tree growing in the Hatay region (Turkey) during the harvest period.

STUDY OBJECTS AND METHODS

Plant materials. For the experiments, olives and olive leaves were collected during the harvest period from the Halhalı olive trees growing in Arsuz District of Hatay, Turkey.

Preparation of samples. Prior to analysis, the leaf samples were cleared of impurities with distilled water and dried in the shade on blotting paper. When the leaves became brittle, they were pounded in a mortar and ground into powder. The seeds were mechanically separated from the fleshy part of the fruit, washed with distilled water, laid on drying paper, and dried overnight. The dried seeds were crushed by pounding in a mortar and turned into a slurry owing to a very small amount of gel-like substances. The olives were separated from their seeds, pounded in a mortar, and then turned into a slurry using an IKA T25 knife homogenizer. On completion of the preliminary preparations, the samples were made ready for Soxhlet extraction.

Soxhlet extraction. In order to obtain extracts from the prepared samples, 30 g of raw olive fruit, 16 g of dried olive leaves, and 40 g of dried olive seeds were placed in the cartridge of the Soxhlet apparatus. Each sample was first extracted at 55°C for 3 h using 400 mL of petroleum ether, with non-polar lipophilic components removed. Afterwards, the extraction process was continued with 400 mL of ethanol used as a solvent. For this, ethanol was evaporated under vacuum in a rotary evaporator, and the high-density ethanol extracts remaining in the balloon were weighed. The prepared extracts were dissolved in distilled water to obtain stock solutions for the measurements. These stock solutions were divided into 10 equal volumes, each poured into tubes with plastic caps and stored in a deep freezer at –40°C until the measurements.

Methods for quantitative determination. In this study, all the measurements for each parameter were performed in duplicate and the data were presented as their arithmetic means.

Determination of total phenolic content. The total amount of phenolic substances contained in the three extracts (fruit, seed, and leaf) was determined according to the method of Slinkard and Singleton using the Folin-Ciocaltaeu reagent [27]. Gallic acid was used as a standard. Total phenols were calculated as gallic acid equivalents using a standard calibration curve obtained from the absorbance values read against the gallic acid solutions in ethanol at 25, 50, 100, 150, 200, 250, and 500 μg/mL concentrations. Spectrophotometric and absorbance measurements were performed at 765 nm on a Shimadzu 1201 device.

Determination of total flavonoid content. The total flavonoid content in the samples was determined according to the method of Moreno *et al.*, which was based on the formation of complexes of flavonoid groups with metal ions [28]. In this study, we used aluminum as a metal ion and quercetin as a standard molecule. A stock solution was prepared with a final concentration of quercetin in methanol reaching 100 μg/mL. Dilute

solutions with concentrations of 15, 30, 45, 60, and 75 µg/mL were prepared from this solution. For this, we placed 100 µL of 10% $AI(NO_3)_3$ in the test tubes, first adding 100 µL of 1 M CH_3COOK and 3.8 mL of methanol, and then adding 1 mL of the standard/extract solutions. The mixture was vortexed and incubated for 45 min in a shaking water bath at 25°C. The absorbance of the formed yellow complex was measured at 415 nm in the spectrophotometer. Total flavonoids were calculated as quercetin equivalents with the help of the quercetin standard curve.

Determination of reducing capacity. The Oyaizu method was used to determine the reducing capacity of the samples [29]. This method is based on monitoring the absorbance at 700 nm for the yellow color that turns green in the experimental environment with the Fe^{3+}/Fe^{2+} conversion, with high absorbance corresponding to high reduction potential. The solutions with 5, 10, 25, 50, 100, and 150 µg/mL concentrations of butylated hydroxyanisole were used as standards. The standard graph was drawn using the absorbances of the butylated hydroxyanisole standards spectrophotometrically measured at 700 nm. The reducing potential of the samples was determined using this calibration graph.

Determination of metal ion chelating activity. The chelating of ferrous ions by the ethanol extracts of Halhali olives was estimated by the method of Dinis *et al.* [30]. For this, the extracts were added to a solution of 2 mM $FeCl_2$. The reaction was initiated by adding 5 mM of ferrozine, and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution was then measured spectrophotometrically at 562 nm. Ethylenediamine tetraacetic acid (EDTA) was used as a positive control. The percentage of inhibition of the ferrozine-Fe^{2+} complex formation was calculated using the formula below:

$$\text{Inhibition} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

where A_0 is the absorbance of the control; A_1 is the absorbance in the presence of the extract samples or standards.

Determination of H_2O_2 scavenging activity. The hydrogen peroxide scavenging activity was determined according to the titration method developed by Zhang [31]. Ascorbic acid was used as a positive control. The sample or the positive control (at concentrations ranging from 50 to 1000 µg/mL) and H_2O_2 (0.10 mM) were added to an Erlenmeyer flask for the titration reaction. Ammonium molybdate (3%), H_2SO_4 (2.0 M), and KI (1.8 M) were added and mixed. The resulting yellow mixture was titrated with 5 mM $Na_2S_2O_3$ until the yellow color disappeared. The hydrogen peroxide removal activity (%) of the extracts and standard substances was calculated according to the formula below:

$$H_2O_2 \text{ Scavenging activity} = [(V_0 - V_1)/V_0] \times 100 \quad (2)$$

where V_0 is the control titrant volume; V_1 is the sample and standard titrant volume.

Determination of DPPH radical quenching activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical quenching activity was determined according to the method of Blois [32]. This long-established method evaluates the potential of DPPH, which is a stable free radical, to react with phenolic molecules with hydrogen donor properties in the reaction medium. The DPPH concentration decreased due to the reaction can be monitored spectrophotometrically at 517 nm. Butylated hydroxyanisole was used as a positive control. Dilute solutions of the standard at concentrations of 5, 10, 25, 50, 100, and 150 µg/mL were prepared. The 0.1 mM solution of DPPH was prepared in 70% methanol. 3 mL of the standard/sample solution was taken and placed in test tubes. After adding 1 mL of the DPPH solution, the mixture was thoroughly vortexed and incubated for 30 min at room temperature in the dark. At the end of the incubation, absorbance was measured in the spectrophotometer at 517 nm. The radical scavenging effects (%) of the solutions were calculated using the equation below based on the absorbance values compared to the DPPH solution used as a control:

$$\text{DPPH radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100 \quad (3)$$

The solution prepared by mixing only 3 mL of methanol and 1 mL of DPPH solution without adding any chemicals was used as a control solution.

A_0 is the absorbance of control; A_1 is the absorbance of sample/standard.

Determination of antimicrobial activity. The antimicrobial activity of the olive leaf, fruit, and seed extracts was determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute. Minimal inhibitory concentrations (MICs) for each extract were tested against standard bacterial (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *S. aureus* MRSA ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *Acinetobacter baumannii* ATCC 19606) and fungal (*Candida glabrata* ATCC 90030 and *Candida albicans* ATCC 14053) strains. The strains were obtained from the American Type Culture Collection (Rockville, USA). The bacterial strains were grown in a Mueller Hinton Broth (Merck), while the fungal strains were grown in a RPMI 1640 Broth (Sigma-Aldrich Chemie GmbH Taufkirchen, Germany).

To obtain a standard inoculum, the turbidity of bacteria and fungi was prepared according to the Mcfarland 0.5 chart. All dilutions were made with distilled water. The final concentrations of the extracts were diluted to 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µg/mL. Fluconazole was used as a standard drug for fungi, while ampicillin, ciprofloxacin, amikacin, ampicillin, tigecyline, and vancomycin were used as standard drugs for bacteria. Standard inoculums of

bacteria and fungi (10^6 CFUs/mL) were inoculated onto agar plates with a sterile plastic ring-tipped loop (0.01 mL). The plates were evaluated after they were kept in an oven at 35°C for 16–20 h for bacteria and 48 h for fungi. Minimal inhibitory concentrations were determined as the lowest concentrations that inhibited the growth of bacteria and fungi [33, 34].

Determination of antithrombotic activity. The antithrombotic activity of the extracts was determined by using a Chronolog aggregometer and the optical aggregometry technique [35]. Optical aggregometers are modified spectrophotometric instruments. They measure changes in light transmittance through the cuvette by adding a stimulating agent (an agonist such as collagen or ADP) while the platelet-rich plasma is mixed at a certain speed and the platelets form aggregates. The system calibrates itself to detect 100 units of transmission difference between the platelet-poor plasma and the platelet-rich plasma.

In our experiments, 75 mL of venous blood taken from a 47-year-old healthy male after 8 h of fasting was poured into tubes with EDTA, 3 mL for each tube. The platelet-poor plasma was obtained by centrifugation at 1000 rpm for 10 min, and the platelet-rich plasma was obtained by centrifuging at 3500 rpm for 10 min. For all the measurements, 950 µL of these plasmas was added to the measuring cuvettes. For the blank measurement, only the platelet-rich plasma was added (without any extract), and the aggregation was measured after adding the agonist (collagen). For the sample measurements, the extracts were added to the platelet-rich plasma samples at varying concentrations and incubated at 37°C for 5 min. The aggregation measurement was performed by adding the agonist at the end of the incubation. All the measurements were carried out in duplicate to calculate the averages. On completion of the measurements, the percentage of aggregation inhibition corresponding to a concentration of 1 mg/mL was calculated for each extract according to the formula below:

$$\text{Antithrombotic activity} = [(T_k - T_1)/T_k] \times 100 \quad (4)$$

where T_k is the transmission of control, %; T_1 is the transmission of sample, %.

Docking studies. The Maestro 12.8 (Schrödinger, New York) program was used in all molecular docking studies. The ligand structures were prepared with the 2D Sketcher. The ligands were minimized using the LigPrep, a utility of the Schrödinger software. The crystal structure of human platelet Glycoprotein VI (GPVI, pdb id: 2GI7), integrin alpha2 I domain/collagen complex ($\alpha 2\beta 1$, pdb id: 1DZI), and the complex of the wild-type von Willebrand factor A1 domain and Glycoprotein Ib alpha (GPIbα, pdb id: 1SQ0) were downloaded from the RCSB Protein Data Bank (www.rcsb.org) [36–38].

Schrödinger's modules (Protein Preparation Wizard Prime, Impact, Epik, Propka, and Prime) were used to remove the ligands and solvent molecules in protein, add hydrogens, assign charges, and delete polar hydrogens for clarity. After the target region of the proteins was determined, a grid box was created with the grid generation panel. Then, the prepared ligands were docked in this grid map 50 times in the standard precision mode using the Glide software [39].

RESULTS AND DISCUSSION

Oxidative damage is involved in the etiopathogenesis of many diseases and antioxidants play an important role in preventing the formation and progression of many diseases [40]. Plants, especially those used as natural resources, provide a diverse set of broad substrates for drug discovery. They have therapeutic properties due to the presence of phytochemicals with antioxidant properties such as phenols, flavonoids, and sterols.

In our study, we aimed to investigate the pharmaceutical potential of the Halhali olive's leaf, fruit, and seed extracts using several different methods. For this purpose, we compared the antioxidant, antimicrobial, and antithrombotic activities of the extracts, and tested the interaction of oleuropein, the most effective molecule in olive composition, with the platelet surface receptors GPVI, $\alpha 2\beta 1$, and GPIbα by the docking technique.

The phenolic contents of the extracts were calculated as gallic acid equivalents (µg gallic acid/mg extract) using the standard graph (Fig. 1). Similarly, the flavonoid contents were calculated as quercetin equivalents (µg quercetin/mg extract) using the standard graph (Fig. 2). The total phenolic and flavonoid equivalent substance contents of the extracts are given in Table 1.

The total phenolic content of the fruit extract was found to be lower than that of the leaf and seed extracts, while its total flavonoid content was the highest at 4.43 µg, compared to 4.24 and 0.86 µg in the leaf and seed extracts, respectively. The percentage of flavonoids in the total phenolic content was determined as 1.51% for the leaf extract, 1.63% for the fruit extract, and 0.27% for the seed extract. These values show that the jelly-oily component, which is found in the interior of the olive pit and contains bioactive components in

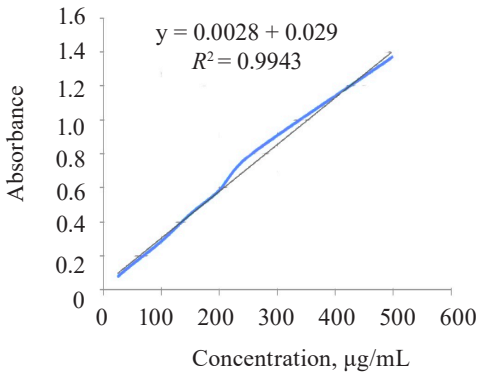

Figure 1 Gallic acid standard curve

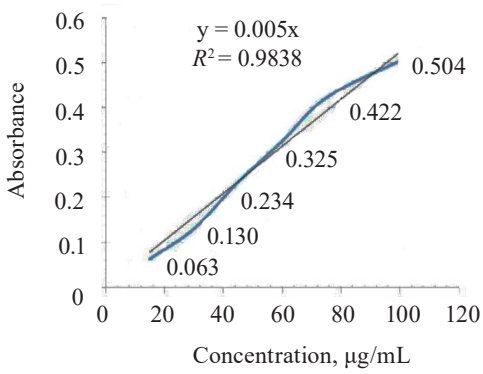

Figure 2 Standard quercetin curve

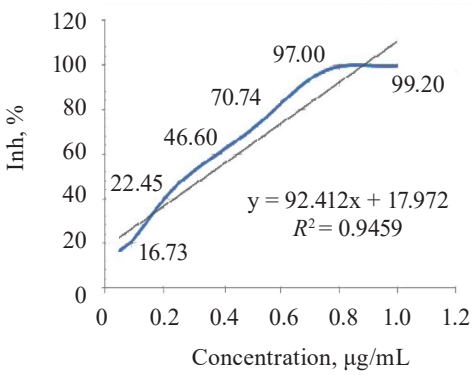

Figure 3 Inhibition-concentration curve for EDTA

Table 1 Total phenolic and flavonoid contents of olive fruit, leaf, and seed

Raw material	Total phenols, µg GAE	Total flavonoids, µg QUE
Leaf extract	280.19 ± 0.40	4.24 ± 0.10
Fruit extract	270.99 ± 0.30	4.43 ± 0.10
Seed extract	317.24 ± 0.20	0.86 ± 0.10

the olive pit extract, is rich in phenolics. However, its percentage of flavonoids in total phenolics is lower compared to that in the leaf and fruit extracts. Çetinkaya and Kulak, in their study of olives from the Kilis region, reported that the ratios between the total phenolic content and the flavonoid composition of this content differed depending on the development period of the olive [41].

The free radical scavenging effect of all the extracts was measured by DPPH assays. The DPPH radical quenching activity was determined according to the method of Blois [32]. The calibration curve was prepared using diluted solutions of 5, 10, 25, 50, 100, and 150 µg/mL of butylated hydroxyanisole, a strong radical scavenging antioxidant used as a standard.

The IC_{50} values calculated for the DPPH radical scavenging activity were 9.22 µg/mL for butylated hydroxyanisole and 3.80, 9.47, and 5.25 mg/mL for the leaf, fruit, and seed extracts, respectively. The antiradical activity values defined as $1/IC_{50}$ were 0.26, 0.11, and 0.19 for the leaf, fruit, and seed extracts, respectively. The strongest DPPH radical scavenging effect was observed in the leaf extracts, followed by the seed and fruit extracts.

Martinez *et al.* reported that olive fruit extracts had strong radical scavenging activity against the DPPH radical [42]. Stankovich *et al.*, who used the DPPH method to determine antioxidant activity in olive leaf extracts from France and Serbia, found IC_{50} values of 113.30 and 94.39 µg/mL, respectively [43]. Orak *et al.* reported the IC_{50} values in Çekişte and Uslu olive leaf extracts to reach 0.63 and 0.65 mg/mL, respectively [44].

The reducing potential of Fe(III) to Fe(II) of the olive leaf, fruit, and seed extracts was determined according to the Oyaizu method [29]. Butylated hydroxyanisole was used as a standard molecule. According to our findings, the reducing potentials of 1 mg/mL extracts in terms of butylated hydroxyanisole equivalents were 257.6, 77.25, and 21.24 µg/mL for the leaves, fruit, and seeds, respectively. We observed the strongest reducing potential in the seed extract. Altemimi *et al.*, who tested antioxidant parameters in methanol and ethanol extracts of olive leaves, found that methanol extracts had the highest phenolic content, while ethanol extracts had higher reducing potential [45]. Fu *et al.* reported the ferric reduction value of the extractable components of olive samples to be 2.70 ± 0.03 µmol Fe(II)/g [46].

The metal chelating potentials of the olive fruit, leaf, and seed extracts and the standard chelator (EDTA) were determined according to Dinis method [30]. The % inhibition-concentration graph (Fig. 3) was created by using dilute solutions of EDTA at varying concentrations.

According to our findings, the highest metal chelating activity (IC_{50}) was seen in the leaf extract (13.33 mg/mL), followed by the seed and fruit extracts (17.37 and 24.76 mg/mL, respectively). Wang *et al.* reported that neuron damage due to lead toxicity in the brain was significantly prevented, and antioxidant capacity increased, in the mice that received olive leaf extracts [47]. Fabiani *et al.* suggested that olive phenolic components protected human mononuclear blood cells and reduced oxidative damage due to their metal chelating abilities among other factors [48].

The hydrogen peroxide scavenging activity was determined according to the method of Zhang *et al.* [31]. Ascorbic acid was used as a standard molecule. We observed that the leaf extract had the strongest activity, with an IC_{50} of 4.27 mg/mL, while the values for the fruit and seed extracts were 36.39 and 30.50 mg/mL, respectively (Fig. 4). Lins *et al.* reported that olive leaf extracts showed protective activity against oxidative stress on erythrocytes in vitro, inhibiting the lysis of erythrocytes and reducing MDA levels formed by oxidation of erythrocyte membrane lipids [49].

The antimicrobial activity of the olive leaf, fruit, and seed extracts was determined using the agar dilution method recommended by the Clinical and

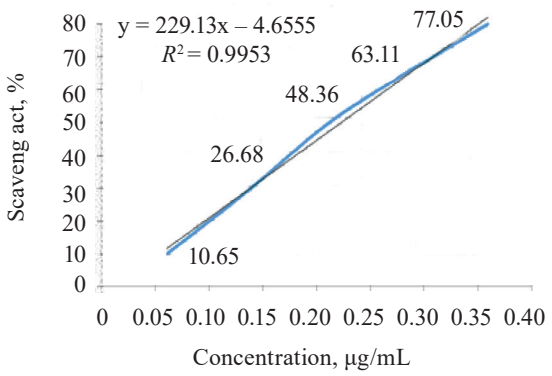

Figure 4 Standard graph of hydrogen peroxide scavenging activity

Table 2 Docking scores of oleuropein against potential target macromolecules

	Docking Scores, kcal/mol
2GI7 (GPVI)	–3.798
1DZI (*α2β1*)	–4.315
1SQ0 (GPIb*α*)	–6.464

Laboratory Standards Institute [33, 34]. The lowest effective concentrations that prevented the growth of bacteria and fungi were determined as minimal inhibitory concentrations (MICs). They were tested against the standard bacterial and fungal strains. Fluconazole was used as a standard drug for fungi, while ampicillin, ciprofloxacin, amikacin, ampicillin, tigecyline, and vancomycin were used as standard drugs for bacteria. The extracts showed varying degrees of antimicrobial and antifungal activity against all tested microorganisms. However, the leaf extract showed stronger antimicrobial and antifungal effects (100–200 µg/mL) compared to the fruit and seed extracts (200–400 µg/mL).

While the leaf extracts were similarly effective on all the tested microorganisms, the fruit and seed extracts were found to be less effective against *Pseudomonas aeruginosa*, Acinetobacter baumannii, and MRSA, which are most commonly isolated from hospital infections (400–800 µg/mL).

Pereira *et al.* analyzed the phenolic compounds in the aqueous extract of powdered olive leaves using HPLC/DAD and investigated their antimicrobial properties. They reported that the inhibitory effects of different concentrations of the obtained extract on microorganisms were respectively found as *Bacillus cereus* ~ *Candida albicans* > *Escherichia coli* > *Staphylococcus aureus* > *Cryptococcus neoformans* ~ *Klebsiella pneumoniae* ~ *P. aeruginosa* > *Bacillus subtilis* [50].

The antithrombotic activity was determined by using the Chronolog system and the turbidimetry technique. According to our data, all three extracts showed antithrombotic activity at certain rates, but the leaf extract had higher values compared to the fruit and seed extracts. The relative antithrombotic activities of the extracts were 23.22, 5.01, and 7.36% for the leaves, fruit, and seeds, respectively, with the control activity of 100%.

Dub and Dugani, in their study on rabbits, reported that the application of repeated amounts of olive leaf extracts reduced thrombus formation and the potential for attachment to the vessel wall by changing the thrombus morphology [51]. Gorzynik-Debicka *et al.* found that olives and olive oil had antiatherogenic and antithrombotic effects, as well as anticarcinogenic effects [52]. Zbidi *et al.* showed that oleuropein and (+)-cycloolivil molecules isolated from the olive tree had strong antithrombotic effects [53]. Similarly, Petroni *et al.* reported that polyphenolic compounds extracted from olives inhibited eicosanoid production and platelet aggregation [54].

The molecular docking technique evaluates the interaction of a molecule with the binding site of an enzyme or a receptor with a protein structure. Certain score algorithms simulate the placement of a molecule in the protein structure by taking into account many factors, including the electro negativities of the atoms, their positions to each other, and the conformation of the molecule to be placed into the protein structure.

In this study, we selected platelet adhesion receptors GPVI, *α2β1*, and GPIb*α* as potential targets and investigated the docking scores and interactions of oleuropein, the olive's major bioactive component, with these targets [55]. We calculated the docking scores of oleuropein against the target molecules (Table 2).

Oleuropein hydrogen bonded with LYS 41, GLN 50, and GLN 48 in the epitope part of the active site of GPVI. It interacted hydrophobicly with ALA 57, PRO 56, ILE 55, PHE 54, and LEU 53, and had polar interactions with SER 43, SER 44, and SER 61. Oleuropein established a metal coordination bond with Co^{2+} in the active site of the *α2β1* protein. Oleuropein hydrogen bonded with GLU 256, SER 257, and GLU 299. The molecule interacted hydrophobically with LEU 220, PHE 224, and LEU 296, and polarly with SER 153, ASN 154, SER 155, THR 221, SER 257, HIE 258, and ASN 295. Oleuropein hydrogen bonded with ARG 64, ASP 83, ASP 106, LYS 132, and GLN 604 in the active site of GPIb*α*. It also interacted with ASN 61, SER 85, HIS 86, SER 108, SER 154, GLN 232, THR 176, and VAL 104. It interacted polarly with PHE 109, TRP 230, ILE 605, TYR 600, MET 239, LEU 178, TYR 130, and LEU 131 (Figs 5 and 6).

CONCLUSION

Since oxidant/antioxidant imbalance is extremely dangerous for the organism and new side effects of synthetic antioxidants are revealed every day, the value of natural antioxidants cannot be overestimated. Our study showed that although the olive leaf extract was more effective, all the extracts (fruit, seed, and leaf) had remarkable antioxidant, antimicrobial, and antithrombotic effects. Our results confirmed their

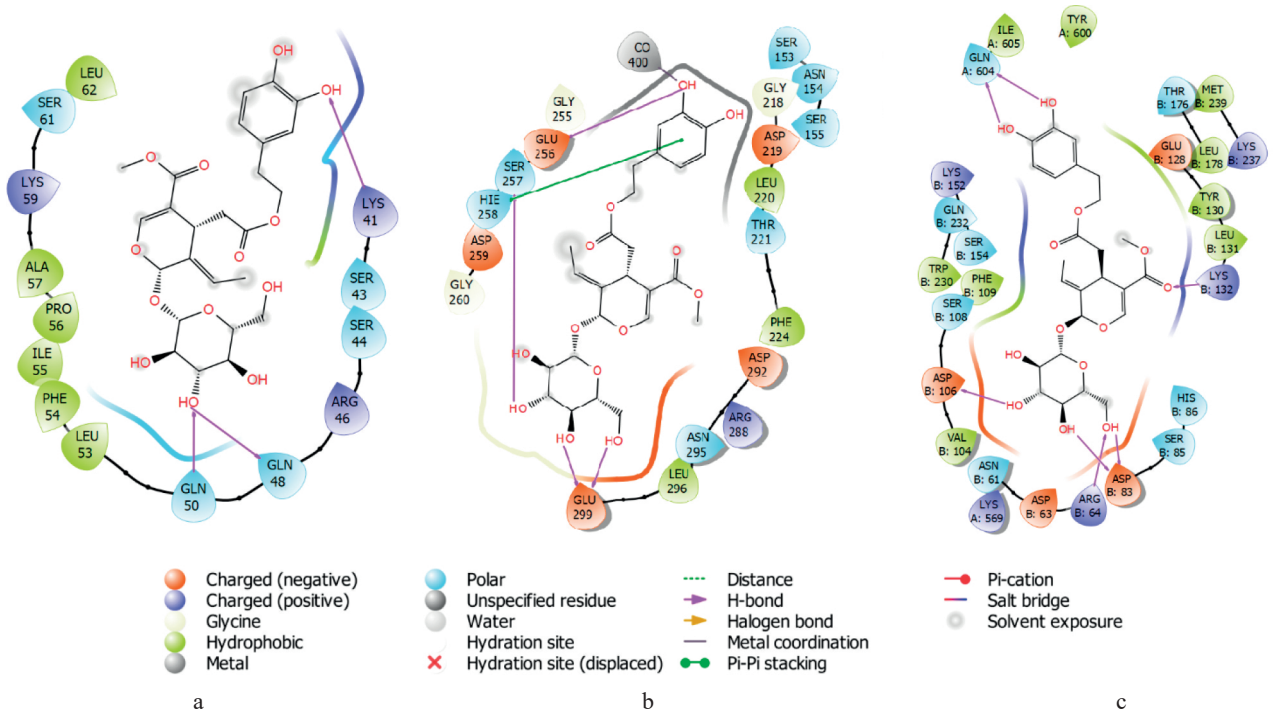

Figure 5 2D interaction of oleuropein against GPVI, *α2β*1, and, GPIb*α*: a – GPVI; b – *α2β*1; c – GPIb*α*

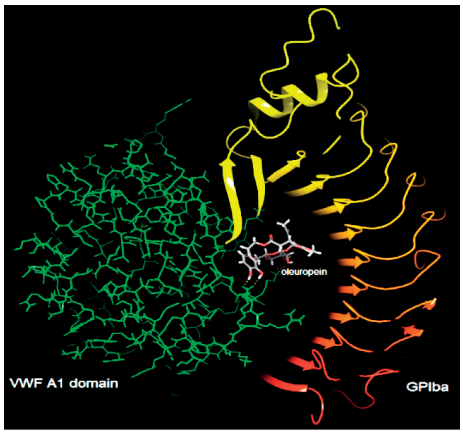

Figure 6 3D interaction of oleuropein against VWF-1A domain and GPIb*α* complex

traditional use in the treatment of various disorders. This should promote the potential use of Halhalı olive leaves as a nutraceutical and pharmacological agent. Further studies can be conducted to isolate and characterize bioactive components in olive leaves, fruits, and seeds, as well as to reveal various drug candidate molecules.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this work.

CONTRIBUTION

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. F. Küçükbay designed the study and supervised the data collection. K. Batçıoğlu, M. A. Alagöz, Y. Yilmaztekin, and S. Günal analyzed and interpreted the data. All of the authors prepared the manuscript for publication and reviewed the draft. All the authors have read and approved the manuscript.

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Liquid products of meat and bone meal pyrolysis: comprehensive assessment by chromatographic methods

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Abstract:
Dorogov's antiseptic stimulators (fractions 2 and 3) are products of meat and bone meal pyrolysis that are used to treat farm animals. However, there is a lack of detailed information about their chemical composition. We aimed to study individual compositions of organic substances in the water- and oil-soluble condensates of these preparations.
Dorogov's antiseptic stimulators ASD-2F and ASD-3F (Agrovetzashchita, Russia) were used as samples of the water- and oil-soluble condensates of meat and bone meal pyrolysis. Volatile substances were identified by gas chromatography and gas chromatography-mass spectrometry, while amino acids were determined by high-performance liquid chromatography.
The initial water-soluble condensate contained ammonium salts, amides of carboxylic acids, N-heterocyclic compounds, hydantoins, amino acids, and dipeptides, with a total content of 8% of the condensate's weight. Its dehydrated concentrate had almost no ammonium salts and amides of carboxylic acids, but its contents of hydantoins, amino acids, dipeptides, and low-volatile nitrogen-containing heterocycles were 10–15 times as high as those in the initial condensate. The condensate contained 13 dipeptides and 19 amino acids with a total content of 2.5%. According to gas chromatography-mass spectrometry, the oil-soluble condensate contained over 30% of nitriles; 7–10% of higher and aromatic hydrocarbons, phenols, and amides (with esters); and 1–3% of N-heterocyclic compounds, naphthalenes, pyridines, and dipeptides. The nitrogen-containing heterocycles, as well as dipeptides, were similar to those in the water-soluble condensate.
We identified 80% of individual organic substances in the water-soluble pyrolytic condensate. Together with its concentrate, they contained more than 220 organic substances divided into 10 main groups. The oil-soluble condensate consisted of over 350 individual organic compounds. The full composition of the preparations can be further identified by three-quadrupole liquid mass spectrometry.

Keywords: Pyrolysis, chemical composition, water-soluble condensate, oil-soluble condensate, meat and bone meal, high-performance liquid chromatography, gas chromatography, gas chromatography-mass spectrometry, triple quadrupole liquid mass spectrometry

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INTRODUCTION

Today, there is a need to determine the chemical composition of liquid products that result from the joint pyrolysis of animal proteins and fats. Performed at temperatures up to 500°C, pyrolysis produces an emulsion of organic substances in an aqueous ammonium buffer solution. The separation of this emulsion results in two fractions: water-soluble and oil-soluble condensates [1–3].

A water-soluble condensate consists of water, carbon ammonium salts (mainly ammonium carbonate), and water-soluble organic substances. An oil-soluble

condensate is a stable water-in-oil emulsion containing up to 90% of organic matter [4, 5].

A number of factors account for the current interest in studying the chemical composition of these products. Firstly, the products of meat and bone meal pyrolysis have been widely used in Russian veterinary practice for more than 60 years. They are known as ASD-2F (Dorogov's antiseptic stimulator, fraction 2 – a water-soluble part) and ASD-3F (Dorogov's antiseptic stimulator, fraction 3 – a non-water-soluble part). ASD-2F is used to prevent and treat a wide range of animal and bird diseases [6, 7]. Recently, it has been produced as a dietary supplement in liquid and capsule forms [2, 8]. ASD-3F is prescribed to animals with skin and hooves pathologies [3, 9]. Secondly, foreign countries consider the oil fraction of pyrolysis products as a potential component of biofuel (pyrolytic fuel), which can be obtained from animal protein waste [10, 11]. For example, pyrolysis can be used to utilize meat and bone meal obtained from animals with spongiform encephalopathy [12]. Krolevets and Bogachev estimated the annual amount of animal meal that can be converted into fuel at hundreds of thousands of tons [13]. However, their estimates were based on the pyrolysis carried out under almost ideal laboratory conditions by condensing products at very low temperatures, which significantly limits their practical application.

In another study, Krolevets *et al.* discussed the indicators of pyrolytic fuel obtained by food waste pyrolysis in a pilot plant [14]. They found that the organic products obtained from the pyrolysis, or at least their individual fractions, can be used as additives to diesel fuel. However, the authors analyzed only gross indicators, such as C, H, N, and S contents, as well as physical-and-mechanical characteristics. Therefore, their results have a limited application. Kukonin and Grek and Yengashev *et al.* [15, 16] considered only the oil-soluble condensate to be of much use, finding the water-soluble condensate to be useful only as an aqueous ammonia fertilizer.

Nozdrin *et al.* identified more than 120 organic substances in ASD-2F, an example of the water-soluble condensate, including 4 cyclic dipeptides [17]. Gurov *et al.* identified more than 170 organic substances in the water fraction by using gas-liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS) on capillary columns with HP-FFAP phases (30 m, 0.25 mm, 0.25 um, PN: 19091F-433, Agilent) and 5% phenylpolymethylsiloxane [18]. The content of 26 individual substances was quantified by the absolute calibration method. The authors found that their total amount in ASD-2F reached 4% of its total mass, which was about 50% of the total mass of organic substances in the preparation. Guzev *et al.* used the simple normalization method to determine carboxylic acid amides and amines (7%), alkyl-hydantoins (30%), and dipeptides (16%), including cyclic dipeptides and their derivatives [19].

Since the chromatograms of the studied preparations had a very high density of peaks, Teshaev and Khasanova analyzed the retention time precision as an identification parameter for gas chromatography (GC) under similar experimental conditions [20]. The authors found that modern gas chromatography with high intralaboratory precision allowed for a high repeatability of the analyte's retention time, ensuring highly reliable identification.

Serba *et al.* studied the amino acid composition of ASD-2F by high-performance liquid chromatography (HPLC) with post-column derivatization with ninhydrin [21, 22]. They reported that the preparation contained 15 free amino acids and 19 bound amino acids with total concentrations of 1 and 5%, respectively. Thus, we can assume that about 4% of the amino acids were present in the preparation as peptides that were converted into amino acids during acid hydrolysis.

Noteworthily, it is relatively easy to isolate an aqueous solution of ammonium carbon salts (distillate) from the water-soluble pyrolysis product by boiling the preparation. The resulting solution can be used as an ammonia fertilizer. Organic substances, which are the distillation residue, can be used as a pyrolytic fuel or a source of bioactive (including pharmaceutical) substances [23–27].

We aimed to study individual and group compositions of organic substances contained in both the water-soluble condensate and the oil-soluble (fuel) fraction of meat and bone meal pyrolysis products. In particular, we identified amino acids and their derivatives (including dipeptides) and quantified them in the water-soluble and oil-soluble condensates.

STUDY OBJECTS AND METHODS

ASD-2F and ASD-3F were used as samples of water-soluble and oil-soluble condensates of meat and bone meal pyrolysis products (Agrovetzashchita, Russia).

To prepare the ASD-2F sample for analysis, we pipetted 0.5 g of the substance (weighed on a laboratory scale with an accuracy of 3 decimal places) into a 15-mL plastic centrifugation tube and added 10 mL of ethanol. Since a suspension formed due to precipitation of ammonium carbon salts, the tube was centrifuged for 10 min at 4000×g, and then the supernatant was taken for analysis.

To concentrate organic substances of ASD-2F and to prepare the organic part, 10 mL of ASD-2F was thermostated in an oven at 80°C for 3 h. The concentrated organic part amounted to 6–8% of the initial mass. The losses due to the evaporation of highly volatile organic substances were estimated at about 20% of their total amounts. Thus, the total content of organic substances in the initial condensate can be reliably estimated as 7–9%. After concentration, organic substances were extracted in 10 mL of ethanol per 0.5 g of the concentrate. After complete dissolution, the sample was centrifuged under normal conditions, and then the supernatant was taken for analysis [25].

To extract organic substances from ASD-3F, we placed 1 mL of the preparation into a 15-mL plastic test tube and added 5 mL of distilled water and a solvent (methylene chloride, butyl acetate, hexane, or o-xylene). The tube was capped and stirred for 1 h. After centrifugation under the same conditions, an extract was taken for analysis – from the lower part of the tube with methylene chloride and from the upper part of the tubes with the other solvents.

The standard samples included 2-pyrollidinone, 2-piperidinone, 3-methylbutanamide, 2-amino-3-methylpyridine, 5,5-dimethylhydantoin, 5,5-ethylmethylhydantoin (Acros Organics, Belgium) with at least 95.0% of the main substance.

Gas chromatography was performed on a Khromatek-Kristall-5000.1 chromatograph with a flame ionization detector (Khromatek, Russia) and a Shimadzu GC-2010 Plus chromatograph with a GCMS-QP2020 mass selective detector (Shimadzu, Japan). Both instruments used VB-1701 30 m×0.32 mm×0.50 µm capillary columns (initial temperature: 120°C; initial isotherm retention time: 5 min; the rate of temperature rise: 10°C/min, final temperature: 230°C). Carrier gas helium was used to maintain constant pressure in front of the column at 100 kPa. The flame ionization detector operated at 250°C, with a hydrogen flow rate of 30 mL/min, an air flow rate of 300 mL/min, and an inert gas (helium) injection at 30 mL/min. The mass detector operated at an ion source temperature of 210°C, an interface temperature of 210°C, a scanning rate of 1425 amu/s, a scanned mass range from 30 to 450 m/z, a cycle's sweep rate of 0.3 s, detector voltage of 0.9 kV, and a solvent effect removal time of 1.5 min. The evaporator worked at 250°C, with a flow split ratio of 1/30, a sample volume of 1 µL, in a gas saving mode. The obtained mass spectra were interpreted using the GCMS Postrun Analysis software (GCMS Solution Version 4.4, Shimadzu, Japan). The components were identified by comparing their mass spectrum in the sample with their mass spectrum in the library (databases NIST-14 and NIST-14s). We also relied on our understanding of animal protein pyrolysis and the synthesis of new compounds from pyrolysis products.

The pH of the oil fraction was determined in the aqueous extract. For this, 2 mL of the oil fraction was placed in a 15-mL test tube and mixed with 10 mL of distilled water. The tube was stirred for 1 h and centrifuged for 10 min at 4000×g. Then, 10 mL of the aqueous solution was taken from the upper part, placed in a beaker for titration, and mixed with 100 mL of distilled water to determine the pH.

Bound and unbound amino acids were determined by high-performance liquid chromatography (HPLC) with pre-column derivatization.

We used the following reagents and solvents: CH_3OH and acetonitrile for HPLC (Panreac, Spain), FMOC (Sigma, USA), o-phthalaldehyde (≥ 99.9%) (Sigma, USA), sodium hydrogen phosphate (≥ 99.9%) (Sigma, USA), hydrochloric acid (≥ 37%), deionized water obtained on a MilliQDirect 8 system (Merck Millipore, Germany), chemically pure trichloroacetic acid (≥ 99.0%), 3-mercaptopropionic acid (≥ 99.0) (Sigma, USA), sodium hydroxide (≥ 99.0), anhydrous sodium tetraborate (≥ 99.0), and sodium tetraborate decahydrate (≥ 99.5%).

To determine protein-bound amino acids, the samples were subjected to acid hydrolysis with hydrochloric acid (6 mol/L) for 24 h at 110°C. The resulting hydrolysate was transferred into a 50-cm^3 round-bottom flask and evaporated under vacuum at 60°C. The dry residue was redissolved in 1 cm^3 of the buffer (pH 2.2) and filtered through a 0.45-µm pore membrane filter into a 2-cm^3 chromatographic vial.

Unbound amino acids were extracted with a saline buffer and 20% trichloroacetic acid to precipitate proteins and peptides. The extract was stirred and kept for 1 h at a temperature from 18 to 25°C. Then, it was centrifuged for 5 min at 2000×g and the aqueous layer was filtered through a 0.45-µm pore membrane filter. Finally, the filtrate was transferred into a vial.

Chromatographic analysis was performed on a 50–150 mm long HPLC column, 2.1–4.6 mm in diameter, with a C18 reversed phase, and a particle size of 1.8–5.0 µm. We also used an Agilent 1260 Infinity LC HPLC system (Agilent Technologies, USA) with a diode array detector.

Derivatization was carried out automatically using a programmable autosampler. For this, 10 mm^3 of orthophthalaldehyde solution (for primary amino acids), 10 mm^3 of fluorenylmethyloxycarbonyl (for secondary amino acids), and 2 mm^3 of the sample's solution were injected into the chromatograph. The volume of the injected sample was 12 mm^3.

The measurements were recorded at a diode array detector wavelength of 338 nm and 262 nm. The parameters of chromatographic analysis were as follows:

Column temperature – 40°C;

Mobile phase A – acetonitrile:methanol:water – 45:45:10;

Mobile phase B – 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2;

Flow rate – 1 mL/min;

Elution mode – gradient (Table 1).

Table 1 Gradient mode parameters

Time, min	Volume of eluent A, %	Volume of eluent B, %
0	2	98
0.5	2	98
20.0	57	43
20.1	100	0
23.5	100	0
23.6	2	98
25.0	2	98

The peaks of the samples' chromatograms were identified by comparing them with the chromatogram of the calibration solution in terms of retention time and spectral ratio.

RESULTS AND DISCUSSION

Table 2 presents the physicochemical parameters of the water-soluble and oil-soluble condensates.

Figure 1 shows a general view of the aqueous condensate's gas chromatogram.

The chromatograms of gas chromatography-mass spectrometry have a large number of peaks and therefore are not informative. For this reason, we did not presented them in the article. The chemical composition of the condensates is analyzed in Tables 3–6.

Table 3 shows the chemical composition of the water-soluble condensate determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) and grouped in accordance with the main component. When analyzing the data, we took into account only peaks with a signal-to-noise ratio of over 10 and a signal area of at least 0.1% of the total area of the target peaks.

In addition to the actual composition of the group (Table 3, column 2), we provided the following data for each of the substances (in brackets): relative signal area, %, in the initial water condensate (by GC-MS)/ actual concentration, %, in the initial condensate (by GC)/relative signal area, %, in the concentrate (organic part) of the water condensate (by GC-MS)/actual concentration, %, in the concentrate (organic part) (by GC).

Columns 3 and 4 (Table 3) show the totals for each group, namely the data for the initial condensate (above) and the data for the concentrate (organic part) of the same condensate (below). The data in column 3 (the number of substances, n1, and their total relative concentration by peak area) were determined by GC-MS, while the data in column 4 (the number of substances, n2, and their total actual concentration) were obtained by GC.

Table 2 Physicochemical parameters of water-soluble and oil-soluble condensates

Indicator	Water-soluble condensate [7]	Oil-soluble condensate
Appearance, smell	Brown liquid with a pungent ammonia-like odor	Thick oily liquid, dark brown to black, with a characteristic smell of burnt bone
Density, g/cm^3	1.09–1.11	0.90–1.00
Water content, %	65.0–70.0	5.0–10.0
Carbon ammonium salts, %	25.0–30.0	1.0–2.0
Organic substances, %	7.0–10.0	88.0–94.0
Number of organic substances, over*	220	350

* Low volatile organic substances are hard to determine by gas chromatography

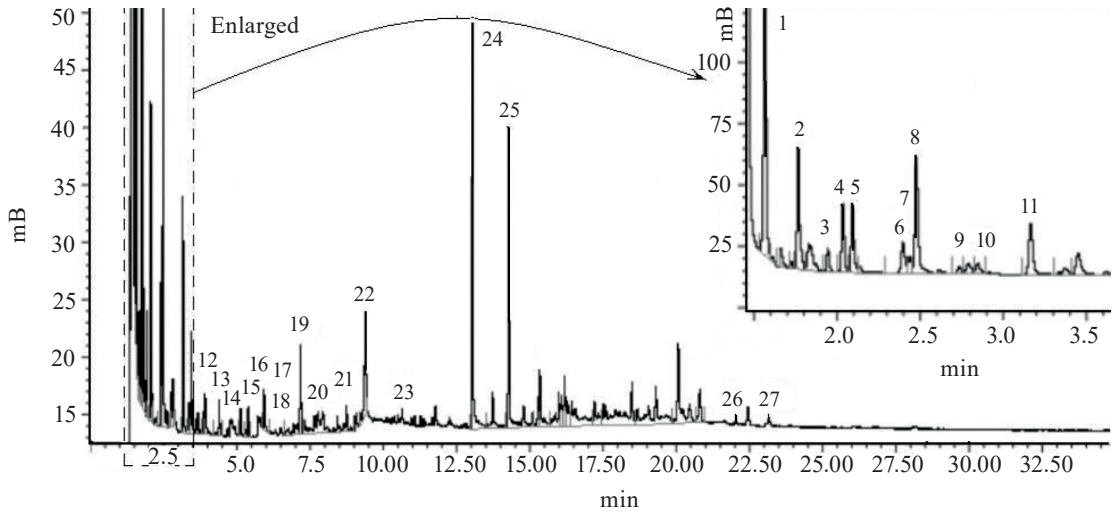

Figure 1 Gas chromatogram of the water condensate: 1 – acetic acid (1.545); 2 – propionic acid (1.743); 3 – acrylic acid (1.804); 4 – butanoic acid (2.068); 5 – dimethylformamide (2.217); 6 – isovaleric acid (2.375); 7 – N-methylformamide (2.419); 8 – acetamide (2.464); 9 – valeric acid (2.713); 10 – N,N-dimethylacetamide (2.786); 11 – propionamide (3.151); 12 – caproic acid (3.861); 13 – butyramide (4.395); 14 – 2-Aminopyridine (4.579); 15 – phenol (5.112); 16 – 3-Methylbutanamide (5.395); 17 – 2-Amino-3-Methylpyridine (6.267); 18 – valeramide (6.529); 19 – 2-Pyrrolidinone (7.175); 20 – caprylic acid (7.780); 21 – urotropin (8.259); 22 – 2-Piperidinone (9.375); 23 – picolinamide (10.305); 24 – 5,5-Dimethylhydantoin (13.047); 25 – 5-Ethyl, 5-Methylhydantoin (14.277); 26 – dipeptide Cyclo (Leu-Pro) (22.107); 27 – dipeptide Leu-Pro (23.037)

Table 3 Chemical composition of the water-soluble condensate by group

Chemical composition by group	Total substances in the group/concentration	
	GC/MS n1/S1, units/%	GC n2/S2, units/%
Carboxylic acids	15/14.7	7/1.42
Acetic (7.03/0.88/1.5/0.23)	8/2.02	7/1.02
Propanoic (1.4/0.22/0.2/0.11)		
2-Propenoic, 2-methyl-Propanoic (0.37/.../…/…)		
Butanoic (1.82/0.11/0.22/0.13)		
3-methyl-Butanoic (0.64/../…/….)		
2-methyl-Butanoic(0.34/0.06/0.1/0.11)		
Pentanoic, 3-methyl- (0.27/0.01/…/….)		
Pentanoic, 4-methyl- (1.12/0.22/…/…)		
5-Hexanoic (0.43/0.13/0.38/0.06)		
4-(Methylamino) butyric (0.19/.../…/…)		
Heptanoic (0.21/../…/….)		
6-Heptenoic (0.41/.../…/….)		
Octanoic (0.27/0.01/0.1/0.32)		
Amides	15/14.4	7/0.65
Formamide (0.17/…/…/)	12/4.31	6/7.67
Acetamide (7.34/0.41/1.47/2.16)		
N,N-Dimethylacetamide (0.31/0.03/0.1/0.15)		
Acetamide, N-methyl- (0.47/…/…/…)		
Propanamide (2.71/0.14/0.71/0.88)		
Propanamide, N-methyl (0.18/…/…/…)		
Propanamide, 2-methyl (0.31/…/…/…)		
Butanamide (0.83/0.03/0.33/0.24)		
Pentanamide (0.1/.../…/…)		
Enanthamide (0.27/../0.21/….)		
2-Propanamid, N, N, 2-trimethyl- (0.25/.../0.14/…)		
Picolinamid (0.21/0.02/0.21/0.07)		
7-Noneamide (0.1/.../…/…)		
N,N-Diethylpropioonamide (0.11/.../0.23/…)		
Imidazoles	10/23.1	2/0.96
1-H-Imidazole, 2-ethenyl (0.52/…/0.54/…)	10/34.1	2/18.65
2,4-Imidazolidinone (retention time 6.929–16.218)		
3,5,5-trimethyl (0.1/…/0.14/…)		
1-methyl-5-piperidin-yl (0.12/…/0.13/…)		
5-methyl- (0.2/…/0.26/…)		
5,5-dimethyl (12.2/0.66/17.06/13.08)		
5-ethyl (0.9/…/0.83/…)		
5-ethyl-5-methyl (6.84/0.31/9.4/5.57)		
5,5-diethyl (0.54/…/0.83/…		
5-isopropyl- (1.24/.../1.71/…)		
5-methyl-5-isopropyl- (0.4/.../2.36/…)		
Pyrroles	7/4.14	1/0.8
Pyrrole (0.38/…/0.33/…)	7/4.08	1/1.24
2-carbonitrile (0.11/…/0.13/…)		
2-oxamide (0.78/…/1.04/…)		
1-methyl-2-(pyrrolidinyl)- (0.26/…/0.26/…)		
1-methyl-2-(pyrrolidinyl)- (0.26/.../0.34/…)		
2-Pyrrolidinone (1.78/0.8/1.6/1.24)		
Pyrrolidine, 1-acetyl (0.56/…/0.28/…)		
3,4-Dimethyl-3-pyrrolin-2-one (0.1/…/0.41/…)		
2-Pyrrolidinone, 4,4-dimethyl-5-methylidene- (0.17/…/0.25)		
Pyridines	6/1.16	–
2-Aminopyridine (0.37/…/0.33/…)	6/0.95	
2-Amino-4-methylpyrimidine (0.2/…/…/…)		
2-Pyridinamine,3-methyl (0.19/…/…/…)		
2-Pyridinamine,5-methyl (0.13/…/0.24/…)		
2(1H)-Pyrididinone, 3,6-dimethyl- (0.1/…/0.14/…)		
3H-Imidazo[4,5-b] pyridine-2-ethanamine, 3-n (0.17/…/0.24/…)		
Lactams	1/5.4	1/0.18
2-Piperidinone (5.4/0.18/5.0/2.38)	1/5.0	1/2.38

Continuation of Table 3

Chemical composition by group	Total substances in the group/concentration	
	GC/MS n1/S1, units/%	GC n2/S2, units/%
Indoles, pyrimidines	6/1.09	–
Indolizine,octahydro (0.1/…/0.1/…)	6/1.88	
2,4(1H,3H)-Pirimididinedione, dihydro-3-methyl (0.22/…/0.43/…)		
1-H-Indazol-5-amine, 3-methyl (0.19/…/0.35/…)		
Thymine (0.11/…/0.13/…)		
5,6-dimethyuracil (0.31/…/0.47/…)		
6-Azathymine (0.16/…/0.2/…)		
Alcohols and O-heterocycles	8/4.13	–
1-Propanol, 2-amino-2-methyl- (0.43/…/…/…)	5/4.37	
2-Hexanone oxime (0.17/…/…/…)		
2,5-Furandione, 3,4-dimethyl- (0.11/…/…/…)		
Oxetane, 2,3,4-trimethyl- (0.58/…/0.4/…)		
2,5-Furandione, dihydro-3-methyl (0.74/…/1.13/…)		
Oxetane, 3,3-dimethyl- (0.23/…/0.32/…)		
Furan, tetrahydro-3,4-dimethyl- (0.18/.../0.16/…)		
Benzoxazole, 2-methyl- (1.69/.../2.36/…)		
Total substances (out of 220) in the groups/concentration	69/68.4	18/4.01
	53/57.3	17/31.0

GC – gas chromatography; MS – mass spectrometry

Substances in bold type are of interest as possible marker indicators that can be used to develop production quality control.

As an example, acetic (7.03/0.88/1.5/0.23) in the carboxylic acids group (Table 3) can be interpreted as follows: acetic acid can be used as a standard substance; its relative content in the initial condensate is 7.03% (by GC-MS), its actual absolute content is 0.88% (by GC); its relative content in the concentrate is 1.5% (by GC-MS); and its actual content is 0.23% (by GC). The omission dots in brackets can be interpreted as follows: the substance was identified and its relative concentration was determined by GC-MS, but it was not analyzed by GC. In addition, some substances can be evaporated during concentration, for example, 2-methyl-propionic acid (2-methyl-Propanoic) is absent in the concentrate.

As can be seen in column 3 (Table 3), we identified 15 carboxylic acids in the water condensate and 8 carboxylic acids in the concentrate with a total relative concentration of 14.7 and 2.02%, respectively (by GC-MS). According to column 4 (Table 3), GLC identified 7 carboxylic acids in the water condensate and the same acids in the concentrate but with a lower concentration (1.02%) compared to the condensate (1.42%).

The organic part of the initial water condensate and its concentrate (Table 3) contained mainly nitrogen-containing substances. In the initial condensate, carboxylic acids (mainly fatty acids and ammonium salts) had a significant concentration of 15 (by GC-MS) and 1.4% (by GC), while in the concentrate, they were under 2 (by GC-MS) and 1% (by GC). A similar picture was observed for amides, which numbered 15 and 12 in the initial condensate and its concentrate, respectively (by GC-MS). Their contents in the group were 14.4 and 2.3% for the initial condensate and its concentrate, respectively. Additionally, the GC method identified 7 substances for the initial condensate and 6 substances for its concentrate at their absolute group concentrations of 0.65 and 7.7%, respectively.

Imidazoles (hydantoins) constitute one of the main groups of organic substances in condensates. They numbered 10 in both samples, with their content increasing from 23% in the initial condensate to 34% in its concentrate (by GC-MS). According to GC, their concentration increased even more significantly: from 1% in the condensate to 19% in its concentrate. The most important substances in this group are 5,5-dimethylhydantoin (0.7 and 13%) and 5-ethyl,5-methylhydantoin (0.31 and 5.6%) in the condensate and its concentrate by GC-MS and GC, respectively.

Of particular interest are pyrroles and lactams mainly represented by 2-pyrrolidinone and 2-piperidinone, whose concentration did not change significantly even after concentration. According to GC-MS, the group of pyrroles consisted of 7 substances with a total content of about 4%. The concentration 2-pyrrolidinone in the initial condensate was 1.78 and 0.8% by GC-MS and GC, respectively, while in the concentrate it amounted to 1.6 and 1.24% by GC-MS and GC, respectively. The group of lactams, however, was represented by only one substance, 2-piperidinone (δ-valerolactam), according to GC-MS. Its concentration in the initial condensate was 5.4 and 0.18% by GC-MS and GC, respectively, whereas in the concentrate it reached 5.0 and 2.38% by GC-MS and GC, respectively.

Such groups as pyridines, indoles, pyrimidines, O-heterocycles, and alcohols were only analyzed by

Table 4 Amino acid derivatives and dipeptides in ASD-2F by HPLC

Group of substances	Type of condensate sample		
	Initial condensate with derivatization, S, %	Initial condensate, S, %	Condensate concentrate (organic part), S, %
Free amino acid derivatives			
l-Lysine, methyl ester	0.12	–	–
Glycine, N-acetyl-	1.20	–	–
Alanine, 2-methyl-	0.88	–	–
L-leucine, N-acetyl, methyl ester	0.22	0.98	2.40
L-alanine, N-acetyl-	–	0.14	0.34
D-Alanine, N-allyloxycarbonyl-, dodecyl ester	–	0.26	0.41
L-Proline, 5-oxo-	1.14	–	–
L-proline, 1-acetyl-	–	0.37	0.52
L-Proline,5-oxo-, methyl-	–	0.36	0.73
Pyrrole-2-carboxylic acid	0.42	–	–
TOTAL (number/signal area)	6/3.98	5/2.11	5/1.48
Dipeptides			
Gly-Gly	0.56	–	–
Gly-Pro	–	0.90	1.24
Cyclo (-Gly-Pro)	–	3.27	4.41
Cyclo (-Ala-Ala)	–	0.20	0.42
Ala-Leu	–	0.41	0.74
Ala-Val	–	0.47	0.67
Cyclo (-Ala-Val)	–	0.45	0.66
Cyclo (-Ala-Pro) N-acetyl-	–	1.67	2.45
Cyclo (-Ala-Trp)	–	0.76	1.20
Val-Val	0.84	–	–
Cyclo (-Val-Val)	–	0.12	0.19
Cyclo (-Leu-Pro)	–	2.22	3.11
Cyclo (-Pro-Pro),Diethyl ester	–	2.03	2.73
Ile-Pro	–	1.03	1.29
Cyclo (-Ile-Pro)	–	0.15	0.30
TOTAL (number/signal area)	2/1.40	13/13.76	13/22.33

GC-MS. In total, we identified more than 20 individual organic substances (Table 3).

Table 4, which continues Table 3, shows the contents of amino acids and dipeptides in the initial water condensate and its concentrate by GC-MS. In addition, it presents the GC-MS data on silyl derivatives of the condensate obtained after derivatization.

Amino acid derivatives and dipeptides make up a significant part of the organic substances of the water condensate (Table 4).

As can be seen in Table 4, the GC-MS method without derivatization identified 5 amino acid derivatives with a total concentration of 1.5 to 2.1% in the water condensate and its concentrate, respectively. Alanine and proline derivatives dominated among the amino acids. Also, we identified 13 cyclic and linear dipeptides with a total relative concentration of 13.8 and 22.3% in the water condensate and its concentrate, respectively. Noteworthily, the dipeptides were mostly represented by alanine (6 dipeptides) and proline (6 dipeptides), and to a lesser extent by valine (3 dipeptides), glycine (3 dipeptides), leucine (2 dipeptides), isoleucine (2 dipeptides), and tryptophan (1 dipeptide).

Table 5 shows the contents of free and bound amino acids in the water condensate determined by HPLC. As we can see, 95% of all amino acids was presented by lysine, phenylalanine, leucine, argininepro line, and hydroxyproine, whose total content exceeded 2600 mg/100 mL (2.4% in the water condensate).

The profile of bound amino acids in the water condensate differed significantly from the profile of free amino acids. This might be due to the effect of hydrolysis or the type and concentration of dipeptides in the initial condensate. Our analysis confirmed that the condensate's organic substances contained significant amounts of dipeptides based on glycine, alanine, valine, histidine, proline, hydroxyproline, cysteine, and methionine.

According to Table 5, the total concentration of the organic substances identified in the study exceeded 90% of all the substances found during the chromatographic analysis of the water condensate and its concentrate.

The analysis of the oil-soluble condensate. The oil-soluble condensate (pyrolytic fuel, ASD-3F) is a mixture of organic pyrolysis products with low solubility in water which, after separation, form a fraction clearly separable from the water condensate. We used various organic solvents to extract organic substances from the oil fraction. According to our results, at least 60% (by mass) of substances could be extracted with butyl acetate, and about 70%, with hexane and ortho-xylene. The most efficient solvent was methylene chloride, which enabled the extraction of 80% of organic substances from the oil fraction. In total, we identified more than 320 organic substances by GC-MS, of which 118 substances had a content (signal area) of over 0.1%. They were grouped in the same way as the data on the water condensate (Table 6).

Table 5 Content of amino acids in the water condensate

Name of amino acid	Free amino acids, mg/100 mL	Bound amino acids, mg/100 mL
Glycine	8.54 ± 1.28	281.11 ± 19.68
Alanine	4.99 ± 0.75	528.07 ± 36.96
Valine	2.69 ± 0.40	175.05 ± 12.25
Leucine	298.00 ± 44.71	80.032 ± 5.600
Lysine	1067.00 ± 160.07	667.47 ± 46.72
Arginine	248.80 ± 37.32	–
Histidine	33.88 ± 5.08	214.21 ± 14.99
Aspartic acid	8.54 ± 2.98	11.19 ± 0.78
Glutamic acid	13.34 ± 2.00	95.26 ± 6.67
Isoleucine	3.90 ± 0.59	24.22 ± 1.70
Proline	109.30 ± 16.39	530.13 ± 37.11
Phenylalanine	689.30 ± 103.39	38.14 ± 2.67
Methionine	1.75 ± 0.26	658.13 ± 46.07
Serene	25.43 ± 3.81	3.05 ± 0.21
Threonine	12.40 ± 1.86	–
Cysteine	14.22 ± 2.13	214.19 ± 14.99
Tyrosine	5.93 ± 0.89	2.05 ± 0.14
Hydroxyproline	185.50 ± 9.30	1855.69 ± 129.89
Hydrolysin	11.10 ± 0.76	11.37 ± 0.79
TOTAL	2744.60 ± 192.00	5389.36 ± 377.25

Table 6 Chemical composition of the oil-soluble (fuel) fraction of pyrolysis products by group

Substances by group (retention time, min)	Total substances in the group/Total signal area
Aromatic hydrocarbons	20/7.69
1-Octene,3.7-dimehyl- (0.12)	
Toluene (1.37)	
2-Decene, 6-mehyl-,(Z) (0.36)	
Ethylbenzene (0.46)	
p-Xylene (0.24)	
o-Xylene (0.33)	
Styrene (1.06)	
Benzene, propyl- (0.31)	
Benzene, 1-ethyl-2-methyl- (0.24)	
Benzene, 1-ethyl-3-methyl- (0.29)	
Mesitylene (0.14)	
Benzene, 1-ethenyl-2-methyl- (0.56)	
Benzene, 2-propenyl- (0.19)	
Benzene, n-butyl- (0.19)	
Benzene, pentyl- (0.34)	
Benzene, (1-methyl-2-cyclopropen-1-yl)- (0.29)	
Benzene, hexyl- (0.12)	
Butylated Hydroxytoluene (0.17)	
Indene (0.78)	
1H-Indene,1,1-dimethyl (0.13)	

Continuation of Table 6

Substances by group (retention time, min)	Total substances in the group/Total signal area
Higher hydrocarbons	15/10.75
1-Decene (0.73)	
1-Undecene (1.06	
1-Tridecene (1.16)	
Tridecane (0.54)	
Cyclododecane (1.17)	
Tetradecane (0.6)	
Pentadecane (1.27)	
n-Tridecan-1-ol (0.66)	
Hexadecane (0.68)	
n-Pentadecanol (0.77)	
3-Heptadecene, (Z)- (0.63)	
Heptadecane (0.70)	
Octadecane (0.23)	
1-Octadecene (0.22)	
Nonadecane (0.28)	
Phenols	10/7.94
Phenol (2.23)	
o-Cresol (0.8)	
Phenol, 2,6-dimethyl (0.15)	
p-Cresol (3.0)	
Phenol, 2-ethyl-(0.18)	
Phenol, 2,4-dimethyl- (0.7)	
Phenol, 2-ethyl-5-methyl- (0.13)	
Phenol, 2,3,6-trimethyl- (0.2)	
Phenol, 2-ethyl-4-methyl- (0.4)	
Biphenyl (0.15)	
Amides, esters	11/10.14
Enanthamide (0.13)	
Hexanamide (0.2)	
Phenylamide, 4-methyl- (0.52)	
Phenylpropanamide (0.16)	
Heptadecanoic acid,methyl ester (0.17)	
Hexadecanamide (3.42)	
Miristamide, N-methyl- (0.92)	
Octadecanamide (2.07)	
Propanamide, cyclopentyl-N-methyl- (1.05)	
Miristamide, N-methyl- (0.3)	
Nitriles	25/31.27
Butanenitrile, 3-methyl- (0.17)	
Heptanonitrile (0.2)	
6-Hepten-1-nitrile (0.32)	
Benzonitrile, 3-methyl- (0.12)	
Octanenitrile (0.24)	
7-Octene-1-nitrile (0.38)	
Benzonitrile, 2-methyl- (0.16)	
2,4-Pentadienenitrile, 2-amino-4-methyl (0.19)	
Nonanenitrile (0.27)	
Benzyl nitrile (0.63)	
9-Decene-1-nitrile (0.17)	
Benzenepropanenitrile (0.75)	
1H-Pyrrole-2-carbonitrile (0.44)	
Undecanenitrile (0.28)	
Benzonitrile, 2,4,6-trimethyl- (0.38)	
1,5-Dimethyl-2-pyrrolecarbonitrile (0.11)	
Tetradecanenitrile (0.32)	
10-Undecenenitrile (0.13)	
Propanenitryle, 3,3-thyobis- (0.49); Pentadecanenitrile (0.1)	
Oleanitrile (9.95)	
Heptadecanenitrile (9.39)	
Octadecynenitrile (5.18)	
Nonadecanenitrile (0.18)	

Continuation of Table 6

Substances by group (retention time, min)	Total substances in the group/Total signal area
Pyridines	6/1.00
Pyridine (0.18)	
Pyridine, 2,6-dimethyl- (0.11)	
Pyridine, 2-ethyl- (0.24)	
Pyridine, 2,4-dimethyl- (0.25)	
Pyridine, 1,2,3,6-tetrahydro-1-(phenylmethyl)- (0.22)	
Naphthalenes	6/2.37
Naphthalene, -tetrahydro (0.11)	
Naphthalene, -dihydro (0.11)	
Naphthalene (0.87)	
Naphthalene, 2-methyl- (0.38)	
Naphthalene, 1-methyl- (0.75)	
Isopropenylnaphthalene (0.15)	
N-heterocycles	16/9.04
Pyrrole (2.03)	
Pyrazine, 2,6-dimethyl- (0.15)	
1-H-Pyrrole-2,5-dione, 3-ethyl-4-methyl- (0.11)	
Indole (2.97)	
Indole, 3-methyl- (1.01)	
1H-Indole, 4-methyl- (0.13)	
1H-Indole, 2-methyl- (0.5)	
1H-Indole, 2,3-dimethyl- (0.46)	
1H-1,3-Benzimidazol-4-amine, 5-methyl- (0.2)	
Benzofuro[3.2-d]pyrimidin-4(3H)-one (0.34)	
1H-Benzimidazole, 2,5-dimethyl- (0.11)	
5H-Indeno[1,2-b]pyridine (0.19)	
9H-Pyrido[3,4-b]indole,1-methyl- (0.2)	
2,4-Imidazolidinone-5,5-dimethyl (0.12)	
2,4-Imidazolidinone-5-methyl-5-ethyl- (0.4)	
2,4-Imidazolidinone-5-methyl-5-(2-methyl)- (0.12)	
Dipeptides	8/1.56
Cyclo (-Val-Val) (0.15)	
Cyclo (-Ala-Trp) (0.16)	
Cyclo (-Gly-Pro) (0.1)	
Leu-Pro (0.12)	
Cyclo (-Leu-Pro) (0.32)	
Cyclo (-Pro-Pro), Diethyl ester (0.23)	
Ile-Pro (0.13)	
Cyclo (-Ile-Pro) (0.36)	
Total (out of 350 substances, 100%)	118/81.5

According to Table 6, the group of nitriles contained the largest number of substances (25) with the highest total content (31%). High concentrations were found for the nitriles of oleic acid ($C_{18}H_{31}N$, about 10%), margaric acid ($C_{17}H_{33}N$, 9.4%), and stearic acid ($C_{18}H_{35}N$, 5.2%), which accounted for over 25% of all organic substances.

Almost equivalent in concentration were the groups of amides and esters (11 substances, 10%), N-heterocycles (16 substances, 9%), and higher hydrocarbons (15 substances, more than 10%). The main substances in the group of amides and esters were palmitic acid amide (3.4%) and stearic acid amide, together accounting for half of all the substances in the group. Indole had the highest concentration in the N-heterocycle group (about 3%), accounting for almost a third of all organic substances in the group. The group of higher hydrocarbons did not have any dominant substances, with most substances making up 0.5–0.7%.

The groups of aromatic hydrocarbons and phenols had a similar total content (about 8%), but differed sharply in the number of substances (20 and 10, respectively). Aromatic hydrocarbons were mainly represented by aliphatic derivatives of benzene, as well as styrene. Only toluene and styrene had concentrations above 1%, with the other substances having lower contents. In the group of phenols dominated phenol itself (2.2%), cresols (2 substances, 3.8%), and xylenol (0.7%).

The naphthalene group contained 6 substances and had a total concentration of 2.4%, where naphthalene and methylnaphthalene made up 1.6%.

The oil-soluble condensate contained relatively few pyridines and dipeptides, accounting for 3% in total. However, the dipeptides in this condensate appeared to be similar to those found in the water condensate of the pyrolysis products.

CONCLUSION

We studied the chemical composition of the water- and oil-soluble condensates of meat and bone meal pyrolysis by gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC). These methods identified up to 80% of individual organic substances in the water-soluble condensate.

The water-soluble condensate and its dehydrated and desalted concentrate contained over 220 organic substances divided into 10 main groups. The condensate was mostly represented by ammonium salts and carboxylic acid amides, N-heterocyclic compounds, hydantoins, amino acids, and dipeptides, with a total content of 8% of the condensate's weight. We used gas-liquid chromatography and analytical standards to determine the contents of 27 substances in the water condensate. Their total concentration was 4% of the condensate's organic substances, or over 50% of the relative content.

HPLC identified 19 free amino acids and peptides in the water condensate, with a total content of 2.5% each. We found that the organic substances identified during our study exceeded 90% of all the substances determined chromatographically in the initial water condensate. Its dehydrated concentrate had almost no ammonium salts or carboxylic acid amides. However, the contents of hydantoins, amino acids (10), dipeptides (13), and low-volatile nitrogen-containing heterocycles were 10–15 times as high as in the initial condensate, accounting for 31% of the total content of organic substances.

The oil-soluble condensate (pyrolytic fuel) contained over 350 individual organic substances divided into 9 main groups, which were determined by GC-MS. More than 40% of the substances were nitriles and amides of fatty acids, which were part of original animal fats, as well as aromatic and higher hydrocarbons, N-heterocyclic compounds, and aliphatic derivatives of phenols. We found that the nitrogen-containing heterocycles and a small amount of dipeptides (up to 10%) were similar to those in the water-soluble condensate.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Top-dressing treatment of spring barley to modify its quality

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Abstract:
New barley products can be developed by modifying the content of bioactive components in the grain through breeding, as well as improving its quality at lower fertilizer costs. We aimed to study the effects of the genotype, growth conditions, and top-dressing application of nitrogen and organo-mineral fertilizers on the chemical composition of barley grain.
The barley varieties Novichok, Rodnik Prikamya, and Pamyaty Rodinoy were grown under normal (2020) and dry (2021) field conditions. The plants were top-dressed with mineral (CAS; SpetsKhimAgro, Kirovo-Chepetsk, Russia) or organo-mineral (Amino Start and Alfastim; Polydon® Agro, Moscow, Russia) fertilizers in the tillering or heading phases. The contents of protein, starch, fat, and crude fiber in the grain were analyzed with an INFRAMATIC 8620 instrument (Perten Instruments, Stockholm, Sweden).
The CAS fertilizer reduced protein, fat, and fiber by 4.5–8.3% (Novichok) during the drought and increased starch by 2.1% (Novichok), fiber by 14.2% (Rodnik Prikamya), and fat by 18.9% (Pamyaty Rodinoy) under normal humidity. Amino Start applied under normal conditions increased starch by 2.9% and reduced protein and fat by 7.8–8.9% in Rodnik Prikamya, as well as increased protein and fat by 14.4 and 6.3%, respectively, but reduced starch by 5.1% in Pamyaty Rodinoy. Alfastim applied under normal conditions reduced the content of protein by 10.7% (Rodnik Prikamya), but increased it by 3.6–7.2% in the other cultivars. It also increased fiber by 22.8% in Rodnik Prikamya, but decreased it by 18.6% in Pamyaty Rodinoy. Finally, this fertilizer decreased fat by 12.7% in Rodnik Prikamya, but increased it by 9.8% in Pamyaty Rodinoy. In the drought, the fertilizers Alfastim and Amino Start increased the protein content by 5.2–12.2% in Rodnik Prikamya and Pamyaty Rodinoy.
Top-dressing barley plants with mineral or organo-mineral fertilizers can modify the grain composition (up to 10.4% of fiber, 3.6% of starch, and 7.5% of protein and fat), depending on the consumer's requirements.

Keywords: *Hordeum vulgare*, cultivar, protein, fiber, starch, fat, yield

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INTRODUCTION

The barley (*Hordeum vulgare* L.) is one of the oldest cereals in the world that was first cultivated in the eighth millennium BC [1]. Currently, it ranks fourth by yield and crop area globally. First used as human food, barley began to be mainly used as animal feed and brewing grain, partly due to the increased importance of wheat and rice [2, 3]. Due to its ability to grow in a wide range of climatic conditions, barley is widely cultivated in those countries where the production of other cereals is difficult [4]. Today, barley is grown in more than 100 countries around the world. In 2020, its world production amounted to 152 Mt, following rice (508.7 Mt), wheat (758.3 Mt), and corn (1207.1 Mt).

In 2019, Europe accounted for over 94.2 Mt (60%) of global barley production, followed by Asia (22.6 Mt, 16%) and North and Central America (14.5 Mt, 9%). Russia, France, and Germany have produced over 10% of the world barley production each over the past ten years [5]. In 2020, the total barley area was 51 Mha, following rice (164 Mha), corn (201 Mha), and wheat (219 Mha) [6]. Barley yield averaged 1.8 t/ha in the 1960–1970s, 2.2 t/ha in the 1980–1990s, and 2.7 t/ha since the 2000s. In 2016, it broke the barrier of 3 t/ha, with the highest yield of 3.1 t/ha in 2019.

In 2020, per capita barley consumption was highest in North Africa, with 19.5 kg in Morocco, 17.2 kg in Ethiopia, and 16.0 kg in Syria, according to FAO. This indicator is much lower in the developed countries, with 0.8 kg in the European Union, 0.6 kg in the USA, and 0.3 kg in Canada. Globally, per capita barley consumption in 2020 was 1 kg compared to 18.4 kg for corn, 53.9 kg for rice, and 67.4 kg for wheat [5].

Barley is commonly used as feed, food, and brewing grain, accounting for 65–75, 15, and 8%, respectively [7–9]. The last decade has seen an increased interest in using barley as food for humans [10]. Human health and well-being are often associated with a balanced diet. Therefore, consumers look for functional foods that have a preventative effect on chronic diseases in addition to replenishing essential nutrients [11, 12].

Chemically, barley has a high content of protein, fiber, and vitamins, as well as a low content of fat and sugar [13]. It is rich in natural antioxidants and beta-glucan, a unique soluble fiber, as well as bioactive compounds including health-benefitting phenols and lipids [14–16].

The rising interest in barley has coincided with increased obesity and chronic diseases, such as cancer and heart disease. According to medical studies, a prolonged intake of food rich in barley flour may protect the body against hyperlipidemia, diabetes, atherosclerosis, and cancer [17, 18]. Cereals such as barley and oats have been shown to reduce the risk of developing type 2 diabetes [19].

The positive characteristics of barley suggest growing possibilities for its use in food products [20, 21]. Currently, the use of barley grain is determined by various factors. The first factor is its content of protein, beta-glucans, starch, and non-starchy polysaccharides [8]. Foreign breeders, selecting new barley varieties to be used as feed, food, and brewing grain, commonly evaluate only two parameters: the hull content (chaffy or hulless grains) and the beta-glucan content. Malt barleys are almost always chaffy and low in beta-glucan. Food barleys are usually hulless and have an average content of beta-glucan. Feed barleys can be both chaffy and hulless, with a low content of beta-glucan. It is common practice that if barley grain grown for brewing does not meet certain standards, it is redirected to the feed market at a substantially lower price [22]. Food barley can also be used as feed if it is of poor quality.

Unlike foreign breeders, the Russian State Standards (R 53900-2010 for feed barley and 5060-86 for brewing barley) take into account the contents of protein, crude fiber, and ash to assess the quality of barley grain and its uses. The quality of feed barley is also determined by its fat content [23]. These components are largely dependent on the weather conditions during the growing season. For example, Bindereif *et al.* showed significant changes in protein and fat contents in climatically contrasting years [24]. Yusov *et al.* reported the effect of weather conditions on the contents of protein, starch, and fat [25]. Bohačenko *et al.* established the effect of humidity in greenhouses and phytotrons on the contents of protein and starch in barley grain [26].

On the other hand, agronomists have practiced split nitrogen application over the last decade. In particular, they first apply nitrogen during sowing and then use it as a top dressing during the critical phase of its consumption by the plants [27]. The effect of top dressing is commonly evaluated by two main indicators, the yield and the protein content in the grain. Therefore, it is of practical significance to study the effect of top-dressing application of nitrogen fertilizers on the contents of nutrients (starch, crude fiber, and fat) in barley grain grown under different humidity conditions. In addition, barley's high genotype diversity provides ample opportunities for identifying and breeding cultivars for specific uses [28, 29]. One of the key priorities in breeding is to identify the grain's genetic ability for more efficient nitrogen use and high yields, as well as to ensure its quality at lower nitrogen costs [30, 31].

To develop new products and breed new cultivars, we need to be aware of variations in the contents of bioactive components in barley grain and their mutual relations [30]. Therefore, we aimed to study the effect of the genotype, growing conditions and top-dress application of nitrogen and organo-mineral fertilizers on the chemical composition of barley grain.

STUDY OBJECTS AND METHODS

Our experiments were carried out in 2020–2021 in the field crop rotation of the Department of Agrochemistry and Crop Farming at the Federal Agricultural Research Center of the North-East (Kirov, Russia). The experimental site had a sod-podzolic, medium loam soil formed on the eluvium of Perm clays. Its arable layer had the following agrochemical parameters: pH_{KCl} = 4.59–5.00 units, mobile phosphorus – 148.0–157.0 mg/kg of soil, mobile potassium – 127.0–140.0 mg/kg of soil, and humus – 1.74–2.00%. The site's area was 10 m^2, with the plots systematically placed with an offset. The experiments were conducted in quadruple. Pre-sowing mineral fertilizers NPKS (25:4:4:2) were applied at an amount of 0.3 t/ha, which ensured at least 3.5–4.5 t/ha of spring barley grain, according to previous studies.

Our study objects were spring barley cultivars named Novichok, Rodnik Prikamya, and Pamyaty

Rodinoy. The cultivars were created for grain-fodder use by I.N. Shchennikova, a Corresponding Member of the Russian Academy of Sciences, in the Federal Agricultural Research Center of the North-East. Rodnik Prikamya and Pamyaty Rodinoy are on the list of the most valuable barley varieties in the Russian Federation.

Polydon® Amino Start and Alfastim® (Polidon® Agro Company, Moscow, Russia) were used as organo-mineral fertilizers. They contain macro-, meso-, and microelements in combination with amino acids and low-molecular-weight peptides to increase the intake of mineral fertilizers at the initial stages of vegetation. Top-dressing application of Alfastim in the second half of the growing season is recommended to increase the yield and quality of the crop. These liquid organo-mineral fertilizers of a new generation are based on humic and fulvic acids, natural growth agents, microelements, amino acids, and polysaccharides. They are widely used in the cultivation of grain crops, winter rapeseed, corn, and soybeans [32].

Polydon® Amino Start contains 200 g/L of L-amino acids, 130 g/L of nitrogen (total N), 75 g/L of phosphorus (P_2O_5), 25 g/L of potassium (K_2O), 15 g/L of magnesium (MgO), 6 g/L of iron (Fe), 3 g/L of manganese (Mn), 3 g/L of zinc (Zn), 3 g/L of copper (Cu), 3 g/L of boron (B), 1 g/L of molybdenum (Mo), and 0.05 g/L of cobalt (Co). It is used to stimulate the growth of the root system and increase the plant's productive tillering, stress resistance, and yield.

Alfastim® contains triterpene acids (100 g/L), L-amino acids (50 g/L), carbohydrates (50 g/L), auxin-cytokinin complex (10 g/L), membrane-active substances (10 g/L), and vitamins B_1, B_7, PP (5 g/L). The fertilizer activates the most important metabolic reactions, regulates the absorption of nutrients, stimulates the excretion of the root system, and increases the permeability its cell walls. In addition, it has immune-stimulating, antioxidant, and adaptogenic effects, increasing resistance to water scarcity, salt and chemical stress, as well as pathogen and pest attacks.

The liquid mineral fertilizer CAS 28 (carbamide-ammonium saltpeter mixture, or urea ammonium nitrate in English literature) (SpetsKhimAgro, Kirovo-Chepetsk, Russia) is the only nitrogen fertilizer that contains nitrate, ammonium, and amide nitrogen and does not contain free ammonia, which can significantly reduce unproductive losses of nitrogen [33, 34]. It is used to activate the growth of the plant during the intensive development of its above-ground part and the formation of flower buds, the key to the harvest.

The experiment's scheme was as follows:
– control (without treatment of vegetative plants with mineral and organo-mineral fertilizers);
– top-dressing with Polidon® Amino Start (1 L/ha) in the tillering phase;
– top-dressing with CAS 28 (30 L/ha) in the tillering phase; and
– top-dressing with Alfastim® (1 L/ha) in the heading phase.

Harvesting was carried out with a Wintersteiger combine (Wintersteiger Seedmech, Ried im Innkreis, Austria) in the phase of full wax ripeness. The contents of crude protein, starch, crude fiber, and fat in barley grain were measured with an INFRAMATIC 8620 analyzer (Perten Instruments, Stockholm, Sweden) according to the manufacturer's guidelines. The data were expressed as a percentage of the grain's dry weight.

Statistical processing of the data was carried out using descriptive statistics, correlation and variance analyzes in Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA).

In May, June, and the first half of July 2020, the weather was unstable in temperature and rainfall – dry in the first ten days of a month, with light, sometimes heavy rains in the second or third ten-day periods (Table 1). The second half of July was moderately warm, with frequent, sometimes heavy rains. August was warm to moderately warm, mostly dry or with little rainfall. On the whole, the prevailing weather conditions in the 2020 growing season were favorable for the cultivation of spring barley.

In May 2021, the weather was predominantly warm and hot, with both dry and rainy periods. June and July were moderately warm to hot, as well as dry with occasional rain. Some places had soil drought. August was warm to hot with local rains. On the whole, the vegetation conditions in 2021 can be described as moderately dry.

Table 1 Weather conditions during the growing season (Kirov weather station)

Month	Average t, °C	Deviation from the norm, °C	Rainfall, mm	% of the norm	Sum of effective temperatures, °C
			2020		
May	12.2	+0.9	89	154	226.6
June	15.3	–1.2	41	47	535.0
July	20.5	+1.6	100	110	1016.0
August	15.1	–0.5	61	73	1327.9
			2021		
May	15.0	+3.1	58	107	320.4
June	19.9	+3.5	63	78	767.3
July	19.2	+0.3	92	113	1207.2
August	18.8	+2.9	38	51	1634.8

Table 2 Effect of top-dressing fertilization on spring barley yield, t/ha

Cultivar	Mineral fertilizers			
	Control	CAS	Amino Start	Alfastim
		2020		
Novichok	2.95[a]	3.38[b]	4.22[c]	4.39[c]
Rodnik Prikamya	5.03[a]	5.37[b]	5.01[a]	4.86[a]
Pamyaty Rodinoy	5.17[a]	5.48[b]	5.99[c]	4.94[a]
		2021		
Novichok	1.25[a]	2.22[b]	2.24[b]	2.02[b]
Rodnik Prikamya	2.19[a]	2.76[c]	2.31[ab]	2.45[b]
Pamyaty Rodinoy	1.75[b]	1.55[ab]	1.75[b]	1.29[a]

[a, b, c] – The parameter values accompanied by the same letters do not differ statistically according to the Duncan criterion at $p > 0.05$

RESULTS AND DISCUSSION

Under normal humidity conditions of the 2020 growing season, Pamyaty Rodinoy was the most productive cultivar, with an average grain yield of 5.40 t/ha, compared to 3.74 t/ha for Novichok (Table 2).

The top-dressing fertilization with CAS 28, Polidon® Amino Start, and Alfastim® increased the barley yield by an average of 8.3, 15.8, and 8.2%, respectively. The pre-sowing application of nitrogen fertilizers is insufficient for barley, since it has low needs in this element in the early stages of growth, and precipitation leads to its unproductive loss from the soil [27].

In a study by Plaza-Bonilla *et al.*, the top-dressing application of CAS 28 resulted in an 18% increase in barley yield compared to the control [35]. The authors recommended top-dressing to increase barley yield in the Mediterranean countries in the years with normal humidity. Glukhovtsev *et al.* reported an 8.4–17.8% increase in barley yield from the top-dressing of four spring barley cultivars with mineral and organo-mineral fertilizers [36]. In contrast to our study and those by the above authors [35, 36], Tanaka and Nakano found no effect from the top-dressing of barley with nitrogen fertilizers at normal air temperatures but they reported a positive effect at high temperatures [37]. This might be due to the fact that the Japanese researchers worked with winter barley, rather than spring barley, which grows predominantly at low air temperatures, with only a 2–3°C difference between the average temperatures during the study years and the average annual data.

The cultivars under study reacted to top-dressing differently. Particularly, Novichok was the most responsive to improved nutrition, showing a statistically significant increase of 14.6–48.8% in barley yield. Rodnik Prikamya and Pamyaty Rodinoy had a significant increase of 6.0–6.7% with the application of CAS 28 in the tillering phase. Pamyaty Rodinoy also showed a significant yield increase of 15.9% when top-dressed with Polidon® Amino Start. The treatment with Alfastim® in the heading phase was effective only for the Novichok cultivar (48.8% increase). Rodnik Prikamya was the least responsive to the application of the fertilizers. Our findings were consistent with those in the previous studies [36, 38].

Under the dry conditions of 2021, top-dressing proved significantly more effective than in normal humidity conditions, which was consistent with the conclusions made by Glukhovtsev *et al.*, Tanaka and Nakano, and Kastury *et al.* [36, 37, 39]. In particular, the application of CAS 28, Polydon® Amino Start, and Alfastim® increased the barley yield by 25.8, 21.4, and 11.0%, respectively, compared to the control. Rodnik Prikamya showed a significantly higher yield (25–52%) than the other cultivars. The top-dressing of this cultivar with Polidon® Amino Start did not lead to higher yield, while the other two fertilizers (CAS 28 and Alfastim®) increased its yield by 12–26%. The Novichok cultivar, just like in 2020, responded positively to all the fertilizers, with an increase in grain yield of 62–79%. The Pamyaty Rodinoy cultivar showed no response to the treatment with CAS 28 and Polidon® Amino Start, and had a 26% decrease in yield when top-dressed with Alfastim®.

According to the two-factor ANOVA, under the normal humidity conditions of 2020, the effects of the cultivar's genotype, top-dressing fertilization, and interaction of factors on yield variability were 64.8, 7.3, and 15.1%, respectively. Under the dry conditions of 2021, these indicators amounted to 47.9, 12.0, and 21.1%, respectively.

Over 90% of barley grain's dry matter consists of three main components: starch (59.1–61.6%), fiber (18.16–21.46%), and crude protein (11.74–13.64%) [40]. Therefore, while increasing the productivity of spring barley cultivars, we should maintain their quality indicators. According to State Standard R53900-2010, the content of crude protein in feed barley of classes 1, 2, and 3 must be at least 130 g/kg (13%), 120–130 g/kg (12–13%), and 120 g/kg (12%), respectively.

Table 3 shows a significant effect of genotypic differences and top-dressing fertilization on the protein content in barley grain, which is consistent with the previous studies [30, 41].

As can be seen, the protein contents in our study were within the typical protein contents for spring barley of 9–13% according to Sterna *et al.*, Ortiz *et al.*, and Filippov *et al.* or 7–30% according to Jaeger *et al.* and Gong [30, 42–45].

According to Table 3, the Polidon® Amino Start and Alfastim® fertilizers raised the quality of the Rodnik Prikamya cultivar from class 3 to class 2 under the dry conditions of 2021. We also found that Polidon® Amino Start raised the quality of the Pamyaty Rodinoy cultivar from class 3 to class 1 under the normal humidity conditions of 2020. In the other cases, the barley grain corresponded to class 3.

Published data indicate that the protein content in barley grain is determined by a combination of genetic, environmental, and agronomic factors [46]. Large amounts of nitrogen and abiotic stress (drought and high temperatures) increase the protein content in

Table 3 Effect of top-dressings on protein content in barley grain, %

Treatment	Barley cultivars		
	Novichok	Rodnik Prikamya	Pamyaty Rodinoy
2020			
Control	10.92[a]	11.96[c]	11.46[a]
CAS 28	10.87[a]	11.93[c]	11.61[a]
Polidon® Amino Start	10.84[a]	11.03[b]	13.11[b]
Alfastim®	11.32[b]	10.68[a]	11.46[a]
2021			
Control	11.92[b]	11.27[a]	10.82[a]
CAS 28	11.38[a]	11.72[a]	10.98[ab]
Polidon® Amino Start	11.59[ab]	12.48[b]	11.72[b]
Alfastim®	11.40[a]	12.65[b]	11.71[b]

[a, b, c] – The parameter values accompanied by the same letters do not differ statistically according to the Duncan criterion at $p > 0.05$

Table 4 Effect of top-dressings on fiber content in barley grain, %

Treatment	Barley cultivars		
	Novichok	Rodnik Prikamya	Pamyaty Rodinoy
2020			
Control	3.49[ab]	2.81[a]	3.45[b]
CAS 28	3.95[b]	3.21[b]	3.06[b]
Polidon® Amino Start	3.64[b]	2.81[a]	2.92[a]
Alfastim®	3.33[a]	3.45[b]	2.81[a]
2021			
Control	5.52[c]	4.83[a]	4.78[a]
CAS 28	5.06[ab]	4.92[a]	4.73[a]
Polidon® Amino Start	5.00[a]	5.03[a]	4.63[a]
Alfastim®	5.31[b]	4.98[a]	4.77[a]

[a, b, c] – The parameter values accompanied by the same letters do not differ statistically according to the Duncan criterion at $p > 0.05$

grain [18, 47]. In our study, we found varietal differences in the cultivars' response to the same fertilizers in the years with contrasting humidity. In 2020 (normal humidity), the Alfastim® fertilizer increased the protein content in the Novichok cultivar and reduced it in the Rodnik Prikamya cultivar. In 2021 (drought), however, this fertilizer had the opposite effect on these cultivars. The Pamyaty Rodinoy cultivar benefited from all the fertilizers in dry conditions. Polidon® Amino Start had a positive effect on Pamyaty Rodinoy in both years, while for Rodnik Prikamya, its effect was positive in dry conditions and negative under normal humidity. Novichok was not affected by this fertilizer in both years. In dry conditions, it had a protein decrease of 4.5% after being treated with the CAS 28 fertilizer.

The dry conditions of 2021 halved the effect of the cultivar's genotype on the protein variability (23.3 and 12.2% for 2020 and 2021, respectively) and doubled the effect of top-dressings (5.4 and 11.4% for 2020 and 2021, respectively). The protein content correlated with barley yield in 2020 (r = 0.701), but their relationship became statistically insignificant under dry conditions.

Crude fiber is a coarse, indigestible component of plants. The higher its content in farm animal feed, the lower is the feed's nutritional value. At the same time, all animals need fiber in moderation to stimulate their intestinal tract [18]. We found that in all the variants of treatment, the resulting grain belonged to class 1, with a content of crude fiber under 70 g/kg (7%), varying within 2.81–5.52% (Table 4).

In our work, fiber contents (Table 4) were significantly lower than those obtained in the studies by Šterna *et al.*, Biel and Jacyno, and Prasadi and Joye, where total fiber in barley ranged within 11–34% [30, 40, 48]. However, our findings were consistent with the data of Ul Ain *et al.* and Sumina and Polonsky, who reported the content of non-starch cell wall polysaccharides of 3–8% [47, 49]. These discrepancies can be partly explained by different expressions of fiber content, i.e., in relation to absolutely dry grain mass (in the above studies) and air-dry grain mass (in our study).

In scientific literature, the term "fiber" often refers to several different, although chemically close, substances. For example, Li and Komarek differentiate between insoluble, soluble, and total fiber [50]. Insoluble fiber includes cellulose, water-insoluble hemicellulose, and lignin, i.e., structural components of the cell wall. Soluble fiber is composed of a variety of non-cellulose poly- and oligosaccharides.

In the study by Prasadi and Joye, the contents of soluble, insoluble (cellulose, arabinoxylan and lignin), and total fiber in barley grain were 2.6–5.0, 12.0–22.1, and 10.1–27.9%, respectively [48]. According to State Standard R 53900-2010, feed barley of class 1 must contain no more than 7% of crude fiber, while that of class 3, at least 9%. Since we studied feed cultivars, rather than brewing grain, they were low in crude fiber. Crude fiber was determined by treating crushed grain samples with concentrated solutions of sulfuric acid (removing starch and hemicellulose) and then with caustic potassium (removing lignin). As a result, only cellulose remained in the sample. With this approach, our fiber contents coincided with the cellulose content (4.3–4.6%) reported by Biel and Jacyno [40].

Under the dry conditions of 2021, the top-dressings did not significantly affect the fiber content in the Rodnik Prikamya and Pamyaty Rodinoy cultivars, while significantly reducing it in the Novichok cultivar (by 3.8–9.4%). Under the normal humidity conditions of 2020, Novichok did not show any statistically significant response to the fertilization. Pamyaty Rodinoy had a reduced fiber content when treated with Polidon® Amino Start and Alfastim® (by 15.3–18.7%), while Rodnik Prikamya, on the contrary, had a higher content when treated with CAS 28 and Alfastim® (by 14.2–22.8%).

The effect of the top dressings on fiber variability was 4.9% in 2020. However, under the dry conditions of 2021, it was statistically insignificant. Yet, the effect

Table 5 Effect of top-dressings on starch content in barley grain, %

Treatment	Barley cultivars		
	Novichok	Rodnik Prikamya	Pamyaty Rodinoy
2020			
Control	52.51[a]	48.47[a]	50.21[b]
CAS 28	53.59[b]	49.38[ab]	50.63[c]
Polidon® Amino Start	53.91[b]	49.89[b]	47.66[a]
Alfastim®	53.68[ab]	50.62[b]	50.43[bc]
2021			
Control	51.00[ab]	48.87[a]	50.78[b]
CAS 28	50.20[a]	49.05[a]	52.04[c]
Polidon® Amino Start	50.95[ab]	48.64[a]	48.76[a]
Alfastim®	51.31[b]	48.51[a]	49.09[a]

[a, b, c] – The parameter values accompanied by the same letters do not differ statistically according to the Duncan criterion at $p > 0.05$

Table 6 Effect of top-dressings on fat content in barley grain, %

Treatment	Barley cultivars		
	Novichok	Rodnik Prikamya	Pamyaty Rodinoy
2020			
Control	1.82[a]	1.57[b]	1.43[a]
CAS 28	1.85[a]	1.58[b]	1.70[c]
Polidon® Amino Start	1.92[a]	1.43[a]	1.52[b]
Alfastim®	1.82[a]	1.37[a]	1.57[b]
2021			
Control	2.44[b]	2.20[a]	2.17[a]
CAS 28	2.29[a]	2.15[a]	2.31[ab]
Polidon® Amino Start	2.30[a]	2.16[a]	2.28[ab]
Alfastim®	2.43[b]	2.20[a]	2.34[b]

[a, b, c] – The parameter values accompanied by the same letters do not differ statistically according to the Duncan criterion at $p > 0.05$

of the cultivar's genotype remained almost unchanged, regardless of humidity (28.8 and 27.2% for 2020 and 2021, respectively).

We found no statistically significant correlations between the contents of fiber and protein in the cultivars ($p \leq 0.05$) in both years. There is no consensus in literature on these two indicators. For example, Biel and Jacyno and Filippov *et al.* reported higher contents of crude protein in barley with lower contents of starch and fiber [40, 43]. However, Šterna *et al.* revealed close positive relationships between these indicators [30]. This might be due to differences in the plant growth conditions and in the specific genotypes selected for study.

Starch is the third dominant component of spring barley grain that has a significant impact on its nutritional quality. Whole grain barley may contain 58–77% of starch [51]. In our study, the content of starch was somewhat lower (Table 5), but it was within the range reported by Doroshenko *et al.* and Izydorczyk *et al.* [15, 18].

The Rodnik Prikamya cultivar reacted to top-dressing fertilization by changing its starch content. With no significant effect observed in 2021 (drought), Polidon® Amino Start and Alfastim® had a slight positive effect (2.9–4.4%) in 2020 (normal humidity). Pamyaty Rodinoy reacted to the top-dressings more strongly, compared to the other cultivars. In both years, the CAS 28 fertilizer significantly increased the starch content in this cultivar (by 0.8–2.4%), while Polidon® Amino Start, on the contrary, decreased this indicator (by 5.1–4.0%). Alfastim® slightly reduced the starch content (by 3.3%) in dry conditions. Novichok's starch content was not significantly affected by the fertilizers in 2021. However, under normal humidity, it increased (by 2.1–2.6%) under the influence of CAS 28 and Polydon® Amino Start. Thus, we observed a significant effect of genotypic differences and growth conditions on the starch content.

According to ANOVA, the top-dressing fertilization had no effect on starch variability under drought conditions and a weak effect under normal humidity (4.7%). The effect of genotypic differences decreased by half in dry conditions (from 60.9 to 24.2%).

As for relationships between starch, protein, and fiber, we found statistically significant negative correlations between protein and starch contents ($r = -0.737$ in 2020 and $r = -0.623$ in 2021), as well as a close positive relationship between starch and fiber contents in 2020 ($r = 0.751$). The negative correlation between protein and starch was confirmed by the studies of Biel and Jacyno and Filippov *et al.*, although it was not always manifested in the works by Yu *et al.* and Zhou *et al.* [40, 43, 52, 53]. On the other hand, in contrast to our data, Memon *et al.* reported a negative relationship between starch and fiber contents [54].

Fat is another important component of barley grain, which affects its nutritional value. According to literature, its content in barley is 1–3% [55, 56]. Our data were consistent with this content (Table 6).

Similarly to the parameters above, the top-dressings had different effects on the fat content in barley grain depending on its genotype (cultivar) and growth conditions. In particular, the fat content in Novichok was not affected in 2020 (normal humidity), but in 2021 (drought), it decreased by 5.8–6.0% under the influence of CAS 28 and Polidon® Amino Start. Rodnik Prikamya, however, had the opposite reaction. Its fat content reduced by 8.9–12.7% when treated with Polidon® Amino Start and Alfastim® in 2020, with no effect in 2021. Pamyaty Rodinoy was affected positively by all the three fertilizers in 2020, with an increase in fat of 6.2–18.9%. However, under the dry conditions of 2021, only Alfastim® had a statistically significant effect on this cultivar, increasing its fat by 7.8%.

According to ANOVA, the effect of the cultivar on fat variability was twice as high under normal humidity compared to dry conditions (69.8 and 35.3%, respectively), with almost the same effect of top-

dressing fertilization (6.1 and 5.6%). Thus, it was the genotypic differences in responding to the drought that had the most significant effect on the fat content. This parameter correlated positively with the starch content (r = 0.576–0.781) and negatively with the yield (r = –0.630–0.662) in both years of the study. Our data were consistent with those of other researchers, who found that the weather conditions can significantly change the effectiveness of top-dressings [35, 57].

Our study revealed significant differences in the effect of the top-dressings on different cultivars of spring barley. In particular, all of the three fertilizers increased the yield of Novichok in both years. Under normal humidity, this increase amounted to 14.5% from CAS 28 and 43–49% from the other two fertilizers. In dry conditions, however, all the fertilizers were equally efficient, increasing the yield by 62–79%. The Rodnik Prikamya cultivar had its yield increased under normal humidity by CAS 28 (6.8%) and in dry conditions by CAS 28 (26.0%) and Alfastim® (13.2%). The Pamyaty Rodinoy was positively affected only under normal humidity by CAS 28 and Polidon® Amino Start, with a yield increase of 6.0 and 15.9%, respectively. In dry conditions, Alfastim® had a negative effect, decreasing the yield by 26.3%. Similar differences were observed in the effect of the fertilizers on the grain quality.

CAS 28 significantly reduced the contents of protein (4.5%), fat (6.1%), and fiber (8.3%) in the Novichok cultivar in dry conditions and increased its starch content (2.1%) under normal humidity. The other two cultivars were positively affected by this fertilizer. Rodnik Prikamya had its fiber increased by 14.2% under normal conditions. Pamyaty Rodinoy had its fat increased by 18.9% under normal humidity and starch increased by 0.8–2.5% in both years.

Polidon® Amino Start decreased the contents of fat and fiber in the Novichok cultivar by 5.7–9.4% in dry conditions and increased its starch by 2.7% under normal humidity. However, this fertilizer had the opposite effect on the contents of protein, fat, and fiber in the other two cultivars under normal humidity. It increased starch (2.9%) and decreased protein (7.8%) and fat (8.9%) in Rodnik Prikamya. In Pamyaty Rodinoy, on the contrary, it increased protein (14.4%) and fat (6.3%) and reduced starch (5.1%). Yet, in dry conditions, both cultivars had a higher protein content (by 8.3–10.7%).

Alfastim® increased the protein content (3.6%) in Novichok under normal conditions but decreased it during the drought (4.4%). It had the opposite effect on Rodnik Prikamya, reducing its protein (10.7%) under normal humidity and increasing it (12.2%) in dry conditions. As for Pamyaty Rodinoy, Alfastim® increased its protein (7.2%) under dry conditions but had no effect on this parameter under normal humidity. We also found that under normal conditions, this fertilizer increased the fiber content in Rodnik Prikamya (22.8%), but decreased it in Pamyaty Rodinoy (18.6%). However, it had the opposite effect on the fat content, decreasing it in Rodnik Prikamya (12.7%) and increasing it in Pamyaty Rodinoy (9.8%). In addition, Alfastim® increased fat in Pamyaty Rodinoy in the drought (7.8%) and starch in Rodnik Prikamya under normal humidity (4.4%), as well as decreased fiber in Novichok in dry conditions (3.8%).

CONCLUSION

According to our results, the top-dressing of spring barley plants with various fertilizers led to a statistically significant increase in grain yield, especially in dry conditions (an average of 10–15%, with a maximum increase of 50–80% for the Novichok cultivar, compared to the control). However, the vegetation conditions (year) and top-dressings (specific fertilizer) can also change the chemical composition of grain, especially its fiber content (the coefficients of variation for Novichok, Rodnik Prikamya, and Pamyaty Rodinoy being 7.37, 10.38, and 9.08%, respectively, in 2020 with normal humidity). The starch content was the least variable: from 0.49% for Rodnik Prikamya to 3.65% for Pamyaty Rodinoy in the dry year of 2021. The variability in protein and fat was the highest for Pamyaty Rodinoy (6.73 and 7.47% under normal humidity; 4.18 and 3.38% in the drought). The lowest variability for all the parameters was found in Novichok.

These data indicate a possibility of both genetic (through traditional breeding) and agronomic (top-dressing treatment) changes in the chemical composition of spring barley grain grown for food or feed. The highest increase in fiber was observed in the Rodnik Prikamya cultivar treated with the fertilizers CAS 28 (14.2%) and Alfastim® (22.8%) under normal conditions of 2020. The protein content can be increased by treating Rodnik Prikamya and Pamyaty Rodinoy with Alfastim® and Polydon® Amino Start in dry conditions (5.2–12.2%), as well as by applying Polidon® Amino Start to Pamyaty Rodinoy under normal conditions (14.4%). Polidon® Amino Star and Alfastim® can also be used to change the fat content in barley grain, namely to increase it (by 8.2–8.3% in Pamyaty Rodinoy) or decrease it (8.9–12.7% in Rodnik Prikamya), depending on the consumer's requirements.

Thus, the top-dressing treatment of particular spring barley cultivars with specific fertilizers can significantly affect their use in the production of functional feeds and foods.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cold chain relevance in the food safety of perishable products

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Abstract:
The food cold chain is an effective tool that allows food markets to maintain food quality and reduce losses. Poor logistics may result in foodborne disease outbreaks and greenhouse gas emissions caused by organic matter decay. The ongoing pandemic of COVID-19 makes it necessary to study the chances of SARS-CoV-2 transmissions in food products.
This study reviews cold chain logistics as a handy tool for avoiding food safety risks, including COVID-19.
The cold chain of perishables and its proper management make it possible to maintain quality and safety at any stage of the food supply chain. The technology covers each link of the food chain to prevent microbial spoilage caused by temperature fluctuations and the contamination with SARS-CoV-2 associated with perishable foods. Given the lack of knowledge in this field in Latin America, the region needs new research to determine the impact of the cold chain on perishable foodstuffs.
The perishable cold chain is only as strong as its weakest link, and the national and international markets require new traceability protocols to minimize the effect of COVID-19.

Keywords: Cold chain, meat, food safety, temperature, COVID-19

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INTRODUCTION

Food safety and environmental protection are one of the most important issues in the global scientific and technological agenda, as well as the subjects of the growing concern and awareness of final consumers. An effective farm-to-table cold chain is a tool that provides food safety by uninterrupted refrigeration. This process maintains a controlled temperature of the perishable product in a given time and space, thus promoting a constant heat transfer coefficient and particular temperature of the dissipation medium [1]. Product temperatures have to remain within a certain range to avoid microbial proliferation, tissue damage, and excessive emission of CO_2 caused by inefficient refrigeration devices and food wastage [2, 3]. Therefore, the industries responsible for cold chain logistics must find better alternatives to reduce CO_2 emissions [4, 5] because perishables require appropriate management, equipment, and facilities at each stage of the food supply chain [3].

Efficient cold chain management uses reliable data on temperature modes for various foods: for instance, low temperatures that are suitable for meat products may damage tropical fruits and reduce their sensory quality [6]. Accurate data on optimal storage temperature for each product could reduce the volume of food waste caused by inadequate cold chain management and mitigate the alarming food loss worldwide [7, 8]. The cold chain technology provides various benefits by limiting the proliferation of microorganisms and avoiding food waste. Efficient cold chain logistics extends shelf life, thus providing the final

consumer with a safe product with an optimal sensory profile [9]. Therefore, companies must provide the necessary conditions and equipment for the entire cold chain with its weakest links, not only in the manufacturer's facilities, which are often certified by international standards, such as ISO 9001, ISO 22000, or HACCP [10].

During the current pandemic, food supply chains must reinforce their hygiene measures because SARS-CoV-2 might be transmitted by fomites, although few reports are available on this subject [11, 12]. Nevertheless, in Qingdao, SARS-CoV-2 was revealed in the packaging of cod imported to China via the cold food chain, where the low temperature and humidity increased the stability of the virus [13]. Consequently, disinfection strategies were implemented for packaging cold-chain imported products [14]. This paper highlights the effect of temperature fluctuations on the perishable food cold chain, as well as on the potential presence of SARS-CoV-2 in perishable foods.

RESULTS AND DISCUSSION

Food supply chain. Food products require a variety of handling throughout the food supply chain, from the primary production to the consumer's plate, and each stage involves a large number of people. Assessing the number of people in food production is a difficult task by itself. The European Union alone has at least 11 million establishments engaged in food production, including 300 000 food and beverage companies. Their products are purchased by 2.8 million distribution and food service businesses, whose principal objective is to supply products to some 500 million EU citizens [15].

Considerable food losses and waste are unavoidable because every single stage of the food supply chain involves numerous difficulties. An estimated one-third of the global food production is wasted for various reasons. Approximately 1.3 billion tons are reported as waste every year. The ratios usually differ, depending on the food supply chain link and the conditions within each country [8, 16, 17]. The United Nations Environment Program has estimated that about 931 million tons of food waste were generated by retail, food service, and household waste [18]. While food waste reduction is on the global agenda, Latin America can hardly offer any data on food wastage in the region. Only four countries in Latin America can provide information on food loss and waste. They are Belize, Brazil, Mexico, and Colombia [19–21].

Food cold chain. Cold chain is one of the main tools of the food industry. It ensures the optimal conditions for food meant for human consumption. Cold chain is a system that keeps food under a controlled temperature from the moment of its production or harvest until it reaches the final consumer [22–24]. However, the International Dictionary of Refrigeration describes it as "a series of actions and equipment applied to maintain a product within a specified low-temperature range, from harvest/production to consumption" [25].

Highly perishable food products retain acceptable safety and quality throughout the whole supply chain provided that certain factors are maintained, e.g., temperature and relative humidity [24]. Perishables may suffer an adverse variation when the storage temperature is outside the ideal temperature range [23]. Therefore, cold chain monitoring is critical to identify any weaknesses for successful intervention [26]. Consequently, food science develops various ways to monitor the temperature of a product in real-time and access its temperature history throughout the process [23]. Some of the frequently employed options include radio frequency identification (RFID) tags, time and temperature integrators (TTIs), and wireless sensor networks (WSNs) [27, 28].

Several studies (Table 1) used various monitoring tools to test food temperature along the cold chain and registered temperature abuse at all chain stages [9, 23]. Therefore, food distribution is a complex process that takes into account the effect of environmental conditions on the food temperature throughout the cold chain [29]. Transportation, storage, and retail stages demonstrated the highest temperature abuse rate [30–32].

Temperature abuse can happen in all cold chain links, especially at the last stages. The logistics management definitely requires improvement, given that temperature fluctuations may occur during inspection procedures at customs or ports [35, 42]. A uniform temperature distribution in the refrigeration equipment is crucial to avoid disruptions related to product position within the refrigerator [47].

Technologies employed. The farm-to-table food product chain requires significant resources. Both developed and developing countries keep increasing their refrigeration energy consumption in an attempt to reduce food spoilage [51]. Monforti-Ferrario *et al.* estimated that about 30% of the electrical energy used in the European Union food industry is spent on cooling and freezing [52].

Such refrigerants as chlorofluorocarbon and hydrochlorofluorocarbon dominated the refrigeration field in the 1930s, and vapor compression refrigeration gained ground as an alternative throughout the food supply chain. In the 1980s, the growing environmental awareness resulted in a number of international agreements with timescale applications for eliminating hydrochlorofluorocarbon. Later, the European Union proposed introducing regulations on fluorinated gases, including all hydrofluorocarbon refrigerants, e.g., R134A, and mixes containing fluorinated gases, e.g., R407C, R410A and R404A [53].

Multiple compressor refrigeration packs or air-cooled condenser racks are regularly used in commercial refrigeration systems in food distribution centers. In the United Kingdom, modern systems normally use scroll compressors and R404A refrigerants [53]. Supermarkets use open refrigerators that have

Table 1 Temperature fluctuations observed throughout the cold chain

Country	Link	Product	Recommended temperature, °C	Temperature abuse	Sensor	Reference
Belgium	Harvest to restaurant	Endive	0	16°C	Data loggers	[34]
Canada	Processing to retail	Lettuce	≤ 5	7°C	Temperature recorders	[35]
China	Processing to distribution	Meat	≤ 4	8–10°C	Data loggers	[36]
	Transport	Fish	−18 ± 2	≤ 18.6°C	RFID	[37]
Finland	Retail	Fish and meat	≤ 4	≤ 105 min with temperatures above recommended	Data from the inspector's office, data logger, infrared thermometer	[32]
France	All links	Dairy and meat products	≤ 4	9°C	Temperature recorder	[38]
	All links	Smoked salmon	≤ 8	Temperatures above the recommended	Data loggers	[39]
	Transport/storage	Milk	≤ 4	≤ 11.7°C	Data loggers	[40]
Iceland	Transport	Fish	5	The temperature was ≥ 5°C during 31.1% of transportation time	Data loggers	[41]
Japan	Farm to retail	Lettuce	≤ 5	3–15	Data loggers	[42]
Slovenia	Retail	Miscellaneos products	Different temperature ranges	Temperatures above recommended for different products	Infrared thermometer	[31]
South Africa	Cold store to haven	Fruits	2	≤ 81% temperature abuse for 1.5 h	Data loggers	[43, 44]
Spain	Storage	Ham	≤ 5	≤ 12.2°C	Data loggers	[45]
	Retail	Meat, dairy, fishery products and vegetables	2–8	Temperatures above the recommended for different products	Infrared thermometer	[46]
Taiwan	Transport to home	Frozen shrimps	–20	≤ 17.2°C	Data loggers	[47]
Thailand	Transport/storage		–20	≤ 17°C at different times	Infrared thermal camera/ data logger	[48]
USA	Harvest to retail	Live oyster	1.7–10	≤ 14.4°C	Temperature sensors	[49]
	Transport to display	Lettuce	≤ 5	≤ 18.2°C	TempTale4 sensors	[50]
	Transport	Fresh-cut leafy greens	≤ 5	5–10°C	Data loggers	[51]

no physical barrier between goods and people, except for an air screen that keeps out humidity and warm air [54].

A refrigerated container, also called an integral reefer container, is required to transport perishable foodstuffs. It usually refers to a metal box with polyurethane insulation and a refrigeration unit, typically approved by the International Organization on Standardization, i.e., it is of ISO quality [55]. The container comprises three main components: an air circulation system, a microprocessor controller, and a cooling system. The microprocessor controls the refrigeration unit, records the data obtained by the maintenance system, performs diagnostics, and generates the temperature database during transportation. The refrigeration unit must have a power source supplied by land equipment or container vessels [56].

Different alternatives are available throughout the cold chain, some of which are low-cost, whereas others are more complex and sophisticated, depending on the product's link. Economical alternatives, e.g., ice, or more expensive options, e.g., forced air, vacuum cooling, or hydro-cooling systems, can be used for pre-cooling at the production site. Storage usually occurs in cold rooms, in small or large warehouses with traditional refrigeration equipment, in controlled air conditioning systems, e.g., CoolBot™, or in cold evaporation rooms. The food processing stage may include some alternatives, e.g., refrigerators, individual quick freezing (IQF), blast freezing, freeze-drying, etc. The transportations stage usually includes such alternatives as ice, refrigeration units in trailers, evaporative coolers, or passive cooling [57].

Cold chain in transportation. Cold chain food transportation involves land, air, and maritime resources. Until 2005, trucks used to be the most popular land transport vehicle. Estimates suggest that more than 90% of foods are moved by land in the United Kingdom [2, 58]. The distance that vehicles travel to deliver a food product to the retailer is highly variable [37, 59]. A lot of different factors must be considered during food transportation to keep the cold chain undisturbed. Seasonal temperature can strongly affect the food supply chain, being as low as –10°C in winter or as high as 35°C in summer [7].

Trucks must be able to provide favorable temperature for the perishable foodstuffs they transport, considering that they travel up to 2500 km [37, 59, 60]. Many studies report temperature rises during transportation of milk, strawberries, and fresh-cut leafy greens, as well as during the loading and unloading of fish [39, 50, 61, 62]. Additionally, temperature control deficiencies can be potentiated by the impact of the environment on the temperature fluctuations of the transported products [60].

The advantage of air transport is that food can be quickly moved to locations far from being produced or harvested. However, this transportation method is not very popular, except for high-cost foods and products with a short shelf life that must reach their destination quickly [2]. The cold chain by air transport is extremely complex. Only half of the time required is actually the flight time: the other half is spent on the management procedures to load and unload the product into and out of the aircraft, which results in poor temperature control [2, 63].

In contrast to air transport, shipping products by sea is slower, although more profitable in cases of meat, dairy products, fish, fruits, and vegetables. Therefore, the type of transportation generally depends on the type of the product to be moved. Specialized vessels are usually used to transport bananas. For most products, reefer containers are more cost-effective and can provide a better logistics [2, 64, 65].

Transport to home, which is the last transportation link in the cold chain, is often the weakest one, with the highest temperature increase [66]. Passive cooling devices are a viable alternative to avoid this problem, e.g., phase change material in easily portable insulated boxes [67, 68].

Cold chain in warehouses. Achieving that perishable food reaches the last link of the food supply chain in optimal conditions is complex and expensive. The food industry invests millions of dollars in order to attain this goal and avoid having to recall a product due to contamination during the cold chain [69]. A food product can remain stored in distribution centers for many days [70]. Its shelf life depends on the right conditions, and the optimal temperature control is essential. Unfavorable temperature deviation during the storage of perishable products is related to the corresponding link in the supply chain. Derens *et al.* revealed that the biggest temperature problems in France were related to display cabinets, transport to the home, and domestic refrigerator storage [37].

Unfavorable fluctuations occur less frequently during storage at the distribution center. Multiple studies conducted on such perishables as meat, dairy products, and vegetables confirmed temperature records below 4°C at the storage stage in distribution centers [37, 60, 70]. However, these data were obtained from developed countries. Developing countries have less favorable conditions in terms of equipment, energy sources, and environment, which is often due to their geographical location.

Retail cold chain. Derens-Bertheau *et al.* reported that such stages of the cold chain as display, transport to home, and domestic refrigerator storage are also highly problematic. Retail distribution link appears to be one of the main problems, which can be related to various reasons. Firstly, product information often lacks temperature specifications [31]. Secondly, open refrigeration equipment sometimes fails to maintain the required temperature as a result of an uneven distribution of the airflow throughout the rack [45, 71, 72]. The latter occurs when the equipment lacks capacity or configuration to achieve an effective air curtain [73, 74]. Therefore, refrigeration equipment with doors is a viable option to achieve greater efficiency in cooling of perishables and electricity consumption [54].

Some recent studies pointed to the need for improving the cold chain control during retail: they detected adverse temperature deviations in meat products, poultry, and pork [30, 32, 72]. Even within retail distributors, position of the product in the refrigeration equipment might result in temperature fluctuations caused by design, configuration, or services. Baldera *et al.* reported a 25, 40, and 57% shelf-life reduction for cheese, salmon, and chicken because they were positioned at the highest point inside the refrigerator.

Within the retail food industry, refrigeration systems are responsible for a significant amount of greenhouse gas emissions, which could be reduced by using improved equipment [75]. The electricity consumed by a supermarket generates approximately 50% of these emissions [76]. Moreover, some supermarkets still use hydrofluorocarbons as refrigerants. This refrigeration method is responsible for 30% of greenhouse gas emissions caused by refrigerant leakage, but it still remains quite popular in the United Kingdom [77]. As a result, standards have been implemented to decrease its use in favor of natural options, such as CO_2 [78].

Food spoilage induced by microorganisms. Shelf life is the time food keeps its physical, chemical, and microbiological properties and is safe for human consumption. Food spoilage can be associated with environment, composition, packaging, human handling, microorganisms, etc. All these factors reduce shelf life [79, 80]. Potential factors that affect shelf life can be divided into intrinsic and extrinsic. Therefore, a microorganism can be considered an extrinsic factor if it

does not belong to the product. Meat products constitute a highly favorable food source for various bacteria since meat has a lot of nutrients and high water activity, which promote microbial growth. As a result, meat products easily acquire unpleasant texture, odor, and flavor [80].

Food contains a great wide variety of microorganisms, e.g., *Campylobacter*, *Pseudomonas*, *Enterococcus*, *Acinetobacter*, *Psychrobacter*, *Moraxella*, etc., as well as numerous kinds of bacteria, fungi, yeasts, protozoa, and viruses [81–85]. Food in general and meat products in particular are susceptible to a wide range of potential spoilage microorganisms. Meat products have been reported as foods with the highest temperature abuse [86]. Therefore, this kind of food must be handled, stored, and transported at a temperature range of 2–7°C, depending on the type of meat product [87].

However, strict temperature control throughout the cold chain is just another tool that minimizes the risk of microbial contamination. Additional measures can help avoid other contamination sources and reduce the chance of microbiological growth. Some psychrotrophs, for example, can grow at temperatures below 7°C [84, 88]. This group of microorganisms includes such high-risk species as *Listeria monocytogenes* and *Yersinia enterocolitica*, both of which can grow at temperatures below 5°C [89]. However, the European Union reports a low incidence of outbreaks related to psychrotrophs compared to bacteria, e.g., *Campylobacter* and *Salmonella* [90]. Meanwhile, a higher incidence of norovirus cases with a meager mortality rate was reported in the USA through 2017. It was followed by *Salmonella* and, with fewer cases, *Listeria*. However, these bacteria could be considered equally dangerous since 3–8 and 3–35 people die from *Salmonella* and *Listeria* each year, respectively [81].

Food loss and waste are not only a concern for economic and environmental reasons, but they also present a high risk of an outbreak due to contaminated food. Pathogens, such as *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Yersinia pestis*, and most *Escherichia coli*, are found within the *Enterobacteriaceae* family [91]. Some of them can grow when temperatures are above 7.2°C, e.g., *E. coli* O157: H7 [82]. Some strains of these bacteria are natural to the digestive tract of warm-blooded animals [92]. However, strains classified as Diarrheal *E. coli* (DEC) are becoming more aggressive as a result of their improved resistance to different antibiotics [93]. They are subdivided by their virulence into shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC).

COVID-19 and its relevance to food safety. Throughout 2019, the world faced a danger that severely impacted public health and hit the worldwide economy. Coronavirus disease (COVID-19) is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Up to early November 2021, it caused 247 968 227 confirmed cases, of which 5 020 204 deaths were reported in at least 200 countries. Consequently, vaccines are reported to have been applied in 7 027 377 238 doses among the world's population [94]. Vaccination works in conjunction with recommended safety measures, but the number of cases reported vary from region to region in each country.

A 55-day period without viral transmission was achieved in the Beijing region, which was interrupted on June 11, 2022 when a SARS-CoV-2 case was reported and continued to spread. The virus was detected on different surfaces on a wholesale seafood and meat market, particularly on a board used to slice salmon imported from Europe. The virus could be transmitted through food in the cold chain, cross-contamination by workers, or packaging, but the sequences found were consistent with those of infected Chinese citizens who returned from Europe [95, 96].

Moreover, the Chinese Center for Disease Control and Prevention carried out different studies that detected the presence of SARS-CoV-2 nucleic acid in samples of chicken and shrimp imported from South America and marketed in various Chinese cities [97]. Consequently, several studies (Table 2) proved the presence of SARS-CoV-2 in food products and raw materials. Whether food is a direct transmission route remains unclear, which does not eliminate the possibility of cross-contamination [98]. Therefore, strict control measures are required to reduce outbreaks, particularly in large slaughtering and meat-processing companies, where outbreaks have been reported in Portugal, Australia, The United Kingdom, and Ghana [99].

Food products receive little scientific attention in COVID-19 research since they are not considered a potential transmission route of SARS-CoV-2. Studies conducted on fresh vegetables, e.g., cucumbers, revealed that the virus might remain infectious for up to 72 h [107]. Castrica *et al*. commented that researchers are increasing the number of studies on wastewater based on the understanding that monitoring the pathogen in this environment could create a real-time pandemic model [110, 111]. Moreover, communal areas must not be overlooked as a source of contact infection, although no positive samples have been found from different regions, potentially due to adequate sanitation measures [106].

Since the jury is still out on the foodborne transmission of COVID-19, inoculations were carried out on pork, beef, and salmon samples with different concentrations of SARS-CoV-2 to investigate the stability of the virus after controlled cold-chain management (4 and –20°C) [112]. Viral RNA was detected in all samples stored at 4°C for 72 h after infection, with a higher average number of RNA copies in the pork meat at the lowest concentration. At the highest concentration, viral RNA could be detected in all samples up to 216 h after infection. Viral RNA remained detectable up to 20 days after infection in the samples stored at –20°C in both inoculum

Table 2 Detection of SARS-COV-2 virus in food-safety-related samples

Country	Sample	Type of study	Detection method	Reference
USA	Water and wastewater	Persistence evaluation	RT-qPCR and Vero E6 cells	[102]
South Korea	Tap water	Stability in water samples	qRT-PCR	[103]
China	Salmon	Viability of SARS-CoV-2 under different conditions	Vero E6 cells	[104]
Bangladesh	Wastewater	Detection of SARS-CoV-2 genetic material	RT-PCR	[105]
Finland	Wastewater influent	Presence and stability of SARS-CoV-2 RNA	RT-qPCR, N2 assay, ABI BigDye™ v3.1 Chemistry	[106]
Brazil	River water	Viability of SARS-COV-2 under different temperature conditions	Plaque assay	[107]
Canada	Surfaces in food retailers	Detection of SARS-CoV-2 (all of them negative)	RT-qPCR	[108]
	Apple, tomato, cucumber, and lettuce	Preservation of infectivity (HCoV-229E)	Plaque assay (MRC-5 cells)	[109]
France	Zebra mussel	SARS-CoV-2 genome detection in zebra mussels as bioindicators of water contamination	RT-qPCR Genes tested: RdRP: RNA-dependent RNA polymerase gene; E, an envelope protein gene and N, nucleocapsid protein gene	[110]
Italy	Polyethylene and polystyrene food trays	Persistence of SARS-CoV-2 (ATCC® VR-1986HK™)	RNA extracted with QIAamp® Viral RNA Mini Kit RT-qPCR using the VETfinder detection kit	[111]

concentrations, being present to a greater measure in pork and beef, probably because of their fat content and texture.

The actual routes of SARS-CoV-2 transmission are not entirely clear [113, 114]. Currently, the closest relationship is to viruses reported in bats [115]. Given the variety of information about possible COVID-19 transmission routes, people reacted by increasing food safety and hygiene measures, e.g., washing and sanitizing kitchen surfaces, food, and hands [116]. Additionally, animal foods should undergo proper cooking procedures before consumption [117]. Unfortunately, people might fail to link security measures to food safety and give up these practices after the pandemic if their importance is not promoted [118]. Another critical point is the correct washing and disinfecting methods, e.g., using of detergent residues or inefficient disinfecting with vinegar, as was observed in Brazil [119–122].

On the other hand, food could be a transmission route for SARS-CoV-2, as it has been linked to meat processing plants, wherefrom viral particles could be transported to meat through the chain links. Some publications reported worrying data on the survival of viral surrogates, depending on the food and temperature. The SARS-CoV-2 viral surrogates remained in chilled and frozen meat and fish for a long time at refrigeration and freezing temperatures, possibly because the low temperatures allowed the virus to survive [123]. Low temperatures and humidity increased the survival of SARS-CoV-2 and contributed to its long-distance spread during logistics and trade [124]. The widespread recommendation to prevent contamination of food and processing surfaces focuses on disinfection protocols at every stage of food processing.

Another relevant factor indicates that contaminated food and packaging imported from areas of active outbreaks of COVID-19 were located in China [125]. In contrast, low levels of SARS-CoV-2 were detected on stainless steel and cardboard surfaces under room temperature and constant humidity [126, 127]. Frozen foods were reported to carry SARS-CoV-2 without human contact, thus necessitating procedures for safe transport. Scientists reported incidents related to cold chains in frozen meat and fish. An improvement system for food cold chain management includes information detection, chain linkage, and credible traceability [128]. We identified two areas of improved cold chain management practices. The first one focuses on the analysis of requirements for the prediction of transmission risk and temperature ranges. The other concentrates on the documentation regarding critical control points throughout the cold chain, thus implementing objective traceability.

For high-quality food to reach the final chain link, the food industry needs qualified staff who are aware of the relevance of their work. All workers along the cold chain should possess a solid food safety culture, be able to identify the hazards of food mishandling, and understand the importance of hygienic measures both before and after the handling of animal products, tools, etc. [129, 130]. In addition, whole and retail vendors must be tightly supervised by government and non-government certification agencies [131, 132]. However,

Nyarugwe *et al*. conducted surveys of employees from diverse areas in food companies located in China, Greece, Tanzania, and Zambia [133]. They found that workers in African compared lacked training and safety culture in comparison to those in China and Greece.

Unfortunately, the statistics of foodborne disease outbreaks in developing countries are scarce, and adequate product resources for safe food are few [134]. Therefore, the safety culture and personal training identified by Nyarugwe *et al*. in African companies could be extrapolated to those in Latin America [133]. In addition, the challenges of the COVID-19 pandemic require better hygiene practices in the general population. For instance, male workers were reported to have a greater sense of risk of contracting SARS-CoV-2, but they took fewer safety measures to protect themselves, compared to female employees [118].

Mohammadi-Nasrabadi *et al*. assessed the knowledge on health and food safety during the COVID-19 pandemic in restaurant personnel only to discover that none of them had received any training on this topic [135]. The authors classified the level of COVID-19 awareness in restaurant workers as low (17%), moderate (35.2%), and good (47.2%). After the training, these percentages increased.

CONCLUSION

Food safety has the potential to have a strong impact on different sectors of each nation. By supplying healthy food to the population, governments can reduce the occurrence of foodborne diseases. Companies that produce, transport, or trade in perishables should raise the awareness of their employees about refrigeration equipment, its maintenance, calibration, and temperature monitoring. This simple measure is especially important in developing countries, where it could improve food handling at each supply chain step.

The present work stresses the need to use refrigeration equipment that ensures temperature control at each stage of the cold chain because various potential microorganisms may affect meat products' safety and shelf life. An appropriate cold chain management could reduce food wastage, especially in developing countries, thus decreasing carbon print. An effective cold chain possesses a reliable traceability and contributes to international efforts against the COVID-19 pandemic. Therefore, effective food cold chain management standards increase food safety and prevent the spread of SARS-CoV-2.

CONTRIBUTION

P. Arriaga-Lorenzo compiled the bibliography and initiated the research. P. Arriaga-Lorenzo, E. de Jesús Maldonado-Simán, R. Ramírez-Valverde, P.A. Martínez-Hernández, D.N. Tirado-González, and L.A. Saavedra-Jiménez participated in the analysis and discussion of each topic. All the authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Agriculture in the Baksan Gorge of the Central Caucasus, Kabardino-Balkaria, Russia

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Abstract:
No agriculture is possible without soil. This article reviews available data on the soils of the Baksan Gorge located in the Kabardino-Balkarian Republic, Russia. The research objective was to collect and analyze information on the soil composition and crop yields in this region of the Central Caucasus.
The review covered the last five years of scientific publications cited in Scopus, Web of Science, and Elibrary. It also featured contemporary and archival documents on the soil composition and periglacial agriculture in the Baksan Gorge.
The agriculture and cattle breeding started in the Central Caucasus in the first millennium BC when the local peoples began to develop these lands as highland pastures and, subsequently, for agricultural farming. During the second millennium BC, crop production became one of the most important economic sectors in the Central Caucasus. Corn, barley, wheat, and millet were the main agricultural crops in the Baksan Gorge. Millet has always been a traditional Kabardian crop, and millet farming occupied the largest flatland areas. Barley was the staple crop in the highlands. Currently, the list of local staple crops includes corn, wheat, and sunflower. Barley, oats, peas, potatoes, vegetables, berries, nuts, grapes, and annual herbs are also popular. The past fifteen years have seen an extensive development of intensive horticulture in the Baksan Gorge.
Agricultural ecology and production problems depend on the localization of agriculture in the Central Caucasus. This research reviewed data on the effect of soil composition on the yield and value of agricultural crops in the Baksan Gorge of the Central Caucasus.

Keywords: Land use, crops, soil, Central Caucasus, Baksan Gorge

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INTRODUCTION

Agriculture has expanded into hard-to-reach regions with a harsh climate [1–3]. This global trend is a result of soil depletion and climate change, which reduce the agro-ecological potential of traditional agricultural regions [4, 5]. Russia is extremely heterogeneous in terms of food security [6]. Russian North Caucasus provides itself with agricultural products by more than 80% [7]. Diversification of agricultural production in the regions is an urgent task in the current conditions of import substitution [8]. As a result, some regions may experience difficulties in complying with FAO food security standards [9]. In this regard, agricultural science focuses on the fallow soils in the Russian Arctic and other hard-to-reach regions [10–12].

The current rapid deglaciation of the Central Caucasus has become a source of new lands [13–16]. The deglaciated areas demonstrate good projective plant cover and herbal diversity [17]. The farmers of the Central Caucasus use these new territories as pastures, hayfields, and cultivated lands, thus increasing the local planted acreage.

Sustainable soil development and environmental management are of highest importance in Russia because soil degradation has already destroyed a third

of its soil resources [18]. Soil depletion is a relevant issue for the Kabardino-Balkarian part of the Central Caucasus. At present, the available lands of the Kabardino-Balkarian Republic cover 1247 thousand hectares, which is 3.5% of the total available lands of the North Caucasian Economic Region. Agricultural land occupies 627.6 thousand hectares, or 50.3% of the active area, including 284.5 thousand hectares (22.8%) of arable lands. More than 38% of agricultural lands are affected by water and wind erosion, as well as salinization [19].

The research objective was to describe the periglacial agriculture in the Baksan Gorge of Kabardino-Balkaria.

The authors analyzed the agricultural history in the Central Caucasus and studied the local soils and their agricultural application under the harsh orographic and climatic conditions of the Central Caucasus. The research also revealed various aspects of food security in the Central Caucasus.

STUDY OBJECTS AND METHODS

The research covered five years of scientific publications in domestic and foreign peer-reviewed journals on the soil and agriculture of the Central Caucasus. The search parameters included English keywords for Web of Science and Scopus databases and Russian ones for the Elibrary database. The study also featured monographs on the development of Caucasian agriculture. The methods included data analysis, systematization, and generalization.

The paper also introduces some results of laboratory physicochemical studies of the Baksan soils, as well as photographs of soil sections obtained by field research.

RESULTS AND DISCUSSION

Agricultural history of the Central Caucasus. The early first millennium BC saw an active rise of highland tribal communities, who began to develop mountain pastures [20]. The North-Caucasian highlands, with their steep slopes and rocky soils, were unsuitable for crop farming but provided excellent pastures for cattle breeding. For centuries, cattle provided the highlanders with everything they needed. Cattle breeding was the main subsistence in the ancient economy of the North-Caucasian peoples [21]. The progress of crop agriculture was less prominent. However, it was also an important sector of the Central-Caucasian economy in the second millennium BC. The arable plow farming was possible in the flatlands, but highland crop farming developed less intensively [22]. The unfavorable climatic conditions resulted in infertile permafrost and seasonally freezing soils. Together with the complex terrain, they were responsible for the poor agricultural development of the Central-Caucasian highlands. The first arable farming reached these areas together with the general technological development and required a lot of draft power. Nevertheless, crop farming was always part of the ancient economy in all the gorge areas of the Central Caucasus.

Plow farming found little application in the Central-Caucasian highlands with its harsh climate and complex terrain. Hoe farming with its artificial terraces was more efficient. All suitable lands were subjected to agricultural processing, including capes, watershed hill tops, thirty-degree slopes, etc. Steep slopes were terraced to provide extra acreage. In fact, terrace agriculture was practiced in the Central Caucasus until the middle of the 20th century.

Terracing was also popular in other parts of the Caucasus, especially in Dagestan [23]. Agricultural terracing has always been the optimal solution for hilly and mountainous terrains all over the world [24–27]. Ancient Italy was especially famous for its terraces, which proved to be the most effective form of land cultivation in the harsh conditions because terraced slopes prevented soil degradation [28, 29].

In the Central Caucasus, some terraces were as big as 1–1.5 hectares. The bottom of the terrace consisted of large stones and boulders, followed by small stones and yellow clay. The upper layer consisted of imported soil and humus, which were compacted by sheep and cow herds. This method provided the maximal moisture retention. Terrace construction depended on such factors as soil composition, slope angle, vegetation, etc. The terraces performed two important functions: they optimized the relief by reducing the chance of avalanches and mudflows, as well as increased in the acreage [30].

The economic structure of the Central Caucasus has always depended on its topography. The Central Caucasus can be divided into flatlands, foothills, highlands, and alpine areas. The highlands and alpine areas had very little land suitable for plowing, which made the life of the local people a constant struggle with nature. It took the local tribes superhuman efforts to turn the mountain slopes into plowed fields. They literally had to conquer every piece of land from nature by chopping down forests, uprooting shrubs, and removing stone boulders. Irrigation and soil fertilization were also extremely effort-consuming, but the gorges of the Central Caucasus eventually developed a complex network of irrigation canals [31].

Despite the enormous efforts to increase productivity, the yields were still small. The seeds vs. harvest ratio was 1:3, 1:5, or even 1:2 in lean years. Sometimes, farmers even failed to reach a 1:1 ratio [32]. The flatlanders used a heavy limber plow, dragged by three or four pairs of oxen. Highlanders preferred a wooden light plow with an iron share. This tool provided shallow plowing as the plow could loosen only the very surface soil, but this method was more rational because the fertile soil layer was thin anyway [33].

Corn, barley, wheat, and millet were the staple agricultural crops in the Central Caucasus. Most flatlands were allotted for millet, a traditional Kabardian crop. Millet covered almost 40% of the entire acreage [22, 34]. Barley was the staple crop in the highlands. Its varieties were frost-resistant and low-

maintenance in terms of soil because of the harsh climate with its frozen or seasonally freezing soils. Wheat did not survive in the Central-Caucasian highlands because it failed to ripen during the short warm season [35].

Glacial dynamics and expansion of arable soil areas in the Central Caucasus. Like many other mountain-glacier regions these days, the Caucasus has been experiencing a stable deglaciation for several thousand years. Glaciers all over the world are gradually decreasing in number, area, and volume. Elbrus is the largest mountain glacier in Russia. It includes 16 major glacial streams [36]. In 2007, its total ice sheet area was 120 km^2. Elbrus has two big glaciers. The Big Azau is located on the southern slope. It is 9.35 km in length with a total area of 20.2 km^2. The vast Jikiuankez ice field on the northern slope consists of two glaciers, Birjalychiran and Chungurchatchiran. Its total area is 23.4 km^2. The Elbrus ice cap covers several altitudes, descending from the peaks (5642 m) to the bottom of the Big Azau glacier (2542 m) [37, 38].

The first instrumental survey of the entire Elbrus glacier took place in 1887–1890. It resulted in a topographic map at a scale of 1:42 000 [39]. This map served as ground zero for every subsequent glacier survey in this region. A lot of data on the Elbrus deglaciation are available for 1957–1997, when the Elbrus glaciers shrank by 12.5 km^2, i.e., an average of 0.25 km^2 per year. Between 1887 and 2007, the area of Elbrus glaciers decreased by 20% [13, 40, 41]. Climate change is not the only enemy of the Caucasian glaciers: cryoconites reduce the surface albedo and thereby accelerate the melting [14, 15].

The mass balance of glaciers on the southern Elbrus dropped by half over the past two decades and amounted to 63 cm w.e., i.e., water equivalent. Between 2010 and 2018, its average value dropped to 90.4 cm w.e. The ice and perennial firns accumulated in the second half of the XX century are melting at an unprecedented rate: they are almost exhausted over a large area at the altitude of 3700–4000 m. The glacier feeding boundary has risen by 200 m, and the firn consumption is increasing. The ablation area has melted down to lava ridges. The cumulative mass balance has reached its minimum value over the past 50 years [42].

As the Central-Caucasian glaciers continue to retreat, the periglacial landscapes of the Baksan Gorge continue to grow. The periglacial Bashkara area increased significantly in 1996–2006. In the ten years it took the grass line to move 10–20 m up the slope, the projective cover and herbal diversity also increased dramatically [17]. Residents of the Baksan Gorge use the former glacier areas that are now covered by grass. These territories still freeze in winter or remain cryosolic, but they serve as pastures and hayfields in warm seasons. In their turn, the lands that were previously used as pastures and hayfields gradually become arable. The agricultural area is expanding, which is very important in mountainous areas where arable soil is scarce.

Soils of the Baksan Gorge and their agricultural application. The Baksan Gorge boasts a wide variety of geomorphological forms and climatic conditions, which affected the local soil-forming processes and agriculture. Foothill farmers mostly grow wheat, corn, and sunflower (Fig. 1), not to mention fruit and vegetables. The highland and alpine areas with their permafrost and seasonal freezing develop meat and dairy farming, sheep breeding, etc., and the local agricultural lands are mostly pastures and hayfields [43, 44].

The soils of the Baksan Gorge are diverse, depending on the altitude and the bank of the Baksan River [44–46]. The right bank of the Baksan River is covered by forests of pine, pine and birch, birch and pine, and birch. They host a wide variety of mountain forest soils (Fig. 2).

Mountain forest-meadow soils develop under the birch crooked forests that cover the northern slopes. These soils have a prominent humus-accumulative horizon and residual weathering. The mechanical

Figure 1 Crops cultivated in the foothills of the Baksan Gorge (photo by R.Kh. Tembotov)

a b c

Figure 2 Profiles of mountain forest soils in the Baksan Gorge: mountain forest-meadow soil (A), mountain brown forest soil (B), and Mountain dark-grey forest soil (C) (photo by R.Kh. Tembotov)

Table 1 Mountain woodland soils of the Baksan Gorge: physical and chemical parameters of upper horizons (0–20 cm)

Soil	pH (H_2O)	Density, g/cm^3	Humus	
			Content, %	Stocks, t/ha
Mountain forest-meadow	6.0	–	5.9	–
Mountain brown forest	6.6	0.9	12.1	199
Mountain dark-grey forest	6.3	0.90	7.4	148

composition reflects the redistribution of chemical elements along the profile. Table 1 contains some physical and chemical characteristics of mountain forest-meadow soils defined as part of this research. These indicators are similar to mountain forest-meadow soils in other regions of Russia. For instance, the humus content in the South Urals is 5–7%, and the soil is slightly acidic. In the South-Eastern Transbaikalia, the humus content reaches 8–9%, and the soil is slightly acidic, too [47]. Mountain forest-meadow soils are of little agricultural use since they occur mostly in forests. In places like the Baksan Gorge, mountain forest-meadow soils are available for grazing and haymaking, and few areas are free from forest vegetation.

Mountain brown forest soils are common in the highlands and in the alpine forests between 800 and 1800 m above sea level, on watershed hill tops, and on the northern slopes of various steepness. These soils are formed by eluvial rocks and talus, e.g., sandstone, clay, limestone, and granite. Mountain brown forest soils have no distinct genetic horizons. They are relatively uniform in color: brown or brownish-fallow. The humus content depends on the depth: its powdery and granular structure becomes nutty and lumpy in B-horizon. The thickness of the humus profile ranges from 23 to 38 cm, while its mechanical composition varies from heavy to light loamy.

Table 1 features the main physical and chemical characteristics of the mountain brown forest soils of the Baksan Gorge. The dense forestation of the terrain prevents its agricultural application: only forest meadows can be used as hayfields. In the Kaliningrad Region, however, mountain brown forest soils are used not only for pastures and hayfields, but also for arable land [48].

Mountain dark-grey forest soils develop in the forest-steppe zone of low-altitude mountains covered by post-forest meadows and broad-leaved forests under partially percolative water regime. The parent-rock material is represented by carbonate clays, talus, and heavy-clay loams. Mountain dark-grey forest soils have a weak morphological differentiation of organic matter in the upper profile, thick A-horizon humus, and no morphologically pronounced podzolization. The A-horizon humus is 25 cm thick. The mechanical composition is loamy with fractions of fine sand and silt. Table 1 features the main physical and chemical properties of mountain dark-grey forest soils of the Baksan Gorge. In other regions, dark-grey forest soils have a similar humus content (4.7–8%) from slightly acidic to neutral [49]. In the Baksan Gorge, forest-free dark-grey forest soils are used for crop farming, gardening, hayfields, or pastures.

Figure 3 illustrates the mountain-meadow soils in the left-bank alpine part of the gorge, covered with under alpine and subalpine vegetation.

The mountain-meadow alpine soils of the Baksan Gorge appeared under alpine meadows under percolative water regime and low temperatures. These soils are located in the alpine zone, on slopes of various steepness

a b c

Figure 3 Profiles of mountain-meadow soils in the Baksan Gorge: mountain-meadow alpine soil (a), mountain-meadow subalpine soil (b), and mountain meadow-steppe subalpine soil (c) (photo by R.Kh. Tembotov)

Table 2 Mountain-meadow soils of the Baksan Gorge: physical and chemical parameters of upper horizons (0–20 cm)

Soil	pH (H_2O)	Density, g/cm^3	Humus	
			Content, %	Stocks, t/ha
Mountain-meadow alpine	4.4	0.7	11.7	162
Mountain-meadow subalpine	5.7	0.66	15.3	202
Mountain meadow-steppe subalpine	5.6	0.95	13.6	256

and exposure. The incomplete set of genetic horizons in the profile is their specific feature. The A-horizon humus profile is up to 20 cm thick. It has a dark-brown color and a powdery structure. The underlying horizons are yellowish-brown and structureless. The parent rock is represented by acidic residual deposits and talus. The soil profile is rock-debris and stones, while the mechanical composition is from medium loamy to light loamy.

Table 2 presents the mean values of the physical and chemical characteristics of this type of soils. The mountain-meadow alpine soils of the Western Caucasus have the same acidity (4.4) as those of the Central Caucasus, but a higher humus content (14.3%). The high humus content is a direct result of the humid climate and the corresponding patterns of organic transformation. The decomposition of organic matter occurs slowly, resulting in a robust accumulation of semi-decomposed organic matter [50]. The Baksan mountain-meadow alpine soils serve as summer pastures.

Mountain-meadow subalpine soils are to be found on the slopes of the Lateral (Bokovoi) and Rocky (Skalisty) Ridges of various steepness. In the subalpine bioclimatic zone, they develop at low temperatures beneath subalpine and post-forest meadow vegetation under percolative water regime. The parent rocks are represented by unsaturated siallitic weathering detritus of non-carbonate dense sedimentary and massive crystalline rocks. As a rule, they are residual deposits and talus of non-carbonate bedrocks, with accidental loose sediments. Mountain-meadow subalpine soils develop in the cold and humid alpine climate. They are usually medium-thick: 20–40 cm. The soil has no distinct genetic horizons, the transitions between the horizons are smooth. The upper horizons are dark brown with a gray tint, which changes to light brown downwards.

Table 2 presents the mean values of the physical and chemical characteristics of these soils. In the Western Caucasus, mountain-meadow subalpine soils are more acidic. Unlike alpine soils, they are more humus-rich than in the Baksan Gorge [50]. In other regions, mountain-meadow alpine and subalpine soils serve as distant pastures [51]. In the Baksan Gorge, they can be used hayfields or pastures.

Mountain meadow-steppe subalpine soils appear in xeromorphic areas among the mountain-meadow soils of the southern, southeastern, and eastern slopes of the Lateral (Bokovoi) and Rocky (Skalisty) Ridges of the Baksan Gorge. The parent-rock material is weathering detritus of dense sedimentary non-carbonate rocks, and the soil cover is represented by combinations of soils of different thickness, stone content, and removal. The soil profile ranges from 20–25 to 40–50 cm. It is grass-covered, with no distinct horizons. A-horizon is dark brown, changing to yellowish-brown downwards; the compaction is weak. Table 2 presents the mean values of the physical and chemical characteristics of this type of soil. In other Caucasian areas, mountain meadow-

a b c

Figure 4 Profiles of highland and foothill soils of the Baksan Gorge: sod-calcareous soil (a), mountain-meadow chernozem-likes soil (b), and mountain leached chernozem (c) (photo by R.Kh. Tembotov)

Table 3 Highland and foothill soils of the Baksan Gorge: physical and chemical parameters of upper horizons (0–20 cm)

Soil	pH(H_2O)	Density, g/cm^3	Humus	
			Content, %	Stocks, t/ha
Mountain sod-calcareous	7.7	0.70	13.2	178
Mountain-meadow chernozem-likes	6.9	0.90	11.0	197
Mountain leached chernozems	7.7	1.01	10.2	197
Mountain leached agricultural chernozems	7.9	1.21	6.5	105

steppe soils are more alkaline (pH 6.1), but their humus content (9.2%) is lower than in the Baksan Gorge [52]. Mountain meadow-steppe subalpine soils are used as pastures in the Baksan Gorge and in the rest of the Caucasus.

Figure 4 shows mountain sod-calcareous, mountain-meadow chernozem-likes, and mountain leached chernozem soils that developed in the highlands and foothills of the Baksan Gorge, in the belt of steppe meadows, and in the meadow steppes proper.

Mountain sod-calcareous soils are common in the forest-steppe zone on the watershed hill tops, as well as on slopes of various steepness and exposure. These soils develop on less weathered and thin limestone residual deposits and talus. They have no distinct profile, as well as a lot of rubble and rocks. The humus horizon is dark and granular or lumpy-granular. The mechanical composition is medium loamy with fractions of coarse and fine dust.

Table 3 presents the mean values of the physical and chemical characteristics of mountain sod-calcareous soils, determined as part of this research. These soils are quite suitable for grain crops. However, the climate is unfavorable, and these soils are located on the steep slopes of the Baksan Gorge. As a result, they serve only as hayfields and pastures. Sod-calcareous soils make excellent vineyards in humid boreal and subboreal conditions [53].

Mountain-meadow chernozem-likes soils develop in the bottom subalpine belt, covered by steppe subalpine meadow and meadow vegetation. The humus horizon is dark gray, with a brownish tint. Their fine-grained structure changes to fine cloddy downwards. These soils are well-compacted and porous. They contain a lot of roots, rubble, and rocks, the number of which increases with depth. The humus profile is 43–48 cm in the thick subtypes, 34.8 cm in the medium-thick subtypes, and 18–12 cm in the thin subtypes. The mechanical composition is medium to heavy loamy. Table 3 presents the mean values of the physical and chemical properties of mountain-meadow chernozem-likes soils. In the Western and Eastern Caucasus, these soils are neutral or slightly alkaline (pH 6–7.1), with a very high humus content (12–15.3%), like in the Central Caucasus [54]. These soils possess a rather high potential fertility. However, they are used as highly productive hayfields and pastures all over the Caucasus because the harsh climate and complex terrain prevent the local farmers from using them for crop cultivation.

Mountain leached chernozems are located on the northern slope of the Chalk (Melovoy) Range. They appear in the mountain-steppe belt, some 700–1200 m above sea level, which is under meadow steppes and steppes proper. These soils are a result of partially percolative water regime. They also develop on watershed hill tops and slopes of different steepness, mainly in the northwestern, northeastern, and northern

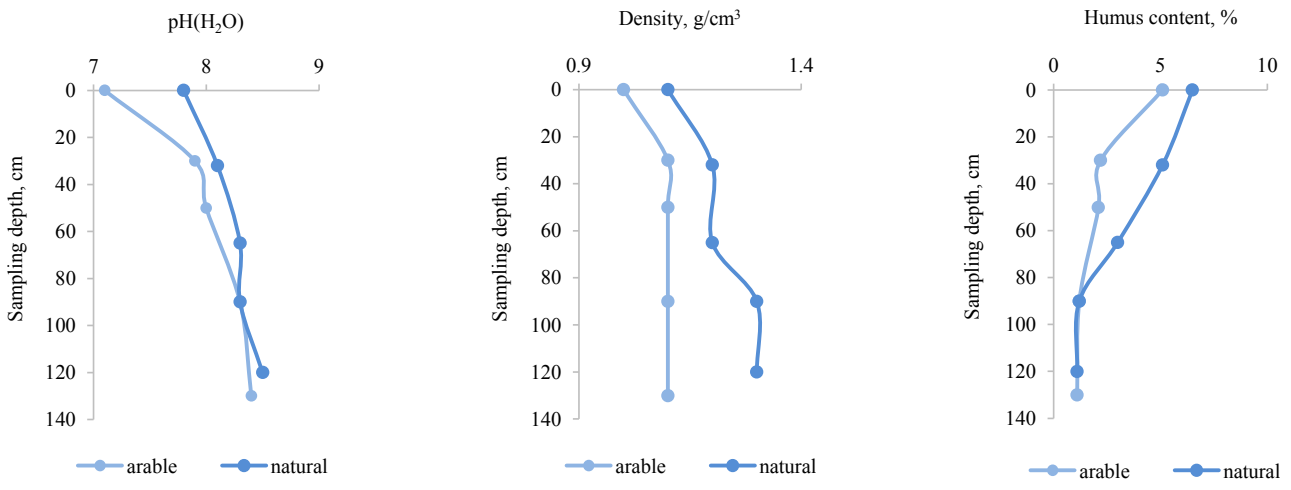

Figure 5 Mountain leached chernozems in the Baksan Gorge: changes in the physical and chemical parameters

exposures. They are located on the residual deposits and talus of limestones, calcareous sandstones, carbonate loams, and clays. Mountain leached chernozems have great agricultural prospects. Their properties make it possible to use them for all zonal crops and perennial plantations.

Table 3 shows the physical and chemical properties of these soils. Mountain leached chernozems are the main arable soils of the Baksan Gorge. That is why Table 3 also presents some physical and chemical properties of mountain agrogenic leached chernozems. A comparative analysis of natural and arable mountain leached chernozems demonstrates that agricultural use results in a greater compaction of the upper soil horizons and a significant loss of humus [55]. The entire soil profile is important for agricultural crops in the Baksan Gorge, especially for sunflower and corn. According to Khakunova *et al*., the profile changes its physical and chemical parameters downwards (Fig. 5) [55]. The humus content goes down sharply, like in leached chernozems elsewhere [56, 57].

Food security in the Central Caucasus. The entire human history is a never-ending search for new methods to meet the basic needs to sustain society, especially in food production. As a result, the existing agricultural systems are adapted to a particular geographical context. They maintain a fragile balance between the basic human needs and the sustainability of the resource [58]. However, people have been trying to adapt the environment for their needs [59]. Extensive agricultural practices changed the landscape, thus becoming a multifunctional agent [60]. This role is especially typical of mountain agriculture with its low-productivity and high-quality focus. These agricultural systems are dominated by grazing and perennial crops, e.g., orchards, vineyards, etc. As a rule, mountain regions have fewer agricultural holdings compared to the national averages. Geographic constraints reduce labor productivity in the highlands by 28% compared to less favorable areas and by 40% compared to valley farming.

The Federal Law on Organic Products and Amendments to Certain Legislative Acts of the Russian Federation was adopted in 2018 and entered into force on January 1, 2020 [61]. This law obliges all Russian regions to localize agricultural products and introduce the best organic farming practices. The transition to a highly productive and green agricultural economy requires a deep analysis of soil resources of fallow and arable lands, including those that appeared as a result of deglaciation [62, 63]. Russian active and fallow agroecosystems are unique systems represented by models of development, degradation, progradation, and evolution of biogeocenosis in space and time as a result of the multidirectional agrogenic impact that occurred in the 20th century [64, 65]. The Central Caucasus is of particular interest because the region is currently experiencing a turbulent agricultural development of lands available after deglaciation [13, 17, 37]. The Baksan lands possess an enormous food security potential for the entire Central Caucasus. The local farmers are eager to develop the ice-free areas as pastures, hayfields, and crop farms, thus increasing the acreage in the region.

Mountain-meadow ecosystems are essential for the sustainable development of mountain areas as providers of various ecosystem services, e.g., regulation, culture, food, etc. [66–68]. Long-term overgrazing leads to soil degradation of mountain meadows, a lower humus content in the fertile layer, a greater compaction of the upper horizons, a lower humidity, and a change in microbial indicators [69, 70]. However, no grazing also destroys the biodiversity of mountain meadows, increases tree and shrub vegetation, develops excessive turfiness, and promotes plants of low nutritional value [71, 72].

The effective management of mountain meadows should be based on sustainable consumption of natural resources, i.e., an optimal ratio between the economic use of resources, their renewal rate, biodiversity, and ecosystem integrity [73–75]. A combination of various

plant, soil, landscape, economic, and organizational indicators helps recognize the stages of pasture change in mountain meadow ecosystems [76–78]. This system needs optimal indicators to assess the state of a particular meadow under certain environmental conditions.

The stages of pasture digression should correspond with particular grazing rates. The lack of clear ideas about this correlation is especially relevant for the highlands of the Central Caucasus. The scale of pasture pressure in the region is so great that researchers recognize all mountain meadows in the Central and North Caucasus as semi-natural pasture ecosystems that have formed after centuries of constant grazing [79]. The rental land use, which is currently operating in the territory of Kabardino-Balkaria, affects food security in the mountain-meadow ecosystems of the Baksan Gorge [80]. This situation is also typical of other regions with pronounced diversification and numerous small farms, especially in the Carpathians [81, 82]. In the Central Caucasus, this type of land use leads to unsystematic exploitation of agricultural land [83].

The Central Caucasus is an intensively developing tourist and recreational cluster. The resulting labor reorientation of the local population means that agriculture does not attract young people any more. The same crisis of traditional trade is observed in mountainous regions all over the world [84]. The agricultural development of the Baksan valleys and foothills suffer from the general improper disposal of land resources in Kabardino-Balkaria. The fertile soils of the Baksan valleys and foothills are now allocated for intensive orchard farming [85]. A more rational approach to Central-Caucasian agriculture requires more fertile flat soils for grain crops, while medium and high-intensity adaptive gardens should be cultivated in the foothills and forest-mountain zones or on hillsides and slopes that are unsuitable for arable land because of the complex terrain and harsh climate.

CONCLUSION

The recent decades have seen a rapid deglaciation of the Central Caucasus. On the one hand, such an unprecedented deglaciation rate is bad for the environment. On the other hand, the area of periglacial landscapes is growing. The new ice-free areas are undergoing an intensive agricultural development. Although they are still affected by seasonal freezing and permafrost, they can serve as pastures and hayfields in the summer. The lands that were previously used as pastures and hayfields gradually turn into arable land, which significantly increases the amount of acreage. This expansion is extremely important for the Central Caucasus, where the amount of arable land is extremely low.

Kabardino-Balkaria is a predominantly agrarian republic with about 55% of arable land. Horticulture has become very popular here in recent years. The republic plants intensive and innovative orchards and fruit tree nurseries. Traditionally, Kabardino-Balkaria is one of the five leading regions in the country in this sphere.

The main threat to food security in the region is that 80% of agricultural products come from its 1500 farms, 6500 land holders and sole entrepreneurs, and more than 115 000 personal subsidiary plots. As a rule, arable land is used on a short-term lease, and small farmers can provide no rational crop rotation, no proper agricultural technology, and no fertilizers. Moreover, the local population gradually turns to the robust sphere of recreation and tourism, which discourages the young generation from engaging in agricultural business.

CONTRIBUTION

The authors were equally involved in the research and are equally responsible for any potential plagiarism. E.V. Abakumov supervised the project and wrote the manuscript. R.Kh. Tembotov collected the materials, performed the analytical work, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Physicochemical properties of kashk supplemented with encapsulated lemongrass extract

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Abstract:
Kashk is a perishable fermented dairy product. Since chemical preservatives are harmful for human health, we aimed to study lemongrass (*Cymbopogon citratus* L.) as a natural preservative.
First, we assessed the phytochemical properties of lemongrass extract. Then, we added lemongrass extract and microencapsulated lemongrass extract to kashk samples. Finally, we analyzed their physicochemical and sensorial properties during 60 days of storage.
Catechin (419.04 ± 0.07 mg/L), gallic acid (319.67 ± 0.03 mg/L), and chloregenic acid (4.190 ± 0.002 mg/L) were found to be the predominant phenolic constituents in lemongrass. Total phenolics, total flavonoids, and antioxidant activity (IC_{50}) values of the lemongrass extract were 26.73 mg GA/g, 8.06 mg Quercetin/g, and 2751.331 mg/L, respectively. The beads were spherical in shape with a 35.03-nm average particle diameter and 47.81% microencapsulation efficiency. The pH of the supplemented kashks decreased during the storage time. They showed lower acid degree values than the control at the end of storage. The peroxide, *p*-anisidine, and thiobarbituric acid values of the sample fortified with microencapsulated lemongrass extract were 6.15, 4.76, and 44.12%, respectively, being the lowest among the samples. This kashk sample had the highest hardness (570.62 ± 21.87 g), adhesiveness (18.10 ± 4.36 mJ), and cohesiveness (0.56 ± 0.25) but the lowest chewiness (72.66 ± 3.08 mJ) among the samples. It also had a better sensory profile than the control samples.
Our results indicated that microencapsulated lemongrass extract could be incorporated into kashk to ensure suitable sensorial and textural properties. Furthermore, it may delay fat oxidation and lipolysis during storage.

Keywords: Polyphenols, lemongrass extract, encapsulation, fat oxidation, kashk

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INTRODUCTION

Kashk, in a dried or liquid form, is a fermented dairy product that is very popular in Iran. It is used as a major protein source by nomadic populations in the Middle East. Salty by-products, such as buttermilk, are traditionally used to produce kashk [1]. It chemical composition includes 84.25% of dry matter, 8.57% fat, 95.9% salt, 53.60% total protein, 11.08% ash, and 1.06% lactose. Kashk also contains amino acids and minerals such as calcium, magnesium, iron, sodium, and potassium. Due to its high moisture and protein content, kashk has a high microbial contamination potential [2].

Two major mechanisms are involved in the spoilage of milk products, lipolysis and oxidation. The release of various lengths of fatty acids during the cleavage of milk fat by lipase and phospholipase can contribute to an intense flavor in milk. In particular, short-chain fatty acids released between C_4 and C_8 account for a rancid flavor and odor, while free medium-chain fatty acids released between C_{10} and C_{12} produce a soapy flavor. Finally, the oxidation of lipids cause off-flavor and odors [3].

Lipid oxidation occurs when free radical chain reactions propagate in food. Antioxidants can significantly prevent or inhibit the oxidation of lipids in foods,

even at low concentrations. Additionally, some studies have shown that mixtures of synthetic antioxidants can cause carcinogenic effects. Thus, the use of these synthetic food additives is tightly controlled.

The demand for new, naturally-derived, and more effective antioxidants as alternatives to synthetic products is driven by their unsafety and toxic effects on health, as well as by negative consumer perceptions of chemical additives [4]. Furthermore, the use of plant extracts in dairy products, such as milk, cheese, yogurt, labneh, ayran, and doogh, have been successfully reported. Nevertheless, the efficacy of microencapsulated plant extracts in controlling the quality of industrial liquid kashk has not been studied yet [5].

Lemongrass typically grows to 1.8 m in height and 1.2 m in the spread. It has a small rhizome and its leaves emerge straight out of the ground without stems. They are 1.3–2.5 cm wide and about 1 m long. The *Cymbopogon* genus includes more than 55 species across the world, with different physical and chemical properties [6].

Lemongrass is used in food preparation, especially in Malaysia, Thailand, Vietnam, Sri Lanka, and in the Caribbean islands. It is a highly nutritional functional ingredient with antioxidant and anticancerous properties [7].

Recent years have seen an increased consumer interest in functional foods, especially dairy products. Lemongrass extract has been reported to improve the color and odor attributes of supplemented cheese and reduce cheese environmental pollution. In addition, lemongrass extract or essential oil is a potential antimicrobial agent against a wide variety of pathogenic Gram-positive and Gram-negative bacterial species [8].

In a study by Hematian *et al.*, extracts from the caper plant (*Capparis spinose* L.) were added to kashk [2]. Caper extract had no adverse effects on kashk's pH. Increasing the extract's concentration and storage time decreased *L** and increased *b**, but did not affect the *a** color parameter. Caper extract not only improved the textural properties of kashk but also reduced its odor at the end of the storage period. However, no differences in taste and mouthfeel were observed between the samples. In general, the kashk samples containing 0.350 mg/mL of caper extract had enhanced antibacterial, antioxidant, and antifungal properties and therefore can be considered a novel functional product [2]. Peppermint essential oil (*Mentha pulegium*) can also be used to enrich kashk in concentrations of 1500 and 2500 ppm [5].

Microencapsulation of essential oil facilitates its application as a food ingredient and is therefore recommended to improve the stability of the product. Encapsulation protects the oil from oxidation, limits unwanted environmental impacts, and promotes long-term release, ensuring extra time for its activity [9]. Emulsion is one of the techniques used in microencapsulation. Emulsions are colloidal dispersions of at least two immiscible liquids, one dispersed in the other. They are stabilized with surfactants that have a proper hydrophilic-lipophilic balance. Emulsions have a potential advantage of encapsulation and protection of water-soluble bioactive nutrients, such as vitamins, minerals, probiotics, amino acids, and caffeine [10].

The preparation of emulsion is one of the crucial steps for successful encapsulation. It depends on the selection of wall materials obtained from a variety of natural and synthetic polymers [11].

Microencapsulated lemongrass essential oil was used in several foods such as Coalho cheese and stirred yogurt [9, 12].

Yet, there has been no research into the microencapsulation of lemongrass extract and its application in kashk. Therefore, we aimed to evaluate the impact of microencapsulated lemongrass extract (MLGE) and lemongrass extract (LGE) on kashk and determine its sensory and physicochemical properties.

STUDY OBJECTS AND METHODS

Plant collection and identification. Fresh lemongrass was collected from Firoozabad, Fars province (south of Iran), in April 2020. It was identified by a senior plant taxonomist at the Department of Botany, Fars Agricultural and Natural Resources Research and Education Center, Shiraz, Iran.

Chemicals and materials. All the solvents were of the HPLC grade. A Millipore Direct-Q UV system was used to prepare deionized water. A Biotek microplate reader El×818 was applied to investigate the antioxidant activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from the Sigma-Aldrich company. Reference standards of 17 polyphenols (p-comaric acid, trans-ferulic acid, catechin, carvacrol, eugenol, chlorogenic acid, rosmarinic acid, quercetin, gallic acid, caffeic acid, ellagic acid, rutin, hesperetin, vanillin, hesperidin, coumarin, and sinapic acid) were also obtained from the Sigma-Aldrich company for HPLC analysis.

Preparation of methanolic extract. A Moulinex AR1100 grinder (Coulsdon, Surrey, UK) was applied to grind dried seeds to a fine powder. The powder was used for methanolic extraction by the maceration technique. For this, a certain amount of powdered seed was soaked in methanol (1:10) for about 24 h, filtrated, and injected to determine polyphenols by HPLC. The residue was concentrated by a rotary evaporator at 40°C for antioxidant activity analysis [13].

Determination of antioxidant activity (DPPH). The DPPH free radical scavenger method was employed to evaluate the standard antioxidant activity of lemongrass extract [13]. Gallic acid was the standard compound for this test.

HPLC analysis of polyphenol. Gradient elution was selected for maximum sensitivity. Various ratios of solvent A (1% formic acid in deionized water) to solvent B (methanol, v/v) were used, namely 10:90, 25:75, 60:40, and 70:30 at 0, 10, 20, and 30 min, respectively, which were then maintained isocratic till 40 min. HPLC

was executed on an Agilent 1200 series, with a Zorbax Eclipse XDB-C18 column (4.6×5 μm i. d.; ×150 mm film thickness, RP) and a photodiode array detector. Elution was monitored at 280 and 230 nm. The column temperature was 30°C. The injection volume was 20 μL. The standard solutions had a linear calibration curve with a good correlation.

Spectrophotometric determination of total phenolics. Folin Ciocalteu's reagent was used to spectrophotometrically measure total phenols. The results were shown as gallic acid equivalents. A gallic acid calibration curve was based on the equation: $c = 1.885 \times A + 2.81$, $R^2 = 0.9953$. Finally, four calibration points were plotted within the range of 6.25–50 mg/mL of gallic acid in the reaction mixture.

Spectrophotometric determination of total flavonoids. The aluminum chloride colorimetric method was used to determine total flavonoids**.** Quercetin was used to make a calibration curve. Ten milligrams of quercetin were dissolved in 80% ethanol and then diluted to 6.25, 12.5, 25, 50, 80, and 100 mg/L. The standard solutions (0.5 mL) were diluted and separately mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of 95% ethanol, and 2.8 mL of distilled water. After incubating at 27°C for 30 min, a Shimadzu UV-160A spectrophotometer (Kyoto, Japan) was used to assess the optical density of the reaction mixture at 415 nm. The blank sample consisted of an equal amount of 10% aluminum chloride that was replaced with an equal amount of distilled water. Similarly, the fifteen flavonoid standard solutions (100 ppm) or 0.5 mL of ethanol extracts were reacted with aluminum chloride to assess the flavonoid content as stated above.

Microencapsulation of extract. All the glassware and solutions used in this protocol were sterilized at 121°C for 15 min. The lemongrass extract was added to the sodium alginate mixture containing 2% (w/v) of maize starch (Starch Australia Ltd.). The mixture was dropped into the oil containing Tween 80 (0.02%). On completion of the dropping, the mixture was stirred vigorously until it was fully emulsified to a creamy form. A solution of 0.1 M calcium chloride was then added fast along the side of the beaker, and the phase separation of oil/water emulsion took place. To separate and settle the emulsion at the bottom of the calcium chloride layer, the mixture was allowed to stand for 30 min. The oil layer was drained and the beads were collected by slow centrifugation (3503 g, 15 min), washed once with 0.9% saline containing 5% glycerol, and stored at 4°C.

Particle size, morphology of beads, and microencapsulation efficiency. The aspect ratio of 20 beads was analyzed using a digital microscope and MicroMeasure software version 1.07 (Eq. (1)).

$$\text{Aspect ratio} = \frac{\text{Large diameter}\,(\text{mm})}{\text{Small diameter}\,(\text{mm})} \quad (1)$$

A dynamic light scattering device (90 Plus, Brookhaven Instruments Corp., Vienna, Austria) was used to measure the particle size distribution. Analyses were performed at a scattering angle of 90 at 25°C.

The efficiency of encapsulation was verified by the determination of phenolic compounds. For this, 800 mg of microencapsulated powder was accurately weighed, added to 4 mL of methanol (as solvent), and gently shaken using a vortex for 2 min at room temperature. The tube was then centrifuged (IEC Centra3M Centrifuge, UK) at 3000 rpm for 5 min. The Folin-Ciocalteu colorimetric method was used to measure phenolic compounds in the slurry, which were eventually called microencapsulated polyphenols (P_{encap}, mg GA/g DE). The total polyphenol content of dried lemongrass extract was assessed based on the Folin-Ciocalteu colorimetric method (P_{total}, mg GA/g DE). The following equation was then applied to calculate the efficiency of microencapsulation (E_{encap}, %).

$$E_{encap} = \frac{P_{encap}}{P_{total}} \quad (2)$$

Kashk samples preparation. To prepare kashk samples, milk fat was initially standardized at 1.3% w/v followed by homogenization at 100–200 kg/cm^2, pasteurization at 90°C for 15 min, and cooling down to 43°C. The lyophilized starter culture, CH1 (50U) DVS, was added to the milk and incubated at 43°C until optimal acidity was achieved (150°D). The produced yogurt was then kept at 4–5°C for 24 h. After that, it was transferred to a cooking tank (heated at 80°C for 4 h) and constantly stirred, to prevent burning, until a light brown color appeared in the product. The kashk's total solids were measured on an MA100 infrared moisture analyzer (Sartorius, Gottingen, Germany) and then adjusted to 20% m/m (SNF 14.28 m/m). Sterile salt (1%) was added and the sample was homogenized at 75°C at 100–150 kg/cm^2. Based on the results of sensory evaluation, lemongrass extract (5%, w/w) was added to the milk. The ranking test was performed by 50 non-trained panelists of both sexes, aged 20 to 45, who were pre-selected according to their interest in the study. Various concentrations of lemongrass extract (1, 3, 5, and 7%) were used to assess the flavor preference. Approximately 20 g of each kashk sample was served to every panelist randomly encoded with a 3-digit number. The panelists were asked to select the most and the least preferred samples based on their overall impression. Finally, the total priorities were calculated. An equal quantity of lemongrass extract at a concentration of approximately 10% (w/w) was added to each sample. The samples free of lemongrass extract or microencapsulated lemongrass extract were considered as a control. All the samples were packed hot (80°C) in plastic containers and stored at 4°C. Their sensory and structural properties were eventually analyzed during storage.

pH and acidity of kashk. The pH values of the kashk samples were recorded using a pH meter (Greisinger Electronic, Germany). The Dornic degree (National Standard of Iran, 2852) was then used to show their titrable acidity [14].

Acid degree value. The acid degree value of the samples was determined as described by Borhanpoor *et al.* using the following equation [15]:

$$\text{Acid degree value} = \frac{5.61 \times \text{Titration}(\text{mL})}{\text{Sample}(\text{g})} \quad (3)$$

Color. The kashk's color was measured using a CR-400 Chroma Meter (Konica-Minolta, Osaka, Japan). The *L** value is an indicator of lightness (black to white). The *a** values indicate green and red, while *b** indicates blue and yellow.

Texture. The texture profile analysis was employed to measure the texture of the samples using a CT3 texture analyzer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The test was performed on the first and the 60th day of the storage period. A sample (diameter 20.0 ± 0.5 mm, height 10.0 ± 0.5 mm) was taken from the center of the kashk. Next, the cylindrical kashk was covered with a stretch film and brought to room temperature of 20 ± 1°C. The analysis conditions were as follows: a TA11/1000 aluminum cylinder probe (25.4 mm in diameter), compression to 20% of the initial height, a test speed of 1 mm/s, a penetration rate of 2 mm/s, a pre-test speed of 2 mm/s, and a retention time of 5 s. The textural parameters included hardness (g), chewiness (mJ), adhesiveness (mJ), gumminess (g), and cohesiveness.

Sensory analysis. The control kashk sample, the kashk with lemongrass extract, and the kashk with microencapsulated lemongrass extract (MLGE) were evaluated by 45 educated panelists. They were divided into two age groups, 18–24 years and 24–51 years (42% of males and 58% of females). A 5-point hedonic scale was applied to assess the flavor, odor, color, texture, and overall acceptability, where 5 indicated "like extremely" and 1 indicated "dislike extremely" in comparison with the control kashk. The samples were evaluated by the panelists every 15 days during the storage period.

Peroxide value. The peroxide value was calculated using a method described by Siddique and Park based on Eq. (4) [16]:

$$\text{POV} = \frac{\left[T_{\text{sample}} - T_{\text{blank}}\right] \times 0.01\text{N} \times 1000}{\text{Sample weight}} \quad (4)$$

where POV is the peroxide value, milliequiv. of Peroxide per 1000 g sample; T_{sample} is the titration of sample, mL; T_{blank} is the titration of blank, mL; sample weight, g.

The *p*-anisidine value. The *p*-anisidine value (AnV) indicates the degree of lipid oxidation and is used to measure stable secondary oxidation products. Measurements were performed on a DR5000 UV-V spectrophotometer (Hach Lange, Germany), with iso-octane used as a blank. AnV was determined according to Eq. (5):

$$p\text{-anisidine value} = \frac{25 \times (1.2As - Ab)}{m} \quad (5)$$

where *As* is the absorbance of the sample; *Ab* is the absorbance of the blank; *m* is the mass of the sample, g [17].

Thiobarbituric acid reactive substances. To determine thiobarbituric acid reactive substances (TBARS), one gram of a kashk sample was mixed with 9 mL of 0.25 N HCl solution containing 0.375% TBA (Sigma-Aldrich, USA) and 15% TCA (Merck, Germany). The mixture was heated in boiling water for 10 min, followed by cooling with running water. The mixture was then centrifuged at 3500 rpm for 15 min. The supernatant was collected, and the absorbance was read at 532 nm using a spectrophotometer. TBARS were calculated from the standard curve of malonaldehyde (0–2 mg/kg) (Merck, Germany) and expressed as mg malonaldehyde per kg of the sample.

Statistical analysis. Experimental data were analyzed using SPSS Statistics Version 21.0 (IBM Corp., Armonk, NY, USA). ANOVA ($p \leq 0.05$) and Duncan's multi-range test were employed to perform a mean comparison. All the experiments were carried out in triplicate, unless otherwise stated in the text. All the graphs were created in Microsoft Excel Version 16 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Polyphenol, total phenolic content, flavonoid, and antioxidant activity. Of 17 polyphenols identified (Table 1), the main phenol components in the lemongrass extract were gallic acid (138.95 mg/L), trans-ferulic acid (20.58 mg/L), and thymol (47.9 mg/L). Sinapic acid, catechin, caffeic acid, quercetin, coumarin, vanillin, hesperidin, ellagic acid, and eugenol were not detected in the lemongrass extract. Total phenolics and flavonoids in the lemongrass extract were 26.73 mg GA/g and 8.06 mg Quercetin/g, respectively.

A similar study by Gazwi reported slightly different results, with total phenols and flavonoids amounting to 65.20 mg GA/g and 46.57 mg Quercetin/g, respectively [18]. Alterations in the values might result from the fact that the raw plant material was taken from different plant parts [15]. Furthermore, the differences can be related to the plant populations that originate from various altitudes, geographic origins, and climates [19].

In our study, the antioxidant activity of the LGE was 512.95 μg/mL, which was different from IC_{50} (1998.10 ± 0.02 μg/mL) reported by Lahlou *et al.* [20]. The structure and contents of polyphenols are associated with different factors including plant varieties, culture

Table1 Phytochemical and antioxidant contents of lemongrass extract

Polyphenolic compound	Amount	Retention time, min
Sinapic acid, mg/L	n.d.	16.5
Gallic acid, mg/L	138.9532	3.3
Catechin, mg/L	n.d.	8.3
Caffeic acid, mg/L	n.d.	11.6
Chloregenic acid, mg/L	14.7	10.5
Quercetin, mg/L	n.d	21.6
p-coumaric acid, mg/L	3.040196	15.6
Coumarin, mg/L	n.d.	17.4
Carvacrol, mg/L	10.8	28.4
Vanillin, mg/L	n.d.	13.5
Trans-ferulic acid, mg/L	20.58616	16.3
Hesperidin, mg/L	n.d.	18.5
Ellagic acid, mg/L	n.d	19.02
Eugenol, mg/L	n.d.	23.7
Hesperetin, mg/L	13.11011	22.4
Rosmarinic acid, mg/L	16.53507	19.2
Thymol, mg/L	47.90072	28.9
Flavonoids, mg Quercetin/g	8.06	–
Total phenolic compounds, mg GA/g	26.73	–
IC_{50}, mg GA/L	2751.331	–

n.d. – not detected

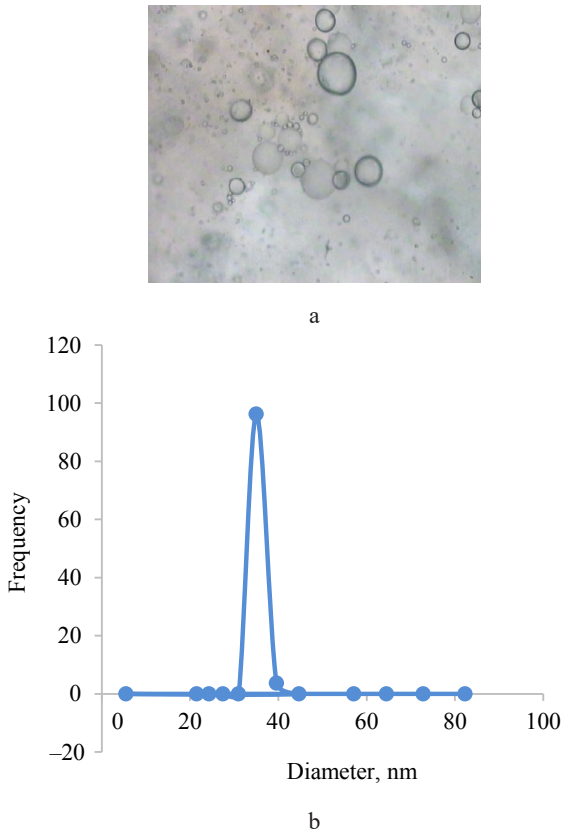

Figure 1 Microencapsulated lemongrass extract: (a) light microscopy image; (b) droplet size distribution

conditions, maturation, and processing, which affect total polyphenols mainly present in the combined form [21].

Particle size, the morphology of beads, and microencapsulation efficienc The beads containing starch had a spherical shape (Fig. 1). They were observed by light microscopy (×100). The type of wall material is an important factor for the bead shape. For example, alginate beads showed a smaller size and a more regular shape compared to pectin [22].

In our study, the average particle diameter was 35.03 nm. Size is an important indicator of the beads' stability and efficiency [23]. We found the efficiency of microencapsulation to be 47.81%. In contrast, Mehran *et al.* reported values of 93.1 and 97.4% for encapsulation efficacy. The main reason for this variation was the composition of the wall material used for encapsulation [24].

pH and acidity of kashk. Changes in the pH and acidity of the kashk samples supplemented with lemongrass extract and microencapsulated lemongrass extract during 60 days of storage at 4°C are presented in Fig. 2a and b. The pH values significantly ($p<0.05$) decreased in both experimental groups on the first day of storage and continued to decrease throughout the whole storage period. The pH ranged from 3.89 to 3.95 and was considered acceptable for the commercial kashk varieties. The presence of lactic acid bacteria in yogurt inevitably leads to post-acidification, which may cause increased post-acidification in kashk [25]. The acidity of different sorts of kashk could effectively increase due to post-acidification during storage. These results reflect those of Kim *et al.*, who reported that the pH values of Gouda cheese supplemented with pepper extract were not significantly different from those of the control cheese at each time point. However, the pH of each sample did not change significantly throughout the aging period [26].

Acid degree value. The levels of free fatty acids in the samples were expressed as acid degree values (Fig. 3). We found that the kashk samples supplemented with lemongrass extract and microencapsulated lemongrass extract had lower acid degree values than the control at the end of the storage time. The ADVs in all kashks increased during the storage time. However, molds and lactic acid bacteria were the main agents responsible for lipolysis in this product [27]. Spontaneous lipolysis happens during cold storage. This view is supported by Khan *et al.*, who enriched cheese with mango kernel oil (*Mangifera indica* L.). They concluded that the amount of free fatty acids increased in all treatments and the control samples over the entire 90-day period. Bacterial lipase and moisture contents are the main causes of free fatty acids [28].

Color analysis. Lightness (L^*), red-green axis (a^*), and yellow-blue axis (b^*) are the factors that evaluate the color of kashk (Table 2). Our results showed the highest values of L^* in the control samples. We hypothesized that the kashk samples containing

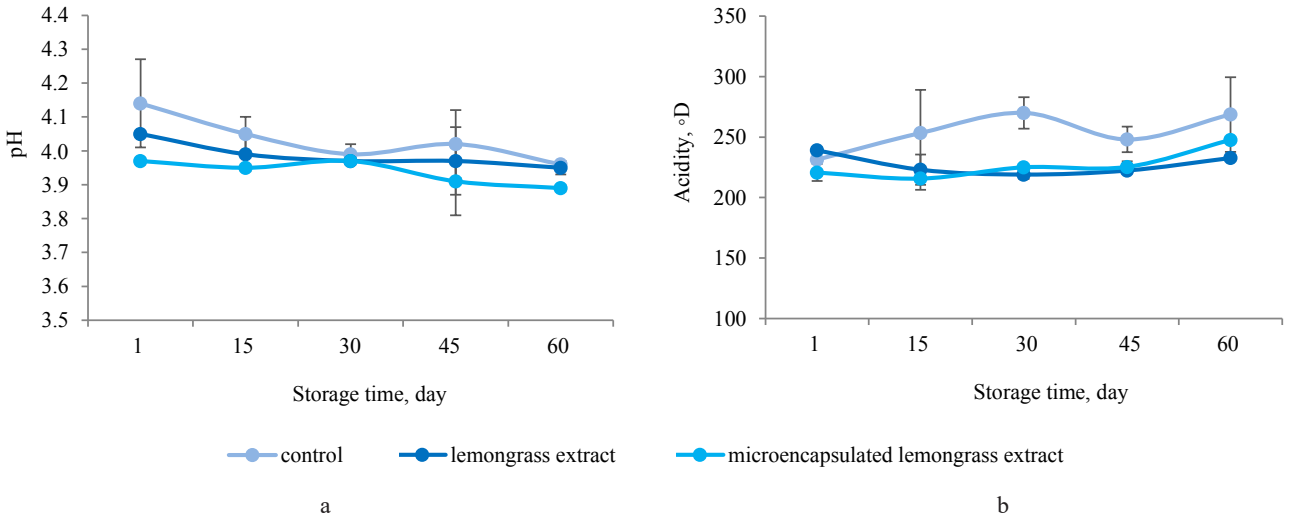

Figure 2 Chemical parameters of kashk samples supplemented with lemongrass extract and microencapsulated lemongrass extract during storage: (a) pH; (b) acidity

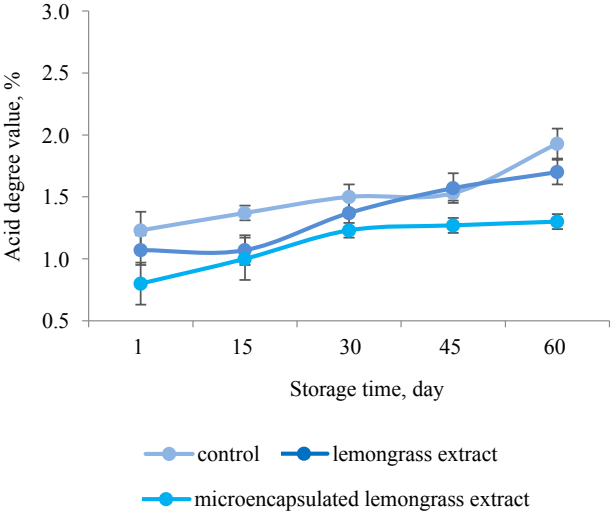

Figure 3 Acid degree values of kashk samples supplemented with lemongrass extracts during storage

lemongrass extract and beads showed lower light scattering and brightness due to water absorption, so the final product looked darker. The white appearance of this product was due to the presence of colloidal particles, such as milk fat globules and casein micelles, that could scatter visible spectrum light. In a similar study of yogurt, the encapsulated polyphenolic compounds extract powder showed lower lightness than the plain yogurt due to the presence of pigments such as anthocyanin [29]. Further, the *a** (redness) decreased but *b** (yellowness) values increased for the kashk samples fortified with microencapsulated lemongrass extract. The more yellowish color was due to an increase in the encapsulated extract in the kashk. Higher greenness of the microencapsulated samples could be related to the green color of LGE.

Texture. In our study, the texture profile analysis of the kashk samples correlated well with their sensory parameters (Table 3). In particular, hardness decreased during storage in the control and lemongrass extract and increased in the microencapsulated lemongrass extract. A similar result was reported by Pérez-Soto *et al.* for the control cheese on the first day of storage in comparison with the other microencapsulated enriched cheese [30]. Furthermore, hardness is related to the pH of the kashk samples. The lower the pH, the higher the stiffness of the kashk structure. At decreasing pH values, the covalent bind of ionic species to the casein strand becomes protonated during curd formation. This phenomenon subsequently increases hydrophobic interactions between protein molecules to finally make the curd harder [31]. Therefore, the sample with microencapsulated extract with the lowest pH (Fig. 2a) revealed the greatest hardness at the end of the storage time.

The results also showed that in the kashk samples, cohesiveness values were equal during the storage time and among the kashk samples on the same storage day. According to results obtained in a previous study for yogurt enriched with iron-entrapped niosomes, adhesiveness had no significant differences between the control and microencapsulated yogurt [32]. Increased water in the gel system could be one of the reasons for downgrading adhesiveness [33]. Further, we found that gumminess increased in the fortified samples during storage. This finding was consistent with a study by Sah *et al.*, where higher gumminess was attributed to the reduction in pH followed by structural shrinking and rearrangement of the kashk structure [34]. Ahmed *et al.* reported a stabilized casein network and improved gumminess due to the interaction between phenolic compounds in the extract and proteins, which could lead to the rearrangement of proteins during storage [33]. In our research, the sample with

Table 2 Color parameters of kashk samples supplemented with lemongrass during storage

Parameters	Days	Control kashk	Lemongrass extract	Microencapsulated lemongrass extract
*L**	1	66.22 ± 5.31^{aA}	61.22 ± 3.53^{aA}	58.33 ± 3.92^{aAB}
	15	65.44 ± 2.92^{aA}	62.89 ± 3.62^{aA}	51.89 ± 3.45^{bB}
	30	64.22 ± 5.09^{aA}	60.56 ± 4.26^{aA}	63.44 ± 2.68^{aA}
	45	65.33 ± 2.91^{aA}	60.33 ± 2.77^{aA}	65.22 ± 3.40^{aA}
	60	66.56 ± 3.83^{aA}	58.89 ± 3.36^{aA}	67.00 ± 2.56^{aA}
*a**	1	-3.33 ± 0.57^{bB}	-2.11 ± 0.48^{abC}	-0.67 ± 0.55^{aB}
	15	-3.22 ± 0.62^{bB}	-3.44 ± 0.29^{bC}	-1.56 ± 0.53^{aB}
	30	0.78 ± 0.83^{aA}	0.44 ± 0.70^{aB}	1.67 ± 0.57^{aA}
	45	-1.00 ± 0.57^{bA}	2.22 ± 0.57^{aAB}	2.33 ± 0.66^{aA}
	60	-1.00 ± 0.62^{bA}	0.78 ± 0.40^{aB}	-0.78 ± 0.52^{bB}
*b**	1	11.33 ± 0.79^{bC}	16.22 ± 0.54^{aB}	11.67 ± 0.62^{bC}
	15	12.33 ± 1.24^{aC}	12.33 ± 0.83^{aC}	13.11 ± 0.97^{aC}
	30	16.44 ± 0.41^{aAB}	14.44 ± 0.93^{bB}	17.67 ± 0.44^{aB}
	45	18.00 ± 0.62^{bA}	19.78 ± 0.59^{abA}	21.22 ± 0.77^{aA}
	60	15.56 ± 0.64^{aB}	16.11 ± 0.26^{aB}	16.44 ± 0.33^{aB}

*L** is the luminance or lightness component, *a** (from green to red) and *b** (from blue to yellow)

[A-B] Means in the same column with different uppercase letters differ significantly ($p \leq 0.05$)

[a-b] Means in the same row with different lowercase letters differ significantly ($p \leq 0.05$)

*Mean ± SD (n = 4)

Table 3 Texture analysis of kashk samples fortified with lemongrass extract and microencapsulated lemongrass extract during storage

Parameters	Samples	Day 1	Day 60
Hardness, g	Control kashk	802.25 ± 83.75^{aA}	309.75 ± 36.00^{bB}
	Lemongrass extract	700.38 ± 66.62^{aAB}	199.54 ± 3.03^{bC}
	Microencapsulated lemongrass extract	494.54 ± 8.50^{aB}	570.62 ± 21.87^{aA}
Adhesiveness, mJ	Control kashk	33.97 ± 10.41^{aA}	12.49 ± 2.17^{aA}
	Lemongrass extract	27.21 ± 21.20^{aA}	11.63 ± 1.09^{bA}
	Microencapsulated lemongrass extract	13.62 ± 2.48^{aA}	18.10 ± 4.63^{aA}
Cohesiveness	Control kashk	0.70 ± 0.01^{aA}	0.45 ± 0.04^{aA}
	Lemongrass extract	0.760 ± 0.015^{aA}	0.36 ± 0.15^{aA}
	Microencapsulated lemongrass extract	0.72 ± 0.04^{aA}	0.56 ± 0.25^{aA}
Gumminess, g	Control kashk	773.05 ± 120.85^{aA}	460.82 ± 26.68^{aB}
	Lemongrass extract	566.20 ± 44.10^{bAB}	838.75 ± 2.25^{aA}
	Microencapsulated lemongrass extract	372.05 ± 9.45^{bB}	472.35 ± 11.25^{aB}
Chewiness, mJ	Control kashk	117.54 ± 29.72^{aB}	135.56 ± 8.67^{aA}
	Lemongrass extract	90.60 ± 3.77^{aB}	273.69 ± 81.29^{aA}
	Microencapsulated lemongrass extract	430.32 ± 10.13^{aA}	72.66 ± 3.80^{bB}

[A-C] Means in the same column with different uppercase letters differ significantly ($p \leq 0.05$)

[a-b] Means in the same row with different lowercase letters differ significantly ($p \leq 0.05$)

* Mean ± SD (n = 4)

microencapsulated lemongrass extract had the greatest gumminess among the samples at the end of the storage time. However, chewiness was the highest in the kashk with lemongrass extract and lowest in the sample with microencapsulated extract on the 60th day of storage. This finding was similar to the results reported by Ojagh *et al.* [35].

Sensory analysis. The results of sensory analysis for color, odor, flavor, texture, and overall acceptability of the kashks stored at 4°C for 60 days are provided in Fig. 4. Significant differences ($p < 0.05$) were observed for flavor and odor between the control and the samples fortified with lemongrass extract and microencapsulated lemongrass extract at the end of the storage time. In a study by Sawale *et al.*, accelerated amounts of herb (0.1 to 0.5%, *Pueraria tuberosa*) added into milk caused lower color, appearance, and mouthfeel scores [36]. In our study, the samples with microencapsulated extract revealed a better sensory profile than the control samples, which verified their high acceptability. These results were in agreement with a study by Balabanova *et al.* [37]. The texture of the kashk supplemented with microencapsulated lemongrass extract increased at the end of storage because sodium alginate,

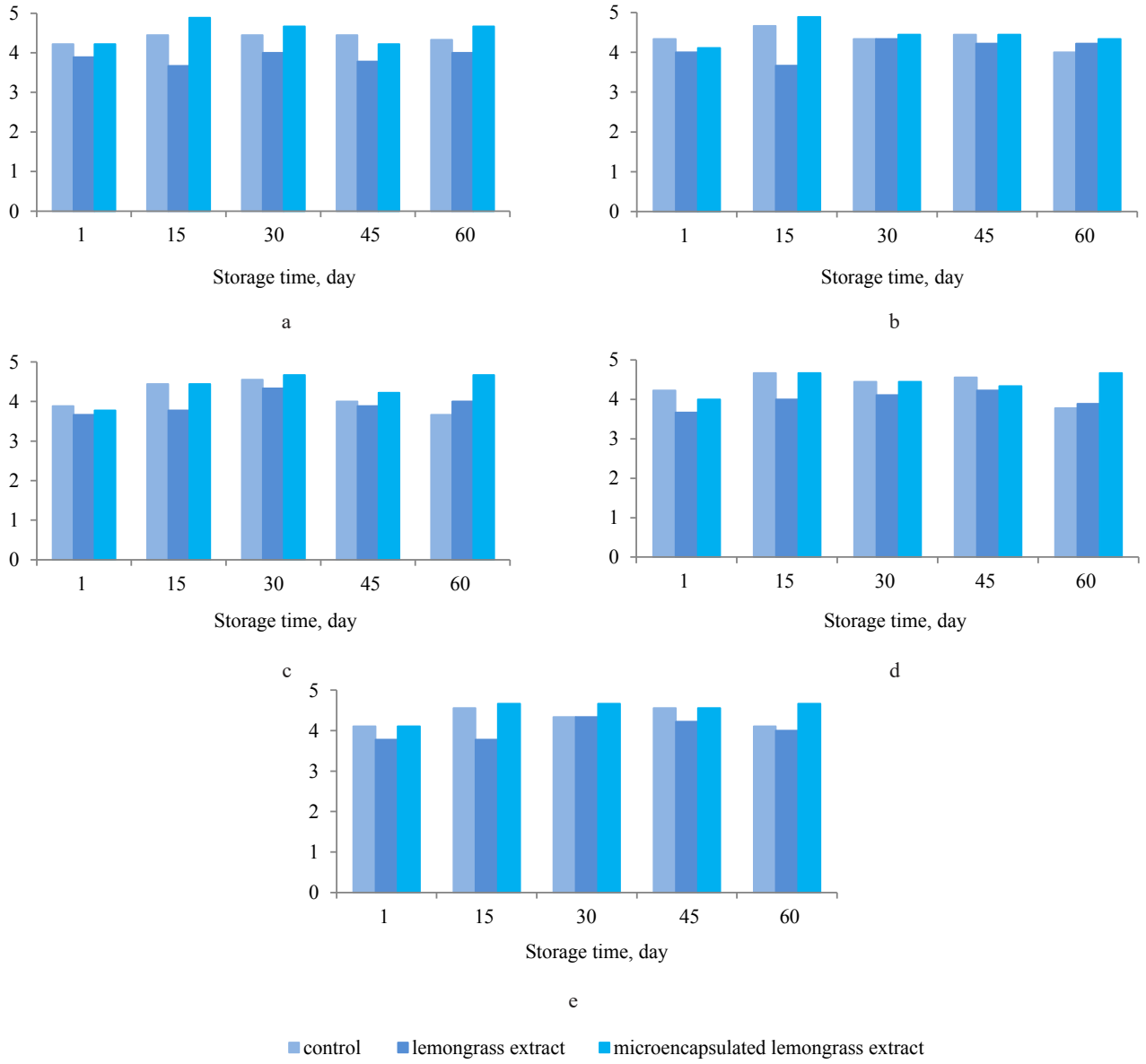

Figure 4 Sensory characteristics of lemongrass extracts during storage for 60 days at 4°C: (a) flavor; (b) color; (c) odor; (d) texture; (e) overall acceptability

an anionic polysaccharide, formed complex coacervates with two milk proteins, *β*-lactoglobulin and bovine serum albumin, through electrostatic interactions under specific conditions, which seemed to strengthen the gel in yogurt [38].

The peroxide value, *p*-anisidine value, and thiobarbituric acid values. Table 4 shows an increasing trend in the peroxide value, *p*-anisidine value, and thiobarbituric acid values during the storage period for all the samples. However, this increase was slower in the samples fortified with lemongrass extract and microencapsulated lemongrass extract, compared to the control. This means that fat oxidation was reduced in the fortified kashks. It might be related to gallic acid, a major antioxidant compound in lemongrass extract [39]. The antioxidant activity of phenolic compounds in lemongrass extract during storage was enhanced by its encapsulation. Therefore, the peroxide value was decreased in the kashk containing emulsion nanoparticles (dispersed droplets in W/O emulsions), compared to simple extract throughout storage.

In contrast, encapsulation can raise the inhibitory ratio of natural antioxidants due to the controlled release of phenolic compounds and continuing availability of the antioxidant [40]. The decomposition of the primary oxidation products of the emulsion lipid increased the *p*-anisidine value of the kashk samples during storage. This value was considerably influenced by the addition of lemongrass extract during 60 days. It was significantly ($p < 0.05$) lower in the experimental samples compared to the control. The antioxidant function of the microencapsulated polyphenols may reduce both the primary and secondary oxidation of lipids [41].

Table 4 Peroxide, *p*-ansidine, and thiobarbituric acid values in kashk samples during storage

Parameters	Days	Control kashk	Lemongrass extract	Microencapsulated lemongrass extract
Peroxide value (milliequiv of peroxide/ 1000 g sample)	1	0.77 ± 0.18^{aA}	0.67 ± 0.25^{aA}	0.65 ± 0.11^{aA}
	60	2.86 ± 1.11^{aA}	2.02 ± 0.98^{aA}	0.69 ± 0.21^{aA}
p-anisidine	1	18.32 ± 1.08^{bB}	26.44 ± 0.97^{aB}	21.24 ± 1.06^{bA}
	60	29.25 ± 0.90^{bA}	40.92 ± 1.04^{aA}	22.25 ± 0.79^{cA}
Thiobarbituric acid content (mg malonaldehyde/kg)	1	0.034 ± 0.002^{aA}	0.031 ± 0.004^{aA}	0.034 ± 0.005^{aA}
	60	0.051 ± 0.009^{aA}	0.048 ± 0.011^{aA}	0.049 ± 0.012^{aA}

[a-b] Data within the same row marked with different lowercase letters are significantly different ($p \leq 0.05$)

[A-B] Data within the same column marked with different uppercase letters are significantly different ($p \leq 0.05$)

* Mean ± SD (n = 3)

Throughout storage, all the samples showed a significant increase in thiobarbituric acid values. The control kashk obtained a higher value (0.034 on the initial day to 0.051 on day 60) followed by the sample with lemongrass extract (0.031 on the initial day to 0.048 on day 60) and the sample with microencapsulated extract (0.034 on the initial day to 0.048 on day 60). The thiobarbituric acid value increases in the lemongrass-fortified samples were lower than in the control. These results were consistent with those of El-Sayed *et al.*, who indicated that the butter fortified with sage and rosemary essential oils had smaller concentrations of secondary oxidative products like malonaldehyde and ketones [42].

CONCLUSSION

Lemongrass extract was successfully encapsulated using the emulsion method. The phytochemical screening of the extract revealed the presence of Gallic acid, Thymol, Rosmarinic acid, Hesperetin, and Trans-ferulic acid as major compounds. The microcapsules were spherical in shape. The acidity and pH of the samples fortified with lemongrass extract were acceptable for commercial kashk varieties. These samples had lower acid degree values than the control at the end of the storage time. The application of microencapsulated lemongrass extract in the kashk matrix did not have a negative effect on the color parameters. The kashk with microencapsulated extract had the highest hardness on the 60th day of storage but the lowest chewiness. The fortified samples were able to reduce fat oxidation compared to the control. High overall acceptability showed that encapsulation could be applied for kashk fortification.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Medusomyces gisevii L.: cultivation, composition, and application

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Abstract:
Tea fungus (*Medusomyces gisevii* L.) is a natural symbiotic consortium of yeast-like fungi and bacteria. Scientific literature provides a lot of information about the consortium, but it is largely fragmentary. We aimed to review and systematize the information on the research topic.
We studied scientific publications, conference proceedings, intellectual property, regulatory documents, and Internet resources on the *M. gisevii* consortium using Scopus, Web of Science, e.LIBRARY.RU, and Google Academy. The methods applied included registration, grouping, classification, comparative analysis, and generalization.
We described the origin and composition of tea fungus, specifying the microorganisms that make up its symbiotic community depending on the place of origin. Then, we reviewed the stages of fermentation and cultivation conditions in various nutrient media and presented the composition of the culture liquid. Finally, we analyzed the antimicrobial effect of *M. gisevii* on a number of microorganisms and delineated some practical uses of the fungus.
The data presented in this article can be used to analyze or develop new methods for the cultivation and application of *M. gisevii*. We specified some possibilities for using not only the culture liquid but also the fruit body of the fungus in various industries.

Keywords: Kombucha, *Medusomyces gisevii*, composition, cultivation, application

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INTRODUCTION

Kombucha, a fermented tea drink, is reported to have been first mentioned as early as 220 BC [1]. Originating in Manchuria (China), it was brought to Japan in 414 AD, and then to Eastern Europe and America. Kombucha appeared in Russia and Ukraine during the Russo-Japanese War of 1904–1905 [2]. During the Second World War, the drink was brought to Germany, and in the 1950s, to France, as well as North Africa, where it became quite popular. In the post-war years, its popularity reached its peak in Italy and Switzerland, where the drink was found as beneficial as yogurt [3].

Literature offers a variety of names for the kombucha drink based on *Medusomyces gisevii* L., including "Manchurian mushroom", "Japanese mushroom", "Japanese sponge", "sea mushroom" or simply "mushroom", "kvass" or "tea kvass", "fango", "kombuha", "Indian tea mushroom", "miraculous mushroom", "tea fungus", "kam-boo-ha", "Scoby", "Hongo", and many others [1, 4, 5]. The book *Kombucha* by Gunter W. Frank boasts as many as 86 synonyms for the drink's name.

Historically, the kombucha culture liquid has long been used in traditional medicine not only as a refreshing drink, but also to heal various diseases.

Today, kombucha is sold in retail stores, and the *M. gisevii* culture can be purchased online [1, 6].

According to State Standard STB 1818-2007 of the Republic of Belarus, "Functional Food Products. Terms and Definitions", a functional food product is a product that is intended for systematic use as part of a diet in all age groups of a healthy population to reduce the risk of developing diseases associated with nutrition, as well as to maintain and improve health due to the presence of physiologically functional ingredients.

Functional food products can be divided into several groups (Fig. 1).

According to Fig. 1, a drink based on the *M. gisevii* microbiological community can be classified as a functional product.

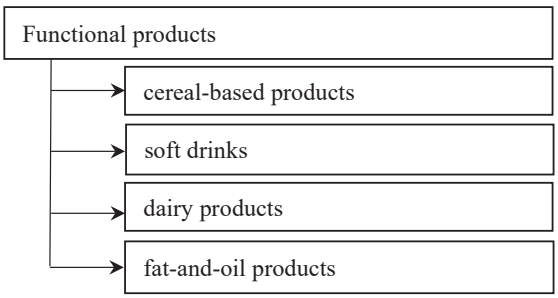

Figure 1 Classification of functional products

M. gisevii (medusomycete) is a symbiotic community of yeast-like fungi and bacteria that forms a thick, leathery, amorphous layered film of tea fungus on the surface of fermentable nutrient solutions (tea extract, juice, etc.) [7].

It is quite common to cultivate tea fugus at home and use its culture liquid, or fermentation product, as a drink (kombucha). This drink is widely known as a valuable preventative and medicinal remedy for various diseases. Its popularity is fueled by recommendations given in popular science articles and publications on traditional medicine. However, scientific literature also reports its negative impact on the body [1, 8]. Therefore, we found it important to study the chemical composition of the *M. gisevii* fermented culture liquid and to systematize the materials available on it.

STUDY OBJECTS AND METHODS

Our study was carried out at the Department of Biotechnology, the Faculty of Technology of Organic Substances, Belarusian State Technological University. We studied scientific articles, conference proceedings, intellectual property, regulatory documents, and Internet resources over a period from 1989 to 2022. They were selected from the bibliographic databases of Scopus, Web of Science, eLIBRARY.RU, and Google Academy by using *Medusomyces gisevii* L. and kombucha as keywords. We analyzed the data by employing such methods as registration, grouping, classification, as well as comparative analysis and generalization.

RESULTS AND DISCUSSION

Composition. The first scientific information about the *Medusomyces gisevii* L. microflora appeared in the articles of Gustav Lindau (1913). Its species composition is diverse and dependent on the conditions, place, and time of cultivation. The *M. gisevii* symbiont is composed of culture liquid, zooglea, mesoglea, and sediment [7, 9, 10].

The zooglea of *M. gisevii* is a complex structural formation of bacterial cellulose in which various microorganisms are immobilized. It is based on the colonies of acetic acid bacteria *Gluconacetobacterim*, *Acetobacter*, *Lactococcus*, *Lactobacillus*, and *Clostridium*, as well as yeasts *Saccharomyces*, *Bretanomyces*, *Torulopsis*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Candida*, and others [4, 7, 9–11].

Amarasinghe *et al.* identified the following components in the zooglea: *Acetobacter xylinum*, *Acanthodica xylinoides*, *Acetobacter aceti*, *Acetobacter pausterianus*, *Bacterium gluconicum*, *Kloeckera* spp., *Schizosaccharomyces pombe*, *Saccharomyces ludwigii*, *Saccharomyces cerevisiae*, *Torulaspora* spp., *Zygosaccharomyces bailii*, and *Pichia* spp.

In the study by Savary *et al*., the zooglea was composed of *Dekkera bruxellensis*, *Hanseniaspora uvarum*, *Acetobacter okinawensis*, and *Liquorilactobacillus nagelii* [13]. The patent by Chekasina *et al.* described the following consortium: *Saccharomyces mandshuricus*, *Hausemaspora* sp., *Torulopsis globosa*, *Torulopsis* sp., *S. ludwigii*, *Saccharomyces lactis*, *A. xylinum*, *A. aceti*, *Gluconobacter subaxydans*, and other microorganisms [14].

Bayramaliyeva *et al.* isolated thirteen strains of yeast from the *M. gisevii* fruit body and culture liquid, as well as characterized their morphological and cytological features [15].

Thus, the *M. gisevii* symbiosis can have a very diverse composition.

Quorum sensing is a mechanism that establishes a balance between the microorganisms of a symbiont [9]. It enables a symbiotic community to form, depending on the combination of certain factors. In addition, the symbiotic community contains about 1–10% of so-called "persister" microorganisms. These are cells which are at rest and represent a protective and adaptive life form.

The color of the zooglea is determined by the tea extract or other coloring components of the nutrient medium. The zooglea is kept on the surface of the culture liquid due to carbon dioxide and partly due to the edge adhesion to the vessel, with a film growing at rest without layers. When the film gets damaged, it forms a new layer on the surface of the old one, resulting in layers. The synthesis of bacterial cellulose begins after the symbiont is placed in the nutrient medium and proceeds through several stages: 1) consolidation of bacteria with associates forming on the surface of the culture liquid; 2) synthesis of cellulose microfibrils and the formation of multilayer structures of bacterial cellulose on the surface of the medium; 3) their colonization by microorganisms; and 4) the activity of symbiont microorganisms. Bacterial cellulose forms throughout the entire cultivation period, although it depends on many factors (substrate, extract, inoculum, temperature, pH, etc.).

Therefore, the metabolic activity of *M. gisevii*'s enzymatic systems depends on the activity of microorganisms in the culture liquid that are immobilized in bacterial cellulose. Bacterial cellulose is synthesized by the acetic acid bacteria *Gluconacetobacter xylinus* with the help of monosaccharides (glucose or its phosphorylated forms) [9, 16]. It is

formed by Gram-negative bacteria of the genera *Komagataeibacter* (*Gluconacetobacter*), *Agrobacterium*, *Achromobacter*, *Enterobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella*, *Azotobacter*, and *Alcaligenes*, as well as by Gram-positive bacteria *Sarcina ventriculi* and *Rhodococcus*. The best known producer of bacterial cellulose is the acetic bacterium *Komagataeibacter xylinus* (*G. xylinus*, *A. xylinum*, *Acelobacter aceti* ssp. *xylinum*, *Acetobacter xylinus*).

The inner part of bacterial cellulose is a multilayer structure of microfibrils, in which nutrient substrates, enzymes, microorganisms and their metabolic products move due to diffusion.

Mesoglea is made of filamentous formations of bacterial cellulose containing symbiont microorganisms. It is most actively formed in extracts of black and green teas at low positive temperatures (< 10°C) [9].

The sediment of the culture medium is usually localized at the bottom of the vessel as a dense brown mass [9, 10]. It consists of resins, tannins, tea dust, yeasts, and bacteria that stop growing in the presence of 4–5% of sugars. This is due to the accumulation of microbial products (e.g., organic acids) in the culture liquid.

The fungal biomass contains crude protein (45–53%), carbohydrates (37–43%), including polysaccharides (23–26%), lipids (8–11%), nucleic acids (7–9%), minerals (8–10%), vitamins (C, B_1, PP – 0.11–0.54%), amino acids (28–33%), and microelements (2.1–3.7%). The microelements (mg/kg) include potassium (14025), sodium (5790), magnesium (1379), iron (1017), copper (31.7), zinc (70.5), manganese (342), and chromium (10.5) [17].

The amino acids in the biomass protein (%) include lysine (2.01), histidine (1.75), arginine (2.05), aspartic acid (3.54), trionine (1.41), serine (1.39), glutamic acid (4.07), proline (1.97), alanine (1.97), cystine (0.21), valine (1.73), methionine (0.45), isoleucine (1.57), leucine (2.23), tyrosine (1.23), and phenylalanine (1.93) [17].

Fermentation process. Kombucha is produced by three types of fermentation: lactic acid, alcoholic, and acetic [4, 7]. During lactic acid fermentation, glucose is decomposed to lactic acid under the action of lactic acid bacteria. Alcoholic fermentation leads to the decomposition of glucose to ethyl alcohol by yeast, with carbon dioxide emitting during the process. Acetic fermentation converts ethyl alcohol into acetic acid and water by oxygen and acetic bacteria [18].

Fermentation also produces several intermediate substances, including phosphoric acid. It plays an important role in the formation of phosphoric acid esters, which are converted into free pyruvic acid.

In addition, fermentation leads to an increase in polyphenols and flavonoids in the culture liquid. Thearubigin turns into theaflavin and the culture liquid changes its color from dark to light during the cultivation of the fungus [19, 20].

Catechins present in tea can be converted by symbiont microorganisms to simpler components, which increases the antioxidant power of the culture liquid [19–21]

Cultivation conditions. Since the *M. gisevii* microorganisms can be found on the surface of plants, they can be grown on artificial nutrient media, using extracts of various types of tea and other plants [9]. Their normal growth requires a nutrient medium that satisfies the needs of the symbiotic community. Such a medium may include leaves of black and green tea, rooibos (*Aspalathus linearis*), fireweed (*Epilobium*), lemon balm (*Melissa officinalis*), common oak (*Quercus robur*), common blueberry (*Vaccinium myrtillus*), fragrant callisia (*Callisia fragrans*), herniaria (*Herniaria*), acacia (*Acacia*), gray myrobalan (*Phyllanthus emblica*), Bengal quince (*Aegle marmelos*), woolly erva (*Aerva lanata*), cassia (*Cassia auriculata*), common barley (*Hordeum vulgare*), mint (*Mentha*), common thyme (*Thymus vulgaris*), nettle (*Urtica*), savory (*Satureja*), turmeric (*Curcuma xanthorrhiza*), and other plants [1, 19, 22–26].

The main substrates of *M. gisevii* are carbohydrates represented mainly by mono- and oligosaccharides (glucose, fructose, galactose, mannitol, xylose, sucrose, maltose, etc.), alcohols, organic acids, and other substances [27]. Galactose is less preferable as a nutrient substrate, while sucrose and lactose are better assimilated. The use of lactose results in minimal formation of bacterial cellulose. Glucose promotes the synthesis of bacterial cellulose and gluconic acid, especially at a concentration of 10–20 g/L. Replacing glucose with maltose leads to a 10-fold decrease in bacterial cellulose [9].

Voon *et al.* analyzed tea fungus cultivation with white refined sugar, coconut sugar, and sugar molasses [28]. They reported a significantly larger fungal biomass when using refined sugar. The culture liquid based on sugar molasses contained the largest amount of organic acids, while the culture liquid based on coconut sugar showed better antioxidant activity and a higher phenol content.

During cultivation, yeast oxidizes carbohydrates to ethyl alcohol and carbon dioxide, and bacteria complete the oxidation of ethanol to acetic acid. The acid accumulates during bacterial cultivation and affects the pH value. During symbiont cultivation, the nutrient medium is saturated with ethanol and acetic acid, thus protecting the symbiotic community from contamination by foreign microflora [9].

When microorganisms actively consume the nutrient substrate, the culture medium becomes acidified. This process depends on the temperature and the presence of various additives in the medium, such as alcohols (ethanol and glycerol). Glycerol in low concentrations serves as a source of carbon for the synthesis of bacterial cellulose, but in high concentrations (> 20%) it inhibits this process [9]. Ethanol (1.0–1.5%) can accelerate

the synthesis of bacterial cellulose and be used as an alternative carbon source [29].

In addition to the above alcohols, ethylene glycol, propanol, butanol, methanol, and mannitol are used to stimulate the biosynthesis of bacterial cellulose. These additives can increase the yield of bacterial cellulose by 13.4–56.0%. However, carbohydrates at concentrations of 5–10% are preferable for production purposes. Also, food industry wastes such as molasses and distillery waste are recommended as cheap substrates [9]. Organic acids (lactic, pyruvic, malic, acetic, succinic, and citric) added to the main substrate increase the yield of bacterial cellulose by 1.4–1.9 times [30]. Specialized media have proven effective for tea fungus cultivation, which, in addition to carbohydrates, contain salts ($(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $FeSO_4$, $CaCl_2$, $NaMoO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$, etc.), vitamins (PP, B_1, B_2, B_3, B_6, H), and aminobenzoic acid [9].

Gladysheva *et al.* produced bacterial cellulose by bioconversion of *M. gisevii* on a synthetic nutrient medium containing sucrose, black tea extract, starch hydrolysate, and enzymatic miscanthus hydrolysate [31, 32]. Although this medium is not optimal for the biosynthesis of bacterial cellulose, IR spectroscopy established that the bacterial cellulose obtained was a chemically pure compound containing only cellulose. This indicated a high adaptive potential of the symbiotic culture of *M. gisevii*.

According to Skiba *et al.*, the highest yield of bacterial cellulose (7.5–8.0%) was provided at an inoculum amount of 10–20% vol., although all the amounts under study produced bacterial cellulose samples with the same three-dimensional microfibrillar structure [33]. The authors found that the inoculum amounts and the duration of biosynthesis affected the degree of polymerization. Thus, the process of biosynthesis can be controlled to synthesize bacterial cellulose with a given degree of polymerization.

Pribilsky *et al.* pointed to the importance of water pretreatment for fungus cultivation. In their study, filtered water was disinfected and treated with natural minerals (flint, carnelian, opal chalcedony, quartz) [34]. The minerals were placed in water for the entire fermentation process to promote fungal growth and inhibit foreign microorganisms.

The patent by Skripitsyna described a method for obtaining drinks from fermented vegetable extracts with spices and salt [6]. For this, vegetable juice was fermented with the culture liquid of *M. gisevii*.

Another method of cultivating fungus to obtain a soft drink was presented by Ogarkov *et al.* [35]. The authors mixed sugar, tea fungus concentrate, water-soluble melanin, and a water-alcohol solution of lemon balm with water in the presence of carbon dioxide and kept the mixture at 7–10°C.

Fungus zooglea can also be cultivated anaerobically, resulting in a drink with maximum biological activity [36, 37]. However, this method has two disadvantages, namely a decrease in dry matter and an increase in the cultivation time up to 150 days [36].

In addition, Skripitsyna and Zajtsev described methods for preparing beverages by successively fermenting sugar-containing products (water with sugar, jam, or honey) with yeast cultures (baking, wine, etc.) and cultivating tea fungus [6, 38].

Rogozhin *et al.* studied the effect of low temperatures on the cultivation of *M. gisevii* [10]. It is a fact that low temperatures inhibit the body's metabolism. A short-term effect of low temperatures usually leads to a higher metabolic and functional activity than under normal conditions. However, their prolonged action, especially with temperatures below 4°C, can cause death in some living organisms. In another study, Rogozhin *et al.* cultivated *M. gisevii* in a black tea extract [39]. Low temperatures completely suppressed the activity of symbiont microorganisms, which manifested in stable pH values and electrical conductivity for 30–240 days. However, raising the temperatures increased the symbiont's productivity, as shown by changes in the above indicators.

In the same study [39], *M. gisevii* was cultivated in coffee extracts at 8°C. During the first 30 days, symbiont microorganisms were at rest and then, during the following 60–240 days, their metabolic activity was increased by lower pH values and higher electrical conductivity of the culture liquid. This indicated that the coffee extract contained components that could activate microorganisms even at low temperatures. However, prolonged exposure to –20°C had a negative effect on the viability of *M. gisevii*, showing individual cryoprotective properties of black tea and coffee extracts. Furthermore, negative temperatures had different effects on the ability of *M. gisevii* to synthesize bacterial cellulose in black tea and coffee extracts. In the black tea extract, the symbiont's enzymatic systems were less active, while in the coffee extract, they exhibited high activity, manifesting in a 1.84–3.92-fold increase in the zooglea mass [39].

Tea fungus should not be stored at low temperatures since it can lead to a so-called "malolactic transformation". This means that malic acid, which is beneficial for the body, is converted into lactic acid, whose excess can cause muscle pain and fatigue. However, the study by Jayabalan *et al.* showed that heat treatment was not appropriate either for preserving tea fungus [40].

Marchenko and Sotnikov calculated the productivity of the fungus film during its cultivation [41]. The acid formation rate of the culture liquid was 0.03–0.08 ΔK/h, where K was the acidity of the fermented drink taken as the volume (cm^3) of 0.1 mol/L of sodium hydroxide solution used to neutralize 100 cm^3 of a culture liquid sample. Therefore, various methods are proposed to stimulate fungal growth, e.g., 8–10 g of dead bees (a source of chitin) per 1 liter of nutrient medium [15, 42].

This additive accelerates the production of bacterial cellulose, increases fungal biomass, and improves the taste of the resulting product.

A culture with hydrophilic properties immobilized on a carrier with a rough surface (chopped twigs, wood chips) can be used to reduce the cultivation time, increase productivity, and improve the quality of kombucha [41]. There is also a method for preparing a non-alcoholic beverage with a pear flavor based on tea fungus and fruit waste used as a substrate [43].

Composition of the *Medusomyces gisevii* culture liquid. During the symbiont cultivation, the culture liquid accumulates a large number of various components, including nutrient substrate residues and products of microbial activity moving due to diffusion. The culture liquid contains organic (acetic, gluconic, citric, oxalic, lactic, kojic, tartaric, pyruvic, L-lactic, D-sugar, usnic, malonic, malic, and succinic) acids, inorganic (phosphoric) acids, proteins, lipids (sterols, phosphatides, fatty acids), carbohydrates, vitamins (C, B, PP), pigments (chlorophyll, xanthophyll), enzymes (catalase, lipase, protease and carboxylase, amylase, tryptic enzymes), nucleic acids, nitrogenous bases, chitin, caffeine, amino acids, purine bases, polyphenols, ethanol, as well as various elements (zinc, copper, iron, manganese, nickel, cobalt) and even a natural antibiotic, jellyfish [4, 7, 17, 21, 23, 25, 26, 42, 44, 45].

During the cultivation of *M. gisevii*, the nutrient medium is saturated with ethanol and acetic acid, creating favorable conditions for natural protection of the symbiotic community from contamination by foreign microflora. However, at the initial stage of cultivation, the emerging bacterial cellulose can be damaged by various types of mold due to a high content of carbohydrates and tea extracts in the medium [9]. Therefore, it is recommended to add a small amount of the fermented culture liquid during the initial period of symbiont growth.

Ivanov *et al.* found glucuronic acid (0.037–1.390%) in the first 5 days of fungal growth, which was not detected later [17]. During 20 days of growth, the culture liquid (100 cm^3) contained citric and malic acids (4 mg) and volatile acids (12 mg). The content of ethyl alcohol ranged from 0.15 to 0.7%. However, alcohol was not detected on the 30th day of growth. In addition, the culture liquid contained vitamins C and B, tannins (0.08%) and purine bases, a large number of resinous and fat-like substances insoluble in water and alcohol, proteins and nucleoproteins (5.24%), and a number of enzymes (amylase and catalase).

Antimicrobial properties of the culture liquid. *M. gisevii* has antimicrobial activity due to the presence of antibacterial substances in its culture liquid, which have both bacteriostatic and bactericidal properties. The culture liquid increases the size and volume of bacterial cells, which leads to changes in their shape, vacuolization, and the appearance of granular inclusions. Further, it decreases the intensity of redox processes in microbial cells, reduces their virulence, and increases immunogenicity [46, 47]. In the study [48] associated the antimicrobial activity of *M. gisevii* with the action of acetic acid (the main product of fermentation) on microorganisms. Many researchers [5, 25, 45, 46, 48–53] established the antibacterial efficacy of the *M. gisevii* culture liquid against various microorganisms (Table 1).

Noteworthily, not only acetic acid and large proteins, but also other molecular structures can be active components of the *M. gisevii* culture liquid [45].

Thus, although numerous studies have shown the antimicrobial effect of the *M. gisevii* culture liquid against a number of pathogenic microorganisms, they have not fully explained the exact mechanism of this effect.

Practical use. Depending on the nutrient substrate, the *M. gisevii* culture liquid can be used as a soft drink to prevent a wide range of diseases, such as hypertension, atherosclerosis, sleep disorders, liver problems, gastrointestinal disorders, and others [1, 2, 4, 5, 9, 12, 55–60]. Kombucha stimulates the endocrine and immunocompetent systems, limits atherosclerotic plaques, corrects body weight, increases the body's resistance to carcinogenic factors, has a sedative effect, prevents and alleviates headaches, reduces alcohol dependence, as well as has antitumorous and probiotic effects in combination with ginger [1, 61, 62]. There is a formulation of a fermented beverage based not only on tea fungus but also on birch chaga fungus [37].

Bondareva *et al.* developed a biologically active substance with a prebiotic effect based on *M. gysevii* [57, 63]. This culture liquid is also used as a plant base for medicines with a pronounced healing and anti-inflammatory effect [2, 64, 65]. The systematic intake of the fungal cultural liquid can improve the well-being of elderly people with severe symptoms of atherosclerosis and have a beneficial effect on intestinal atony and gastrointestinal diseases. The culture liquid can also reduce blood cholesterol levels due to a high content of gluconic acid. However, quite a few publications [8, 26] point to its negative effects on human health.

M. gisevii can be used as a biosorbent to remove heavy metals through the ion exchange mechanism [66, 67]. The symbiont is used to produce acetic acid on an industrial scale for the food industry, as well as to produce starter cultures for fermented milk products [10, 68]. Kombucha enriched with sea grapes (*Caulerpa racemosa*) has been proposed as a functional drink to combat obesity [69].

Bacterial cellulose is widely used in the production of pulp, paper, and paints, as well as in the fine chemical industry and electronics [10, 11, 32, 70]. Fine powders obtained from bacterial cellulose are used in the food industry as thickeners and gelling agents [9, 10]. Unlike plant cellulose, bacterial cellulose is pure and free of lignin, hemicellulose, and other impurities [53]. Therefore, a biofilm can serve as a matrix for immobilizing various inorganic compounds (ions of silver, selenium, magnesium, cobalt, manganese, etc.)

Table 1 Antibacterial efficacy of *Medusomyces gisevii* culture liquid

Microorganism	Source of information	Microorganism	Source of information
Aspergilus niger	[46, 50]	*Helicobacter pylori*	[45]
Bacillus anthracis	[52]	*Pasteurella multocida*	[52]
Bacillus aureus	[46]	*Pasteurella avium*	[52]
Bacillus cereus	[45, 46, 52]	*Penicillium aurantiogriseum*	[50]
Bacillus mycoides	[52]	*Proteus vulgaris*	[52]
Bacillus sp.	[50]	*Pseudomonas aeruginosa*	[45, 46, 50, 52]
Bacillus pumilus	[52]	*Pseudomonas fluorescens*	[52]
Bacillus subtilis	[25, 52]	*Salmonella abortusequi*	[52]
Brucella abortus	[52]	*Salmonella sp.*	[5, 54]
Brucella melitensis	[52]	*Salmonella dublin*	[52]
Brucella suis	[52]	*Salmonella enteritidis*	[50]
Campylobacter jejuni	[45]	*Salmonella gallinaran*	[52]
Candida albicans	[45, 46]	*Salmonella paratyphi*	[52]
Candida tropicalis	[46]	*Salmonella pullorus*	[52]
Candida parapsilosis	[46]	*Salmonella typhimurium*	[25, 45–50]
Candida glabrata	[46]	*Sarcina lutea*	[50]
Candida dubliniensis	[46]	*Sarcina maxima*	[52]
Candida sake	[46]	*Serratia marcescens*	[52]
Clostridium botulinum	[52]	*Staphylococcus aureus*	[5, 45, 46, 48–50, 53, 54]
Clostridium novyi	[52]	*Staphylococcus epidermidis*	[46, 52]
Clostridium histolyticum	[52]	*Staphylococcus saprophyticus*	[52]
Clostridium tetani	[52]	*Streptococcus caseolyticus*	[52]
Clostridium septicum	[52]	*Streptococcus cremoris*	[52]
Escherichia coli	[5, 25, 45, 46, 49, 50, 52–54]	*Streptococcus pyogenes*	[52]
Leoconostoc monocytogenes	[55]	*Shigella dysenteriae*	[25, 46]
Listeria monocytogenes	[46, 48]	*Shigella flexneri*	[52]
Malassezia sp.	[46]	*Shigella sonnei*	[45]
Micrococcus sp.	[52]	*Vibrio cholerae*	[25]
Micrococcus luteus	[46]	*Yersinia enterocolitica*	[45]
Microsporum gypseum	[46]		

and biogenic molecules (peptides, amino acids, proteins, enzymes, vitamins, hormones, antibiotics, etc.). Depending on the immobilized components, the film can then be used in medicine and/or pharmaceutics, e.g., to treat burns and ulcers, as well as postoperative, purulent, and traumatic wounds [16, 56, 59, 71]. Biofilms with immobilized bioactive substances can also be applied in cosmetology to restore skin elasticity. The Siberian Federal University in cooperation with the Institute of Biophysics (Siberian Branch of the Russian Academy of Sciences) use bacterial cellulose as a substrate for growing various tissue engineering structures. Since bacterial cellulose is non-toxic and non-allergic, as well as has a high absorption capacity, it can be used as an adsorbent to stimulate digestion.

In bakery, the *M. gisevii* culture liquid is added to the dough to activate its maturation and increase the calorie content of the finished product [10, 72, 73].

Tea fungus can be used as a feed additive for animals. For example, in Tatarstan, a preparation containing *M. gisevii* biomass and/or culture liquid is used for feeding birds [17]. Dried biomass can also be applied for the same purpose [13].

The fruit body of tea fungus is used to create eco-friendly clothing and to produce valuable bacterial cellulose by recovering hydrocarbon waste [74].

The Department of Biotechnology at the Belarusian State Technological University (Minsk, Republic of Belarus) has been developing a preparation based on the fruit body of tea fungus to stimulate plant growth.

CONCLUSION

Based on the above, we can conclude that the *Medusomyces gisevii* L. culture liquid is an excellent prophylactic against cardiovascular and gastrointestinal diseases. It can help treat atherosclerosis, acute tonsillitis, arterial hypertension, and other diseases. However, there is still a lack of research into the composition and properties of the culture liquid, as well as methods for its cultivation. Therefore, new works are published each year that open up new possibilities for the use of *M. gisevii*. We analyzed the prospects for using not only the culture liquid but also the fruit body of *M. gisevii* in the food, pharmaceutical, and other industries. In addition, the materials collected from numerous studies can be used to create an optimal technology for its cultivation.

CONTRIBUTION

O.S. Ermakova collected information and E.A. Flyurik processed it.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Evaluating extremophilic microorganisms in industrial regions

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Abstract:
Abiotic and biotic stresses have a major impact on crop growth. Stress affects the root system and decreases the amount of nutrients in fruits. Modern agricultural technologies help replace mineral fertilizers with new generation biopreparation. Unlike chemical fertilizers, biofertilizers reduce the risk of adverse environmental impacts. Of special interest are extremophilic microorganisms able to survive in extreme conditions. We aimed to study the phytostimulating ability of extremophilic bacteria isolated from disturbed lands in the coal-mining region.

We isolated microorganisms from disturbed lands and studied their cultural, morphological, and biochemical properties. Then, we determined their ability to synthesize indole-3-acetic acids. The extremophilic bacteria were identified and subjected to biocompatibility testing by co-cultivation. Next, we created consortia of pure cultures and analyzed biomass growth. Finally, the biopreparation was experimentally tested on *Trifolium prantense* L. seeds.

We isolated 10 strains of microorganisms that synthesized 4.39 to 16.32 mg/mL of indole-3-acetic acid. The largest amounts of the acid were produced by *Pantoea* spp., *Enterococcus faecium*, *Leclercia* spp., *Rothia endophytica*, and *Klebsiella oxytoca*. A consortium of *Pantoea* spp., *E. faecium*, and *R. endophytica* at a ratio of 1:1:1 produced the largest amount of indole-3-acetic acid (15.59 mg/mL) and accumulated maximum biomass. The addition of 0.2% L-tryptophan to the nutrient medium increased the amount of indole-3-acetic acid to 18.45 mg/mL. When the *T. prantense* L. seeds were soaked in the biopreparation (consortium's culture fluid) at a concentration of 2.5, the sprouts were 1.4 times longer on the 10th day of growth, compared to the control.

The consortium of *Pantoea* spp., *E. faecium*, and *R. endophytica* (1:1:1) stimulated the growth of *T. prantense* L. seeds. Our findings can be further used to develop biofertilizers for agriculture.

Keywords: Microbial consortium, biopreparation, soil, extremophilic microorganisms, seed germination, *Trifolium prantense* L.

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INTRODUCTION

By 2050, the world's population is expected to reach 9.8 billion people [1]. This population growth, as well as industrialization and a decline in agricultural areas, puts enormous pressure on the decreasing supply of food raw materials [2–4]. To solve this problem, agriculture needs to be modernized [5]. Moreover, there is a shortage of macro- and micronutrients in food products. This problem has been caused by environmental pollution, which depletes nutrients in the soil and, consequently, in agricultural crops.

Abiotic and biotic factors have a significant impact on the quality, growth, and yield of agricultural crops [6, 7]. Abiotic effects include drought, salinity, and pollution (e.g., heavy metals or pesticides) [8–10]. Every year, they cause significant economic losses during plant growth. Due to stress, the roots do not absorb the sufficient amount of minerals from the soil, which leads to a lack of nutrients necessary for normal plant growth.

To increase yields, advanced technologies are used in soil cultivation, including land reclamation (drainage and irrigation) and chemical fertilizers. On the one hand, these fertilizers have a positive effect on plant growth and development [11]. Yet, they can also lead to soil acidification, as well as air and water

pollution. Moreover, their excessive use worsens the quality of food [12]. For example, Potetnya *et al.* studied nitrogen and complex fertilizers. They found that nitrogen fertilizers have the greatest effect on plant growth by affecting plant proteins and chlorophyll. Complex fertilizers contain phosphorus and potassium. Although phosphorus is an important biogenic element, it can sometimes provoke slow plant growth and stem development, as well as pigmentation disorders (dirty green or reddish color). Potassium can also have negative effects, causing a lack of water at the cellular level, as well as wilting and twisting of leaves. Therefore, using complex fertilizers is not a universal solution, since the need for nitrogen, phosphorus, and potassium varies from crop to crop [13].

Biofertilizers are considered quite promising fertilizers [14, 15]. They are substances containing living organisms and/or their metabolites. When applied to seeds, plant surfaces, or soil, they colonize the rhizosphere, which promotes growth and availability of primary nutrients [16]. The use of biofertilizers is on the rise, especially in sustainable agriculture and organic farming. As well as being cost-effective, they improve soil fertility, increase plant growth and yield, and make plants more stress-resistant. In addition, biofertilizers help reduce the burden on the environment and improve soil quality [17]. Kha *et al*., who treated beans with the culture fluid of the bacterial strain *Rhizobium tropici*, reported its positive effect on the formation of tubercles [7].

Microbial biostimulators are an effective tool for sustainable agriculture. They can increase the yield and quality of agricultural crops by activating physiological and molecular processes in the plants [18]. The phytohormones produced by microbial biostimulators are an effective alternative to the phytohormones industrially produced by chemical synthesis. These substances regulate the physiological processes in plants, including their growth, development, release of nutrients, and adaptation to the environment [19].

Modern agricultural technologies help replace mineral fertilizers with new generation biological products. Of particular importance are native microbiota adapted to specific climatic conditions [20, 21].

Extremophilic microorganisms (extremophiles) are bacteria that can survive under extreme environmental conditions, such as high or low temperatures, lack of oxygen, adaptation to acidic or basic pH values, high salt concentrations, etc. [22]. As a rule, extremophiles are highly resistant to a number of environmental factors, which makes them resistant to specific climatic conditions [23].

In this study, we used consortia of extremophilic microorganisms, which were isolated from the disturbed lands of Kuzbass coal mines, as biofertilizers, or growth phytostimulants. Our aim was to study the ability of these extremophilic bacteria to exhibit phytostimulating properties.

STUDY OBJECTS AND METHODS

Extremophilic microorganisms were isolated from the surface layer of the technozem in Kuzbass (Kemerovo Oblast). The sampling was performed in the northwestern part of the Prokopievsk-Kiselevsk geological region of Western Siberia (54°14″ north latitude, 86°26″ east longitude). The soil in the study area is represented by overburden rocks of sandy loamy granulometric composition, lacking a fertile layer.

The soils were sampled at a depth of 0–10 cm according to State Standard 17.4.4.02-2017.

The soils in Kuzbass have acidic and slightly alkaline pH values [24, 25]. In addition, the region has a continental or sharply continental climate with low average annual temperatures [26]. Therefore, to isolate extremophilic microorganisms characteristic of this region, soil samples were cultivated under unfavorable conditions according to Zenov *et al.* [27]. For this, 1 g of soil was added to 5 mL of meat-peptone broth (pH 5.3 and 9.0) consisting of 10 g of meat peptone (LenReaktiv, Russia), 11 g of meat extract (ChemExpress, Russia), 5 g of sodium chloride (ChemExpress, Russia), and up to 1 L of distilled water. Microorganisms were cultivated in an LSI-3016A/LSI-3016R shaker-incubator (Daihan Labtech, South Korea) at 15°C, 100 rpm for 24 h. Further, suspensions were prepared from the subculture diluted to 10^{-10}, 10^{-11}, and 10^{-12}. They were then reinoculated in meat-peptone agar in Petri dishes by the pour plate method at pH 5.3 and 9.0 and cultivated at 15°C for 24 h. The agar consisted of 10 g of dry peptone, 11 g of meat extract, 5 g of sodium chloride, 20 g of agar-agar (LenReaktiv, Russia), and up to 1 L of distilled water. The reinoculation was repeated three times to obtain pure cultures using the streak plate method [28, 29].

To determine cultural properties of the isolated strains, we prepared suspensions of pure cultures at low concentrations, followed by deep plating on meat-peptone agar. Microorganisms were cultivated in a TCO-1/80 SPU thermostat (Smolensk SKTB SPU, Russia) at 25°C for 24 h. Their morphological features were determined by microscopy of a stained fixed smear with methylene blue [30]. The cultures were classified into Gram-positive or Gram-negative according to Belkin *et al.* [31]. Sporulation was determined according to Pereira *et al.* and mobility, according to Molofeyeva *et al.* [32, 33].

Promising strains with phytostimulating activity were selected based on their ability to synthesize indole-3-acetic acid. Indole-3-acetic acid is a phytohormone that stimulates the proliferation of plant cells and enhances the absorption of minerals and nutrients from the soil [21, 34]. To determine its amount, a suspension of microorganisms in meat-peptone broth containing 0.1% L-tryptophan (ChemExpress, Russia) was cultivated for 48 h at 25°C and then centrifuged for 5 min at 10 000 rpm. After that, 1 mL of the supernatant was added to 1 mL of the Salkowski reagent and incubated for 30 min at room temperature.

Next, the optical density was measured on a PE-5Z00VI spectrophotometer (UNITEK, Russia) at 535 nm. Based on the results, a calibration graph of the standard solution was created to determine the amount of indole-3-acetic acid [35].

The isolated bacteria were identified using a Vitek 2 Compact automatic microbiological analyzer (BioMerieux, France) with ID-GP and ID-GN cards for Gram-positive and Gram-negative microorganisms, respectively. The cultures were grown on Columbia agar with blood for 48 h at 25°C. Then, a suspension of strains was prepared with a McFarland density of 2.70–3.30 using a Densichek plus densitometer (BioMerieux, France) [36].

Biocompatibility was determined by co-cultivation. For this, pure cultures were grown on meat-peptone broth for 48 h at 25°C. Then, they were centrifuged for 5 min at 5000 rpm. The test culture was applied to Petri dishes with meat-peptone agar, and the supernatant was added to the wells. Microorganisms were cultivated for 24 h at 25°C [5].

To create consortia, suspensions of pure cultures were prepared in physiological saline with a McFarland density of 0.8–1.1. Then, 5% of the medium containing consortium microorganisms was added to the meat-peptone broth. The resulting consortia of extremophilic bacteria are shown in Table 1.

The amount of indole-3-acetic acid synthesized by the consortia was determined by the method described above.

To select the optimal medium composition for the highest biomass yield, microorganisms were cultivated on different media at 25°C for 48 h (Table 2).

Biomass growth was measured according to Zandanova and Gogoleva [37].

To maximize the yield of indole-3-acetic acid, L-tryptophan was added to the previously selected nutrient media in the amount of 0.1, 0.2, and 0.5% of the medium. Microorganisms were cultivated at 25°C for 24 h. The amount of indole-3-acetic acid was measured by the method described earlier.

Red clover (*Trifolium pretense* L.) is a fodder plant widespread in Kemerovo Oblast. It is a promising plant model for assessing the destructive activity of extremophilic microorganisms against heavy metals and organic contaminants, as well as their phytostimulatory activity [38]. *T. pratense* L. seeds were prepared in two ways: 1) by soaking in 5 mL of a consortium of microorganisms at a McFarland density of 1.5 and 2.5 for 24 h at 5–8°C; 2) by soaking in 5 mL of distilled water for 24 h at 5–8°C.

Ten seeds were sown into the soil at a depth of 1 cm. They were germinated for 10 days at 18–25°C and relative humidity of 80%. The seeds soaked in the consortia were irrigated with distilled water, while those soaked in distilled water were irrigated with consortia solutions.

Statistical processing was carried out using the Statistica for Windows v. 12.0, (StatSoft, Inc.) at a statistical significance of $P = 0.95$. The tables and figures show the arithmetic mean values of the indicators under study. All the experiments were carried out in triplicate.

Table 1 Consortia of extremophilic bacteria

Consortium	Ratio of extremophilic microorganisms in the consortium		
	Pantoea spp.	*Enterococcus faecium*	*Rothia endophytica*
A	1	1	1
B	2	1	1
C	1	2	1
D	1	1	2

Table 2 Composition of nutrient media for the highest biomass yield

Medium components	Content of components in nutrient media, g/L		
	Medium 1	Medium 2	Medium 3
Molasses (KhimKomplekt, Russia)	0.60	–	–
KH_2PO_4 (Ural-OTsM, Russia)	0.03	0.40	0.70
$MgSO_4 \times 7H_2O$ (KhimKomplekt, Russia) $\times 10^{-1}$	0.35	8.00	8.00
NaCl (ChemExpress, Russia) $\times 10^{-1}$	0.35	–	5.00
KNO_3 (LenReaktiv, Russia)	0.35	4.00	–
$CaCO_3$ (Ural-OTsM, Russia)	0.50	–	–
Na_2HPO_4 (KhimKomplekt, Russia)	–	1.40	–
$(NH_4)_2HPO_4$ (Ural-OTsM, Russia)	–	–	0.50
Corn extract (LenReaktiv, Russia)	0.25	–	–
Sucrose (ChemExpress, Russia)	–	10.00	–
Yeast extract (LenReaktiv, Russia)	–	–	0.10
Glucose (ChemExpress, Russia)	–	–	10.00

The equipment for the study was provided by the Instrumental Methods of Analysis in Applied Biotechnology Center at Kemerovo State University.

RESULTS AND DISCUSSION

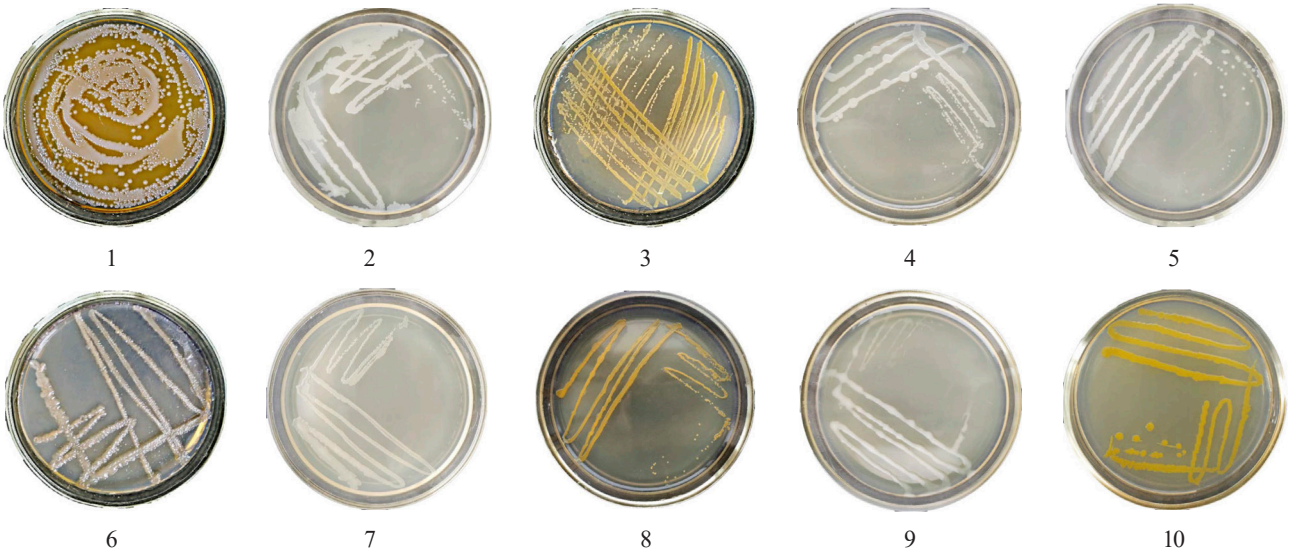
We isolated 10 pure cultures from the soil samples with unfavorable cultivation conditions (acidic and alkaline pH, low temperature). Their cultural and morphological characteristics are presented in Table 3.

Figure 1 shows the growth of the microorganisms in Petri dishes.

The microscopy results for the isolated strains are shown in Fig. 2.

Table 3 Cultural and morphological characteristics of microorganisms

No. of native microorganism	Characteristics	
	Cultural	Morphological
1	Transparent, oily, convex, rounded with smooth edges, 1 mm in diameter	Bacilli, in groups of 0.551×0.305 µm, Gram-negative, spore-forming, immobile
2	Whitish, glossy, convex, rounded with smooth edges, 1 mm in diameter	Cocci, 0.653 µm, Gram-positive, spore-forming, motile
3	Yellow, glossy, raised, rounded with smooth edges, 2 mm in diameter	Diplococci, 0.496×0.271 µm, Gram-negative, spore-forming, immobile
4	Transparent, glossy, flat, rounded with smooth edges, 2 mm in diameter	Bacilli, 0.496×0.271 µm, Gram-positive, spore-forming, immobile
5	Whitish, oily, raised, rounded with smooth edges, 2 mm in diameter	Bacilli, 0.596×0.238 µm, Gram-negative, spore-forming, immobile
6	Whitish, oily, convex, rounded with smooth edges, 2 mm in diameter	Diplobacilli, 0.596×0.385 µm, Gram-positive, non-spore-forming, immobile
7	Transparent, glossy, raised, rounded with smooth edges, 2 mm in diameter	Diplobacilli, 1.032×0.415 µm, Gram-positive, spore-forming, immobile
8	Orange, glossy, raised, rounded with smooth edges, 1 mm in diameter	Cocci, 0.693 µm, Gram-positive, spore-forming, motile
9	Transparent, oily, raised, rounded with smooth edges, 1 mm in diameter	Bacilli, 0.673×0.520 µm, Gram-negative, spore-forming, immobile
10	Yellow, oily, raised, rounded with smooth edges, 3 mm in diameter	Cocci, 0.569 µm, Gram-negative, spore-forming, motile



Note: the microorganisms were inoculated on meat-peptone agar by the streak plate method

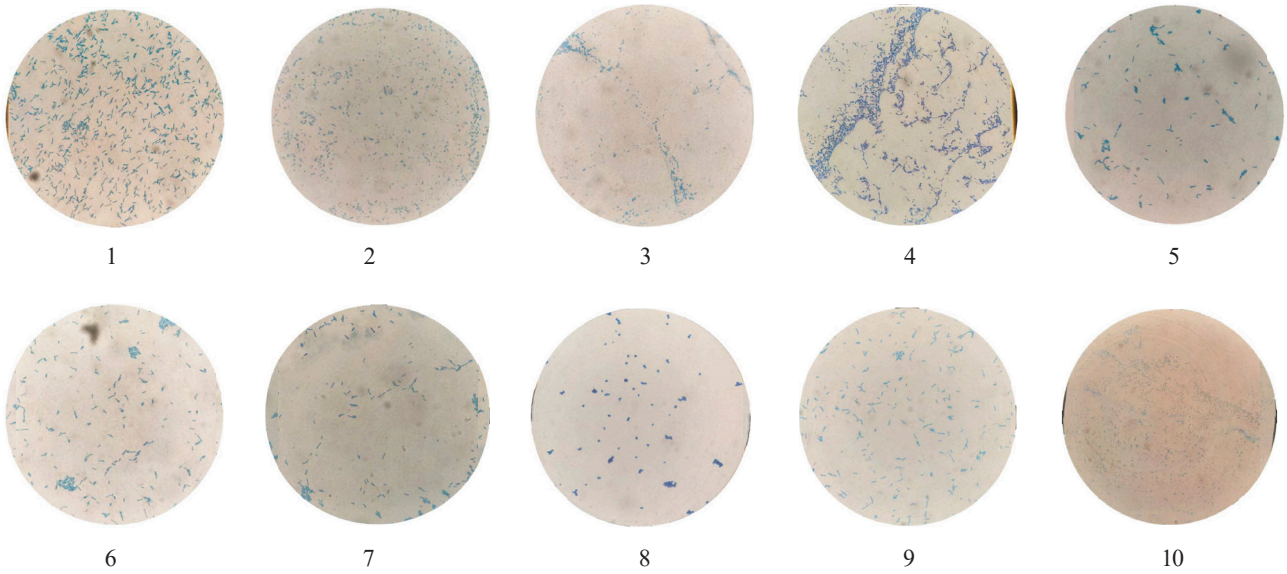
Figure 1 Growth of the microorganisms in Petri dishes, where 1–10 are the numbers of the strains

Table 4 shows the amounts of indole-3-acetic acid synthesized by the isolated microorganisms.

As can be seen from Table 4, the amounts of synthesized indole-3-acetic acid varied from 4.39 to 13.32 mg/mL. The highest and the lowest efficiency was exhibited by strains No. 3 (13.32 mg/mL) and No. 9 (6.39 mg/mL), respectively. As a result, we selected 5 promising strains (No. 1, 2, 3, 4, and 10), which synthesized > 10 mg/mL of indole-3-acetic acid.

To identify the selected isolates, we studied their physical and biochemical characteristics. According to the results, strain No. 1 was *Pantoea* spp. (probability 0.98), No. 2 was *Enterococcus faecium* (probability 0.88), No. 3 was *Leclercia* spp. (probability 0.88), No. 4 was *Rothia endophytica* (probability 0.86), and No. 10 was *Klebsiella oxytoca* (probability 0.89). Their biochemical characteristics are presented in Table 5.

According to Luziatelli *et al.*, the *Pantoea* species includes agronomically significant strains that are able to stimulate plant growth [39]. In their study, *Pantoea* strains accumulated 0.12 mg/mL of indole-3-acetic acid. In another study [40], Lee *et al.* analyzed the effect of *E. faecium* on plant growth. The authors found that this strain enhanced plant growth and produced 3.63 mg/mL of indole-3-acetic acid. Snak *et al.* reported that the *Leclercia* strain stimulated plant



Note: microscopy of fixed smears of test organisms stained with methylene blue, using immersion oil

Figure 2 Microscopy of isolated microorganisms, where 1–10 are the numbers of the strains

Table 4 Amounts of indole-3-acetic acid synthesized by the isolated microorganisms

No. of strain	Amount of indole-3-acetic acid, mg/mL
1	10.51 ± 0.49
2	12.41 ± 0.62
3	13.32 ± 0.61
4	11.69 ± 0.52
5	7.78 ± 0.39
6	6.80 ± 0.34
7	5.54 ± 0.26
8	7.05 ± 0.35
9	4.39 ± 0.19
10	10.71 ± 0.53

growth and synthesized 4.8 mg/mL of indole-3-acetic acid [41]. The study by Shurygin *et al.* proved that the bacteria of the genus *Rothia* produced 5.4 mg/mL of indole-3-acetic acid [42]. According to Poveda and Gonzalez-Andres, *K. oxytoca* showed the maximum yield of indole-3-acetic acid (17.4 mg/mL) [14]. Our results contradicted these published data. This may be due to the fact that we isolated the microorganisms from the Siberian soils and therefore they had greater resistance to adverse environmental conditions and a better ability to produce metabolites.

To create consortia, the microorganisms were tested for biocompatibility (Table 6).

As we can see in Table 6, *Leclercia* spp. inhibited the growth of *Pantoea* spp., but, at the same time, exhibited active joint growth with *R. endophytica*, *E. faecium*, and *K. oxytoca*. The *K. oxytoca* strain was biocompatible only with *E. faecium* and *Pantoea* spp., inhibiting the growth of the other strains. *Pantoea* spp. showed active growth together with *E. faecium* and *R. endophytica*, and no growth with *Leclercia* spp. *R. endophytica* and *E. faecium* were biocompatible with all the strains under study. Based on these results, we compiled 4 consortia from *Pantoea* spp., *E. faecium*, and *R. endophytica*.

According to Table 7, the largest amount of indole-3-acetic acid was synthesized by consortium A (15.59 mg/mL). For consortia B, C, and D, the results did not differ significantly. We found that the consortia produced on average 1.4 times more indole-3-acetic acid that the individual microorganisms included in the consortia and about 1.2 times more than *Leclercia* spp. Thus, the optimal ratio of *Pantoea* spp., *E. faecium*, and *R. endophytica* was 1:1:1.

Then, we selected the optimal composition of the nutrient medium for the highest yield of microbial biomass (Fig. 3). The compositions of the nutrient media are presented in Table 2.

In the first hours of cultivation on media No. 1, 2, and 3, the numbers of colony-forming units were 3.2, 3.9, and 2.9 CFU/mL×10^6, respectively. The largest number was observed on medium No. 1 after 24 h (9.7 CFU/mL×10^6), and on media No. 2 and 3 after 28 h (14.4 and 7.8 CFU/mL×10^6, respectively). Thus, the phase of death began earlier on medium No. 1 than on media No. 2 and 3. However, the number of colony-forming units on medium No. 3 was approximately 2 times lower than on medium No. 2. Thus, we recommend medium No. 2 as optimal for accumulating the largest amount of biomass.

Jahn *et al.* reported that L-tryptophan is a precursor of indole-3-acetic acid [43]. Therefore, to increase the yield of this phytohormone, we had to select an optimal amount of L-tryptophan. For this, 0.1, 0.2, 0.5% of L-tryptophan was added to medium No. 2. Then, we analyzed biomass growth and the amounts of indole-3-acetic acid synthesized (Table 8).

Table 5 Physical and biochemical characteristics of selected microorganisms

Microorganism	Physical and biochemical characteristics
Pantoea spp.	L-pyrrolydonyl arylamidase, D-glucose, Fermentation/glucose, Beta-glucosidase, D-mannitol, D-mannose, D-sorbitol, Saccharose/sucrose, D-trehalose, Malonate, L-Lactate alkalinisation, Phosphatase, Coumarate
Enterococcus faecium	Arginine dihydrolase 1, Beta-galactosidase, Cyclodextrin, L-Aspartate arylamidase, L-Pyrrolydonyl-arylamidase, Tyrosine arylamidase, Polymixin b resistance, D-galactose, D-ribose, Lactose, N-Acetyl-D-Glucosamine, D-maltose, Bacitracin resistance, Novobiocin resistance, Growth in 6.5% NaCl, D-mannose, Methyl-B-D-Glucopyranoside, O/129 Resistance (comp.vibrio), Salicin, D-trehalose, Arginine dihydrolase 2, Optochin resistance
Leclercia spp.	Adonitol, L-pyrrolydonyl arylamidase, D-Cellobiose, Beta-galactosidase, Beta-N-acetyl-glucosaminidase, D-glucose, Fermentation/glucose, Beta-glucosidase, D-maltose, D-mannitol, D-mannose, Beta-xylosidase, Saccharose/sucrose, D-trehalose, L-Lactate alkalinisation, Beta-N-acetyl-galactosaminidase, Coumarate, ELLMAN
Rothia endophytica	Arginine dihydrolase 1, Alpha-glucosidase, Ala-Phe-Pro Arylamidase, Leucine arylamidase, L-Proline arylamidase, L-Pyrrolydonyl-arylamidase, Alanine arylamidase, Tyrosine arylamidase, D-ribose, D-maltose, Saccharose/sucrose, D-trehalose, Optochin resistance
Klebsiella oxytoca	Adonitol, L-pyrrolydonyl arylamidase, L-Arabitol, D-Cellobiose, Beta-galactosidase, D-glucose, Gamma-glutamyl-transferase, Fermentation/glucose, Beta-glucosidase, D-maltose, D-mannitol, D-mannose, Beta-xylosidase, Palatinose, D-sorbitol, Saccharose/sucrose, D-tagatose, D-trehalose, Citrate (sodium), Malonate, 5-keto-D-gluconate, L-Lactate alkalinisation, Succinate alkalinisation, Alpha-galactosidase, Phosphatase, Glycine arylamidase, Lysine decarboxylase, L-histidine assimilation, O/129 resistance (comp. vibrio), L-malate assimilation, L-Lactate assimilation

Table 6 Determination of biocompatibility between the microorganisms

Strain	*Pantoea* spp.	*Enterococcus faecium*	*Leclercia* spp.	*Rothia endophytica*	*Klebsiella oxytoca*
Pantoea spp.		+	–	+	+
Enterococcus faecium	+		+	+	+
Leclercia spp.	–	+		+	–
Rothia endophytica	+	+	+		–
Klebsiella oxytoca	+	+	+	+	

"+" – compatible; "–" – incompatible

Table 7 Amounts of indole-3-acetic acid synthesized by the consortia of *Pantoea* spp., *Enterococcus faecium*, and *Rothia endophytica* in different proportions

Consortium	Amount of indole-3-acetic acid, mg/mL
A (1:1:1)	15.59 ± 0.76
B (2:1:1)	12.06 ± 0.63
C (1:2:1)	11.57 ± 0.58
D (1:1:2)	12.52 ± 0.62

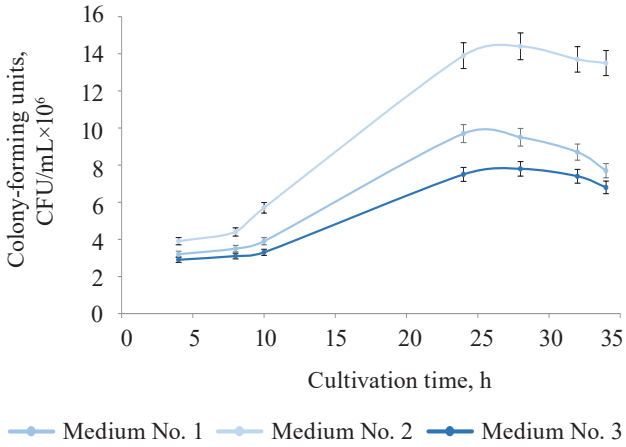


Figure 3 Growth of consortium A on nutrient media with various compositions

Table 8 Amounts of indole-3-acetic acid synthesized on medium No. 2 with addition of L-tryptophan

Medium	Amount of indole-3-acetic acid, mg/mL
Medium No. 2	15.50 ± 0.77
Medium No. 2 + 0.1%	16.26 ± 0.81
Medium No. 2 + 0.2%	18.45 ± 0.92
Medium No. 2 + 0.5%	16.89 ± 0.84

According to Table 8, 0.2% of the volume of medium No. 2 was the optimal amount of L-tryptophan to synthesize 18.45 of mg/mL of indole-3-acetic acid. Thus, we recommended the following composition of the nutrient medium: 0.4 g KH_2PO_4, 0.8 g $MgSO_4 \times 7H_2O$, 4 g KNO_3, 1.4 g Na_2HPO_4, 10 g sucrose, 2.1 g L-tryptophan, and up to 1 liter of distilled water.

We used *Trifolium pretense* L. to experimentally evaluate the ability of this biopreparation to enhance plant growth (Table 9).

As can be seen from Table 9, samples 1, 2, and 3 had 9 sprouts on the 10th day, while sample 4 had 10 sprouts. Sample 2 had the largest sprout size on the 10th day (56 mm) and sample 3 had the minimum size (5 mm). Figure 4 presents the growth of sprouts during the sprouting time by average length.

Table 9 The size and number of *Trifolium pretense* L. sprouts

No. sample	Description	Indicator	Sprouting time, days					
			1	3	5	7	9	10
1	Seeds soaked in the consortium solution (1.5) and watered	Number of sprouts	7	8	8	8	9	9
		Minimum sprout size	0	2	3	5	6	7
		Maximum sprout size	0	18	31	36	42	47
2	Seeds soaked in the consortium solution (2.5) and watered	Number of sprouts	9	9	9	9	9	9
		Minimum sprout size	3	12	28	13	26	31
		Maximum sprout size	29	46	49	52	53	56
3	Seeds soaked in water and irrigated with the consortium solution (1.5)	Number of sprouts	9	9	9	9	9	9
		Minimum sprout size	0	1	1	2	4	5
		Maximum sprout size	0	13	30	32	35	35
4	Seeds soaked in water and irrigated with the consortium solution (2.5)	Number of sprouts	8	9	9	9	9	10
		Minimum sprout size	0	16	25	29	32	36
		Maximum sprout size	0	28	35	38	43	40
Control	Seeds soaked in water and irrigated with water	Number of sprouts	8	8	8	8	8	8
		Minimum sprout size	0	7	15	16	18	20
		Maximum sprout size	0	13	30	33	35	36

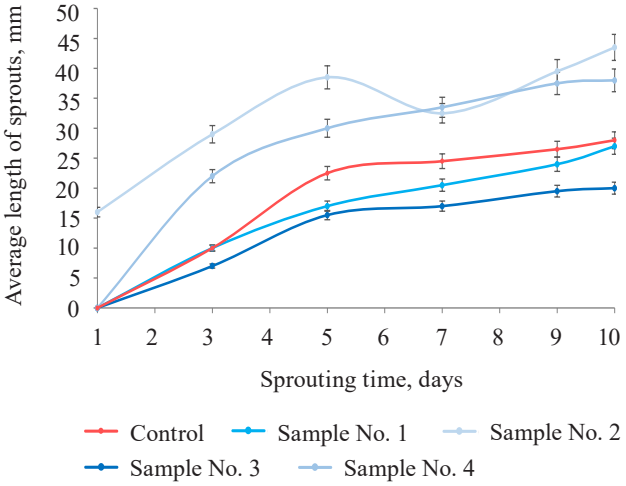


Figure 4 Comparison of average sprout lengths (n = 3)

On average, the sprouts germinated from the seeds soaked in the consortium solution (2.5) were 6 mm longer than the control on the 3rd day. On the 7th and 10th days, their growth was still effective, with an average length being 14 mm and 1.4 times longer, respectively, than the control. However, when irrigated with the consortium in a lower concentration (1.5), the sprouts were 4 mm shorter than the control. Their growth was less effective and the average length was 7 mm shorter than the control. The minimum length was 1.4 times shorter than that of the control. When the sprouts were irrigated with a higher concentration of the consortium (2.5), they were by 13 mm longer than the control on the 3rd day and 1.3 times as long on the 10th day.

As can be seen from Fig. 5, when soaked in the consortium with a concentration of 2.5, 10 seeds of *T. pretense* L. developed a root, which is 2 seeds more than the number of seeds soaked in the consortium with a concentration of 1.5. It should be noted that the seeds soaked in water did not develop a root.

According to Fig. 6, only 2 of the sprouts soaked in the 1.5 consortium had a second cotyledon on the 10th day, compared to 7 sprouts of the control sample. Thus, the consortium with a concentration of 2.5 proved most effective to soak the seeds in, since almost all the seeds (8 out of 10) developed a second cotyledon.

CONCLUSION

We isolated 10 strains of microorganisms from the soil samples and described their cultural and morphological characteristics. Based on the amounts of indole-3-acetic acid they synthesized, the most promising strains for consortia were No. 1 (10.51 mg/mL), No. 2 (12.41 mg/mL), No. 3 (16.32 mg/mL), No. 4 (11.69 mg/mL), and No. 10 (10.71 mg/mL). The selected extremophilic bacteria were then identified as *Pantoea* spp. (No.1), *Enterococcus faecium* (No. 2), *Leclercia* spp. (No. 3), *Rothia endophytica* (No. 4), and *Klebsiella oxytoca* (No. 10). Based on bio-compatibility testing, we created four variants of consortia containing *Pantoea* spp., *E. faecium*, and *R. endophytica*. The largest amount of indole-3-acetic acid was synthesized by a consortium with a 1:1:1 ratio of these extremophilic bacteria (15.59 mg/mL).

The most optimal nutrient medium for biomass accumulation contained 0.4 g KH_2PO_4, 0.8 g $MgSO_4 \times 7H_2O$, 4 g KNO_3, 1.4 g Na_2HPO_4, 10 g sucrose, and up to 1 liter of distilled water. After 4 h of cultivation, the number of colony-forming units on this medium was 3.9 CFU/mL×10^6, and the stage of death began after 28 h (14.4 CFU/mL×10^6).

To increase the content of indole-3-acetic acid, L-tryptophan was added to the nutrient medium in an amount of 0.1, 0.2, and 0.5%. We found that with 0.2% L-tryptophan, the biopreparation synthesized 1.2 times more indole-3-acetic acid than the control.

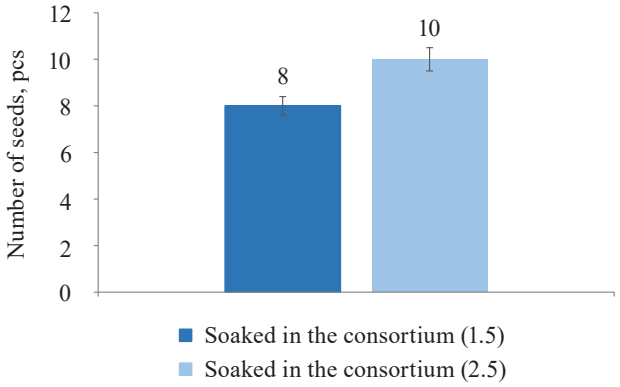


Figure 5 The numbers of germinated seeds soaked in consortium solutions (n = 3)

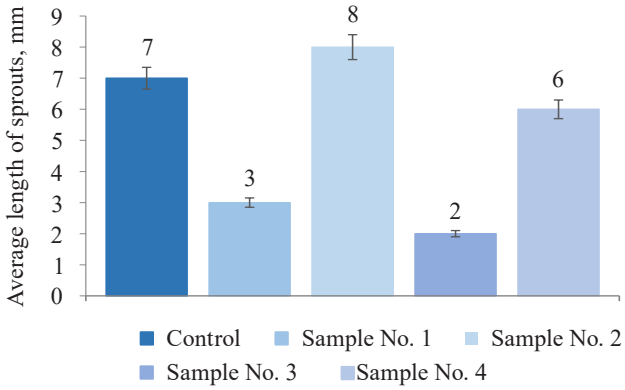


Figure 6 The numbers of sprouts with a second cotyledon from the seeds soaked in the consortium (n = 3)

Trifolium prantense L. was used to assess the ability of the consortium's culture fluid to enhance plant growth. In the first experiment, the seeds were soaked in the consortium solution at concentrations of 1.5 and 2.5. In the second experiment, the seeds soaked in water were irrigated with the consortium solution at concentrations of 1.5 and 2.5. According to the results, the largest number of sprouts (9 sprouts) was formed from the seeds soaked in the 2.5 consortium solution and from the seeds irrigated with the 1.5 solution.

As for sprout length, the seeds soaked in the biopreparation at a concentration of 2.5 proved the most effective on the 10th day (the sprouts were 1.4 times longer compared to the control). Yet, the sprouts germinated from the seeds irrigated with the biopreparation at the same concentration were 1.3 times shorter compared to the control. We can conclude that soaking is the most effective way to treat seeds.

Thus, plant growth was enhanced by soaking the seeds in the culture fluid of the consortium at a concentration of 2.5 made from *Pantoea* spp., *E. faecium*, and *R. endophytica* (1:1:1) cultivated on medium No. 2 (0.4 g KH_2PO_4, 0.8 g $MgSO_4 \times 7H_2O$, 4 g KNO_3, 1.4 g Na_2HPO_4, 10 g sucrose, 2.1 g L-tryptophan, and 1000 mL distilled water). Further research will focus on developing biofertilizers to increase the yield and nutritional value of crops.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this publication.

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Biologically active compounds in *Scutellaria baicalensis* L. callus extract: Phytochemical analysis and isolation

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Abstract:
Plant cells and tissue cultures are sources of secondary plant metabolites. Substances produced by callus cultures can expand the raw material base in pharmacy and food production. However, isolating biologically active substances from medicinal plants is a labor- and time-consuming process. As a result, new and efficient technological processes adapted for extraction from callus cultures are in high demand, and new algorithms of isolation and purification of biologically active substances remain a relevant task.

This research featured callus cultures of *Scutellaria baicalensis* L. The procedures for phytochemical analysis and isolation of biologically active substances involved such physicochemical research methods as high-performance chromatography (HPLC), thin-layer chromatography (TLC), UV spectrometry, and IR spectrometry.

The high performance liquid chromatography confirmed the presence of flavonoids represented by baicalein (5,6,7-trioxyflavone), baicalin (baicalein 7-O-glucuronide), scutellarein (5,6,7,4-tetraoxyflavone), scutellarin (7-O-glucuronide scutellarein), vagonin, and oroxylin. The spectral analyses also detected skutebaicalin. The highest total content of diterpene belonged to the samples extracted with 70% ethanol at 70°C. The content of diterpene was 0.09 mg/cm^3 in terms of betulin. The biologically active substances were isolated from the callus extracts of *S. baicalensis* with a recovery rate of ≥ 80%. The purification scheme made it possible to obtain highly-pure individual biologically active compounds: trans-cinnamic acid, baicalin, and oroxylin A had a purity of ≥ 95%; baicalein had a purity of ≥ 97%; scutellarin and luteolin reached ≥ 96%.

The new technological extraction method made it possible to obtain extracts from *S. baicalensis* callus cultures, which were tested for the component composition. The developed isolation algorithm and purification scheme yielded biologically active substances with a purification degree of ≥ 95%.

Keywords: *Scutellaria baicalensis*, callus cultures, antioxidant activity, geroprotective properties, highly effective chromatography, biologically active substances

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INTRODUCTION

Scutellaria baicalensis L., known as Baikal or Chinese skullcap, is a valuable medicinal plant, historically used in Tibetan medicine [1]. It grows in Mongolia and Manchu; in Russia, it is to be found in Eastern Siberia, Dauria, and Primorye, as well as in the valleys of the Angara and the Amur and in the Sayan mountains [2]. *S. baicalensis* has a diverse chemical composition andis known to be rich in flavonoids of the flavone group: their content reaches 10% in the roots [3–7]. The plant is included in the pharmacopoeias of China, Japan, the Republic of Korea, and Great Britain, as well as in the ninth edition of the European Pharmacopoeia [8]. The qualitative and quantitative analyses of its components and phytopreparations revealed a multicomponent composition and a matrix effect [9, 10]. Based on the instrumental

analysis, the standardization depends on the content of the main active components, namely baicalein and scutellarein, together with their glycosidated forms [11].

Both total extracts and individual substances obtained from *S. baicalensis* are known to have a positive effect on human health. Baicalin is a flavone isolated from the roots of *S. baicalensis*. It possesses antimicrobial, anti-inflammatory, antitumor, antioxidant, and immunomodulatory properties. Baicalin can be used alone or as a stimulant with other drugs to treat various diseases [12–14]. The anti-inflammatory and antioxidant properties mean that extracts and individual substances of *S. baicalensis* can be used in the treatment and rehabilitation of COVID 19 [15, 16].

The biological activity of flavonoids obtained from *S. baicalensis* depends on their structure [17]. *S. baicalensis* is one of the most promising plant raw materials for neuroprotectors that treat concomitant cerebral vascular insufficiency and dementia because it protects vascular endothelial cells and prevents atherosclerosis [18, 10]. These pharmacological properties are especially beneficial for senior population. Extracts and individual components obtained from *S. baicalensis* can be used in geriatrics as part of innovative drugs and functional foods that prevent or inhibit premature aging.

S. baicalensis is a highly-demanded raw material but a limited natural resource. Pharmacy and food industry need alternative sources to satisfy the growing demand for its valuable extracts and individual substances [20]. The existing isolation algorithms are labor-, time-, and resource-consuming. They hardly take into account individual characteristics of the plant component and the matrix effect of the extract. As a result, novel extraction procedures adapted to callus cultures and new effective isolation and purification algorithms remain a relevant task.

The research objective was to develop a new method to extract and accumulate key components obtained from the callus culture of *S. baicalensis*. Isolating individual biologically active substances from total extracts is a promising approach, which is especially important for processing biotechnological extracts. This approach usually relies on different concepts of isolation and purification, including liquid-liquid post-extraction, recrystallization, and sorption-chromatographic technologies.

This research offers a complete cycle for *S. baicalensis* callus extracts, followed by isolation and purification of individual biologically active substances. The developed procedure provides a sequential use of various types of sorbents to reduce the number of stages, increase the process efficiency, and obtain high-purity individual substances from *S. baicalensis* callus extracts, which proved to be an excellent source of pharmacologically active compounds.

Pure substances of plant origin are very important for basic research, but modern efficient recovery and purification processes must meet the so-called green chemistry criteria, i.e., safety, sustainability, feasibility, and efficiency [21–25].

STUDY OBJECTS AND METHODS

The study featured *in vitro* callus cell cultures obtained from seeds of *Scutellaria baicalensis* L. of the *Labiaceae* family.

To obtain sterile material, the seeds were kept at 4°C for two weeks for stratification. After that, they were soaked in 96% ethanol for 30 s and a 6% sodium hypochlorite solution for 30 min. Upon sterilization, the material was soaked three times under distilled sterile water for 20 min. To obtain sterile seedlings, the seeds were planted on an agar medium in 60- and 90-mm Petri dishes. The first seedlings appeared after 4–5 weeks. The sterile seedlings with 2–4 true leaves were used to induce callus cell cultures. The leaves and stems were cut into pieces and planted on agar media in 60- and 90-mm Petri dishes, as well as in jars with ventilated lids. The primary calli were registered on cultivation days 7–14.

The media had a Gamborg (B5) mineral base with casein hydrolyzate, inositol, sucrose, and agar. Indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, and 6-benzylaminopurine (BAP) served as growth regulators.

Studying the biologically active components. To obtain callus extracts from *S. baicalensis*, we selected rational parameters for the extraction of a bioactive complex with potential geroprotective properties from the callus biomass. Ethyl alcohol served as extractant. The dried callus was crushed in a mill and sieved through a 1-mm sieve. The resulting callus powder (3.0 g) was extracted in 260 cm^3 of ethanol. The extraction was carried out in a water bath under reflux at extraction frequency mode 2. The extractant concentration (C, %), extraction temperature (t, °C), and extraction time (t, h) were independent variables. The optical density of the extracts served to assess the efficiency of the process (Table 1).

Isolating the biologically active substances. Figure 1 shows the process of isolating individual biologically active substances from the callus extract of *S. baicalensis*.

Purifying the biologically active compounds. The purification of individual biologically active substances isolated from *S. baicalensis* callus extract included the following stages:

1. We evaporated the ethanol extract under vacuum at ≤ 50°C, added diethyl ether to the evaporated residue, and processed it three times;

2. The resulting ether fractions were combined and evaporated using an IKA RV 8 rotary evaporator;

3. The dry residue (stage 1) was treated with water at 70°C, while the aqueous fraction was treated first with n-butanol and then three times with ethyl acetate;

4. We isolated baicalein from the n-butanol fraction (stage 3);

Table 1 Optical density of *Scutellaria baicalensis* callus extracts

Temperature, °C	Volume fraction of ethanol in extractant, %		
	30	50	70
	Extraction time, 2 h		
	Sample		
30	1	2	3
	0.3903 ± 0.0058	0.5553 ± 0.0051	1.4150 ± 0.0225
50	4	5	6
	0.4365 ± 0.0066	0.5658 ± 0.0062	1.1146 ± 0.0099
70	7	8	9
	0.3674 ± 0.0030	0.5323 ± 0.0048	1.3870 ± 0.0235
	Extraction time, 4 h		
30	10	11	12
	1.0529 ± 0.0100	1.4339 ± 0.0311	1.6221 ± 0.0423
50	13	14	15
	1.1727 ± 0.0132	1.3836 ± 0.0226	1.6446 ± 0.0459
70	16	17	18
	1.1653 ± 0.0126	1.4085 ± 0.0301	1.8900 ± 0.0627
	Extraction time, 6 h		
30	19	20	21
	1.3974 ± 0.0321	1.9995 ± 0.0665	2.3913 ± 0.0725
50	22	23	24
	1.2333 ± 0.0299	1.7362 ± 0.0420	1.8839 ± 0.0621
70	25	26	27
	1.8233 ± 0.0356	1.5790 ± 0.0310	2.1652 ± 0.0632

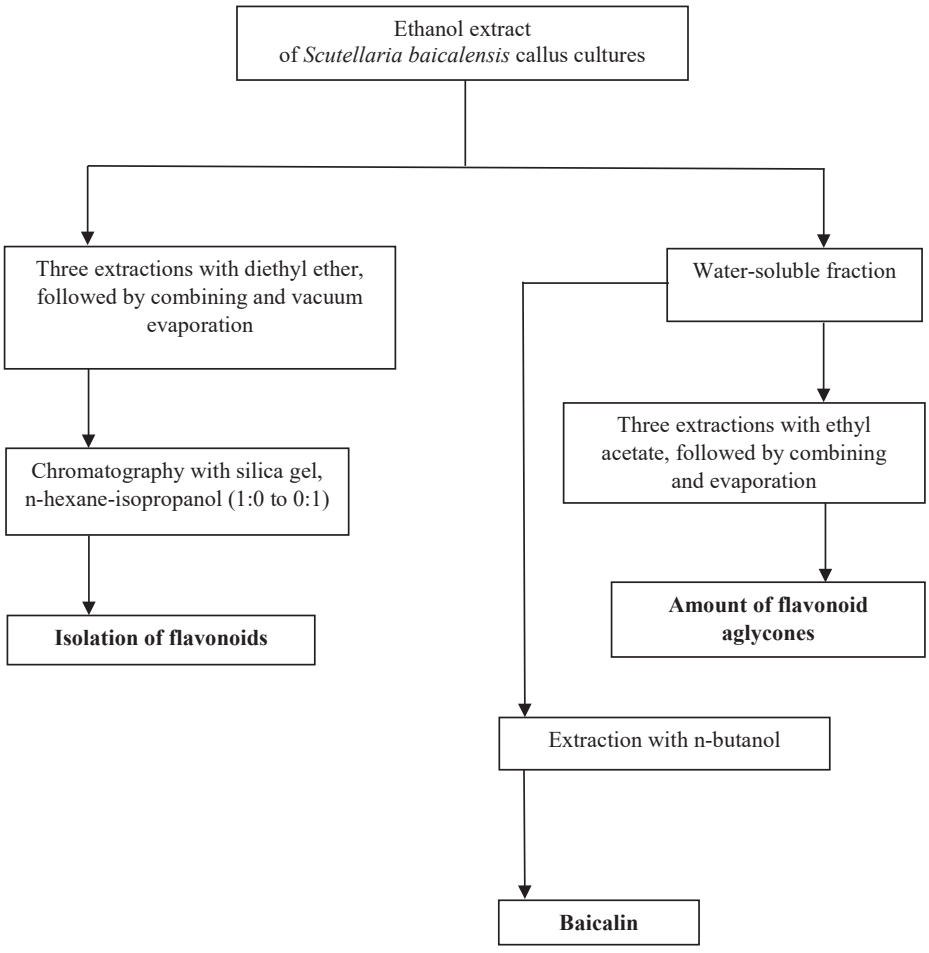


Figure 1 Isolating biologically active substances from *Scutellaria baicalensis* callus extract

5. The ethyl acetate fraction (stage 3) was combined and evaporated;

6. The fraction (stage 5) was chromatographed on Sephadex LH-20 in the mobile phase, gradient H_2O-MeOH (1:0 to 1:2);

7. 97% baicalein was isolated after additional purification on sephadex LH-20 in the mobile phase, gradient H_2O-MeOH (1:0 to 1:2);

8. The ethyl acetate fraction of flavonoids (stage 3) was combined and evaporated;

9. The ether fraction (stage 1) was chromatographed with silica gel, gradient n-hexane-isopropanol (1:0 to 0:1). After that, we isolated flavonoids and hydroxycinnamic acids; and

10. Rechromatography with silica gel; a mobile phase was n-hexane-chloroform (1:0 to 0:1). Rechromatography made it possible to isolate fractions with trans-cinnamic acid, baicalin, and oroxylin A. Their purity reached ≥ 95%.

Figure 2 illustrates the purification scheme for biologically active substances obtained from the *S. baicalensis* callus extract.

This purification scheme made it possible to obtain individual biologically active substances with a purification rate that exceeded 95%.

Spectrophotometry. The spectral (UV) profiles of the total extracts and individual components were recorded using a spectrophotometer (OKB Spectr, St. Petersburg, Russia) in the wavelength range of 190–600 nm with a resolution of 0.5 nm in liquid cuvettes with a long optical path of 10 mm. Photometry was applied to both pure components and their mixes with reagents. This approach made it possible to r eveal the general and specific properties of flavonoid compounds and differential spectra after specific reagents were added. The list of reagents included $AlCI_3$/HCI, NaOMe, NaOAc, and NaOAc/H_3BO_3.

IR spectrometry. Infrared spectra were obtained from a disk with potassium bromide in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 50 accumulation cycles using an FSM-2202 Fourier spectrometer.

Pre-HPLC treatment. The acid hydrolysis test followed the procedure described below. We placed 2 cm^3 of *S. baicalensis* callus extract in a 100-cm^3 conical flask with 20 cm^3 of methanol (Ecos-1, ChDA, Russia) and 2 N HCI (Sigma Tech, Russia) at a ratio of 1:1. The mix was sonicated for 5 min, hydrolyzed in a boiling water bath under reflux for 20 min, evaporated under vacuum to a dry residue, and dissolved in 2 cm^3 of the mobile phase.

We calculated the relative standard deviation for the peak areas of baicalin (CAS no. 21967-41-9 Product No. Y0001273, Sigma-Aldrich, Germany) and scutella-

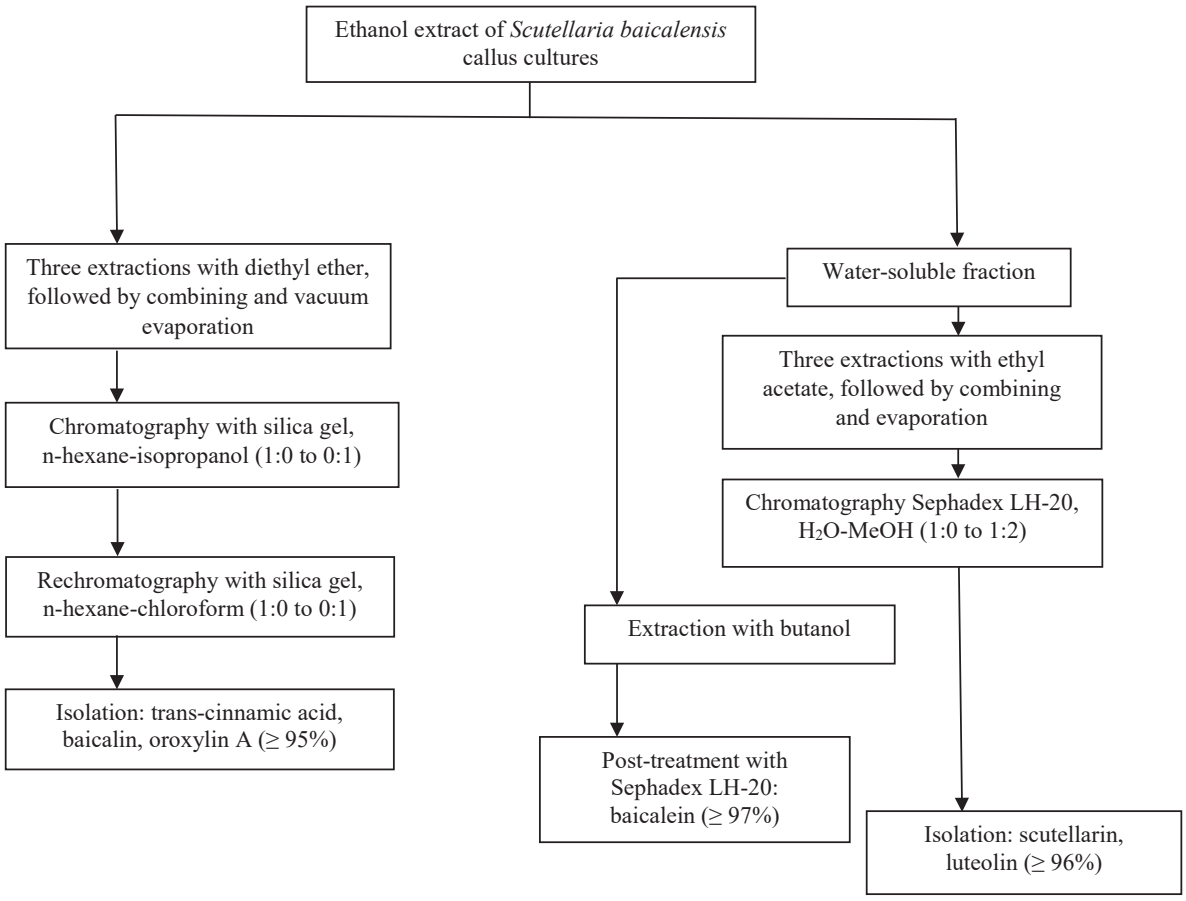


Figure 2 Purifying biologically active substances obtained from *Scutellaria baicalensis* callus extract

rein (CAS: 529-53-3 Product No. C0327, Sigma-Aldrich, Germany). The procedure involved five chromatograms, and the deviation was below 0.5%. The efficiency of the chromatographic column exceeded 10 000 theoretical plates.

Thin-layer chromatography (TLC). The thin-layer chromatography test included Sorbfil PTSKh-AF-A and HPLC Silica gel 60 RP-18 plates (Merk) followed by a densitometry using the Sorbfil TLC plate. The densitometer was equipped with a Sony photofixation system (Handycam HDR-CX405, IMID, Russia). The photofixation was conducted at wavelengths of 254 and 365 nm in the visible radiation range after specific derivatization. The elution involved several systems of mobile phases:

1) chloroform (chemical purity grade, Ecos-1, Russia):methanol (standard pure, Ecos-1, Russia): water: glacial acetic acid (chemical purity grade, Ecos-1, Russia) at the ratio of 100:10:1:0.3;

2) benzene:ethyl acetate:acetic acid (standard pure, Ecos-1, Russia) at a ratio of 100:60:0.5;

3) chloroform:methanol:water:formic acid (standard pure, Ecos-1, Russia) at a ratio of 25:8:1:0.5;

4) ethyl acetate:dimethyl ketone:water:formic acid (standard pure, Ecos-1, Russia) at a ratio of 6:3:1:1; and

5) n-butanol:acetic acid (standard pure, Ecos-1, Russia):water at a ratio of 60:15:25.

We used diluted sulfuric acid or a 25% ethanolic solution of phosphotungstic acid as developers.

High performance liquid chromatography (HPLC). The HPLC followed the procedure described in [26]. The following eluents served as the mobile phase:

1) tetrahydrofuran:acetic acid (standard pure, Ecos-1, Russia):5% H_3PO_4:water at a ratio of 19:20:2:59; and

2) tetrahydrofuran:dioxane:methanol:acetic acid:5% H_3PO_4:water at a ratio of 14.5:12.5:5:2:2:66.

The substances were separated using a Shimadzu LC-20 Prominence chromatograph with a Shimadzu SPD20M diode array detector and a Hyper Clone 5 µm BDS 130Å, C-18 250×4.6 mm column [27].

Statistical processing. Random errors were evaluated by the method of mathematical statistics described by K. Derffel in his Statistics in Analytical Chemistry.

For a limited number of parallel measurements n (n < 20, sample data set), we applied the Student's distribution, which links the sample size and the probability that a certain value falls into a given confidence interval.

The mean value for a number of parallel determinations

$$\bar{x} = \sum x_i / n$$

was accepted as the most probable.

A random error (reproducibility) involves such characteristics as sample variance (S^2), standard deviation (S), and relative standard deviation (S_r, %):

$$S^2 = \frac{\sum_{i=1}^{i=n}(x_i - \bar{x})^2}{n-1}; \quad S = \sqrt{\frac{\sum_{i=1}^{i=n}(x_i - \bar{x})^2}{n-1}}; \quad S_r = \frac{S \cdot 100}{x}.$$

When processing the data, the boundaries of the confidence interval were determined as $(\bar{x} - \mu)$. This is the interval that encompasses the true value for a given confidence probability P and the number of degrees of freedom f (f = n – 1):

$$\Delta x = x - \mu = \pm \frac{t_{Pf} \cdot S}{\sqrt{n}} = C$$

The confidence probability was 95%, or 0.95, i.e., 95 out of 100 values fell into the calculated interval. In the equation below, coefficient t_{Pf} was the Student's coefficient of standard deviations. The dependence of t_{Pf} on f showed that the accuracy of the analysis increased together with the number of degrees of freedom, i.e., the number of parallel results. It happened because the confidence interval characterized the reproducibility and, to some extent, the accuracy. Based on the confidence interval, the true value of the result obtained was represented as the following equation:

$$\mu = \bar{x} \pm \Delta x = \bar{x} \pm \frac{t_{Pf} \cdot S}{\sqrt{n}} = \bar{x} \pm C$$

Table 2 Total flavones

Sample	Total flavones, mg/cm^3
1	19.64 ± 0.03
2	31.01 ± 0.05
3	79.82 ± 0.03
4	22.70 ± 0.07
5	31.97 ± 0.06
6	63.13 ± 0.06
7	19.31 ± 0.03
8	29.28 ± 0.08
9	80.68 ± 0.06
10	57.54 ± 0.11
11	79.71 ± 0.03
12	92.57 ± 0.08
13	65.71 ± 0.13
14	0.77 ± 0.02
15	93.86 ± 0.07
16	64.09 ± 0.06
17	79.94 ± 0.06
18	109.11 ± 0.08
19	76.41 ± 0.11
20	104.62 ± 0.03
21	121.07 ± 0.08
22	66.24 ± 0.03
23	91.99 ± 0.07
24	100.64 ± 0.05
25	95.22 ± 0.08
26	85.65 ± 0.06
27	113.05 ± 0.12

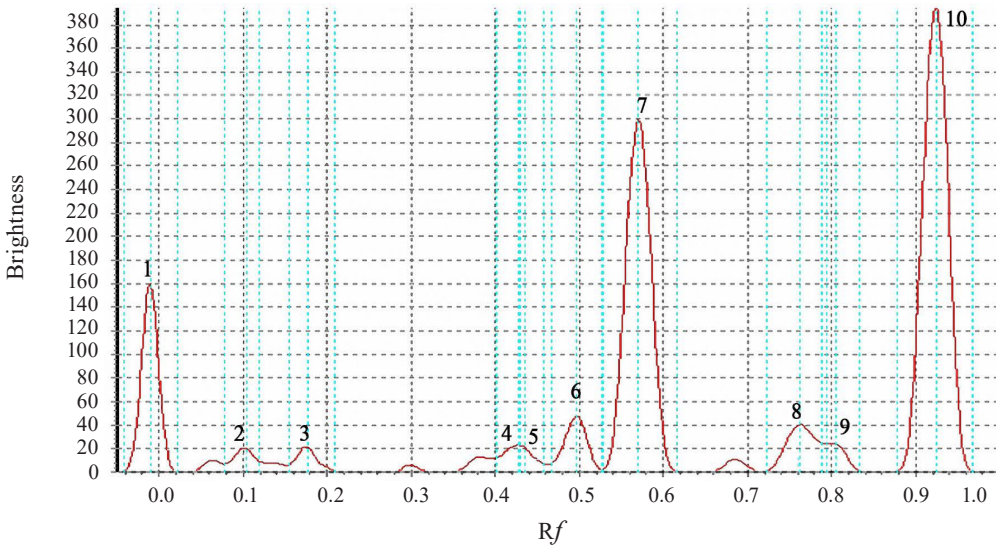


Figure 3 Densitogram of *Scutellaria baicalensis* callus extract: 1 – scutebaicalin, 6 – scutellarin, 7 – vagonin, 10 – baicalinb, 2–6, 8, and 9 – not identified

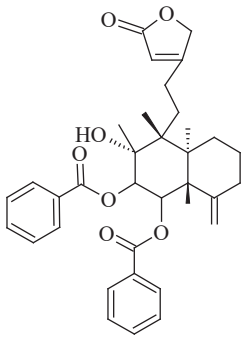


Figure 4 Structure of skutebaicalin

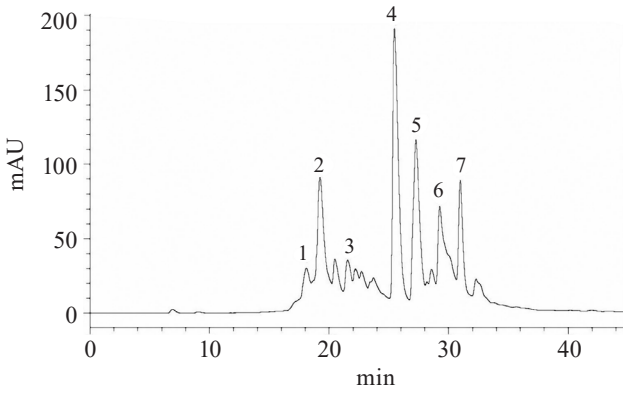


Figure 5 HPLC chromatogram of Sample 21 of aqueous-alcoholic *Scutellaria baicalensis* callus extract: 1 – hedithol-glucopyranoside, 2 – scutellarein, 3 – chrysin, 4 – baicalin, 5 – neobaicalein, 6 – isoscutellarein, 7 – 5,7-dihydroxy-6-methoxyflavone

RESULTS AND DISCUSSION

We recorded the spectrum of the native water-alcohol extract to analyze the composition of water-alcohol callus extracts obtained from *Scutellaria baicalensis* L.

The samples contained flavonoids of the flavone group: baicalein (5,6,7-trioxyflavone), baicalin (7-O-glucuronide of baicalein), scutellarein (5,6,7,4-tetraoxyflavone), scutellarin (7-O-glucuronide scutellarein), vagonin, and oroxylin. Table 2 represents their total content in the samples.

Figure 3 shows a sample densitogram of *S. baicalensis* callus extract. The separation procedure involved a Sorbfil plate under conditions: n-butanol (PanReac, Germany):acetic acid (standard pure, Ecos-1, Russia):water at a ratio of 60:15:25. The plate was developed with a solution of 25% phosphotungstic acid (standard pure, LenReaktiv, Russia) and heated at 95°C for 10 min.

The thin-layer chromatography revealed the following patterns. After the plate was treated with a solution of phosphotungstic acid and heated, lilac spots appeared on the start line (R*f* value = 0). The spectral analysis identified the spot as skutebaicalin (Fig. 4).

The content of diterpene was 0.09 mg/cm^3 in terms of betulin. The highest total content of diterpene belonged to the samples extracted with 70% ethanol at 70°C. The components with R*f* = 0.5, 0.57, and 0.92 (Fig. 3) were identified as scutellarin, vagonin, and baicalin, respectively. When we used 80 and 90% ethanol as extractant, the amount of the extracted target substance increased slightly. The total extract then had a lot of ballast substances of a lipid nature, including fatty acids. The separation of these components turned out to be more expensive and labor-consuming. The complete extraction of the target components was achieved by triple treatment with 70% ethanol, followed by combining the obtained extracts.

Table 3 Retention times of individual components of HPLC chromatogram: Sample 21 of aqueous-alcoholic *Scutellaria baicalensis* callus extract

Peak	Retention time, min	Component	Quantity, μg/cm^3
1	18.15	Hedithol-glucoside	3.90 ± 0.14
2	19.30	Scutellarein	14.87 ± 0.72
3	20.35	Chrysin	5.66 ± 0.50
4	25.70	Baicalin	19.31 ± 0.98
5	27.30	Neobaicalein	15.33 ± 0.32
6	28.70	Isoscutellarein	3.50 ± 0.50
7	31.03	5,7-dihydroxy-6-methoxyflavone	8.95 ± 0.37

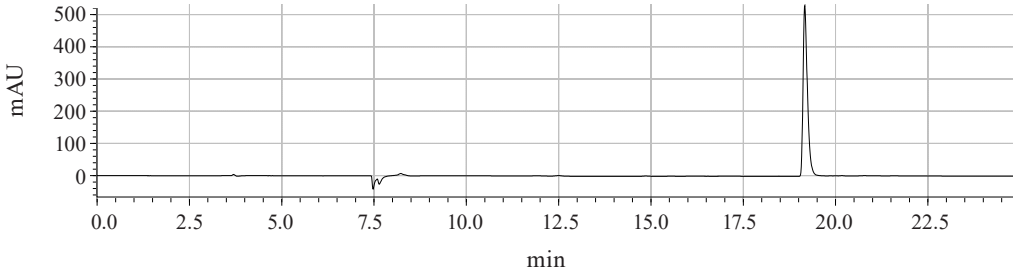


Figure 6 HPLC chromatogram of purified scutellarein isolated from *Scutellaria baicalensis* callus extract

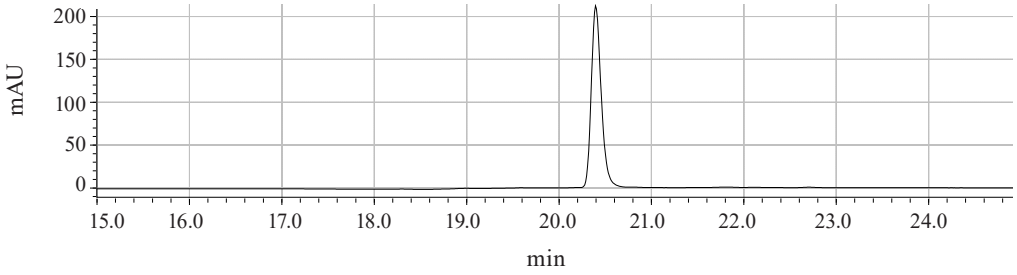


Figure 7 HPLC chromatogram of purified chrysin isolated from *Scutellaria baicalensis* callus extract

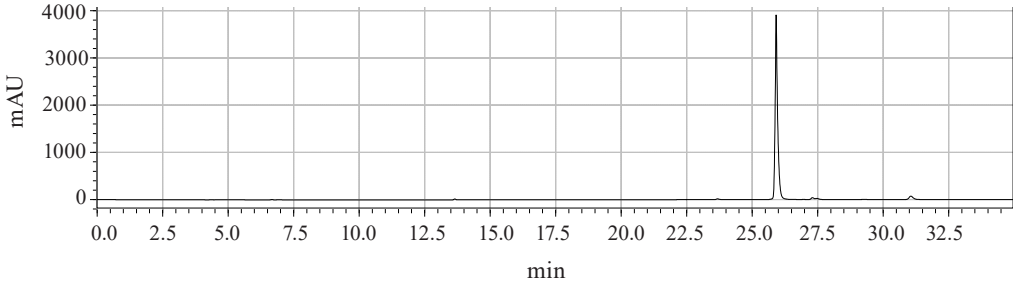


Figure 8 HPLC chromatogram of purified baicalin isolated from *Scutellaria baicalensis* callus extract

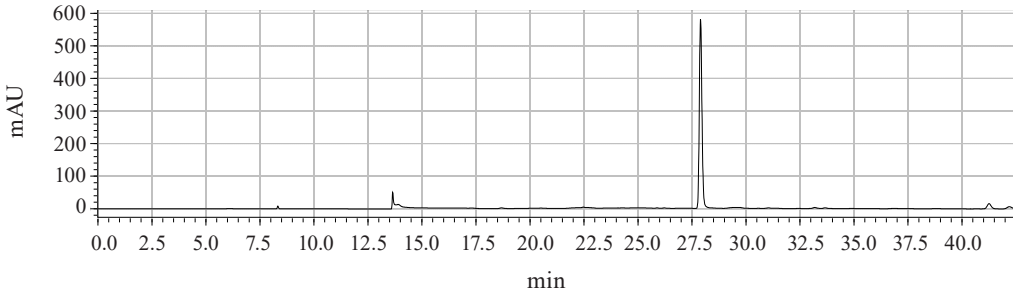


Figure 9 HPLC chromatogram of purified neobaicalein isolated from *Scutellaria baicalensis* callus extract

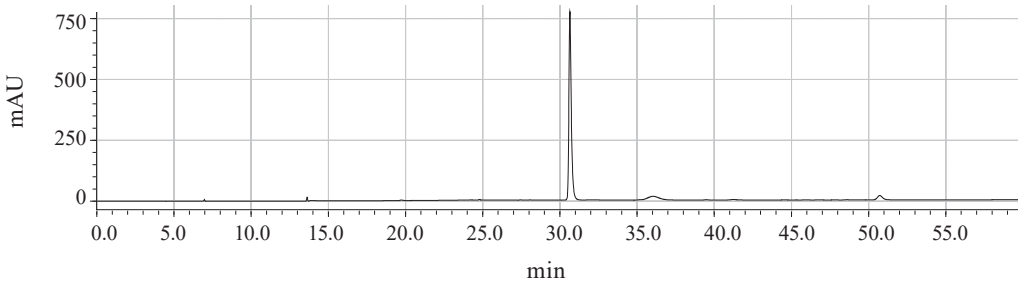


Figure 10 HPLC chromatogram of purified oroxylin A (5,7-dihydroxy-6-methoxyflavone) isolated from *Scutellaria baicalensis* callus extract

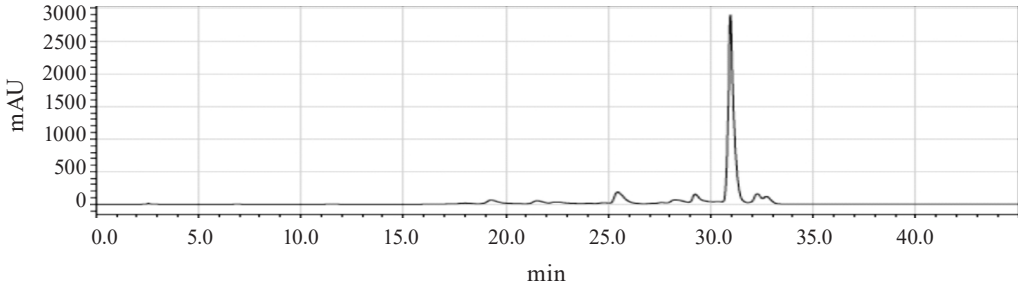


Figure 11 HPLC chromatogram of residue from Sample 3

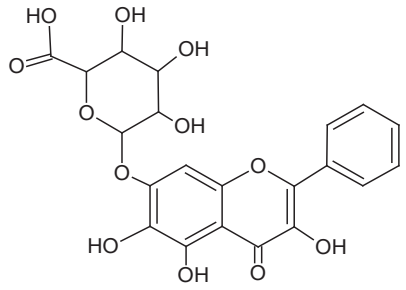


Figure 12 Baicalin: structure

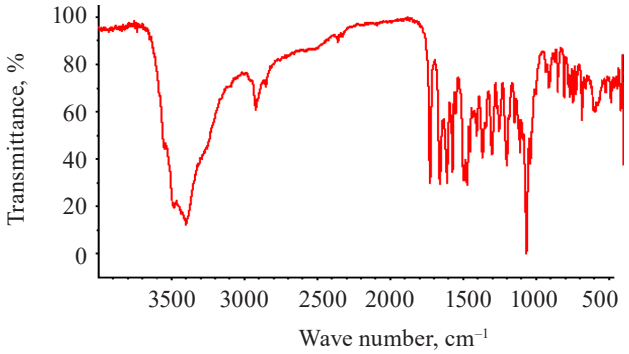


Figure 13 Baicalin: infrared spectrum

The analytical HPLC test detected such flavonoids as scutellarein, wogonin, baicalin, chrysin, 5,7-dihydroxy-6-methoxyflavone, and neobaicalein. The preparative HPLC test involved a fraction collector and made it possible to accumulate individual biologically active substances. The chromatograms in Figs. 5–10 illustrate their high purity.

Samples 1–8 demonstrated strong precipitation. The HPLC analysis showed that the component composition of the residue was represented mainly by baicalein (Fig. 11).

The retention time and spectral profile corresponded to the baicalein standard (98.75%, Herbest, China).

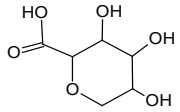
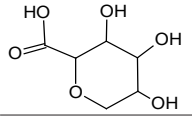
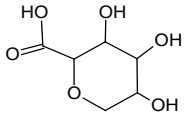
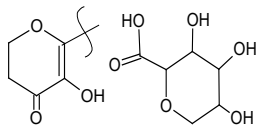
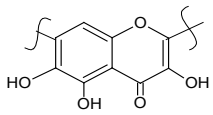
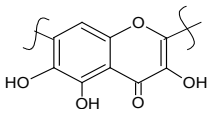
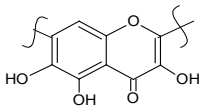
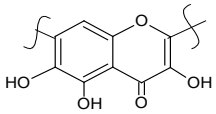
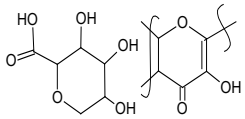
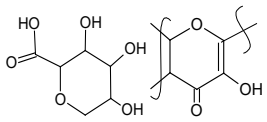
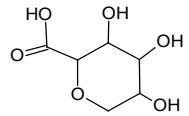
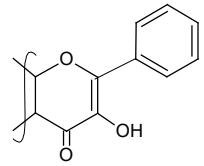
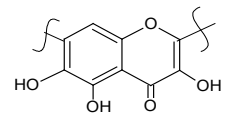
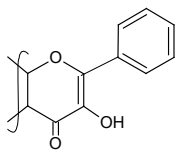
We chose the amount of active compounds in their native state as the main factor in selecting the rational parameters for isolating individual biologically active substances. This factor incurred high-quality purification from ballast components. The isolated biologically active substances demonstrated an extraction degree of ≥ 80%. Figures 12–21 depict two purified samples of biologically active substances as structural formulae and the infrared spectra of individual biologically active substances with 95% purification. The infrared spectra coincided with the standard absorption bands.

The infrared spectrum of baicalin (5,6-dihydroxy-4-hydroxy-2-phenyl-4H-1-benzopyran-7-*β*-D-glucuronide) had the following features (Table 4). The band with the absorption maximum at 3398 cm^{-1} was typical of the associated OH groups. The bands at 2922 and 2853 cm^{-1} appeared because of the symmetric and antisymmetric stretching vibrations of the tertiary carbon in the carbohydrate fragment.

The C=O bond in the carboxyl group corresponded to the 1725 cm^{-1} band.

The bands at 1657 and 1607 cm^{-1} appeared as a result of stretching vibrations of the C=O bond, as well as the effect of OH groups in positions 3 and 5 in the heterocyclic fragment of the molecule. The latter happened due to the formation of an intramolecular hydrogen bond with C=O. It was the effect of this

Table 4 Features of the infrared spectrum of baicalin

No.	Structural fragments	Wave number, cm^{-1}	Type of vibrations/bonds of structural fragments
1		3398	Associated OH groups
2		2922 and 2853	Antisymmetric and symmetric stretching vibrations
3		1725	C=O bond in carboxyl group
4		1657 and 1607	Stretching vibrations of C=O bond
5		1550	Planar stretching vibrations in OH group
6		1571, 1496, and 1462	Stretching vibrations of C=C bond in aromatic systems of rings A and B
7		1408	Deformation vibrations of O-H bond at tertiary carbon atom
8		1364 and 1304	Planar deformation vibrations of O-H bond
9		1254, 1200, and 1147	Antisymmetric vibrations of C-O-C and C-C-O bonds in heterocycle structure
10		1083, 1064, and 1012	Symmetrical stretching vibrations of C-O-C and C-C-O bonds
11		910	C-O-C bond of pyranose ring
12		849	Out-of-plane bending vibrations in hydroxyl group of ring B
13		788, 764, and 745	Deformation vibrations of C-H bond in aromatic structures
14		726	Out-of-plane deformation vibrations of C-C bond

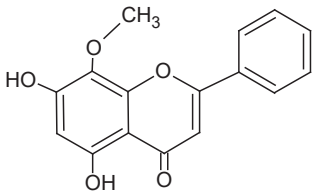


Figure 14 Wogonin: structure

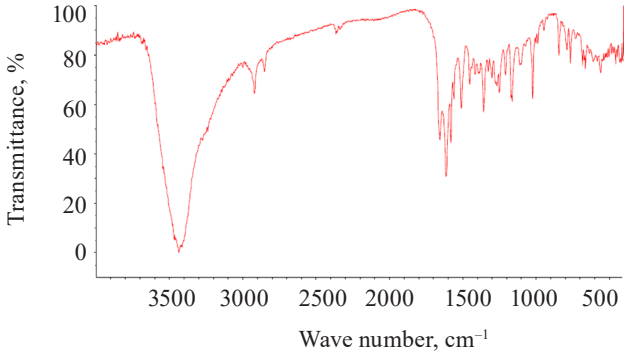


Figure 15. Wogonin: infrared spectrum

hydroxyl that distorted the planar arrangement of the pyran fragment and the bond, thus triggering a doublet resonance. Finally, the OH group at carbon atom 3 in the unsaturated pyran fragment (ring C) was responsible for the weak 1550 cm^{-1} band.

The bands at 1571, 1496, and 1462 cm^{-1} were associated with the stretching vibrations of C-C bonds of aromatic systems in rings A and B. The 1408 cm^{-1} band occurred as a result of the OH group at the tertiary carbon. The bands at 1364 and 1304 cm^{-1} were triggered by the in-plane deformation vibrations of O-H bonds in baicalin structure. The bands at 1254, 1200, and 1147 cm^{-1} appeared as a result of two interacting antisymmetric vibrations of the C-O-C and C-C-O bonds in the heterocycle structures, i.e., ring C and the carbohydrate fragment. The symmetrical stretching vibrations of the C-O-C and C-C-O bonds were responsible for the bands at 1107 and 1064 cm^{-1} in the carbohydrate fragment.

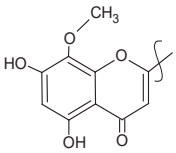
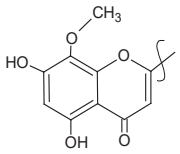
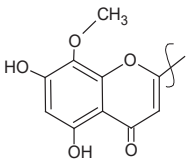
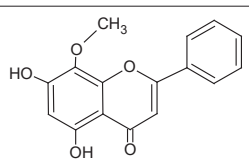
The band at 910 cm^{-1} was specific to the pyranose ring. A relatively weak 849 cm^{-1} band indicated out-of-plane C_2-H bending vibrations, which hinted at an α-anomer with an equatorial arrangement of un substituted hydrogen atoms in ring bonds B-C. Tri-substituted ring A was connected with the deformation of the out-of-plane and in-plane vibrations of the C-H bond. It had bands at 788, 764, and 745 cm^{-1}. Monosubstituted ring B had an out-of-plane bending vibration of the C-C bond at 726 cm^{-1}.

The infrared spectrum of wogonin (5,7-dimethoxy-8-methoxyflavone) had the following features (Table 5). The band with an absorption maximum at 3436 cm^{-1} was typical of the OH group in positions 3 and 7 position of ring A.

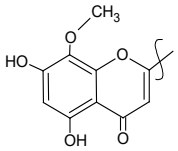
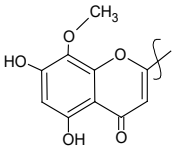
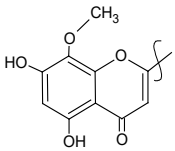
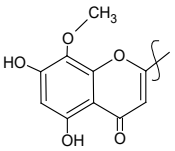
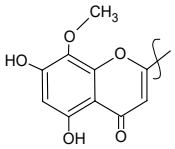
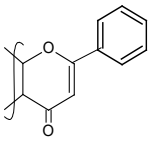
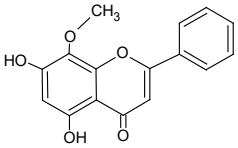
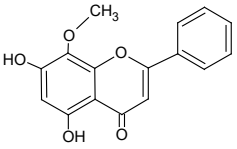
Stretching vibrations of methyl groups C-H were registered at 2922 and 2853 cm^{-1}.

The bands at 1657 and 1612 cm^{-1} occurred as a result of the stretching vibrations of the C=O bond, as well as the effect of OH groups in position 5 in the heterocyclic fragment of the molecule. The latter happened due to the formation of an intramolecular hydrogen bond with C=O. It was the effect of this hydroxyl that distorted the planar

Table 5 Features of the infrared spectrum of wogonin

No.	Structural fragments	Wave number, cm^{-1}	Type of vibrations/bonds of structural fragments
1		3436	Associated OH groups
2		2922 and 2853	Antisymmetric and symmetric stretching vibrations
3		1657 and 1612	Stretching vibrations of C=O bond
4		1581, 1562, 1507, and 1453	Stretching vibrations of C=C bond in aromatic systems of rings A and B

Continuation of Table 5

No.	Structural fragments	Wave number, cm^{-1}	Type of vibrations/bonds of structural fragments
5		1408	Deformation vibrations of O-H bond at tertiary carbon atom
6		1391 and 1384	Planar deformation vibrations of O-H bond
7		1278, 1268, and 1162	Antisymmetric vibrations of C-O-C and C-C-O bonds in heterocycle structure
8		1083, 1064, and 1012	Symmetrical stretching vibrations of C-O-C and C-C-O bonds
9		946	C-O-C bond of pyranose ring
10		844	Out-of-plane bending vibrations in hydroxyl group of ring B
11		787 and 766	Deformation vibrations of C-H bond in aromatic structures
12		730	Out-of-plane deformation vibration of C-C bond

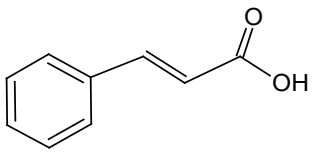


Figure 16 Trans-cinnamic acid: structure

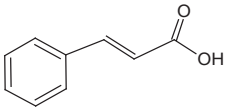
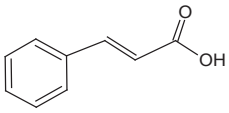
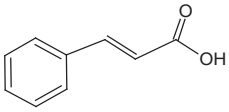
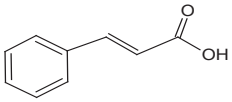
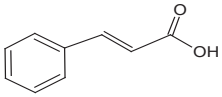
arrangement of the pyran fragment and the bond, thus triggering a doublet resonance.

The bands at 1581, 1562, 1507, and 1453 cm^{-1} were caused by the stretching vibrations of the C-C bond in the aromatic systems. The 1415 cm^{-1} band was related to the in-plane bending vibration of the O-H bond in tertiary carbon. The bands at 1391 and 1384 cm^{-1} occurred as a result of the in-plane deformation vibrations of the O-H bond in the wogonin structure. The bands at 1278, 1268, and 1162 cm^{-1} marked two interacting antisymmetric vibrations of the C-O-C and C-C-O bonds in the heterocycle structures and the methoxyl fragment of ring A. The symmetric stretching vibrations of the C-O-C and C- C-O were responsible for bands 1109 and 1024 cm^{-1}.

The 910 cm^{-1} band was specific to the pyranose ring.

A relatively weak 844 cm^{-1} band indicated out-of-plane bending vibrations of the C_2-H bond, which hinted at an α-anomer with the equatorial arrangement of unsubstituted hydrogen atoms of ring bond B-C. Trisubstituted ring A was characterized by bands at 787 and 766 cm^{-1} due to the deformation of the out-

Table 6 Features of the infrared spectrum of trans-cinnamic acid

№	Structural fragments	Wave number, cm^{-1}	Type of vibrations/bonds of structural fragments
1		3064	Stretching vibrations of bond = C-H in diene fragment
2		3026	Stretching vibrations of C-H bond
3		1680	The C=C bond in diene fragment
4		1631	Stretching vibrations of C=O bond in carboxyl group
5		1576, 1451, 1420, and 1176	Planar stretching vibrations of C-C bond in aromatic fragments
5		1332, 1313, and 1221	Planar deformation vibrations of O-H bond
6		1285	Stretching vibrations of C-O bond
8		979	Planar bending vibrations of hydroxyl groups in the ring
9		766	Out-of-plane deformation vibration of C-C bond

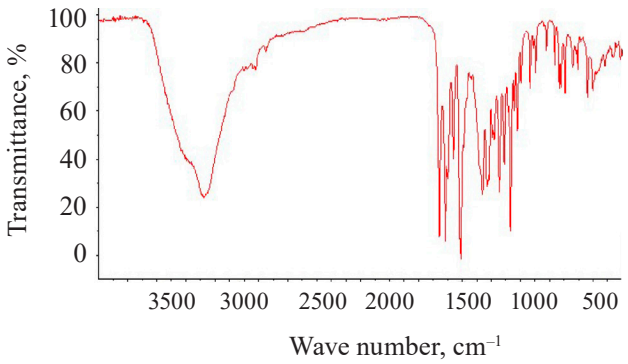


Figure 17 Oroxylin A: infrared spectrum

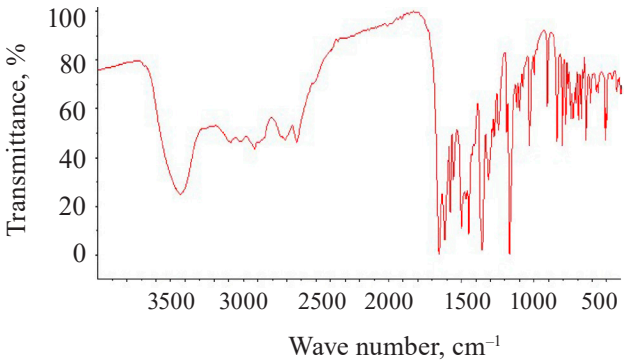


Figure 18 Chrysin: infrared spectrum

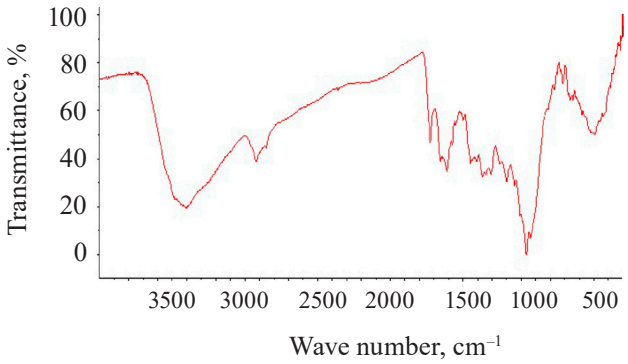


Figure 19 Baicalein: infrared spectrum

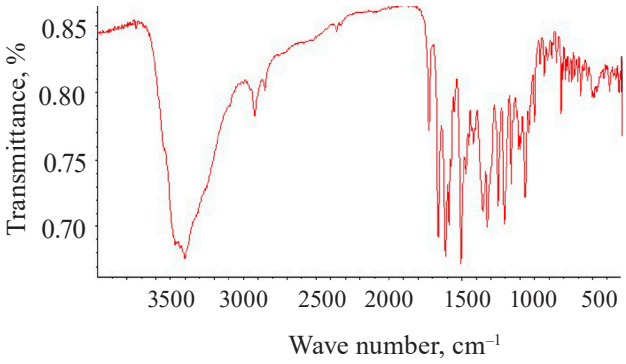


Figure 20 Scutellarin: infrared spectrum

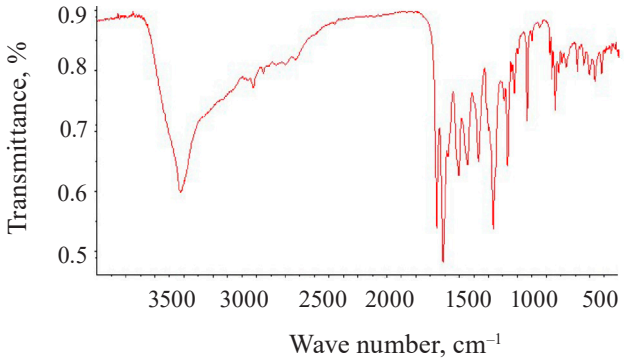


Figure 21 Luteolin: infrared spectrum

of-plane and in-plane vibrations of the C-H bond. Ring B demonstrated out-of-plane deformation vibrations of the C-C bond at 730 cm^{-1}.

The infrared spectrum of *trans*-cinnamic acid (phenylpropenoic acid) demonstrated the following distinctive features (Table 6).

The 3064 cm^{-1} band appeared as a result of the stretching vibrations in the diene fragment of the = C-H bond of phenylpropenoic acid. The stretching vibrations of the C-H bond in the benzene ring were responsible for the 3026 cm^{-1} band. These bands were typical of *trans*-cinnamic acid.

The band at 1680 cm^{-1} could also be considered as the C=C bond in the diene fragment. The 1631 cm^{-1} band was caused by the stretching vibrations in the carboxyl fragment. The bands at 1576, 1451, 1420, and 1176 cm^{-1} resulted from the stretching vibrations of C-H bonds in the aromatic fragment. The absorption 1451 cm^{-1} band was associated with the bending vibrations of the C-O-H bonds in the carboxyl fragment. The bands at 1332, 1313, and 1221 cm^{-1} occurred as a result of bending vibrations of the O-H bond and the stretching vibrations of the C-O bonds, including those in the carboxylic fragment of *trans*-cinnamic acid. The 1285 cm^{-1} band was associated with the stretching vibrations of the C-O bond.

The 979 cm^{-1} band could be explained by the diene fragment in the trans form. The monosubstituted ring demonstrated out-of-plane deformation vibrations of the C-C bond at 766 and 711 cm^{-1}.

CONCLUSION

This article introduced a novel technology for the isolation and purification of target biologically active components from callus cultures obtained from *Scutellaria baicalensis* L. The results are of practical interest for the pharmaceutical and food industries, which demonstrate a high demand for both total extracts and individual highly-pure secondary metabolites of this plant raw material. The new biocultivation method proved effective for secondary metabolites and triterpenoids. The optimal extraction conditions involved 70% ethanol as extractant at 70°C for 6 h and provided a total extract with a high yield of target substances and a minimal content of ballast components. The proposed algorithms for the isolation of individual components using sorption-chromatographic technologies also provided efficient scaling. Therefore, the proposed extraction and sorption-chromatographic methods can be used in laboratory practices and industrial production of pharmaceutical substances and functional foods.

In this attempt to rationalize the isolation of individual biologically active substances from *S. baicalensis* callus extracts, the main task was to isolate physiologically active substances in their native state and purify them from ballast.

CONTRIBUTION

The authors are equally responsible for the research results and the manuscript.

CONFLICTS OF INTEREST

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

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