

A comprehensive review of phytochemicals of *Withania somnifera* (L.) Dunal (Solanaceae) as antiviral therapeutics

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Abstract

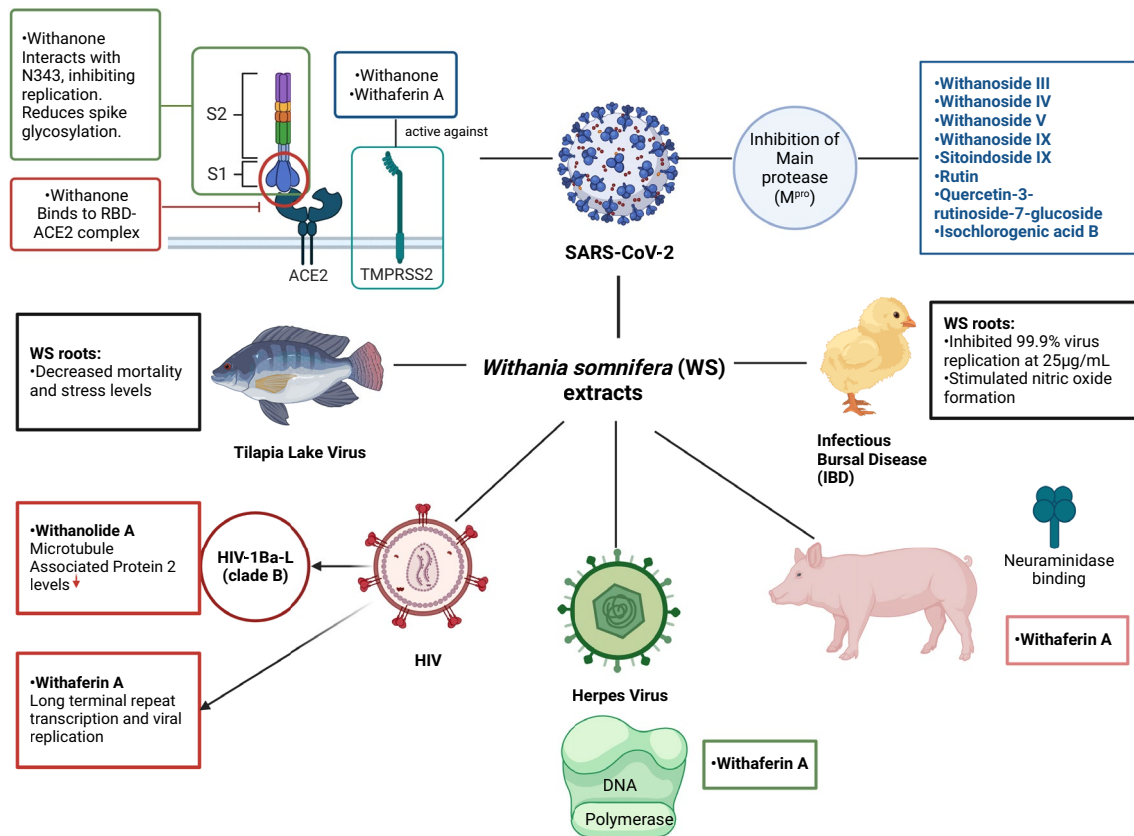
Viruses have caused millions and billions of infections and high mortality rates without successful immunization due to a lack of antiviral drugs approved for clinical use. Therefore, the discovery of novel antiviral drugs is impertinent and natural products are excellent alternative sources. *Withania somnifera* (L.) Dunal (Solanaceae) is recognized as one of the most significant herbs in the Ayurvedic system and it had been utilized in various biological actions for more than 3000 years. This review aimed to discuss the therapeutic effects and associated molecular mechanisms of *Withania somnifera* (WS) and its phytochemicals, withanolides against various viruses in preclinical and clinical settings towards developing potential inhibitors which could target virus proteins or their respective host cell receptors. WS was reported to attenuate coronavirus disease 2019 (COVID-19), serve as a potential ligand against the herpes simplex virus (HSV) DNA polymerase, suppress Alzheimer's disease progression by inhibiting the cytotoxicity induced by the human immunodeficiency virus 1 (HIV-1)-activated beta-amyloid (A β), and attenuate the neuraminidase activity of H1N1 influenza. WS root extracts have also reduced the mortality rates and stress levels in tilapia infected with tilapia lake virus (TiLV), and stimulated antiviral

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nitric oxide formation in chicks infected with infectious bursal disease (IBD). With increasing evidence from previous literatures, further in vitro and in vivo investigations of WS against other viral infections may provide promising results.

Graphical Abstract



Antiviral activity and underlying molecular mechanisms of *Withania somnifera* and its phytochemicals

Article highlights

- Withanolides have been predicted to inhibit SARS-CoV-2 viral proteins.
- Withaferin A has been predicted to alter HSV DNA polymerase's binding conformation.
- Withaferin A also suppressed HIV-1 transcription and replication.

Keywords Phytochemical · Antiviral · *Withania somnifera* · Withanolides · Molecular mechanism · Nanoparticles

1 Introduction

Global health is considerably influenced by viruses and it is clearly noticeable that a prominent reason for deaths worldwide is attributed by virus-borne pandemics/epidemics in the past and present. The most recent virus-borne pandemic which accumulated a total of over 600 million cases and 6.5 million deaths by late August 2022 is the coronavirus disease 19 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1, 2]. Although there are numerous antiviral treatments available, merely a few of them possess effective therapeutic potential. Expensive synthetic antiviral drugs, drug resistance, and associated side effects are key obstacles to the administration of effective

antiviral treatments. Infrequent side effects and a broader therapeutic window are the chief benefits of traditional Indian medicines over the modern medicinal system [3].

Some clinically significant viral diseases include sexually transmitted diseases such as herpes simplex virus (HSV) infecting 3.7 billion people under the age of 50 (HSV-1) and infecting 491 million individuals between the ages of 15 and 49 (HSV-2), and the human immunodeficiency virus (HIV) which cause 40.1 million deaths thus far, as well as respiratory infections such as SARS-CoV-2, which has taken more than 6,584,104 lives since the start of the pandemic in 2019 and the most widespread and adaptive influenza viruses [4]. Viruses such as the tilapia lake virus (TiLV) with mortality rates ranging from 20 to 90% and infectious bursal disease (IBD) virus in chicks with a mortality rate of 90% in very virulent genotypes, have significantly impacted the aquaculture and farming industries. For most of these major viral diseases, there are either no available treatments or the efficacy of available vaccines or drugs are limited and receding [4, 5].

Humans have been utilizing natural products derived from various sources such as plants, animals, minerals, and microorganisms for the treatment of various diseases for at least 60,000 years [6, 7]. In recent decades, natural products have been drawing the attention of researchers as they provide an extensive range of chemical diversity responsible for various biological effects and their drug-like properties. Besides, natural products has gained importance as resources for the development of new lead compounds and drugs for treating various diseases [8]. Their functional efficacy is concerned with three-dimensional chemical and steric properties and presents many leads in terms of their selectivity and efficiency of molecular targets. A vast amount of research has revealed the significant contribution of natural products in terms of drug development [9, 10].

Withania somnifera (L.) Dunal (Solanaceae) is recognized as one of the most significant herbs in the Ayurvedic system, which has been utilized mainly in the management of stress, anxiety [11], depression [12], improvement in cognition and strength elevation [13], combat inflammation [14], and cortisol level for more than 3000 years [15]. Various parts of *Withania somnifera* (WS) such as roots, leaves, flowers, and stems have various therapeutic values due to the presence of an assortment of phytochemicals [12–16]. WS roots have been extensively used in Ayurvedic and Unani medicinal systems. It contains 0.13–0.31% of alkaloids, glycosides, amino acids, steroids, volatile oils, starch, and reducing sugars [17]. Along with the roots, its leaves were also utilized for the treatment of swelling and fever, whereas the flowers were used for astringent and diuretic treatment. WS fruits are active against skin ulcers and tumors, while its seeds are used in increasing sperm count and testicular growth [18].

Pharmacologically, WS extracts and its phytochemicals have a plethora of therapeutic activities as authenticated by various studies. Studies have reported its anticancer, anti-inflammatory, antidiabetic, immunomodulatory, cardioprotective, anti-Alzheimer, anti-microbial, antioxidant, anxiolytic, antidepressant, adaptogenic, neuroprotective, anti-Parkinson's, and anti-Huntington's activities as well as protective effects against drug-addiction, cerebral ischemia, and epilepsy [17, 19]. Some of the most potent antiviral phytochemicals of WS discussed in previous literatures include withanone, withaferin A, and withanolide A along with other WS derivatives with potential in inhibiting against viral transcription, viral replication, and viral entry [20–25].

The paper presents a detailed and updated review of therapeutic effects and associated molecular mechanisms of WS extracts and isolated phytochemicals against different viral diseases in preclinical and clinical settings. In the next section, we discussed the distribution, taxonomic description, phytochemicals, pharmacokinetics, and bioavailability of WS as well as its toxicological effectiveness in preclinical and clinical settings. In Sect. 3, apart from the antiviral mechanistic studies of WS, we also discussed the prediction of the molecular targets derived from both virus and host cells using the in silico molecular docking approach. Lastly, in Sect. 4, we also provided insights on the potential strategies to enhance WS delivery and active targeting while reducing the off-target toxicity. Figure 1 illustrates the complete overview of the entire manuscript. Based on the findings from relevant studies, WS and its phytochemicals could be promising agents for antiviral prophylactic and clinical care.

2 *Withania somnifera*

2.1 Distribution and taxonomic description

WS is the most widespread species in its genus and is an evergreen, xerophytic, woody, short, tender, and perennial shrub that grows about 2 m tall and 1 m wide [17]. WS has fine and short hairs that are silver-grey in color covering its branches which protrude outward from an upright, brownish central stem. The leaves have dense hair on its underside, are green and almost hairless on the upper surface, and grow in alternating positions (opposite to flowering shoots) [17].

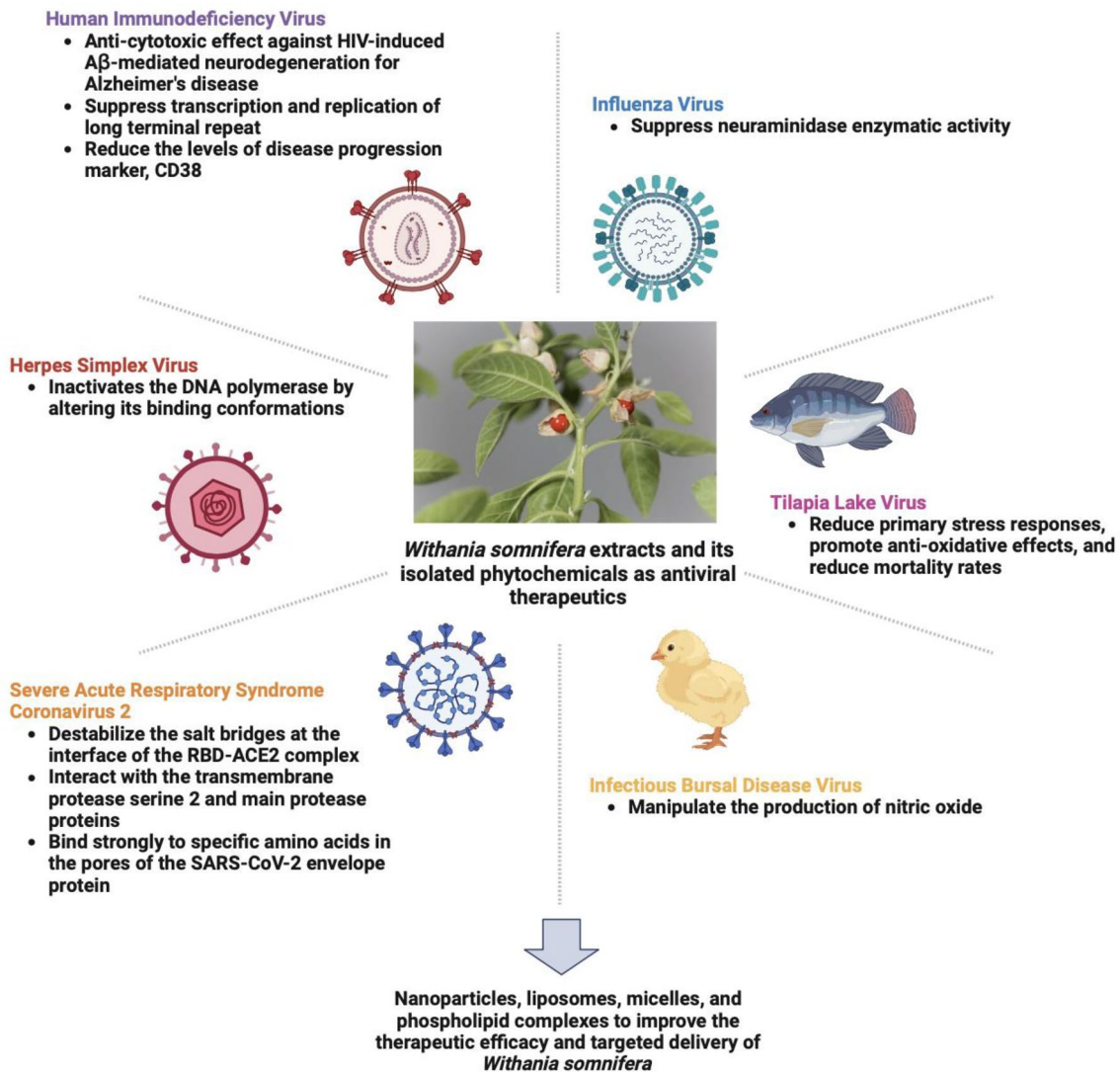


Fig. 1 Summary of the therapeutic effects and associated molecular mechanisms of *Withania somnifera* extracts and isolated phytochemicals against different viral diseases

The flowers are dull, simple, glabrous, small, green, and bell-shaped with green elliptic leaves. The fruits contain seeds which are kidney-shaped and pale brown in color, compressed on a rough, netted surface. The roots are fleshy, short and possess fibrillary secondary branches extending from the main root. The roots exhibit a strong odor and an acrid, bitter taste [26]. It is found naturally in arid locations spreading from tropical Africa and South Africa to the Mediterranean, as well as from the Middle East and Arabia to the Canary and Cape Verde Islands. WS is also broadly distributed in Baluchistan, Afghanistan, Sri Lanka, Pakistan, Nepal, China and India, particularly in West Bengal, Punjab, Uttar Pradesh, Gujarat, Rajasthan and Maharashtra [17, 19]. In various countries like India, it is grown as a medicinal crop mostly for its fleshy roots that possess a plethora of phytochemicals having several pharmacological implications. Although WS has become a naturalized weed in New South Wales and South Australia, the warmer parts of Europe cultivate WS in gardens [17, 27].

2.2 Phytochemicals of *Withania somnifera* and their classification

A countless number of phytochemicals have been extracted from different parts of WS using various solvents such as aqueous methanol, n-hexane, ethyl acetate, and water, with the assistance of diverse separation methods like column chromatography (i.e., reverse phase and size exclusion), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Chlorinated withanolides obtained from an ethyl acetate-soluble fraction of the crude extract

were subjected to repeated column chromatography and a single crystal X-ray diffraction experiment to establish their structures [28]. Besides, nuclear magnetic resonance (NMR) studies have identified other chlorinated compounds [28].

WS fruit extracts made in aqueous methanol, n-hexane and water along with a combination of gas chromatography-mass spectroscopy (GC-MS) and NMR were used to identify metabolites that varied chemically containing fatty acids, organic acids, aliphatic and aromatic amino acids, phenolic acids, withanamides, sterols, polyols, tocopherols and sugars. Based on previous reports, over 12 alkaloids, 35 withanoloids, and many sitoindosides were among several phytoconstituents found in different parts of WS by various phytochemical studies. Only a small number are the main players in displaying pharmacological characteristics [19].

Withanolides are steroidal compounds of the ergostane type with a δ -lactone present between the C22 and C26 atoms and a C1 oxidized position [12, 29, 30]. They are regarded as marker compounds because they are characteristically found in Solanaceae members, particularly in the *Withania* genus. Furthermore, withanolides are similar to phytoconstituents present in *Panax ginseng* in terms of the chemical structure, thus WS is also known as the “Indian Ginseng” [19]. It was discovered that several alkaloids, such as withanine, withananine, somniferine, somniferinine, tropine, pseudotropine, pseudowithanine, choline, 3- α -gloyloxytropine, cuscohygrine, anahydrine, anaferine and isopelletierene, are also present in WS. From the roots of WS, two acylsterylglucosides (i.e., sitoindoside VII and sitoindoside VIII) along with two glycowithanolides (i.e., sitoindoside IX and sitoindoside X) were isolated [19]. Other polyphenols like catechin, naringenin, syringic acid, and p-coumaric acid were also found in significant quantities in WS extracts [31]. Table 1 summarizes various classes of phytochemicals present in WS, while the organ-specific distribution of phytochemicals in WS is presented in Fig. 2.

2.3 Pharmacokinetics and bioavailability

The pharmacokinetics and bioavailability of a substance should be known before its administration for disease treatment. However, the pharmacokinetic and pharmacodynamic (PK/PD) relationships remain a consistent challenge due to the difficulty in the identification and characterization of phytochemicals in medicinal plants [32, 33]. Consequently, after oral administration, it is of great interest to investigate the characterization of absorbed compounds, tissue distribution, pharmacokinetics and elimination patterns of phytochemicals from the body [32–34]. This collective data serves as a critical goal for determining clinical and pharmacological efficacy, resulting in limited information on how the body reacts to the drug/extract consumed. However, differentiating factors can be observed among chemical compounds, comparative and/or synergistic absorption, distribution, metabolism, and excretion (ADME), when the entire plant extract is administered to a human or an animal as opposed to the individual constituents. The phytochemicals of WS present effective and relevant biological activities in preclinical and clinical studies. Interestingly, the pharmacokinetics, dosage, route of administration, and biopharmaceutical profile of WS have been reported [35].

The prediction of the absorption, distribution, metabolism, excretion, toxicity (ADMET), and molecular properties of WS phytochemicals have been reported in an *in silico* study using various online tools, such as admetSAR [36–38]. Similarly, molecular descriptors of WS phytochemicals were examined for Lipinski’s rule of five (ROF). The molecular weight of most detected phytochemicals ranged between 450–500 g/mol, with the exception of withanoside IV at 782.92 g/mol and withanoside V at 766.92 g/mol [35]. Molecular weight is an indispensable aspect when considering drug bioavailability, because as the molecular weight increases, the bulkiness of the compounds also increases thereby affecting bioavailability.

Table 1 Classification of phytochemicals present in *Withania somnifera* [19]

Active constituents	Phytochemicals
Alkaloids	Withanine, withananine, withasomnine, somniferine, somniferinine, somninine, nicotine pseudowithanine, scopoletin, tropeltigloate, and visamine
Flavonoids	Kaempferol and quercetin
Steroids	Cholesterol, sitoinosides VII, VIII, XI, X, diosgenin, stigmasterol, β -sitosterol and stigmastadien
Steroidal lactone	Withaferin-A, withanolide A, E, F, G, H, I, J, K, L, M, and withanone
N-containing compounds	Withanol, somnitol, and somnisol
Glycosides	Sitoindoside VII and VIII
Salts	Cuscohygrine, tropine, pseudotropine, anahygrine, and anaferine

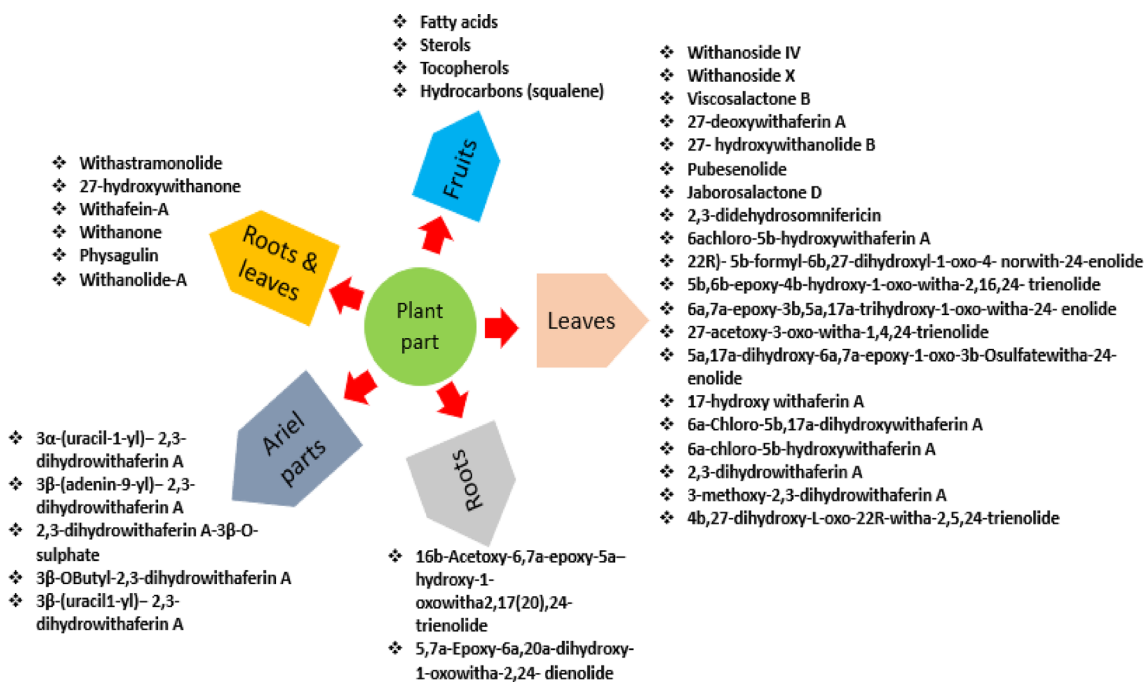


Fig. 2 The organ-specific distribution of phytochemicals in *Withania somnifera*

Adequate bioavailability was exhibited by WS during preclinical assessment [39, 40]. In a recent study, male rats were administered with withaferin A at a dose level of 5 mg/kg intravenously and 10 mg/kg orally, and the absolute bioavailability was reported to be $32.4 \pm 4.8\%$ [39].

Additionally, an *in vitro* study demonstrated that withaferin A was freely absorbed through Caco-2 cells and did not act as a P-glycoprotein substrate [39]. Usually, drugs given through the oral route interact with intestinal bacteria, and withaferin A is sensitive to degradation by bacterial enzymes. Therefore, the stability of withaferin A was evaluated in male rats and human intestines [39]. It was found that the stability of withaferin A in male rats was not significantly different as compared to humans. Withaferin A was stable in simulated gastric fluid, moderately metabolized in intestinal fluid containing bacteria, and rapidly biotransformed (half-life of 5.6 min) in hepatic microsomes. Rat intestine-live *in situ* perfusion was performed to assess the preliminary metabolism of withaferin A. The results demonstrated that the concentration of withaferin A fell swiftly and was reported to be 27.1% in one hour, whereas the concentrations of three main metabolites (i.e., M1, M4, and M5) were increased [39].

The concentrations of withaferin A and withanolide A were also determined by Patil et al. (2013) in mice plasma using HPLC/MS/MS [40]. This method exhibited r^2 value > 0.997 demonstrating a fine linearity within the concentration range of 0.484–117.880 ng/mL for withaferin A and 0.476–116.050 ng/mL for withanolide A. The values of lower bounds of quantification (LLOQs) for withaferin A (0.484 ng/mL) and withanolide A (0.476 ng/mL) were well below $C_{max}/20$, depicting that the method shows acceptable susceptible to perceive withanolides in plasma. This established method was efficiently utilized in the determination of withanolide A and withaferin A pharmacokinetics in mice plasma after oral administration of aqueous root extract of WS. It was documented that withanolides were quickly bioavailable after oral administration, whereas the relative absorption of withaferin A was almost twice as compared to withanolide A [40].

Drug solubility is one of the essential parameters that influences pharmacodynamics and pharmacokinetics throughout, from the administration, absorption into the systemic circulation, movement in the blood, to even the excretion process [41]. The ADMET features primarily represent the likelihood in predicting the certainty of a characteristic between 0 and 1, providing information on a drug candidate and its pharmacokinetics via the prediction database [42]. Based on the literature, all three potent antiviral withanolides from WS extracts (i.e., withanone, withaferin A, and withanolide A) can be absorbed from the intestine. Additionally, an *in vivo* pharmacokinetic study has shown that in the systemic circulation they exhibit a maximum oral bioavailability [35–37].

2.4 Toxicological effectiveness of *Withania somnifera* in preclinical and clinical settings

A total of 36 clinical studies from both indexed and non-indexed journals were found on [clinicaltrials.gov](#) [41, 43]. These studies have showed that root preparation is effective in treating conditions such as schizophrenia, chronic stress, anxiety, hypothyroidism, insomnia, type 2 diabetes, obsessive–compulsive disorder, cognitive improvement, rheumatoid arthritis and male infertility in humans. Additionally, WS root extract demonstrated adaptogenic, tonic, and growth-promoting activity in healthy adults and children, as well as tiredness reduction and improvement in quality of life in chemotherapy-treated cancer patients, as well as an increase of fertility and sexual performance in healthy females. In a randomized, double-blind, placebo-controlled trial in which individuals with subclinical hypothyroidism were treated with standardized aqueous WS root extract containing 5% withanolides, the results showed that serum thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) levels were significantly decreased [44]. Importantly, the patients' responses to the treatment in terms of physical, hematologic, and vital signs, including blood pressure, pulse rate, respiratory rate, and body temperature, demonstrated no substantial safety issues [44]. A case study reported that the patient's serum T3 and T4 levels remained within the normal range during treatment [45]. After 20 days of daily administration of WS root extract (1.4 g/kg body weight), T4 levels in a preclinical study on female mice were considerably raised ($p < 0.001$) but not the T3 concentration [46], indicating its prothyroidic or thyroid stimulatory role (with little or no role in the T3 generation). Therefore, WS is highly effective and may heal a variety of illnesses.

Several parts of WS have been used in Ayurvedic medicine from ancient times and have been shown to be safe. Several toxicological studies have been conducted to support the safety and efficacy of WS. The results of acute and subacute toxicity tests have indicated that WS is safe and effective. Sharada et al. (1993) investigated the acute (24 h) and subacute (30 days) toxicity of alcoholic WS root extract in typical Swiss albino mice and Wistar rats. The mice in this study showed a comparatively high level of tolerance to WS whereby at 1100 mg/kg, there was no acute mortality observed; however, when the dosage was increased, the death rate was rapidly increased. After receiving a 1500 mg/kg dose, no animal survived. Thus, 1260 mg/kg was determined to be the fatal dosage of 50% of the extract. In addition, "acute oral toxicity study" was carried out and it was discovered that 2000 mg/kg dosage did not induce any clinical symptoms of toxicity in any of the rats tested. All animals were sacrificed at the end of the experiment, and no abnormalities in organs were found. Furthermore, an external inspection of rats sacrificed at the conclusion revealed no abnormalities with pathological consequences. There were no deaths in either the treatment or control groups during the research. When all animals (treated and control groups) had completed the 90-day dosage period (120-day dosing period for the recovery groups), they were sacrificed and evaluated to ascertain the overall pathological outcomes [47].

The acute and chronic toxicity of the hydro-alcoholic extract of WS roots were evaluated in female albino rats at a dosage of 1000 mg/kg body weight. According to the observation, an initial exhilaration was followed by dullness, sadness, decreased motor neuron activity, and decreased breathing [48]. Another sub-chronic toxicity study was conducted in accordance with the Organization for Economic Cooperation and Development criteria for chemical testing (No. 408). In this study, three groups of 20 rats (10 males and 10 females) were administered with pure WS extracts orally seven days a week for 90 days at dosages of 100 mg/kg body weight (low dose), 500 mg/kg body weight (medium dose), and 1,000 mg/kg body weight (high dose), respectively. There were no significant changes in body weight increase or feed intake between the treatment and control groups of animals [49].

In summary, WS is still a relatively new plant in current research and there have not been many advancements in the study of its toxicity even though its therapeutic effects are highly promising.

3 Preventive and therapeutic antiviral activities and associated molecular mechanism of *Withania somnifera* and its phytochemicals

Researchers have reported antiviral, immunomodulatory, and anti-inflammatory activities of WS dating as early as 1979 [50]. Withanolide A and withaferin A are steroidal lactones obtained from WS that possess good anti-tumor and anti-inflammatory activities. Withaferin A has been shown to reduce the release of several cytokines in ovarian cancer which included interleukin (IL)-6, IL-8, and IL-18, and tumor necrosis factor- α (TNF α) [51, 52]. Several studies have found that withaferin A treatment can reduce the severity of cytokine syndrome/storm, justifying its anti-inflammatory actions [53].

WS has been recognized for its strong antiviral activities against several viral infections, such as SARS-CoV and SARS-CoV-2 [52, 54–56], chikungunya, human papillomavirus (HPV), HSV, hepatitis c virus, and parainfluenza-3 [57, 58]. Specifically, withanone, withaferin A, and withanolide A from WS have been reported as potent antiviral agents with further elaborations in the following sections. The chemical structures of phytochemicals isolated from WS exhibiting promising therapeutic potential against several pathogenic virus infections are depicted in Fig. 3. The therapeutic effects and associated molecular mechanisms for reported virus infections are summarized in Table 2.

3.1 Herpes simplex virus

HSV is a clinically significant sexually transmitted disease categorized through oral sex contributed by HSV-1 or through genital herpes through HSV-2. According to the World Health Organization (WHO), there have been an estimated total of 3.7 billion individuals (67%) under the age of 50 reported to be infected with HSV-1, whereas a total of 491 million (13%) individuals between ages of 15–49 were reported to be infected with HSV-2 globally [4].

WS has frequently been reported to treat sexually transmitted infections (STIs) [59]. For instance, the aqueous extract of WS exhibited promising in vitro antiviral efficacy against HSV-1 in Vero cell culture [59]. An in silico study conducted by Grover et al. (2011) demonstrated that withaferin A also possessed antiviral activity against HSV [21]. The possible

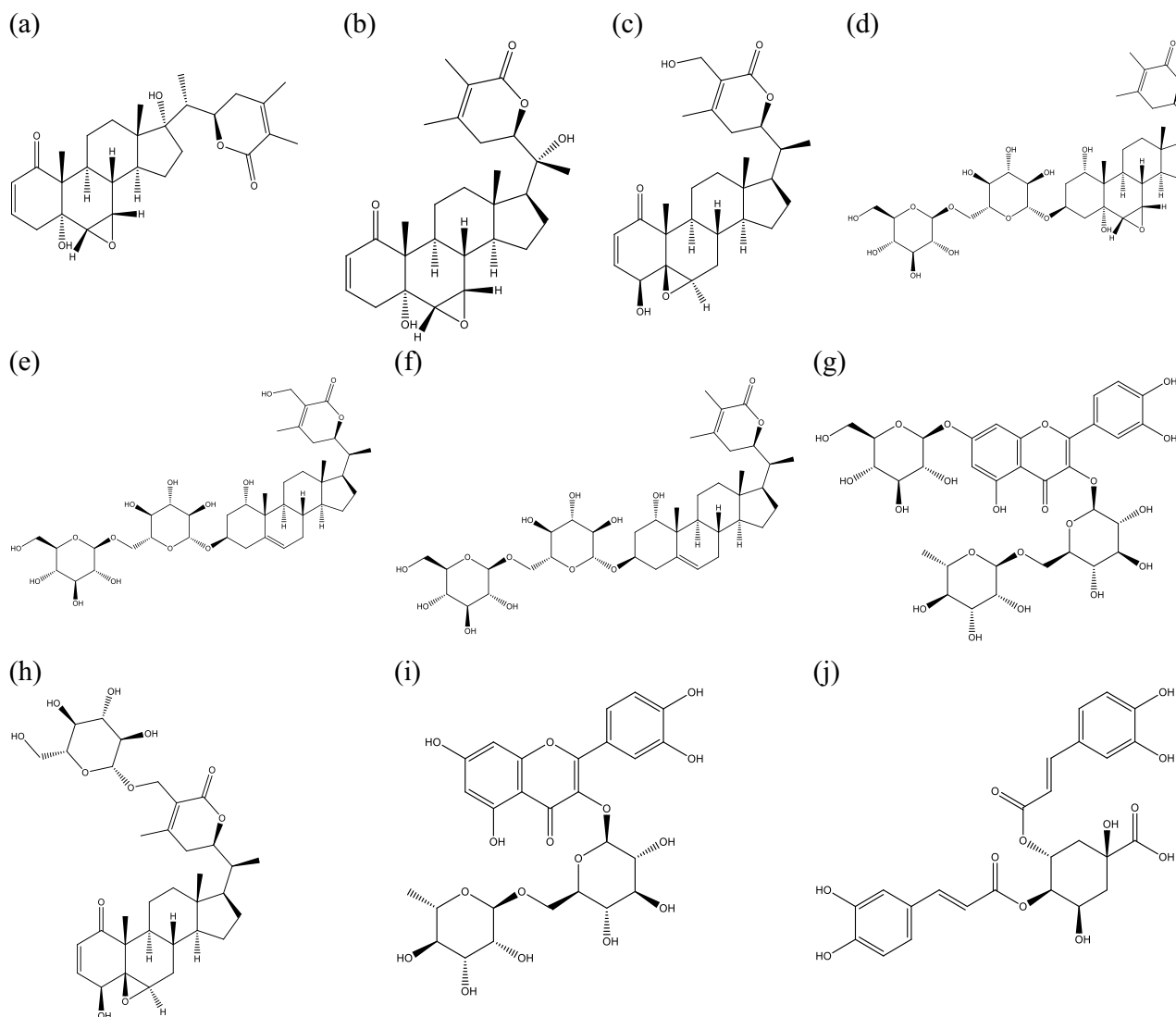


Fig. 3 Chemical structures of promising WS phytochemicals: **a** withanone; **b** withanolide A; **c** withaferin A; **d** withanoside II; **e** withanoside IV; **f** withanoside V; **g** quercetin-3-rutinoside-7-glucoside; **h** sitoindoside IX; **i** rutin; **j** isochlorogenic acid B

Table 2 Antiviral activities and associated molecular mechanisms of *Withania somnifera* extracts and phytochemicals

Type of viral infection	Experimental design	Phytochemical	Therapeutic effect/molecular mechanism	References
SARS-CoV-2	In silico and humanized zebrafish model through SARS-CoV-2 recombinant spike (S) protein In silico	Withanone	Binding with the RBD-ACE2 complex and energy destabilization Inhibit the replication of virus by interaction with Asn343 and reduces spike glycosylation Active against TMPRSS2 Inhibits main protease M ^{pro}	[67]
HIV-1 Ba-L (clade B) infection	In vitro	Withanolide A	Improved % cell viability and MTT exocytosis comparable to control	[23]
SARS-CoV-2	In silico		Strong binding to spike glycoprotein Strong binding to Nsp10/Nsp16 complex Strong binding to spike glycoprotein	[25]
H1N1 influenza virus	In silico	Withaferin A	Acts on neuraminidase binding	[20]
Herpes simplex virus			Act on DNA polymerase	[21]
SARS-CoV-2			Active against TMPRSS2	[22]
SARS-CoV-2			Inhibits RBD from interacting with human ACE2 receptor	[67]
HIV			Reduction in HIV long terminal repeat transcription and viral replication	[21]
SARS-CoV-2	In silico	Withanolide B	Binds strongly to host ACE2 receptor and PL ^{pro}	[25]
SARS-CoV-2	In silico	Withanoside II	Inhibits main protease M ^{pro}	[58]
SARS-CoV-2	In silico	Withanoside IV	Inhibits main protease M ^{pro}	
SARS-CoV-2	In silico	Withanoside V	Inhibits main protease M ^{pro}	
SARS-CoV-2	In silico	Sitoindoside IX	Inhibits main protease M ^{pro}	
SARS-CoV-2	In silico	Withanolide R	Inhibits main protease M ^{pro}	[71]
SARS-CoV-2	In silico	CID 44,423,097	Strong binding to D and E chains of Envelope protein	[73]
		CID 10,100,411		
		CID 101,281,364		
		CID 3,035,439		
SARS-CoV-2	In silico	2,3-dihydrowithaferin A	Inhibition of spike protein of SARS-CoV-2	[71]
SARS-CoV-2	In silico	Rutin	Inhibits main protease M ^{pro}	[72]
SARS-CoV-2	In silico	quercetin-3-rutinoside-7-glucoside	Inhibits main protease M ^{pro}	
SARS-CoV-2	In silico	Isochlorogenic acid B	Inhibits main protease M ^{pro}	
SARS-CoV-2	In vitro nanoparticle-based RNA oligonucleotide biochip system	(-)-catechin gallate and (-)-galloocatechin gallate	Inhibition against nucleocapsid protein of SARS-CoV-2	[74]
Tilapia lake virus	In vivo	Aqueous root extract of WS roots	Improved stress responses, antioxidant effects and decreased mortality rates in highly infected tilapia	[76]

Table 2 (continued)

Type of viral infection	Experimental design	Phytochemical	Therapeutic effect/molecular mechanism	References
Infectious bursal disease (IBD) virus	In vivo—embryonated chicken egg	Methanol:chloroform:water (12:5:3) extract of WS roots	Strong binding affinities between withaferin A and residues in crucial active sites of neuraminidase in IBD virus	[79]
Chicken infectious anaemia virus (CIAV)	In vivo chicks	Powdered herbal WS extracts (Natural Remedies Pvt. Ltd., Bangalore, India)	Increased CD4 + and CD8 + T cell counts, with decreased CIAV viral load	[81]

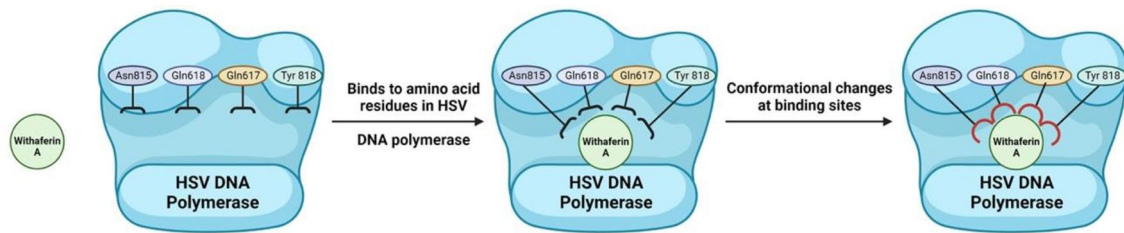


Fig. 4 Mechanism of withaferin A on inactivating DNA Polymerase in HSV

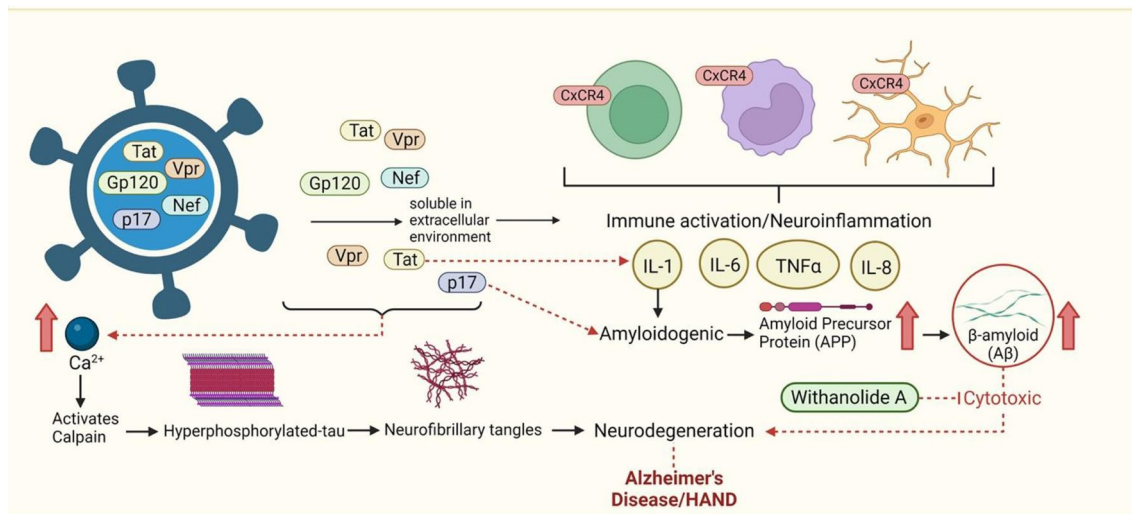


Fig. 5 HIV mechanism of infection in the brain that leads to Alzheimer's Disease or HANDs. Withanolide A inhibits the cytotoxic effects of β -amyloids preventing neurodegeneration

mechanism underlying the anti-HSV properties of WS had been predicted using molecular docking and dynamic simulation analyses, demonstrating that withaferin A can be a potential ligand with high binding affinity to the DNA polymerase structure of HSV at Asn815, Gln618, Gln617, and Tyr818 residues, with alterations to binding conformations [21]. The mechanism of withaferin A on inactivating DNA Polymerase in HSV is illustrated in Fig. 4.

3.2 Human immunodeficiency virus

HIV is recognizable as one of the most historically significant diseases having taken 40.1 million lives thus far, with no confirmed cure up to date. By the end of 2021, there have been an estimated 38.4 million people infected with HIV and 650,000 deaths from HIV-associated causes [4].

One of these HIV-associated causes has been found to be affiliated with Alzheimer's disease (AD) patients. Intriguingly, withanolide A has indirectly exerted its therapeutic effect against HIV-induced AD. A main causative component of AD is the accumulation of insoluble beta-amyloid peptides ($A\beta$) at neuronal synapses. Neuronal degeneration has been linked to HIV through observed increments of amyloid- β precursor protein ($A\beta$ PP) in subcortical white matter axons from previous studies [23]. Even during highly active antiretroviral therapy (HAART), HIV has persistently replicated in the brain of AD patients potentially leading to inflammatory responses involving increased $A\beta$ PP and $A\beta$ [23]. HIV viral proteins, such as HIV-1 transcription transactivator (Tat), HIV-1 viral protein R (Vpr), negative regulatory factor (Nef), and envelope glycoprotein 120 (Gp120), have been found to be soluble in the extracellular matrix further inducing immune activation and neurodegeneration through the assisted production of neurofibrillary tangles (NFT) and $A\beta$, as well as p17 protein found to induce amyloidogenic constructs that are cytotoxic [60]. The anti-cytotoxic effect of withanolide A against HIV-induced $A\beta$ -mediated neurodegeneration for Alzheimer's disease is illustrated in Fig. 5.

Kurapati et al. (2014) reported neuroprotective effects of WS root extract (methanol: chloroform) through improved percentage of cell viability ($p < 0.0001$) with withanolide A against β -amyloid-induced toxicity in HIV-1Ba-L (clade B)

infections on human neuronal cell lines (SK-N-MC) and against substance abuse (cocaine and methamphetamine) [23]. Furthermore, cell death was analyzed using MTT formazan exocytosis, wherein needle-like crystals were formed on the cell surface representing exocytosed MTT formazan. The WS extracts with and without A β treatment showed no MTT formazan exocytosis with even intracellular distribution of formazan crystals, which were comparable to the uninfected control. Additionally, withanolide A showed increased cell viability as compared to control ($p < 0.0001$) and in cells treated with HIV, cocaine, methamphetamine and in combination (HIV + cocaine + withanolide A, HIV + methamphetamine + withanolide A) which were comparable to the controls [23]. In addition, a combined test drug known as Immu-25, which included WS as one of the four total extracts, showed antiretroviral activity against HIV by reducing the mean viral load from 326,438 copies/mL to 180,495 copies/mL ($n = 13$) in 6 months of treatment and 22,069 copies/mL ($n = 9$) after 12 months of treatment while also showing improved symptoms [61]. The study also showed increased mean CD4 + cell count from a baseline of 243–336 cells/ μ L, 527 cells/ μ L and 618 cells/ μ L after treatment for 6, 12, and 18 months, respectively ($p < 0.001$). Additionally, CD8 + cell counts were also increased from a baseline of 786–1079 cells/ μ L, 1347 cells/ μ L, and 1536 cells/ μ L after treatment for 6, 12, and 18 months, respectively [61]. Furthermore, withaferin A was also found to combat HIV-1 by suppressing the transcription and replication of associated long terminal repeat [7, 21].

WS extract was also shown to reduce the levels of disease progression marker, CD38, on CD8 + T lymphocytes in an ex vivo experiment performed on peripheral blood mononuclear cells (PBMCs) of HIV-infected patients, indicating the potential anti-HIV property of WS [62]. In detail, the HIV patient groups studied under antiretroviral therapy (ART) presented a general decline in percentages of CD38 in CD8 + T cells; asymptomatic cases after 6 months of ART ($13.2\% \pm 8.2\%$, p value paired T test 0.005), ART naïve symptomatic and AIDS indicator cases ($15.6\% \pm 9.7\%$, p value paired T test 0.001), ART naïve asymptomatic cases ($18.9\% \pm 11.6\%$, p value paired T-test 0.004) had significant declines of CD38 in CD3+/CD8+ cells than patients of immunological failure ($13.4\% \pm 14.4\%$, p value paired T test 0.01) [62].

3.3 Severe acute respiratory syndrome coronavirus 2

COVID-19 is an infection caused by SARS-CoV-2 and has taken the world by storm since December 2019 [63]. In light of the recent pandemic and the fatalities caused thus far, it is crucial to develop a prospective treatment to prevent and treat COVID-19 patients [64].

According to current research, WS phytochemicals can be used to develop effective COVID-19 therapeutic agents [56]. Comparative molecular docking studies have also been performed to compare the possible therapeutic efficacy between WS phytochemicals with docked scores ranging from -8.37 to -11.30 kcal/mol compared to the co-crystallized ligand standard called N3 at -8.12 kcal/mol [58].

In order for SARS-CoV-2 to be granted entry into a human cell, its spike glycoprotein (S protein) has to form a complex with the human host cell angiotensin converting enzyme 2 (ACE2) receptor. The amino acid Asn343 of the S protein in particular present at the active site may potentially be responsible for the glycosylation of SARS-CoV-2, increasing the S protein binding affinity to ACE2 and thus promoting viral transmission and entry [65]. Previously, literatures have suggested that withaferin A can interact with ACE2, and withanone inhibits the main protease (M^{pro}) of SARS-CoV-2 [22], alternatively known as nonstructural protein-5 (NSP5) or 3CL-pro, which is essential for viral replication [66–68]. In support, V. S. Patil et al. (2021) reported that withanone inhibited SARS-CoV-2 replication by interacting with Asn343 and perturbing spike glycosylation, therefore reducing viral transmission as well [65].

Withanone, an eminent steroidal withanolide, defends cells from elevated pro-inflammatory cytokine levels such as IL-6, IL-1 beta and TNF α [69], which may help in treating COVID-19. Moreover, the two salt bridges at the interface were destabilized by the involvement of withanone in the RBD-ACE2 complex. Molecular docking analysis showed that in the presence of withanone, the salt bridge occupancies at Glu12-Lys87 and Glu20-Arg73 residues decreased from 47.2 to 24.3% and 93.8–80%, respectively. A decrease of 4.3 kcal/mol was observed in the electrostatic components of binding free energy wherein the final trajectories further decreased by 0.6 kcal/mol [67]. The disruption of electrostatic forces between ACE2 and RBD blocked the virus from entering host cells and its consequent infectivity. Similarly, withaferin A also demonstrated strong binding energy of -11.242 kcal/mol with the active site of M^{pro} at Cys 44 and Glu 166 residues, along with strong binding energy (-9.631 kcal/mol) with the Glu 35 and Gln 42 residues in the spike protein/ACE2 [24].

Withanone was also predicted to interact with other proteins, such as transmembrane protease serine 2 (TMPRSS2) and M^{pro} SARS-CoV-2 protease [22, 70]. V. Kumar et al. (2021) described that withanone inhibited the M^{pro} of SARS-CoV-2 demonstrating free binding energy of 34.51 ± 9.63 kcal/mol as compared to the model N3 inhibitor (60.80 ± 5.04 kcal/mol). The inhibition or interference of SARS-CoV-2 M^{pro} is an effective treatment method for COVID-19 [56].

Additionally in favor of using an *in silico* approach, Parida et al. (2020) predicted that M^{Pro} was inhibited by withanolide R, and the S protein of SARS-CoV-2 was inhibited by 2,3-dihydrowithaferin A, with the comparatively lowest total relative binding energies of -141.96 ± 18.39 kJ/mol and -87.60 ± 22.68 kJ/mol, at 500 step bootstrap \pm standard error, respectively [71]. Similarly, withanolide A showed significant interactions with M^{Pro} and RNA dependent RNA-polymerase (RdRP), with binding energy values of -10.292 kcal/mol and -9.668 kcal/mol, respectively [24]. In another *in silico* study conducted by Tripathi et al. (2021), the highest docking energies recorded included sitoindoside IX (-8.37 kcal/mol), withanoside II (-11.30 kcal/mol), withanoside IV (-11.02 kcal/mol), and withanoside V (-8.96 kcal/mol) with the SARS-CoV-2 M^{Pro} [58]. Among the four phytochemicals, withanoside V possessed hydrogen bonding and strong binding affinities with M^{Pro} active sites elucidating active site stability with the highest binding free energy score of -87.01 ± 5.01 kcal/mol, suggesting withanoside V to be an effective antiviral compound as an M^{Pro} inhibitor anticipated to have effective antiviral profiles [58]. Conversely, non-characteristic compounds (i.e., rutin, quercetin-3-rutinoside-7-glucoside and isochlorogenic acid B) obtained from WS showed better antiviral activity with lower binding scores at the active site of SARS-CoV-2 M^{Pro} in comparison to withanone and withanolide [72].

An *in silico* screening study demonstrated the effective use of prospective WS phytochemicals (i.e., withanone, withanolide A, and withanolide B) against SARS-CoV-2 S glycoproteins, M^{Pro}, Papain-like protease (PL^{Pro}) and host ACE2 receptor via their binding energies (BE) and dissociation constants (Kd) [25]. In summary, withanone presented significant binding to SARS-CoV-2 M^{Pro} (BE: 6.14 kcal/mol, Kd: 31.77 mM) as compared to the reference drug oberadilol (BE: 2.23 kcal/mol, Kd: 23.18 mM). Withanolide B exhibited strong binding affinity to human ACE2 receptor (BE: 10.21 kcal/mol, Kd: 32.78 nM) as compared to reference drugs losartan (BE: -6.72 kcal/mol, Kd: 11.86 mM) and Arbidol (BE: 6.69 kcal/mol, Kd: 12.47 mM), as well as papain-like protease of SARS-CoV-2 (BE: 10.3 kcal/mol, Kd: 28.32 nM) as compared to the reference drugs cinacalcet (BE: 6.44 kcal/mol, Kd: 19.17 mM) and procainamide (BE: 5.03 kcal/mol, Kd: 206.96 mM). Comparatively, withanolide A showed expansive effects against SARS-CoV-2 S glycoprotein (BE: 7.18 kcal/mol, Kd: 5.48 mM) as compared to reference drugs hydroxychloroquine (BE: 2.48 kcal/mol, Kd: 15.11 mM) and Arbidol (BE: 3.14 kcal/mol, Kd: 4.99 mM), as well as SARS-CoV-2 Nsp10/Nsp16 complex (BE: 10.38 kcal/mol, Kd: 24.67 nM) as compared to reference drugs hydroxychloroquine (BE: 4.93 kcal/mol, Kd: 244.14 mM) and losartan (BE: 6.49 kcal/mol, Kd: 17.54 mM), along with additionally further effects against SARS-CoV S glycoprotein (BE: 9.78 kcal/mol, Kd: 67.23 nM) and SARS-CoV M^{Pro} (BE: -8.93 kcal/mol, Kd: 285.01 nM) [25].

Interestingly, there were four WS phytochemicals (i.e., CID 44,423,097, CID 10,100,411, CID 101,281,364, CID 3,035,439) that were reported to bind strongly with the amino acids of D (Arg74 and Asn77) and E chains (Thr43, Cys46, Ile59, and Val60) in pores of the SARS-CoV-2 envelope (E) protein and subsequently reduced virus replication [73].

Other than those mentioned above, WS catechin derivatives (i.e., (-)-catechin gallate and (-)-gallocatechin gallate) exhibited a potential inhibitory effect of greater than 40% at a concentration of 0.05 μ g/mL against SARS-CoV-2 nucleocapsid (N) protein identified via a nanoparticle-based RNA oligonucleotide biochip system [74]. These results indicated powerful anti-SARS-CoV-2 capabilities, and in extension this novel method may be used as a target/inhibitor screening assay that has high sensitivity, is cost-effective, and can be miniaturized. Consequently, there is verified indication of the efficacy of WS as a potential medicinal plant for the treatment of COVID-19 and has been suggested to be consumed as a herbal cocktail as an alternative therapeutic [24]. Figure 6 summarizes the overall discussed mechanisms of inhibition against SARS-CoV-2.

3.4 Tilapia lake virus

TiLV is a negative sense, single-stranded RNA virus, which has affected aquaculture farming of tilapia fish with mortality rates ranging from 20 to 90% globally since its discovery in the summer of 2009 [75]. TiLV possesses little to no available treatments, except for previously suggested host resistance achieved through selective breeding [76]. Aqueous extract of WS roots, on the other hand, presented reduced primary stress responses identified through a steady but significant recovery in triglyceride (133.72%), total protein (101.70%), albumin (84.02%), and hemoglobin (32.29%) levels as well as significantly decreased glucose levels (99.26%) when compared to untreated heavily infected tilapia. Furthermore, WS root extract treatment of heavily infected tilapia could further decrease 33.23% of

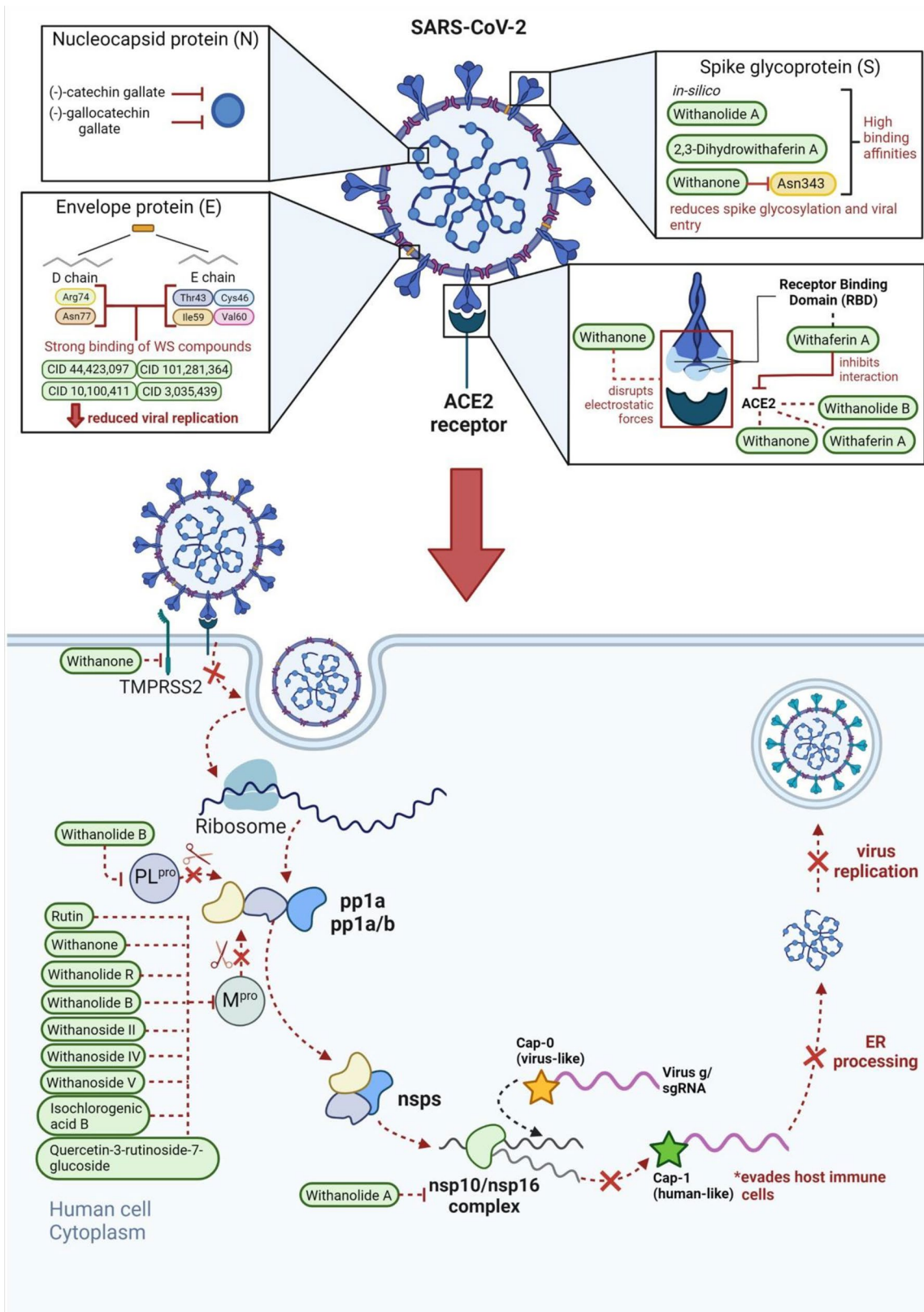


Fig. 6 Summary of inhibition mechanisms of various WS phytochemicals against SARS-CoV-2

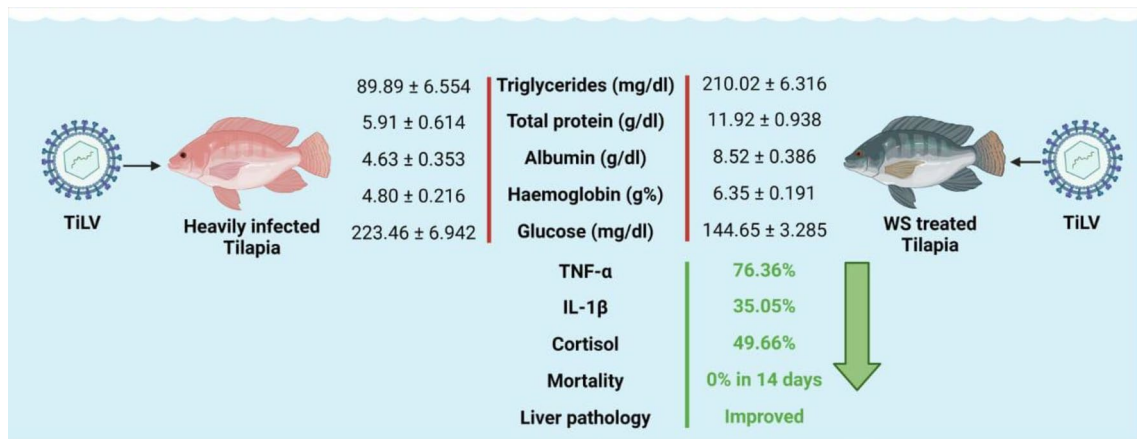


Fig. 7 WS root extracts exhibiting decreased stress responses in tilapia against TiLV

lipid peroxidation, promote antioxidative effects by reducing ROS generation by 184.80%, significantly decrease the levels of proinflammatory cytokines such as TNF-α by 76.36%, IL-1β by 35.05%, and cortisol by 49.66%, improve liver pathology upon treatment, and reduce mortality with a continual decrease to a rate of 0 after 2 weeks [76]. Figure 7 illustrates WS root extracts exhibiting decreased stress responses in tilapia against TiLV.

3.5 Infectious bursal disease virus

The IBD virus is a double-stranded, non-enveloped RNA virus that occurs in young chicks between the ages of 3–6 weeks. Serotype 1 of the virus is infectious and can be categorized based on mortality. Out of which, 60–70% of IBD viral isolates are of the very virulent genotype capable of causing 90% mortality rates and severe economic losses to the poultry meat industry. IBD has been around for 60 years with short-lived success from vaccination treatments [5, 77].

Several antiviral studies have also revealed the potential therapeutic effects of WS against IBD virus infection. For instance, hydroalcoholic extract of WS roots was shown to inhibit 99.9% IBD virus replication at 25 µg/mL concentration, as evident in the cytopathic effect reduction assay [77]. Although nitric oxide (NO) is required for the initial infectivity of the host, NO has also been reported to have inhibitory effects against a wide range of viruses, including IBD, through various mechanisms involving the production of peroxy nitrite radicals, inhibitory effects on RdRP enzyme, viral proteinases, and transcription factors, leading to disruptions in viral replication and entry into host cells [78, 79]. An in vitro study involving chicken embryo fibroblasts (CEF) identified that methanol:chloroform:water (12:5:3) root extract of WS promoted an early and rapid rise in NO formation irrespective of IBD infection and in turn

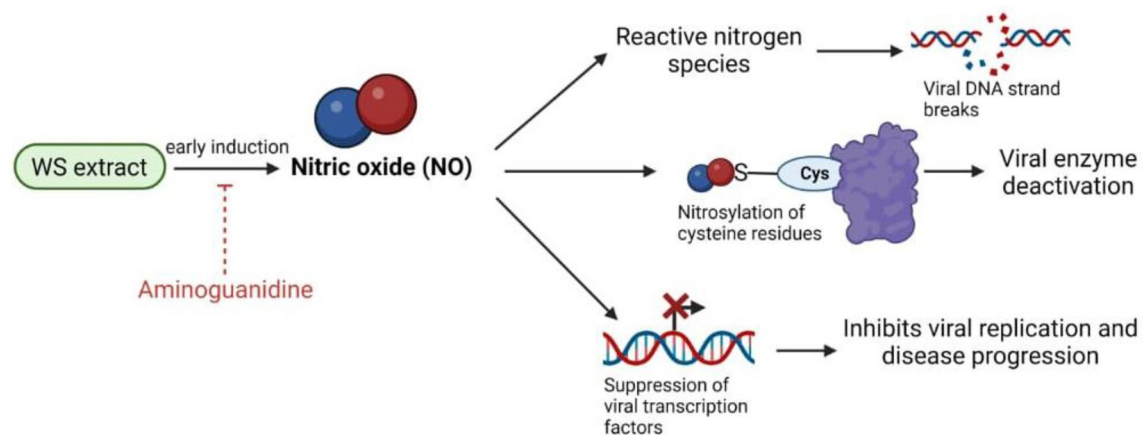


Fig. 8 Inhibition pathways of nitric oxide against viral replication and entry induced by WS extract

inhibited IBD-induced cytopathy [79]. Therefore, the manipulation of NO production by WS root extracts may present a potential mechanism for the inhibition of IBD. The inhibition pathways of nitric oxide against viral replication and entry induced by WS extract is illustrated in Fig. 8.

3.6 Influenza virus and others

Influenza viruses are one of the most easily transmissible diseases among humans and animals alike. There are four main types, which are; A (humans and animals), B (humans, seasonal), C (humans and pigs), and D (cattle). The most well-known epidemics include avian influenza or bird flu; A(H5N1) and A(H9N2) subtypes, as well as swine influenza/flu; A(H1N1) and A(H3N2) subtypes [80]. Moreover, the number of oxygen atoms in the withanolide backbone and structural rearrangements play a critical function for effectual binding [25]. Likewise, withaferin A exerts inhibitory activities against influenza and HPV viruses [20, 81].

Withaferin A has also been reported to possess anti-influenza virus infection. For instance, it could suppress neuraminidase enzymatic activity, an essential enzyme in influenza virus life cycle, of H1N1 influenza virus as predicted in an *in silico* study [20]. The results showed a strong binding affinity between withaferin A and H1N1 neuraminidase enzyme with high binding energy (-7.51 kcal/mol) as compared to the binding energies of approved drugs; oseltamivir (ranging between -4.57 and -7.98 kcal/mol) and zanamivir (ranging between -4.00 and -6.45 kcal/mol). Additionally, withaferin A provided a low 50% inhibitory concentration (IC_{50}) value of 3.14 μ M as compared to prediction values of oseltamivir (38.13 μ M) and zanamivir (18.76 μ M) against neuraminidase, demonstrating the potent inhibitory potential of withaferin A against neuraminidase. The stable molecular dynamic (MD) simulation showed that withaferin A interacted with target residues of H1N1 neuraminidase present in essential sites, such as the catalytic active site, glycosylation site, and metal binding site exhibiting good potential towards enzyme inhibition [20].

Lastly, WS pure extract (Natural Remedies Pvt. Ltd., Bangalore, India) was claimed to decrease the load of chicken infectious anemia virus (CIAV) affecting hematopoietic and lymphoid tissues (thymus and spleen). Besides, it also showed the highest improved CD4+ and CD8+ T-cell counts in chicks as compared to other medicinal plants, such as *Azadirachta indica* (Neem) and *Tinospora cordifolia* (Guduchi) [81].

4 Enhancement of therapeutic efficacy of *Withania somnifera* via effective delivery

Numerous *in vitro* and *in vivo* studies have revealed that WS has antiviral effects. However, due to its low water solubility, poor biodistribution, and multi-targeting capabilities, it is likely to produce systemic toxicity. It has been reported that WS is therapeutically effective when taken orally in high doses of about 800 mg/kg per day [57, 82]. Newer delivery techniques, such as nanoparticles (NPs), liposomes, micelles, and phospholipid complexes, are being investigated to further improve the therapeutic efficacy and targeted delivery of WS [57, 83–85]. Approaches utilizing nanotechnology could prevent such unintentional side effects and enhance clinical translation. Due to its enhanced capacity to transport payloads to precise therapeutic areas and its potential to change cellular permeability, absorption, and pharmacokinetic profiles, nanotechnology has recently attracted a lot of attention [86, 87].

The bioinspired generation of NPs using various biological systems, including bacteria and plants, has become more well-known in recent years. Gold nanoparticles (AuNPs) are favored over other nanoparticle carriers (i.e., iron oxide, silicone, and quantum dots) because of their high biocompatibility, efficiency at quenching, ease of manufacture, wide range of applications, and adaptable optical properties [88]. Currently available nano-formulations include liposomal paclitaxel, liposomal vincristine, and polymeric micelles of paclitaxel [89, 90]. Drug delivery methods that are actively targeted can outperform those that are passively targeted and have enhanced permeability and retention (EPR) effects. As a result, WS needs to be properly planned out and formulation-optimized. Besides, intranasal administration is a dependable method for administering vaccines and certain types of anti-infective treatments, and it can be used instead of oral and parenteral methods [91]. This is primarily attributable to the nasal cavity's highly vascular, permeable, and weakly enzymatic environment, which enables the avoidance of hepatic first-pass metabolism, resulting in more rapid action and mucociliary clearance [92]. In the global cerebral ischemia model, withanolide A was effectively administered by intranasal injection to the olfactory mucosa to have a neuroprotective effect [55]. Thus, researchers predict that the next development in WS therapy will use targeted nanoparticles. In order to better understand and improve the antiviral activity of WS, substantial preclinical and clinical research is needed.

5 Conclusion and future perspectives

Viral diseases have caused significant infectious pandemics and have reduced the number of the global population. The reported infectious viral diseases discussed in this review include human pathogens such as HSV, HIV, SARS-CoV-2, influenza viruses, as well as economically impactful aquaculture and food industry pathogens such as CIAV, TiLV, and IBD virus. Despite their widespread effects across the world, assigned drugs and available viral vaccines have limited efficacy.

Various studies have demonstrated that WS has antiviral activity and pharmacological characteristics to act as an effective and safer alternative. As a majority of the reviewed studies conducted thus far against SARS-CoV-2, influenza viruses, HSV, and HIV were investigated using *in silico* means, future studies may attempt *in vitro* or *in vivo* experiments for further confirmation and progression towards clinical trials. To date, the antiviral potentials of WS are limited to withaferin A, withanolide A, withanone, withanosides, and several non-characterized phytochemicals, thus more phytochemicals that are responsible for pharmacological activities of WS should be tested, with the aim of searching for the key phytochemical(s) in preventing and treating virus infections. Besides, the strategies to enhance its therapeutic efficacy while avoiding off-target toxicity using nanotechnology-assisted delivery or alternative delivery such as intranasal administration should be further investigated and addressed. Given its promising antiviral potentials, WS and its phytochemicals should also be evaluated against different virus infections caused by different strains in order to widen its applicability as antiviral agents.

The discussions in the above sections amply demonstrate that the use of WS and its components have a reasonable and scientific foundation. To promote WS as a potential drug candidate for consumer products, more compelling scientific information addressing its pharmacological assessment, including drug-like properties, is needed. Clinical studies of WS phytochemicals alone and in synergistic combinations are the primary field that requires more research. Further research and clinical trials are needed to determine the precise processes involved as well as the ideal dosage range.

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Declarations

Ethics approval Not applicable.

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