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

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Abstract:
Ashwagandha (*Withania somnifera* (L.) Dunal) is an Ayurvedic medicinal herb that has been known for its therapeutic properties for millennia. Ashwagandha contains several bioactive compounds, including withanolides, alkaloids, and saponins. They make ashwagandha a potent adaptogen and a versatile herb that can maintain optimal health and overall well-being. Ashwagandha reduces stress and anxiety, as well as boosts the immune system. Its anti-inflammatory properties treat arthritis, asthma, diabetes, and inflammatory bowel disease.

Ashwagandha produces an immunomodulatory effect on natural killer cells, lymphocytes, and leukemia cells. It enhances the activity of natural killer cells, increases lymphocyte function, and induces apoptosis in leukemia cells. However, its mechanism of action still remains understudied.

Ashwagandha has an impact on COVID-19: phytochemical withanone blocks or weakens the interaction between S-protein and Angiotensin-converting enzyme 2. Withanoside V and somniferine inhibit viral transcription and replication caused by SARS-CoV-2 M^{pro}.

This review explores the potential utilization of ashwagandha in the food industry, i.e., its safety and toxicity, as well as the mechanism behind its immunomodulatory effect.

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

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INTRODUCTION

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

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

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

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

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

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

RESUITS AND DISCUSSION

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

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

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

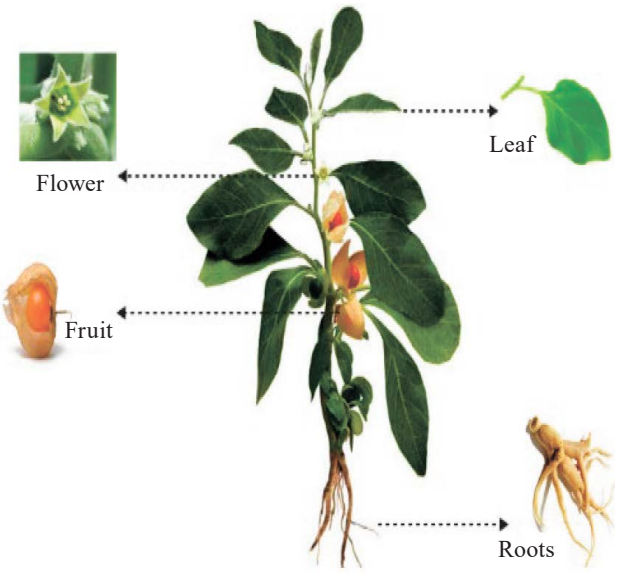
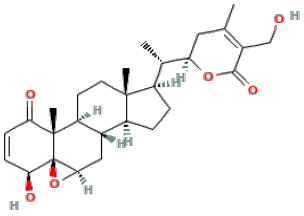
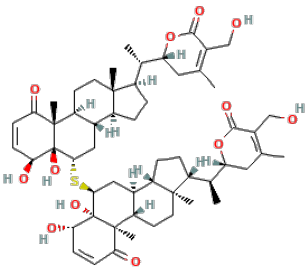
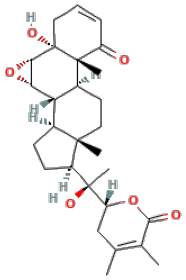
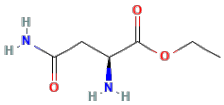
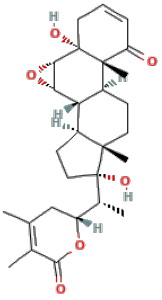
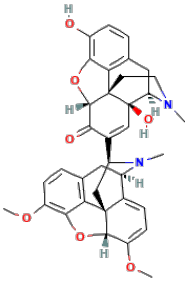
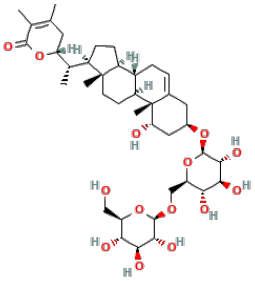


Figure 1 Different parts of ashwagandha plant

Table 1 Bioactive immunomodulatory molecules in Ashwagandha

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

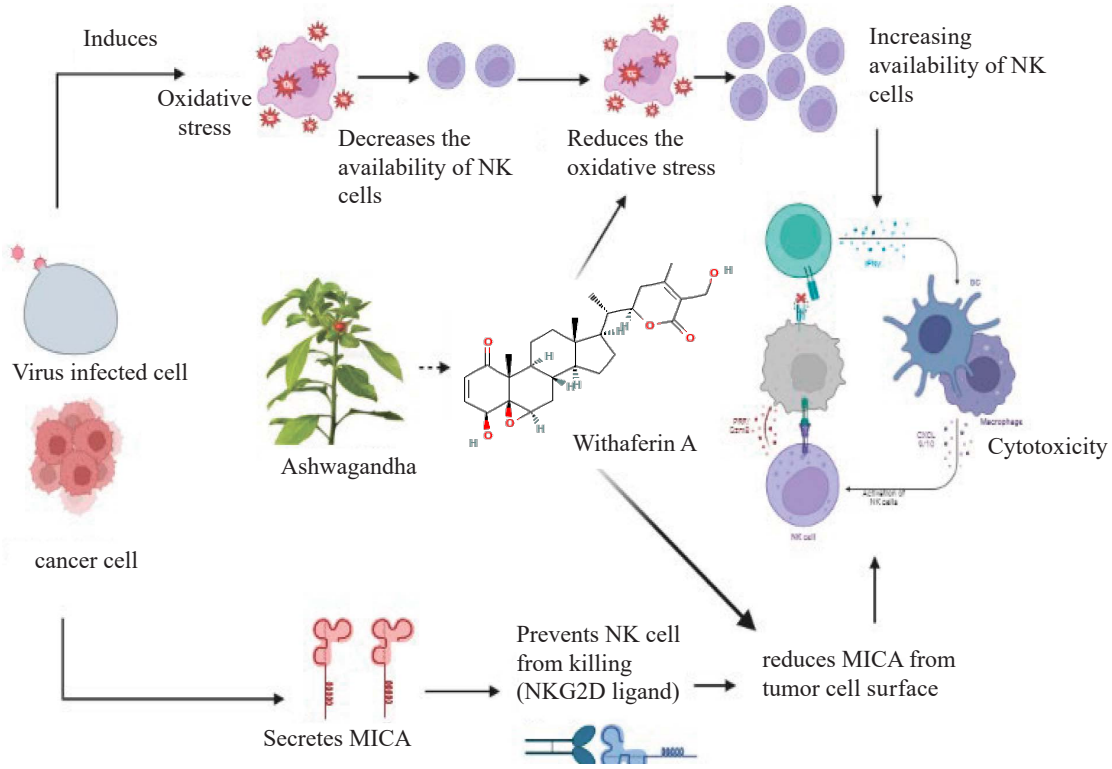


Figure 2 Effect of ashwagandha on natural killer cells

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

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

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

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

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

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

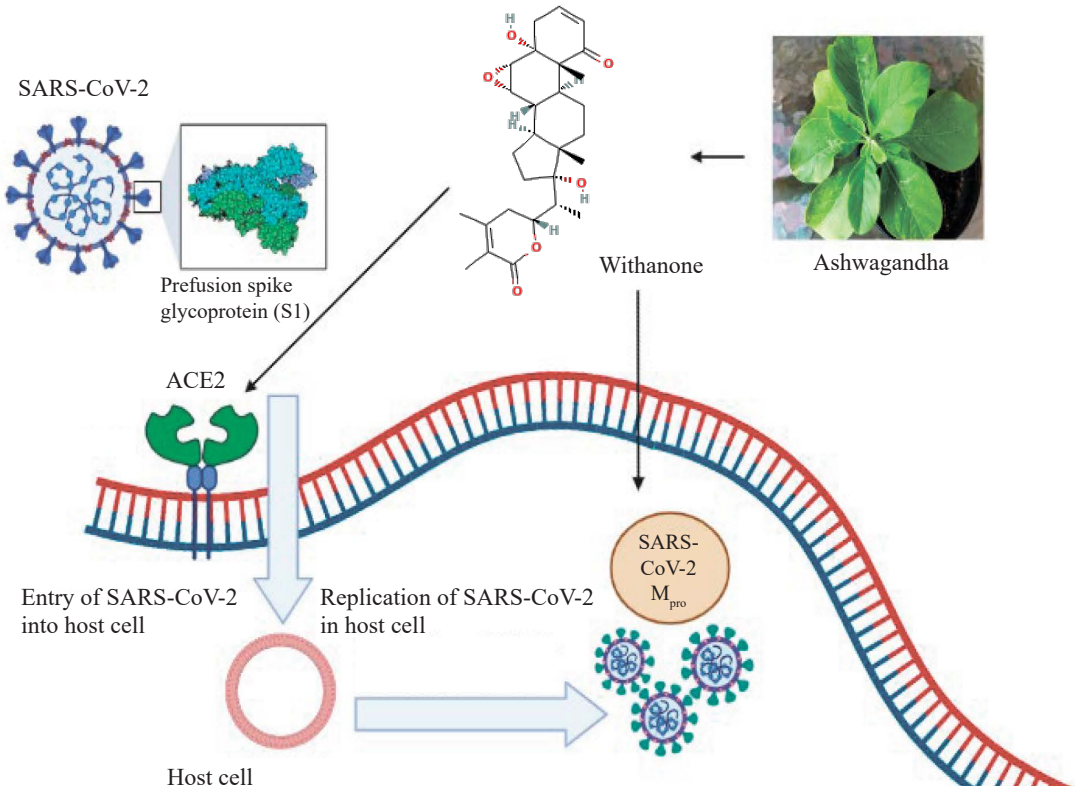


Figure 3 Effect of ashwagandha on SARS-CoV-2

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

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

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

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

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

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

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

Table 2 Application and utilisation of ashwagandha

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

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

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

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

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

CONCLUSION

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

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

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

CONTRIBUTION

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

CONFLICT OF INTEREST

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

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REFERENCES

1. Amritha N, Bhooma V, Parani M. Authentication of the market samples of Ashwagandha by DNA barcoding reveals that powders are significantly more adulterated than roots. Journal of Ethnopharmacology. 2020;256. https://doi.org/10.1016/j.jep.2020.112725
2. Bonilla DA, Moreno Y, Gho C, Petro JL, Odriozola-Martínez A, Kreider RB. Effects of Ashwagandha (*Withania somnifera*) on physical performance: Systematic review and bayesian meta-analysis. Journal of Functional Morphology and Kinesiology. 2021;6(1). https://doi.org/10.3390/jfmk6010020
3. Wal A, Wal P, Rai AK, Tiwari R, Prajapati SK. Adaptogens with a special emphasis on *Withania somnifera* and *Rhodiola rosea*. In: Bagchi D, Nair S, Sen CK, editors. Nutrition and enhanced sports performance. Muscle building, endurance, and strength. Academic Press; 2019. pp. 407–418. https://doi.org/10.1016/B978-0-12-813922-6.00034-5
4. Mahato A, Chakraborty I, Baidya BK. Preparation and evaluation of fruit candy from unripe mango. International Journal of Chemical Studies. 2020;8(1):2727–2731. https://doi.org/10.22271/chemi.2020.v8.i1ao.8682
5. Moharana D, Bahadur V, Rout S, Prusty AK, Sahoo RK. Ashwagandha: The miracle ginseng. Food and Scientific Reports. 2020;1(10):37–42.
6. Paul S, Chakraborty S, Anand U, Dey S, Nandy S, Ghorai M, *et al. Withania somnifera* (L.) Dunal (Ashwagandha): A comprehensive review on ethnopharmacology, pharmacotherapeutics, biomedicinal and toxicological aspects. Biomedicine and Pharmacotherapy. 2021;143. https://doi.org/10.1016/j.biopha.2021.112175
7. Bharti VK, Malik JK, Gupta RC. Ashwagandha: Multiple health benefits. In: Gupta RC, editor. Nutraceuticals. Efficacy, safety and toxicity. Academic Press; 2016. pp. 717–733. https://doi.org/10.1016/B978-0-12-802147-7.00052-8
8. Zahiruddin S, Basist P, Parveen A, Parveen R, Khan W, Gaurav, *et al.* Ashwagandha in brain disorders: A review of recent developments. Journal of Ethnopharmacology. 2020;257. https://doi.org/10.1016/j.jep.2020.112876
9. Bhatt N. Drug discovery from Ayurveda: Biological advances and integrative synergies. In: Bhatt N, editors. Integrative perspectives: Ayurveda, phytopharmaceuticals and natural products. Pune: Continental Prakashan; 2020. pp. 496–512.
10. Mandlik DS, Namdeo AG. Pharmacological evaluation of Ashwagandha highlighting its healthcare claims, safety, and toxicity aspects. Journal of Dietary Supplements. 2021;18(2):183–226. https://doi.org/10.1080/19390211.2020.1741484
11. John J. Therapeutic potential of *Withania somnifera*: A report on phyto-pharmacological properties. International Journal of Pharmaceutical Sciences and Research. 2014;5(6):2131–2148. https://doi.org/10.13040/IJPSR.0975-8232.5(6).2131-48

12. Ageyeva NM, Khrapov AA, Shirshova AA, Chemisova LE, Ulyanovskaya EV, Chernutskaya EA. The elemental profile of ciders made from different varieties of apples. Foods and Raw Materials. 2024;12(2):273–282. https://doi.org/10.21603/2308-4057-2024-2-604
13. Ojileh PC, Okechukwu QN. Value-added zobo drink with date juice. Food Processing: Techniques and Technology. 2023;53(3):545–553. https://doi.org/10.21603/2074-9414-2023-3-2453
14. Gaurav N, Kumar A, Tyagi M, Kumar D, Chauhan UK, Singh AP. Morphology of *Withania somnifera* (distribution, morphology, phytosociology of *Withania somnifera* L. Dunal). International Journal of Current Science Research. 2015;1(7):164–173.
15. Meher SK, Das B, Panda P, Bhuyan GC, Rao MM. Uses of *Withania somnifera* (Linn) Dunal (Ashwagandha) in Ayurveda and its pharmacological evidences. Research Journal of Pharmacology and Pharmacodynamics. 2016;8(1):23–29. https://doi.org/10.5958/2321-5836.2016.00006.9
16. Nile SH, Nile A, Gansukh E, Baskar V, Kai G. Subcritical water extraction of withanosides and withanolides from ashwagandha (*Withania somnifera* L) and their biological activities. Food and Chemical Toxicology. 2019;132. https://doi.org/10.1016/j.fct.2019.110659
17. Shah N, Singh R, Sarangi U, Saxena N, Chaudhary A, Kaur G, *et al.* Combinations of Ashwagandha leaf extracts protect brain-derived cells against oxidative stress and induce differentiation. PLoS ONE. 2015;10(3). https://doi.org/10.1371/journal.pone.0120554
18. Maurya SP, Das BK, Singh R, Tyagi S. Effect of *Withania somnifer* on CD38 expression on CD8+ T lymphocytes among patients of HIV infection. Clinical Immunology. 2019;203:122–124. https://doi.org/10.1016/j.clim.2019.04.003
19. Oza VP, Parmar PP, Kumar S, Subramanian RB. Anticancer properties of highly purified L-asparaginase from *Withania somnifera* L. against acute lymphoblastic leukemia. Applied Biochemistry and Biotechnology. 2010;160:1833–1840. https://doi.org/10.1007/s12010-009-8667-z
20. Balkrishna A, Pokhrel S, Singh J, Varshney A. Withanone from Withania somnifera may inhibit novel coronavirus (COVID-19) entry by disrupting interactions between viral S-protein receptor binding domain and host ACE2 receptor. 2020. https://doi.org/10.21203/rs.3.rs-17806/v1
21. Shree P, Mishra P, Selvaraj C, Singh SK, Chaube R, Garg N, *et al.* Targeting COVID-19 (SARS-CoV-2) main protease through active phytochemicals of ayurvedic medicinal plants – *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Giloy) and *Ocimum sanctum* (Tulsi) – A molecular docking study. Journal of Biomolecular Structure and Dynamics. 2022;40(1):190–203. https://doi.org/10.1080/07391102.2020.1810778
22. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Allergy, Asthma and Clinical Immunology. 2018;14(2). https://doi.org/10.1186/s13223-018-0278-1
23. Tharakan A, Shukla H, Benny IR, Tharakan M, George L, Koshy S. Immunomodulatory effect of *Withania somnifera* (Ashwagandha) extract – A randomized, double-blind, placebo controlled trial with an open label extension on healthy participants. Journal of Clinical Medicine. 2021;10(16). https://doi.org/10.3390/jcm10163644
24. Esmaealzadeh N, Iranpanah A, Sarris J, Rahimi R. A literature review of the studies concerning selected plant-derived adaptogens and their general function in body with a focus on animal studies. Phytomedicine. 2022;105. https://doi.org/10.1016/j.phymed.2022.154354
25. Sharma L. Immunomodulatory effect and supportive role of traditional herbs, spices and nutrients in management of COVID-19. 2020. https://doi.org/10.20944/preprints202009.0026.v1
26. Singh B, Sharma HK, Kaur R. Storage stability of osmo-convective dried beetroot candy. Journal of Pharmacognosy and Phytochemistry. 2020;9(5):2704–2708.
27. Saleem S, Muhammad G, Hussain MA, Altaf M, Bukhari SNA. *Withania somnifera* L.: Insights into the phytochemical profile, therapeutic potential, clinical trials, and future prospective. Iranian Journal of Basic Medical Sciences. 2020;23(12):1501–1526. https://doi.org/10.22038/ijbms.2020.44254.10378
28. Mofed D, Ahmed W, Zekri A-R, Said O, Rahouma M, Faraag AHI. The antiviral efficacy of *Withania somnifera* (Ashwagandha) against hepatitis C virus activity: *In vitro* and *in silico* study. Advances in Microbiology. 2020;10(9):463–477. https://doi.org/10.4236/aim.2020.109035
29. Grunz-Borgmann E, Mossine V, Fritsche K, Parrish AR. Ashwagandha attenuates TNF-α- and LPS-induced NF-κB activation and CCL2 and CCL5 gene expression in NRK-52E cells. BMC Complementary Medicine and Therapies. 2015;15. https://doi.org/10.1186/s12906-015-0958-z
30. Pal A, Kumar KH, Bhushan B, Saharan V. Ashwagandha root extract inhibits acetylcholine esterase, protein modification and ameliorates H_2O_2-induced oxidative stress in rat lymphocytes. Pharmacognosy Journal. 2017;9(3):302–309. https://doi.org/10.5530/pj.2017.3.52
31. Cordo' V, van der Zwet JCG, Canté-Barrett K, Pieters R, Meijerink JPP. T-cell acute lymphoblastic leukemia: A roadmap to targeted therapies. Blood Cancer Discovery. 2021;2(1):19–31. https://doi.org/10.1158/2643-3230.BCD-20-0093

32. Turrini E, Calcabrini C, Sestili P, Catanzaro E, de Gianni E, Diaz AR, *et al. Withania somnifera* induces cytotoxic and cytostatic effects on human T leukemia cells. Toxins. 2016;8(5). https://doi.org/10.3390/toxins8050147

33. Yang J, Wang L, Guan X, Qin J-J. Inhibiting STAT3 signaling pathway by natural products for cancer prevention and therapy: *In vitro* and *in vivo* activity and mechanisms of action. Pharmacological Research. 2022;182. https://doi.org/10.1016/j.phrs.2022.106357

34. Cook KD, Waggoner SN, Waggoner SN, Whitmire JK. NK cells and their ability to modulate T cells during virus infections. Critical Reviews™ in Immunology. 2014;34(5):359–388. https://doi.org/10.1615/CritRevImmunol.2014010604

35. Ochando J, Mulder WJM, Madsen JC, Netea MG, Duivenvoorden R. Trained immunity – basic concepts and contributions to immunopathology. Nature Reviews Nephrology. 2023;19:23–37. https://doi.org/10.1038/s41581-022-00633-5

36. Trivedi MK, Jana S. *In vitro* evaluation of immunomodulatory effects of the test formulation by the estimation of natural killer cells and phagocytosis activities. Cell and Cellular Life Sciences Journal. 2019;4(1). https://doi.org/10.2174/1871523017666181116092934

37. Khan MA, Ahmed RS, Chandra N, Arora VK, Ali A. *In vivo*, extract from *Withania somnifera* root ameliorates arthritis *via* regulation of key immune mediators of inflammation in experimental model of arthritis. Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry. 2019;18(1):55–70. https://doi.org/10.2174/1871523017666181116092934

38. Barua A, Bradaric MJ, Bitterman P, Abramowicz JS, Sharma S, Basu S, *et al.* Dietary supplementation of *Ashwagandha* (*Withania somnifera*, Dunal) enhances NK cell function in ovarian tumors in the laying hen model of spontaneous ovarian cancer. American Journal of Reproductive Immunology. 2013;70(6):538–550. https://doi.org/10.1111/aji.12172

39. Ciotti M, Ciccozzi M, Terrinoni A, Jiang W-C, Wang C-B, Bernardini S. The COVID-19 pandemic. Critical Reviews in Clinical Laboratory Sciences. 2020;57(6):365–388. https://doi.org/10.1080/10408363.2020.1783198

40. Fraser N, Brierley L, Dey G, Polka JK, Pálfy M, Nanni F, *et al.* Preprinting the COVID-19 pandemic. bioRxiv. 2020. https://doi.org/10.1101/2020.05.22.111294

41. Srivastav S, Singh P, Mishra G, Jha KK, Khosa RL. Achyranthes aspera – An important medicinal plant: A review. Journal of Natural Product and Plant Resources. 2011;1(1):1–14.

42. Aboyewa JA, Sibuyi NRS, Meyer M, Oguntibeju OO. Green synthesis of metallic nanoparticles using some selected medicinal plants from southern Africa and their biological applications. Plants. 2021;10(9). https://doi.org/10.3390/plants10091929

43. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, *et al.* SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020;181(2):271–280. https://doi.org/10.1016/j.cell.2020.02.052

44. Munir M, Nadeem M, Qureshi TM, Jabbar S, Atif FA, Zeng X. Effect of protein addition on the physicochemical and sensory properties of fruit bars. Journal of Food Processing and Preservation. 2016;40(3):559–566. https://doi.org/10.1111/jfpp.12635

45. Panda AK, Kar S. Ayurvedic immuno booster: Is it myth or reality in COVID-19 pandemic. International Journal of Current Research and Review. 2021;13(1):134–140. https://doi.org/10.31782/IJCRR.2021.13140

46. Sharifi-Rad J, Quispe C, Ayatollahi SA, Kobarfard F, Staniak M, Stępień A, *et al.* Chemical composition, biological activity, and health-promoting effects of *Withania somnifera* for pharma-food industry applications. Journal of Food Quality. 2021;2021. https://doi.org/10.1155/2021/8985179

47. Baghel S, Saraugi SS, Kumar A, Rathore A, Soni DK. Sensory and physical evaluation of herbal biscuit incorporated with Ashwagandha (*Withania somnifera*) and Ragi (*Eleusine coracana*). International Journal of Chemical Studies. 2020;8(5):136–142. https://doi.org/10.22271/chemi.2020.v8.i5b.10290

48. Abayathilaka WS, Perera GAAR, Rathnayake AMGN, Subasinghe UGPP. Developing a low-calorie green tea based ready-to-drink beverage using Brahmi (Bacopa monnieri), Ashwagandha (Withania somnifera), Stevia (Stevia rebaudiana) & Ceylon Cinnamon (Cinnamomum zeylanicum). 2021.

49. Dhole RR, Undratwad DT, Khadse PN, Meshram TA. Studies on sensory evaluation and cost structure of low-fat flavoured milk incorporated with ashwagandha (*Withania somnifera*) root powder. The Pharma Innovation Journal 2022;11(8):950–954.

50. Sinha M, Mani A, Sinha P. Value addition of guava cheese cv. Allahabad safeda by medicinal herbs. Journal of Pharmacognosy and Phytochemistry. 2017;6(6):856–859.

51. Indu PC, Awasthi P. Development and evaluation of cereal-legume based ladoo supplemented with Ashwagandha (*Withania somnifera*). The Pharma Innovation Journal. 2018;7(7):358–362.

52. Prabu PC, Panchapakesan S, Raj CD. Acute and sub-acute oral toxicity assessment of the hydroalcoholic extract of *Withania somnifera* roots in Wistar rats. Phytotherapy Research. 2013;27(8):1169–1178. https://doi.org/10.1002/ptr.4854
53. Kushwaha S, Betsy A, Chawla P. Effect of *Ashwagandha* (*Withania somnifera*) root powder supplementation in treatment of hypertension. Ethno-Medicine. 2012;6(2):111–115. https://doi.org/10.31901/24566772.2012/06.02.05
54. Xing D, Yoo C, Gonzalez D, Jenkins V, Nottingham K, Dickerson B, *et al.* Effects of acute ashwagandha ingestion on cognitive function. International Journal of Environmental Research and Public Health. 2022;19(19). https://doi.org/10.3390/ijerph191911852
55. Lopresti AL, Smith SJ, Malvi H, Kodgule R. An investigation into the stress-relieving and pharmacological actions of an ashwagandha (*Withania somnifera*) extract: A randomized, double-blind, placebo-controlled study. Medicine. 2019;98(37). https://doi.org/10.1097%2FMD.0000000000017186

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