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Review

Recent Advancements in Extraction Techniques of Ashwagandha (*Withania somnifera*) with Insights on Phytochemicals, Structural Significance, Pharmacology, and Current Trends in Food Applications

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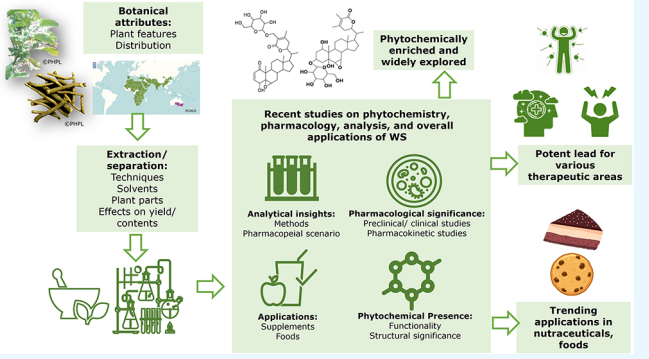
ABSTRACT: Ashwagandha, also known as *Withania somnifera* (WS), is an ayurvedic botanical plant with numerous applications in dietary supplements and traditional medicines worldwide. Due to the restorative qualities of its roots, WS has potent therapeutic value in traditional Indian (Ayurvedic, Unani, Siddha) and modern medicine recognized as the "Indian ginseng". The presence of phytochemical bioactive compounds such as withanolides, withanosides, alkaloids, flavonoids, and phenolic compounds has an important role in the therapeutic and nutritional properties of WS. Thus, the choice of WS plant part and extraction solvents, with conventional and modern techniques, plays a role in establishing WS as a potential nutraceutical product. WS has recently made its way into food supplements and products, such as baked goods, juices, beverages, sweets, and dairy items. The review aims to cover the key perspectives about WS in terms of plant description, phytochemistry, structural significance, and earlier reported extraction methodologies along with the analytical and pharmacological landscape in the area. It also attempts to iterate the key limitations and further insights into extraction techniques and bioactive standardization with the regulatory framework. It presents a key to the future development of prospective applications in foods such as food supplements or functional foods.



1. INTRODUCTION

Historically, herbs have been used in food, cosmetics, and fragrances.[1] They frequently serve as a source of traditional medicine for treating various illnesses.[2,3] One of the most important perennial plants for healing in Indian traditional medicine is *Withania somnifera* (L.) (WS) Dunal, popularly known as ashwagandha or Indian ginseng.[4,5] It belongs to the Solanaceae family. Since the roots of the species exhibit restorative qualities like *Panax ginseng*, it is sometimes known as "Indian ginseng".[6] One of the key herbs in Ayurveda is WS, which is categorized under the Rasayana category and in Ayurvedic medicine refers to methods for extending life and promoting happiness.

WS roots have several steroidal lactones (withanolides), which give this herb a number of therapeutic properties such as immunomodulation, well-being, neuro-regeneration, anticancer, antistress, rejuvenation, geriatric problems, and anti-inflammatory.[7−9] It is also used as medhrasayana, meaning brain tonic and memory enhancer. It is used as a powder or extract and taken with ghee or milk. When taken with milk, it helps to increase weight and proteins in the body. Ghee preparations are taken for medicinal use in the brain. Roots as well as leaves have been studied for their anticancer properties.[10]

Several clinical studies have been reported for the clinical trial of different kinds of extracts in cancer. Clinical trial investigations are required to determine whether these products have any activity in humans. WS is well-known for having a wide range of medicinal characteristics because of the

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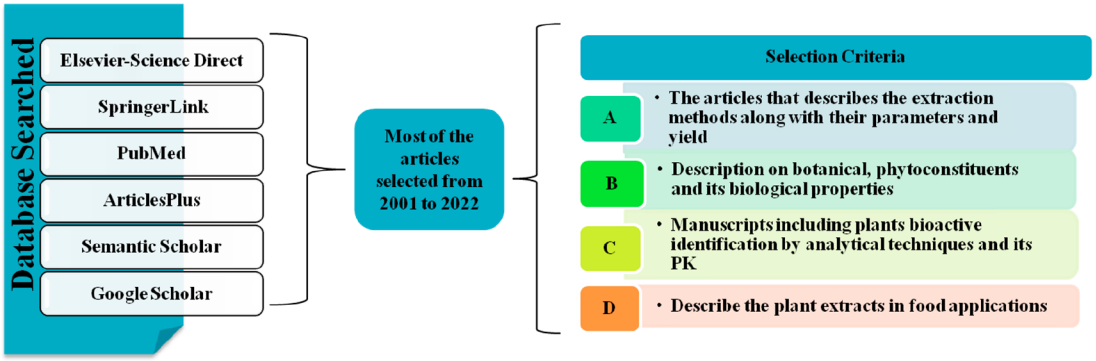

Figure 1. Schematic approach for a review on *Withania somnifera*.

clear presence of certain phytoconstituents such as withanosides or withanolides, alkaloids, flavonoids, sterols, phenolics, and others.[11−13] The occurrence of this secondary metabolite varies per tissue type (leaves, roots, stems, fruits), developmental stage, and chemotype.[14,15] Extraction is the initial stage in every study of a medicinal plant and has a big, important impact on the outcome. An effective extraction technique is crucial for both qualitative and quantitative research of bioactive chemicals produced from plant sources.[16] This Review presents an overview of phytochemical investigations on the extraction, fractionation, and identification of plant bioactive components' ethnopharmacology. This review also provides an overview of phytochemistry and information on extraction and fractionation methods that have been demonstrated to be effective for enriching phytoconstituents. The prime objective of the review is to investigate the importance of WS from various perspectives like its botanical description and reported phytochemical presence, which causes further segregation based on the major classes such as withanolides and their derivatives, alkaloids, flavonoids, phenols, phenolic acids, phytosterols, fatty acids, lipids, coumarins, triterpenes, etc. The compounds of each class were further tabulated with relevant data such as source plant parts and molecular weights with respective references. This also includes brief explanations of the structural features and characteristics.

The aim is also to generate a basis for thorough coverage of important aspects of this remarkable herb. So far, the extraction methodologies and techniques have been taken up for a deeper examination regarding their reported outcomes, observed functionalities, and compounds. The analytical and pharmacological landscape of WS is covered with key recent references.

Although much research has been conducted so far on WS, the need for further investigation in terms of standardization, marker profiling, and optimization of extraction processes is iterated here. Additionally, profound safety and efficacy studies with a strict regulatory framework will enhance the applicability of this potent herb and its derived products in medicines, supplements, and food products.

2. COLLECTION OF DATA SETS

For the present Review, we used Elsevier-Science Direct, SpringerLink, PubMed, ArticlesPlus, Semantic Scholar, and Google Scholar to conduct a literature search mostly from 2001 to 2022. The keywords included in the search were "*Withania somnifera*", "Indian ginseng", "withanolides", "extraction", and "traditional use", which were cross-referenced with "functional foods", "nutraceuticals", "phytochemistry", "applications", and "biological activities".

The reference articles selected for this work are those that (A) describe the extraction methods along with their parameters and yield; (B) describe the botanical description, phytoconstituents, and their biological properties; (C) describe plants' bioactive identification by analytical techniques and its pharmacokinetics; and (D) describe the plant extracts in food applications [Figure 1]. If at least three of the aforementioned points were met by the literature on the plants, they were chosen to be discussed in detail. The references discovered during the search were examined for more information on the extraction methods with respect to their yield and phytoconstituents content.

3. TRADITIONAL USES

India has long been known for its traditional medicinal practices, such as Ayurveda, Siddha, and Unani. Ashwagandha (WS) is a well-known Rasayana herb and among the most prominent Indian herbs used in Ayurvedic medicinal practice.[17,18] It is extremely popular for revitalizing the nerves, bone marrow, and reproductive system. In the Samhita period, the Indian Traditional Ayurvedic System of Medicine expounded on using Ashwagandha:[19] in inflammation of the joints, nerve disorders, and epilepsy;[20] tumors, heart diseases, and Ulcers;[21,22] and cardiopulmonary disorder, rheumatic pain, and arthritis. The species' root and root powder and paste are beneficial.[23] The pulverized leaves of WS are used to treat exterior pain, inflammatory processes, and pubonic pains.[24]

4. PHYTOCHEMICAL COMPOSITION

WS is rich in phytochemicals, but the primary bioactive phytoconstituents of WS are withanolides (steroidal lactones), withanosides, alkaloids, flavonoids, sterols, phenolics, and other phytoconstituents.

4.1. Withanolides and Derivatives. With an ergostane skeleton and polyoxygenated steroid classification, withanolides are a chemical class of naturally occurring steroidal lactones. Withanolides typically have oxygen atoms at positions C-1, C-22, and C-26, as the main chemical components of WS. However, there are some exceptions where the oxygen atom at position C-22 is not functionalized. These compounds can be divided into two major groups based on the arrangement of their side chain: those containing a δ-lactone or δ-lactol at positions C-22 and C-26 and those containing a γ-lactone, which typically involves positions C-23 and C-26[25,26] [Table 1].

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ACS Omega XXXX, XXX, XXX−XXX

Table 1. Withanolides and Derivatives

Withanolides and Derivatives					
sr. no.	structure no.	name	mol. wt.	plant part	references
1	1	withaferin A	470.3	roots, shoot, leaves, whole plant	28−30
2	2	17α-hydroxy withaferin A	486.6	leaves	27
3	3	witharistatin	470.6	leaves	31
4	4	3α,6α-epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide	488.6	roots	46
5	5	2,3-dehydrosomnifericin	488.6	roots	46
6	6	somnifericin	490.6	whole plant	32
7	7	4-deocywithaperuvin	504.6	fruits	38
8	8	coagulin H	520.6	roots	33
9	9	coagulin S	538.6	roots	33
10	10	withaoxylactone	502.6	whole plant	32
11	11	withasomniferol A	486.6	roots	30
12	12	withasomniferol B	472.6	roots	30
13	13	14α,17α-dihydroxywithanolide R	502.6	fruits	38
14	14	iso-withanone	470.6	berries	39
15	15	withanolide A	470.6	roots, shoot, leaves	28−30, 34, 40
16	16	withanolide B	454.6	roots, shoot, leaves	28−30, 34, 40
17	17	6α,7α-epoxy-3β,5α,20β-trihydroxy-1-oxowitha-24-enolide	488.6	whole plant	27
18	18	2,3-dihydro-3β-hydroxy withanone	504.6	whole plant	27, 55
19	19	2,3-dihydro-3β-hydroxy withanone-3β-O-sulfate	584.6	leaves	55
20	20	6α,7α-epoxy-1α,3β,5α- trihydroxy-witha-24-enolide	474.6	berries	39
21	21	withanoside II	798.9	roots	40
22	22	withanoside I	636.7	roots	
23	23	withanoside III	652.8	roots	
24	24	withasomniferol C	470.6	roots	30
25	25	withanolide Z	491.1	leaves	43
26	26	withanoside IV	782.9	roots	40
27	27	withanoside V	766.9	roots	35, 40
28	28	withanoside VI	782.9	roots, leaves	40, 45
29	29	withanoside VII	798.9	roots, leaves	40
30	30	withanoside VIII	945	roots, leaves	45
31	31	withanoside IX	1092.1	roots, leaves	45
32	32	withanoside X	782.9	roots, leaves	45
33	33	withanoside XI	636.8	roots, leaves	45, 46
34	34	coagulin Q	620.4	leaves	47
35	35	withanolide C	523.1	leaves	44
36	36	withanolide R	470.6	leaves	41, 42
37	37	withanolide D	470.6	roots, leaves, aerial part, shoot	12, 13, 28, 34−37
38	38	withanolide E	486.6	leaves, fruits and berries	48−51
39	39	phyperunolide A		leaves and fruits	52
40	40	physagulin D	620.8	leaves	45, 56
41	41	withangulatin A	526.6	leaves	53
42	42	16β-acetoxy-6,7α-epoxy-5α-hydroxy-1-oxowitha-2,17 (20), 24-trienolide	544.6	leaves	34
43	43	withanolide F	470.6	leaves	48−51
44	44	withanolide J	470.6	leaves	49, 50, 54
45	45	withanolide G	454.6	leaves	41, 49, 51, 54
46	46	withanolide H	470.6	leaves	49, 54
47	47	withanolide I	470.7	leaves	49, 54
48	48	withanolide K	470.6	leaves	49, 54
49	49	withanolide L	452.6	leaves	41, 49
50	50	withanolide M	468.6	leaves	49
51	51	withanolide N	452.6	leaves	41
52	52	withanolide O	452.6	leaves	41, 51
53	53	withanolide P	454.6	leaves, fruits and berries	39, 41
54	54	27-hydroxy-withanolide D	472.2	leaves	41
55	55	14α-hydroxy withanolide D	486.6	leaves	41
56	56	17α-hydroxy withanolide D	486.6	leaves	41
57	57	withanolide Q	470.6	leaves	42
58	58	withanolide T	486.6	roots, leaves	51, 57

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Table 1. continued

Withanolides and Derivatives					
sr. no.	structure no.	name	mol. wt.	plant part	references
59	59	withanolide U	486.6	leaves	44, 51
60	60	withanolide Y	486.6	leaves	44

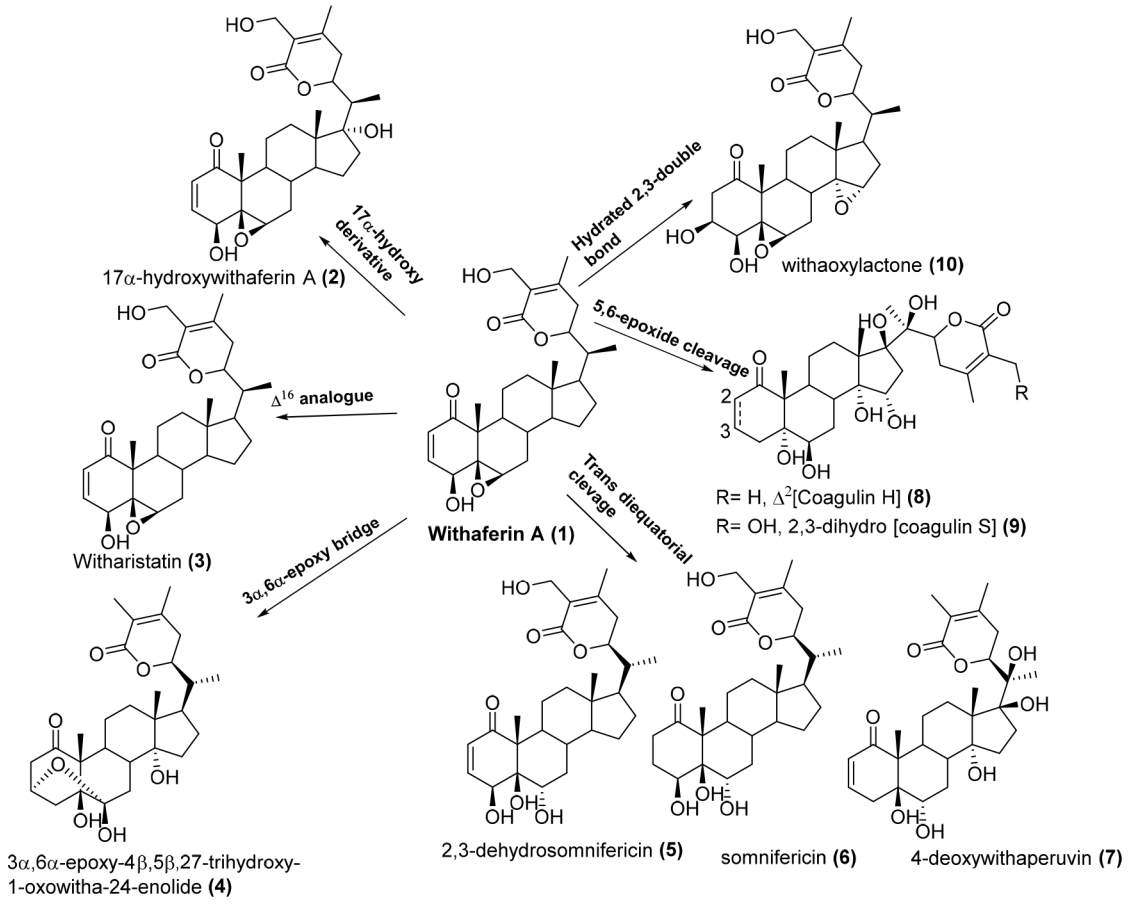

Figure 2. Withanolides having a parent skeleton of withaferin A.

The majority of known withanolides are categorized as δ-lactone or δ-lactol, which can be further broken down into several subgroups, such as withanolides that have the same parent skeleton as withaferin A, withaphysalins, physalins, acnistins, withajardins, withametelins, sativolides, subtriflora-δ-lactones, spiranoid-δ-lactones, norbornane-type withanolides, ring-D aromatic withanolides, ring-A aromatic withanolides, and taccalonolide-δ-lactones[25,26] [Table 1].

The formation of a δ-lactone bond between a carboxyl group at position C-26 and a hydroxy group at position C-22 is the most common withanolide configuration. Other variations include lactone formation with a hydrated carbonyl at C-22 and δ-lactol formation between an aldehyde at C-26 and a hydroxy group at C-22. The stereochemistry at position C-22 may differ when substituents at positions C-23 or C-22 change the relative priorities of groups around the asymmetric center; however, all currently known withanolides share the same stereochemistry at C-22, which corresponds to the (22*R*) configuration.[25,26]

Due to the existence of an oxygenated group at C-23, a γ-lactone with a carboxyl group at C-26 can form; such withanolides are classified into a total of five subgroups: spiranoidwithanolides, trechonolides, subtriflora-γ-lactones, ixocarpalactones, and taccalonolide-γ-lactones. Perulactones are a sixth subgroup with a γ-lactone side chain involving C-26 and C-28.[25,26]

A substantial quantity of withanolides with the parent skeleton of withaferin A (1), known for numerous additional entities with slight modifications, have been described [Figure 2]. These are mostly due to different hydroxylated substitution combinations and glycosylated derivatives. The essential moiety of withaferin A (1) can be seen across different new molecules isolated from WS, including the 17α-hydroxy derivatives (2)[27−30] and witharistatin (3).[31] The fundamental structure is similar to withaoxylactone (10),[32] where the 2,3-double bond has been hydrated. Among the most common withaferin variants are the hydrolytic cleavage of the 5,6-epoxide to give the *trans*-diaxial 5α,6β-diol, as in coagulin H (8) and coagulin S (9) there is a substitution pattern.[33] Similarly, the 20β-hydroxy derivative, like withanolide D (37), has also been reported[12,13,28,34−37] [Table 1].

The 5α-hydroxy-6α,7α-epoxy substitution sequence can be observed in withanolides of WS [Table 1, Figure 3]. Basic modifications in withsomniferol A (11)[30] and B (12),[30]

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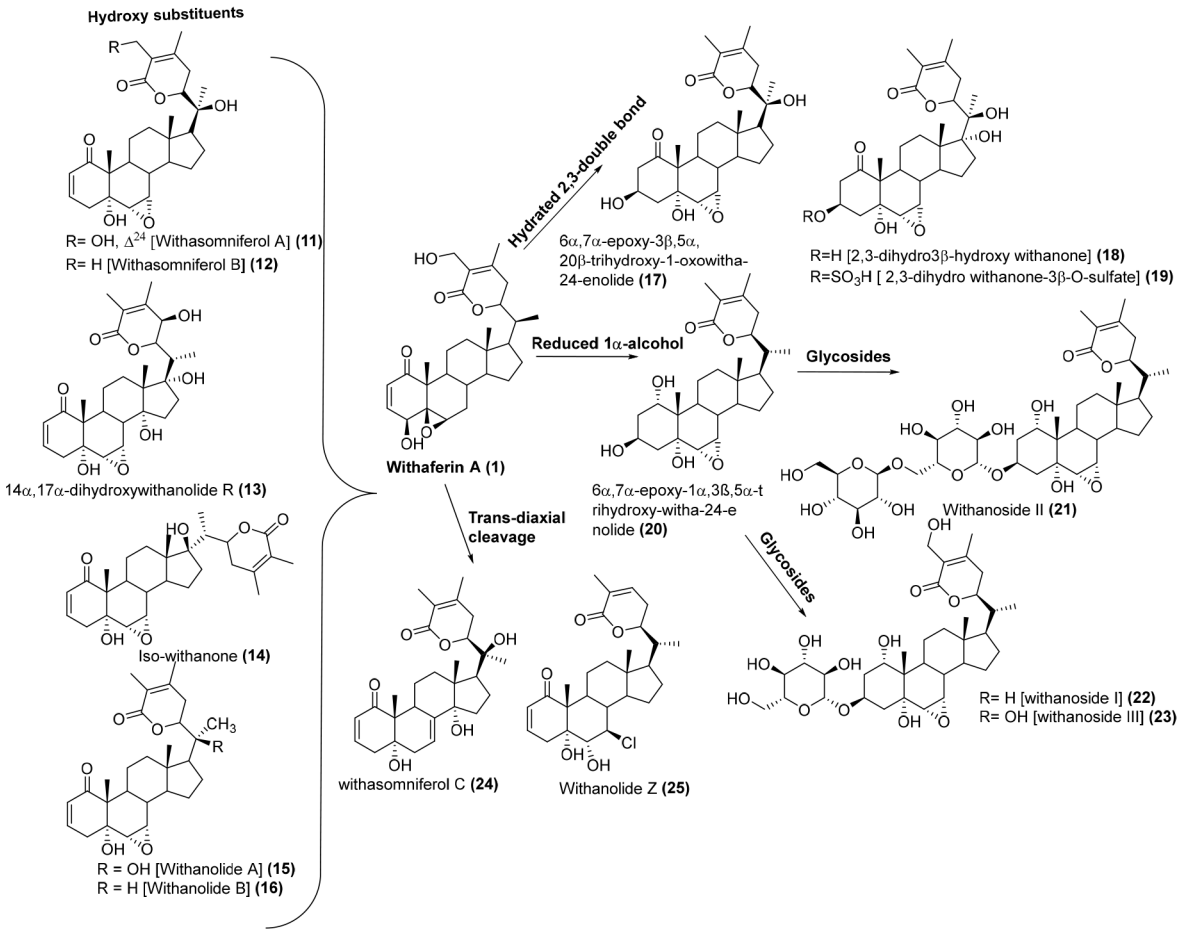

Figure 3. Substitutions such as 5α-hydroxy-6α,7α-epoxy with others on the withaferin A skeleton.

14α,17α-dihydroxywithanolide R (13),[38] and iso-withanone (14)[39] appear to be due to the combination of hydroxyl substituents at both typical positions (C-14, C-17, C-20) and some less common ones such as C-16 or C-23. Structures 17, 18, and 19 showed hydration in the 2,3-double bond, and the last of them was obtained as a 3-sulfate, which is uncommon in withanolides. Withanolide A (15) and B (16) are 5-hydroxy-substituted 6−7 epoxide derivatives.[28−30,34,40] The structures of both molecules are identical, but A differs from B by having an additional hydroxyl group on the C-20 atom. Kirson et al. reported withanolide R (36), as a 23β-hydroxy derivative of withanolide B.[41,42] The reduced 1α-alcohol (20)[39] along with the associated glycosides withanoside II (21), withanoside I (22), and withanoside III (23) were also discovered, indicating that the 1α,3β-dihydroxy arrangement was relatively prevalent among withanolides.[40] In addition, the chlorinated compounds withanolide Z (25)[43] and withanolide C (35)[44] were formed by trans-diaxial cleavage of a 6,7-epoxide and the δ7-withanolide (24).[30]

The 1α,3β-dihydroxy arrangement is a recognized structural distinction between withanolides, with multiple instances depicted in conjunction with 5β,6β- or 6α,7α-epoxides [Table 1, Figure 4]. In ring B, the 1α,3β-dihydroxy arrangement with a 5,6-double bond is reported from the 3β-O-glycosides isolated from WS and includes compounds 26 to 29.[35,40] This group also includes three withanolides with an additional sugar moiety at C-27, withanosides VIII (30), IX (31), and X (32) and an analog of withanoside VI (28) with a monosaccharide at C-3 called withanoside XI (33).[45,46] Coagulin Q (34) has the same aglycone as withanoside VI, but at C-3, there is a monosaccharide unit. All carbohydrate units attached are always β-D-glucose.[47]

As previously stated, many withanolides containing the basic skeleton of withaferin A (1) were separated. Having a couple of exemptions, the substitution patterns of rings A and B in new withanolides relate to those described earlier. The primary variation in structure has combinations of the oxygenated group (hydroxyl or carbonyl groups) at various positions in the steroid nucleus (most commonly at positions 12, 14, 16, 17, and 18) and the side chain (in the majority at C-20, C-21, and C-27). These functions may also exist in cyclic entities, such as lactones, lactols, and cyclic ethers.

Many withanolides exhibit C-14, C-17, and C-20 hydroxylation [Figure 5]. The 14-hydroxy attachment is usually α-oriented, yet there is also an increasing number of 14-β-hydroxy withanolides. As previously stated, hydroxyl replacement at C-17 can occur in either the α or β orientation, with the α orientation being more frequent. One of such a withanolide F (43) was isolated from the leaves of an Israelean chemotype III of WS with a 17-α-oriented alkyl structure.[48−51] Several structures have been identified and reported with various combinations of hydroxy groups at the aforementioned site, sometimes combined with hydroxy groups at positions C-15, C-16, and C-18. Withanolide E (38) is one such withanolides with 14α,17α,20β-hydroxyl substitution reported from WS.[48−51] There are multiple reports of δ16−14α-hydroxy withanolides. These consist of phyperunolide A (39)[52] and 15-acetyloxy withanolides such as withangulatin A (41).[53] Glotter

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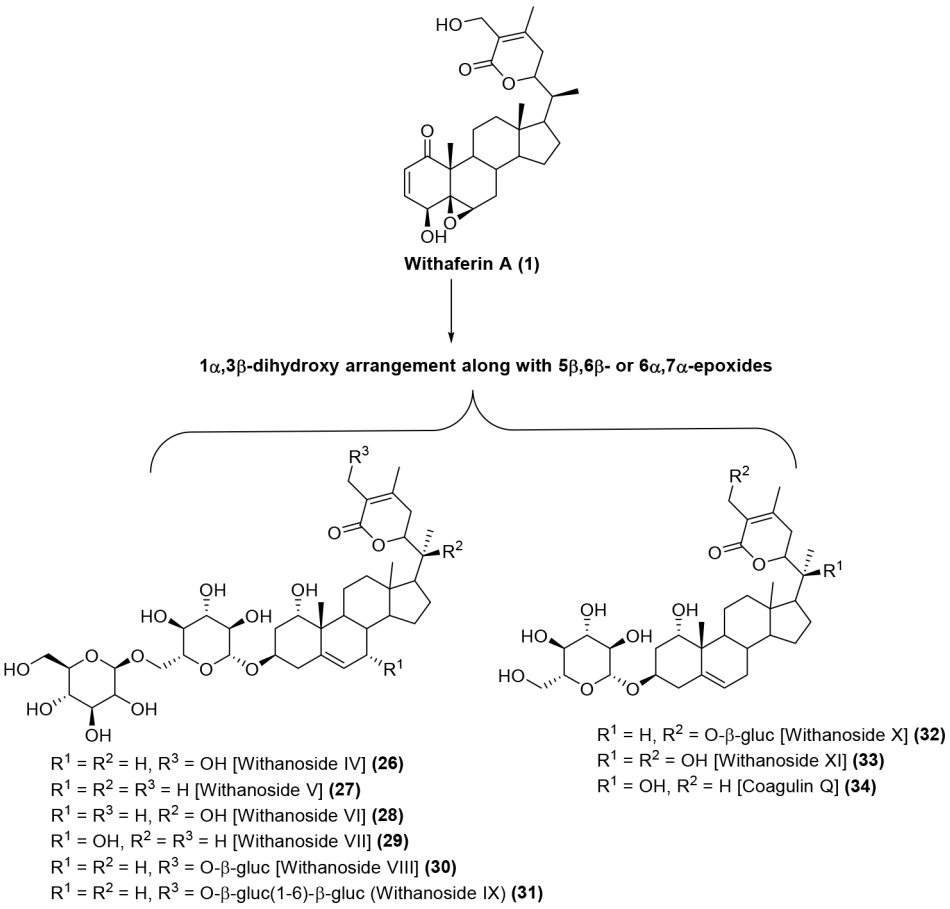

Figure 4. Modifications such as the 1α,3β-dihydroxy arrangement with others on a withaferin A skeleton.

et al. isolated seven compounds, as withanolides G (45) and I (47) are 20-α-hydroxy derivatives, whereas withanolide H (46) contains a 20α,27-dihydroxy moiety. On the other hand, the compounds that have 17-α and 20-α dihydroxy substitutions are withanolide J (44), withanolide K (48), withanolide L (49), and withanolide M (50).[49,50] Withanolide K is a 17α-OH derivative of withanolide I. Among the isolated molecules, withanolides G, H, and I have 20*R*,22*R* configuration, whereas withanolides J, K, L, and M have 20*S*,22*R* configuration.[49,50,54]

Abraham et al. isolated steroidal lactones of the withanolide series from the leaves of WS chemotypes I and II. From chemotype I, they isolated eight compounds, including withanolides N (51), O (52), and P (53). In contrast, from chemotype II they isolated withanolides D (37) and G (45), as well as three new molecules: 27-hydroxy-withanolide D (54), 14α-hydroxy withanolide D (55), and 17α-hydroxy withanolide D (56). Withanolide P (53) was found to have a unique 17α-oriented side chain. All withanolides isolated from chemotype I are unsubstituted at C-20 (20α-H), whereas molecules from chemotype II have a hydroxyl at this position (20α-OH).[41] Kirson et al. isolated two new steroidal lactones of the withanolide type from WS leaves identified by an unusual C-23 hydroxy group. The isolated compounds were named withanolide Q (57) and withanolide R (36).[42] Withanolide T (58), a 20-hydroxy withanone derivative, and withanolide U (59), a 4-hydroxy withanolide G derivative, were further isolated from WS[51] [Table 1].

4.2. Alkaloids. While WS has a number of alkaloids throughout the plant, the roots are where most of them are concentrated. Alkaloids such as anahygrine (64), berberine (63), and cuscohygrine (65) have reportedly been found in roots.[58,59] Alkaloids such as caffeine (68), choline (61), and withanamides A−I (79−87), on the other hand, can be found in other parts as well[58,60] [Table 2, Figure 6].

Withanamides make up a group of naturally occurring compounds belonging to the alkaloid class. They are composed of a pyrimidine ring attached to a lactam ring containing an amide linkage. The lactam ring is further fused to a steroid-like structure containing a five-membered lactone ring and a six-membered ring. The structure of withanamides is characterized by multiple functional groups, including hydroxyl, methoxy, and carbonyl groups.[61,62] Jayaprakasam et al. isolated withanamides A−I (79−87) from the fruits of WS[62] [Table 2, Figure 6].

4.3. Flavonoids. Flavonoids are diverse plant secondary metabolites with various biological activities. They are synthesized from phenylalanine or tyrosine via the phenylpropanoid pathway in WS. Flavonoids have been found in all parts of the plant, including WS's fruit, berries, leaves, and roots[66,67] [Table3, Figure 7].

Most of these flavonoids have chemical structures distinguished by a C6−C3−C6 carbon skeleton with various functional groups attached to the ring structures. The structural diversity of these compounds is caused by the various functional groups, including the hydroxyl (−OH) groups, methoxy ($-OCH_3$) groups, and glycosyl (−O-glucoside) groups attached to the flavonoid skeleton. The flavonoid kaempferol (93) has a ring structure with four

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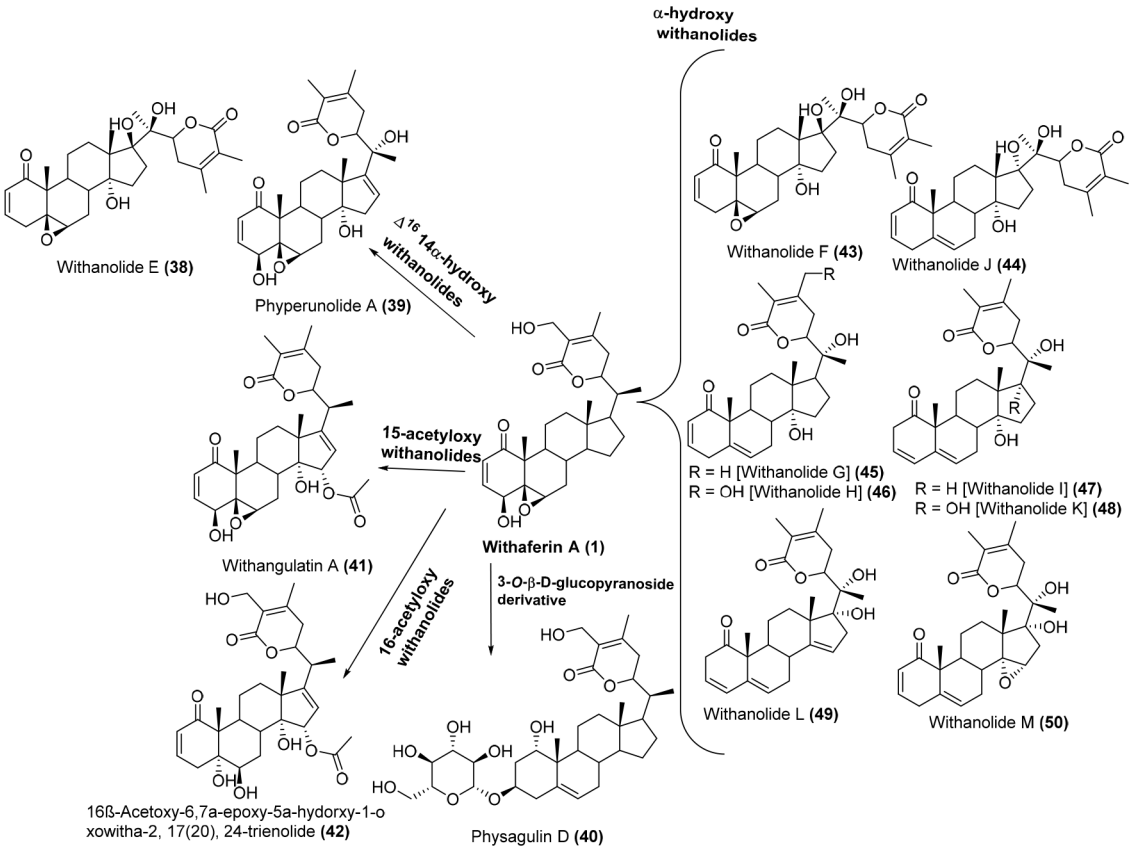

Figure 5. Hydroxylated derivatives of withaferin A.

hydroxyl groups (−OH) at positions 3, 5, 7, and 4′ and a carbonyl group (C═O) attached. WS contains the major kaempferol glycoside kaempferol 3-robinobioside-7-glucoside (95).[63,66−68] A flavone backbone with three hydroxyl groups (−OH) at positions 5, 7, and 4′ and a carbonyl group (C═O) attached to the ring structure also makes up apigenin (90). WS contains a significant amount of apigenin-7-O-*β*-D-glucopyranoside (103), an apigenin glycoside[66] [Table 3, Figure 7].

Another prominent flavonoid found in plants is quercetin (98), which has a flavone backbone with five hydroxyl groups (−OH) at positions 3, 5, 7, 3′, and 4′ and a carbonyl group (C═O) attached to the ring structure.[63,69] The two main quercetin glycosides present in WS are quercetin-3-O-D-glucopyranoside and quercetin-3-rutinoside (102) (rutin)[63,66,70,71] [Table 3, Figure 7].

4.4. Phenols, Phenolic Acids, Phytosterols, Fatty Acids and Lipids, Coumarins, Triterpenes, and Phenyl. *Propanoid Esters and Others.* The abundance of phenolic compounds and others in WS did not go unnoticed.[60,63] Many compounds from the same class have been reported to date, and some are listed below. Campesterol (119), cycloartenol (120), and stigmasterol (122) have all been reported from WS plant parts.[34,52,74] Similarly, the plant contains fatty acids, such as linoleic acid (129) and oleic acid (131). Fruits contain coumarins such as aesculetin (135) and scopoletin (136), which serve as a distinguishing feature in the detection of adulteration[38] [Table 4].

5. EXTRACTION AND FRACTIONATION TECHNIQUES

WS contains a wide range of bioactive compounds, including alkaloids, withanolides, and flavonoids, which are responsible for their therapeutic properties. Various extraction techniques are employed to extract these bioactive compounds from plant material. Extraction techniques play a crucial role in extracting bioactive compounds from WS. The choice of extraction technique depends on various factors, including the type of bioactive compounds to be extracted, the desired purity of the extract, and the intended use of the extract. Reflux extraction, maceration, Soxhlet extraction, ultrasonic extraction, supercritical fluid extraction, and microwave-assisted extraction are some of the commonly used extraction techniques for WS. The use of these extraction techniques has enabled the isolation of bioactive compounds from WS, which can be used for various therapeutic purposes [Table 5, Figure 8].

Extraction involves the separation of desired actives from the given matrices using suitable solvents and extraction techniques.[2,79,80] The type of extraction process used in herbal processing can have a significant effect on the final natural products obtained. The chosen strategy should be fast, modest, harmless to the ecosystem, reproducible, and high-yielding.[80]

Conventional methods for extracting phytochemicals from herbal plants include maceration, infusion, reflux, and Soxhlet extraction, which involve the diffusion of the solvent into plant cells, solubilization of phytochemicals within the plant matrix, and the diffusion of phytoconstituent solvents from plant cells.[81,82] Maceration extraction involves soaking the herb in a solvent for a prolonged period. During this time, the solvent gradually dissolves the herb's active components. This method is commonly used for delicate herbs and for extracting compounds that are not heat-stable.[83] Maceration extraction is also preferred for extracting WS as it is a gentle method that can preserve the delicate phytochemicals and antioxidants

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Table 2. Presence of Alkaloids in Different Parts of *Withania somnifera*

Alkaloids					
sr. no.	str. no.	name	mol. wt.	plant part	references
1	61	choline	104.2	roots/leaves	15, 58
2	62	anaferine	224.3	roots	58, 59
3	63	berberine	336.4	roots	60, 63
4	64	anahygrine	224.3	roots	58, 59
5	65	cuscohygrine	224.3	roots	58, 59
6	66	harmane	180.3	roots	60
7	67	harmine	212.3	roots	60
8	68	caffeine	194.2	fruits/roots	60, 63
9	69	isopelletierine	141.2	roots	58, 59
10	70	nicotine	162.2	roots	64
11	71	noscapine	413.4	roots	60
12	72	papaverine	339.4	roots	60
13	73	pseudotropine	141.2	roots	58, 59
14	74	sedridine	143.2	roots	65
15	75	somniferine	608.7	roots	59
16	76	theobromine	180.2	roots	60
17	77	theophylline	180.2	roots	60
18	78	3α-tigloyloxytropane	222.3	roots	58
19	79	withanamide A	778.9	fruits	61, 62
20	80	withanamide B	754.9		
21	81	withanamide C	754.9		
22	82	withanamide D	783.0		
23	83	withanamide E	783.0		
24	84	withanamide F	780.9		
25	85	withanamide G	752.9		
26	86	withanamide H	774.9		
27	87	withanamide I	941.1		
28	88	withasomnine	184.2	roots	58, 59
29	89	yohimbine	354.5	roots	60

present in the herb.[84,85] Because of their superior performance in terms of high output, self-sufficiency, and pricing, relatively modern techniques such as supercritical fluid extraction and pressurized liquid extraction have been introduced and widely used.[79,81,86] The New Millennium Indian Technology Leadership Initiative variety (NIMTLI-118) of WS was compared for seasonal variation in withanolide content and the effect of temperature on withanolide content.[87] According to the research, the withania shrub grown in the winter has more withanolide content than the shrub grown in the summer.[88] Thus, temperature and environment significantly impacted withanolide content.[89]

Reflux extraction is a method of extraction that involves boiling the herb in a solvent, which is then condensed and recycled back into the boiling vessel. This process is repeated several times, allowing the solvent to extract the active components from the herb. Reflux extraction is a popular method for extracting WS as it can efficiently extract the desired components and can be easily scaled up for larger batches.[83,85] It involves recycling the solvent for extraction and provides batchwise or continuous extraction setup as required. Although it takes a longer cycle time for the process to complete, the economic viability and scalability make it a highly popular technique in large processing platforms. Another key benefit is that it ensures the complete pull of actives from the plant matrices. By this, active loss can be easily curtailed.

On the other hand, ultrasound-assisted solvent extraction (UASE) is a nonthermal extraction method that uses high-frequency sound waves to disrupt the cell walls of the plant material, thereby facilitating the release of bioactive compounds. The plant material is immersed in a solvent, such as ethanol or water, and subjected to ultrasonic waves. Ultrasonic extraction is a rapid and efficient method of extraction that results in high yields of bioactive compounds.[83,90,91]

Microwave-assisted extraction (MAE) is a rapid and efficient method that uses microwave energy to heat the solvent and facilitate the extraction of bioactive compounds from WS. MAE is a relatively new technique, but it has shown promising results in the yield and purity of the extract.[83,84,92] In one study, WS powdered root raw material was extracted using methods like (reflux) ultrasound-assisted solvent extraction (UASE) and microwave-assisted solvent extraction (MASE) with different proportions of extraction solvent as water, ethanol:water (9:1), and water. UASE and MASE were extracted for 5, 10, and 20 min, respectively, while reflux extraction took 5 h. When compared to those of MASE (13.74%) and UASE (11.85%), the yield of the conventional extraction technique (9.51%) is significantly lower. UASE (8.66 μg/mg) and MASE (5.73 μg/mg) ethanol extracts are also higher in total withanolides than conventional ethanolic extracts (4.79 μg/mg).[83] In another study, the root raw material is extracted using conventional (reflux and Soxhlet) and nonconventional [microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE)] methods with ethanol−water in various proportions such as 0, 50, 70, and 100. The extracts are analyzed based on their total phenolic content (TPC). The amount of TPC is higher in MAE and UAE than in conventional methods.[84] These observations indicate the difference in the interface efficiency between raw materials and solvent media. Larger and effective penetration ensures rapid extraction of bioactivity at relatively faster rates, as observed in UAE and MASE techniques compared to conventional ones.

In one study, shade-dried and ground material of WS leaves, stems, and roots were macerated with methanol for 14 days. The methanol extract had higher yields as compared with stem and root extract. Similarly, flavonoid content was higher in leaves (43.51 ± 0.346 mg/g) than in stem (42.82 ± 1.189 mg/g) and root (39.13 ± 0.607 mg/g) extract.[93] Another antiadipogenic withanolide was isolated from the root of WS via reflux extraction with 80% aqueous methanol.[94] The dried root and fruits of WS were homogenized separately at room temperature with methanol:water (8:2) to yield 19% and 22% extractive yields, respectively.[95] A change in bioactive levels as per the plant parts makes it an important selection criterion for studies. Any combinations performed without considering individual profiling will impact the accuracy and precision of the exercise.

Supercritical fluid extraction (SFE) is a modern technique that uses supercritical fluids such as carbon dioxide as the solvent. Supercritical fluids have the unique properties of liquids and gases, making them ideal solvents for extracting bioactive compounds from WS. SFE is a selective and efficient method of extraction that results in a high-purity extract.[96,97] Pulverized and dried WS seeds were extracted with food-grade liquid carbon dioxide with backflow pressures of 450/80 and 555/40 bar/degree Celsius and CO_2 flow of 60 g/min for 22 h and 20 min to produce fatty acids with a 13% yield.[96] This also provides a cleaner alternative for extracting bioactives from

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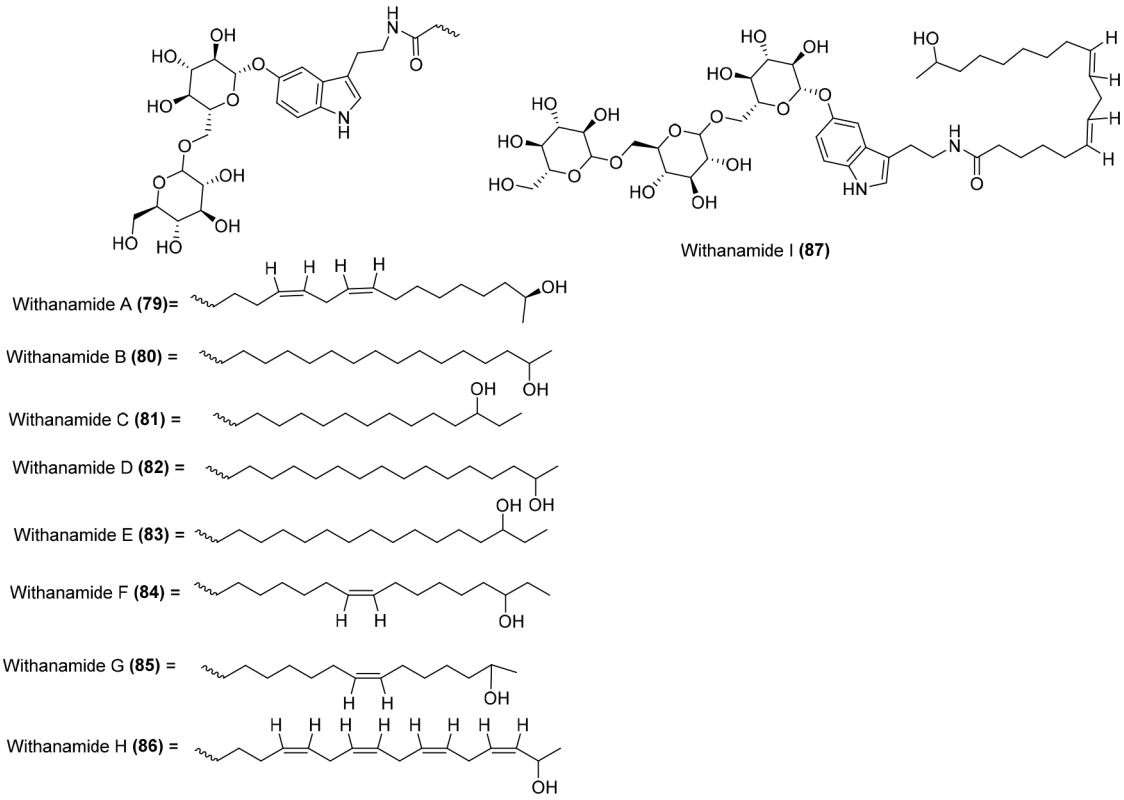

Figure 6. Chemical structure of alkaloids present in *Withania somnifera*.

Table 3. Flavonoids from *Withania somnifera*

Flavonoids					
sr. no.	str. no.	name	mol. wt.	plant part	references
1	30	apigenin	270.2	berries	66
2	31	catechin	290.3	fruits, leaves, roots, berries	66, 67
3	32	6,8-dihydroxykaempferol 3-rutinoside	626.5	leaves	70
4	33	kaempferol	286.2	berries/fruits/root	63, 66, 67
5	34	hyperoside	464.4	roots	60
6	35	kaempferol 3-robinobioside-7--glucoside	756.7	aerial parts	72
7	36	naringenin	272.3	whole plant/fruits	67, 73
8	37	naringin	580.5	whole plant	73
9	38	quercetin	302.2	whole plant	63, 69
10	39	quercetin-3-O-D-glucopyranoside	464.1	leaves	69
11	40	quercetin-3-rutinoside-7-glucoside	772.7	leaves, aerial parts	70, 72
12	41	rhamnetin	316.3	roots	60
13	42	rutin	610.5	roots/shoots/fruits/whole plant/leaves/berries	63, 66, 71
14	43	apigenin-7-O-β-D-glucopyranoside	432.1	berries	66

plant matrices. The skilled operational setup is the primary requirement of the technique.

Two traditional extraction techniques (A. maceration and B. Soxhlet) and two modern extraction techniques (C. microwave-assisted extraction and D. subcritical water extraction) were compared using the raw material of WS, leaves, and roots. In comparison to traditional procedures (A. 20.8% and B. 25.7), modern approaches have higher extraction yields (C. 30.2% and D. 65.6%) and have higher content.[76]

Total flavonoids are key molecules in antioxidant activity; the root of the plant is taken and extracted with different solvents at room temperature for 48 h. The methanol extract (5.8%) was found to have a higher yield, trailed by water extract (4.2%), chloroform extract (0.7%), acetone extract (0.65%), and hexane extract (0.4%). The total flavonoid content is also higher in methanol extract (105.09 ± 8.5 mg QCE/g) as compared to other extracts, and the antioxidant activity is also higher.[98] The dried cut roots of WS were pulverized and extracted exhaustively with hot water, HPLC grade methanol, ethanol, and ethyl acetate using an orbital shaker and mechanical agitation. The extract from each solvent is evaporated at 45−50 °C using a freeze-dryer and stored at room temperature. The methanolic extract produced the highest yield (11.08%), followed by the aqueous solvent extract, ethanol extract (5.92%), and ethyl acetate extract (0.75%).[99]

Acetone and MCW (methanol, chloroform, and water), which were previously compared against six other solvents and solvent mixtures, were shown to produce the best extraction.[100] Also, the root material of WS is immersed in water, a mixture of methanol, chloroform, and water (12:5:3), acetone, or aqueous methanol for 48 h at 40 °C (1:1). Aqueous, acetone, hydroalcohol and methanol−chloroform−water are the different extracts that are produced after the concentrates are filtered and evaporated at 40 °C. The hydromethanolic extract, with a yield of 16.82%, had the highest yield of any extract.[95] The desired polarity of the bioactive can be extracted according to the solvent polarity. Better yields ensure efficient

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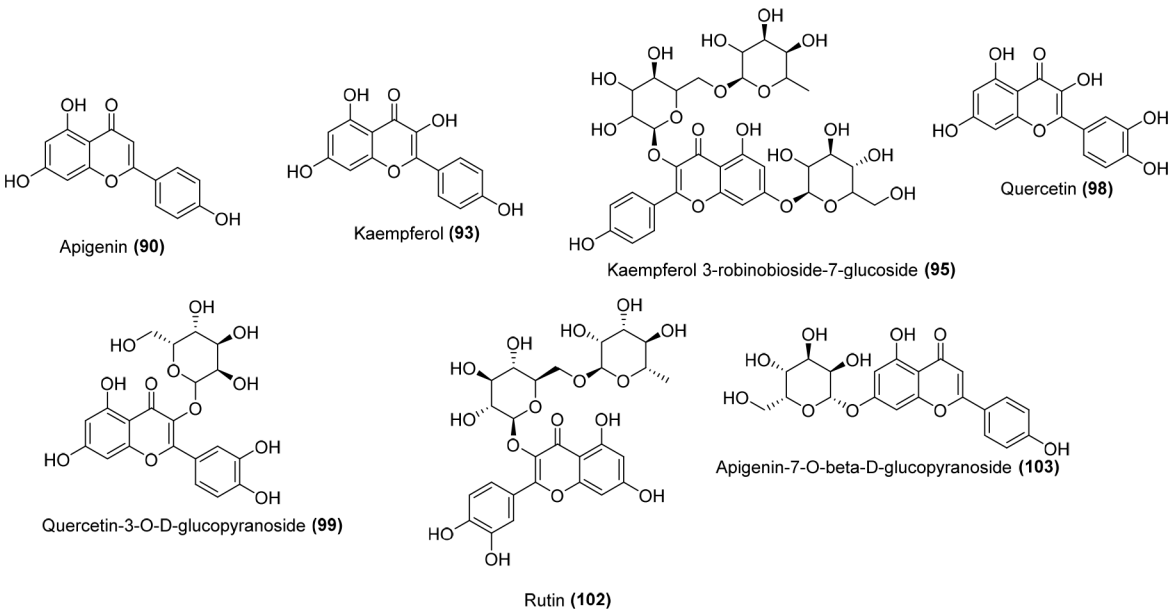

Figure 7. Chemical structure of flavonoids in *Withania somnifera*.

processing, although the concentration levels of desired bioactives also need to be considered.

Aqueous methanol may produce the best results when used to dissolve both hydrophilic and lipophilic components (1:1). In the past, the hydroethanolic extraction of WS roots produced an average yield of 15.40%.[101,106]

The fresh unripe fruits of WS were gathered, ground, and extracted with methanol at 30 °C. The yield of methanol extract was 1.54%, and afterward, it was subjected to liquid−liquid partitioning with hexane, dichloromethane (DCM), and ethyl acetate in a specific order. The methanol layer (37%) yielded the most, trailed by the DCM (3.2%) layer, the ethyl acetate layer (2.75%), and the hexane layer (1.46%).[102] Essentially, in another extract of the WS leaves, the raw material was extracted with 80% aqueous methanol and then exposed to liquid−liquid partitioning with specific solvents such as chloroform and butanol. The investigation revealed that the butanol layer (26.48%) produces higher yields than the other parts.[103]

Soxhlet extraction is a conventional technique that employs a Soxhlet extractor, a glass instrument used to extract compounds from solid herb materials. The sample is extracted with a solvent, such as ethanol or methanol, using a thimble. The solvent is subsequently evaporated, leaving the extract. Soxhlet extraction is a time-consuming but reliable approach for extracting various chemicals from WS.[104]

In addition to Soxhlet extraction, defatting with *n*-hexane and liquid partitioning with diethyl ether are applied to extract the leaves.[105] Also, the leaves and roots of WS were extracted using *n*-hexane, followed by methanol−water (90−70%, MeOH) at 35 °C. After liquid−liquid partitioning of the methanolic water layer with $CHCl_3$ and *n*-BuOH, the plant metabolite variety was separated into four fractions of varying polarity (*n*-hexane, aqueous methanol, chloroform, and *n*-butanol). The quantity of metabolite in leaves was significantly more than in roots, particularly in the aqueous methanolic fraction.[15]

According to the presented data, variables such as extraction solvent, techniques employed, and plant part used significantly affect the yield and assay. Even though techniques such as MASE and UASE have demonstrated promising results, they lack the economic and practical viability for large-scale operations when compared to the conventional extraction mode. To overcome the limitations of traditional methods, further development along these lines is needed to make them viable. Another advantageous platform for obtaining clean extracts is supercritical extraction, which reduces the requirement for a substantial amount of solvent. This is also crucial from a regulatory standpoint. However, when looking at the operational setup and its workings, it demands increased resources and skilled human resources in the operations. This might be a hindrance to its rather low-paced propagation.

The use of distinct plant parts for different therapeutic areas is a well-established practice found in various traditional medical systems. The present work also examines the effect of these plant parts of WS, when treated with various techniques, on outcomes such as bioactive yields. WS has been studied for its pharmacological effects for quite a long time. The relation between plant parts subjected to various techniques and solvent systems and that of pharmacological effects has been enlisted, as shown in Table 5. This can be useful for future comparative and related investigations on this topic. It also accounts for the advantages and limitations of techniques or solvent systems in efficiently extracting the bioactive from the different matrices. As witnessed in prior works, it also focuses on determining the optimal approach for any such studies under consideration.

6. ANALYTICAL TECHNIQUES

Recent upgrades in phytoanalytical research have led researchers to identify, characterize, and quantify the withanolides and their derivatives sourcing from WS using various chromatographic techniques that include TLC, HPTLC, HPLC-PDA, GC-MS, and LC-MS/MS.[15,83,95,99,102,110,111] The thin layer chromatography (TLC) analysis is a versatile technique that can be used to investigate the withanolide profiling in the WS extract.[110] High-performance thin-layer chromatography (HPTLC) is a modern version of TLC used

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ACS Omega XXXX, XXX, XXX−XXX

Table 4. Some Other Phytochemicals Present in *Withania somnifera*

sr. no.	str. no.	name	mol. wt.	plant part	references
		Phenols or Phenolic Acids			
1	104	4-O-caffeoylquinic acid	354.3	leaves	70
2	105	caffeic acid	180.2	fruits, roots, leaves	60, 63, 75
3	106	*p*-coumaric acid	164.2	fruits, roots, leaves	60, 63, 67
4	107	curcumin	368.4	whole plant	73
5	108	4,5-O-dicaffeoyl quinic acid	516.5	leaves	70
6	109	ferulic acid	194.2	fruits, roots, leaves	60, 63, 75
7	110	*p*-hydroxy benzoic acid	138.1	roots	60
8	111	gallic acid	170.1	fruits/roots/leaves/berries/whole plant	60,63,73,75
9	112	2-hydroxycinnamic acid	164.2	fruits	63
10	113	phenyl acetic acid	136.2	leaves and roots	15
11	114	quinic acid	192.2	leaves	70
12	115	syringic acid	198.2	leaves	67
13	116	vanillic acid	168.1	fruits and leaves	63, 67
14	117	3,4,5-trihydroxycinnamic acid	196.2	roots	15
15	118	vanillin	152.1	fruits	63
		Phytosterols			
16	119	campesterol	400.7	roots	15
17	120	cycloartenol	426.7	fruits	76
18	121	cholesterol	386.7	roots	76
19	122	stigmasterol	412.4	roots and fruits	15, 34, 74
20	123	stigmasterol glucoside	574.8	roots	34
21	124	*β*-sitosterol	414.7	roots and fruits	34, 38
22	125	stigmasterone	410.7	roots	34
23	126	*β*-sitosterol glucoside	576.9	roots and leaves	34, 52
		Fatty Acids and Lipids			
24	127	arachidic acid	312.5	fruits	76
25	128	hydroxypalmitic acid diglucoside	580.7	fruits	77
26	129	linoleic acid	280.5	roots and leaves	15
27	130	linolenic acid	278.4	roots and leaves	15
28	131	oleic acid	282.5	roots and leaves	15, 74
29	132	palmitic acid	256.4	roots and leaves	15
30	133	stearic acid	284.5	roots	74
31	134	vaccenic acid	282.5	fruits	52
		Coumarins			
32	135	aesculetin	178.1	fruits	38
33	136	scopoletin	192.2	fruits	38, 59
		Triterpenes			
34	137	*β*-amyrin	426.7	fruits	38
35	138	oleanolic acid	456.7	roots	74
		Phenyl Propanoid Esters			
36	139	withaninsams A	294.3	roots	78
37	140	withaninsams B	294.3	roots	78

for the qualitative analysis and identification of multiclass metabolites present in root and fruit hydroalcoholic extracts.[95] HPLC is a robust and accurate chromatographic technique used to identify and quantify bioactive compounds present in WS extracts. In several studies, withanolide derivatives and withanosides have been identified and quantified using HPLC-PDA methods. The refractive index detector is the choice when one needs to detect analytes with restricted UV absorption such as alcohols, sugars, carbohydrates, fatty acids, and polymers. It has also been reported that these multiclass compounds are identified and quantified with ELSD detection.[112] Gas chromatography−mass spectrometry (GC−MS) analysis was performed with ESI mode at 70 eV to generate mass spectra. The GC−MS running conditions were the same as those mentioned earlier. Metabolite was quantified using its percentage peak area that appeared at the total ion chromatogram in GC−MS analysis.[15] Liquid chromatography coupled with PDA and MS detectors can be used to obtain essential information about WS extracts, such as probable phytocompounds present in the extract. Based on their mass, m/z transitions (MS/MS fragments), UV maxima of each peak, and relative retention periods, compounds can be identified using online databases and literature.[113]

7. PLANT DESCRIPTION

The genus Withania, belonging to the family Solanaceae with 26 species, is widely distributed from the southern Mediterranean to the Canary Islands to south and east Africa and some parts of Asia, including India[13,114,115] [Figure 9]. *Withania somnifera* (L.) Dunal (WS) and *Withania coagulans* (Stocs.) Dunal (WC) species are found in India. The WS, commonly known as Ashwagandha, winter cherry, Nagouri,

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ACS Omega XXXX, XXX, XXX−XXX

Table 5. Different Extraction Techniques for *Withania somnifera* along with Pharmacological Activity

plant part	extraction method	solvent treatment	extract yield (%)	pharmacological activity	reference
roots	reflux	ethanol	9.08	antioxidant	83
		water:ethanol (9:1)	9.43		
		water	9.51		
	UASE	wthanol	3.17		
		water:ethanol (9:1)	9.74		
		water	11.85		
	MASE	ethanol	10.01		
		water:ethanol (9:1)	13.75		
		water	13.02		
leaves	maceration	methanol	7.5	antioxidant	93
stems			5.2		
roots			4.7		
roots	reflux	methanol:water (80:20)	2.19	antiadipogenic	94
seed	super critical CO_2 extraction	liquid CO_2	13	anti-inflammatory	96
root and leaves	maceration	water	20.8	antioxidant and anticancer	104
	Soxhlet extraction	ethanol:water (80:20)	25.7		
	MAE	methanol:water (80:20)	30.2		
	subcritical extraction	water	65.6		
roots	maceration	water	4.2	antioxidant	98
		methanol	5.8		
		chloroform	0.7		
		acetone	0.65		
		hexane	0.40		
roots	exhaustive extraction	aqueous	11.08	cytotoxicity and antimycobacterial	99
		methanol	12.22		
		ethanol	5.92		
		ethyl acetate	0.75		
roots	maceration	water	11.64	antioxidant	101, 106
		acetone	0.162		
		water:methanol (1:1)	16.82		
		methanol:chloroform:methanol (12:5:3)	14.39		
fruits	reflux:mechanical stirring:liquid−liquid partitioning	methanol	1.54	antiproliferative	102
		hexane	1.46		
		DCM	3.2		
		ethyl acetate	2.75		
leaves	maceration:liquid−liquid partitioning	80% methanol	17.57	antidiarrheal	103
		chloroform	20.85		
		n-butanol	26.48		
roots	maceration	ethanol	3.4	regulator of the melanogenesis	107
roots	maceration:liquid−liquid partitioning	hexane	4.44 (mg/g Dw)	-	15
		chloroform	10 (mg/g Dw)		
		n-butanol	11.11 (mg/g Dw)		
		methanolic water	15 (mg/g Dw)		
leaves		hexane	34.29 (mg/g Dw)		
		chloroform	35.7 (mg/g Dw)		
		n-butanol	28.57 (mg/g Dw)		
		methanolic water	228.57 (mg/g Dw)		
roots	maceration	80% methanol	10	prenatal developmental toxicity study	108
roots	maceration	methanol:water (8:2)	19	antileishmanial and anticancer	95
fruits			22		
roots	maceration	methanol	8.45	hypothyroidism	109
		water	8.33		

and Punir, is believed to be of medicinal value in traditional systems of medicines like Siddha, Unani, and Ayurveda.[116] Locally, it is also referred to as "Indian Ginseng" because of its therapeutic and restorative properties.[117]

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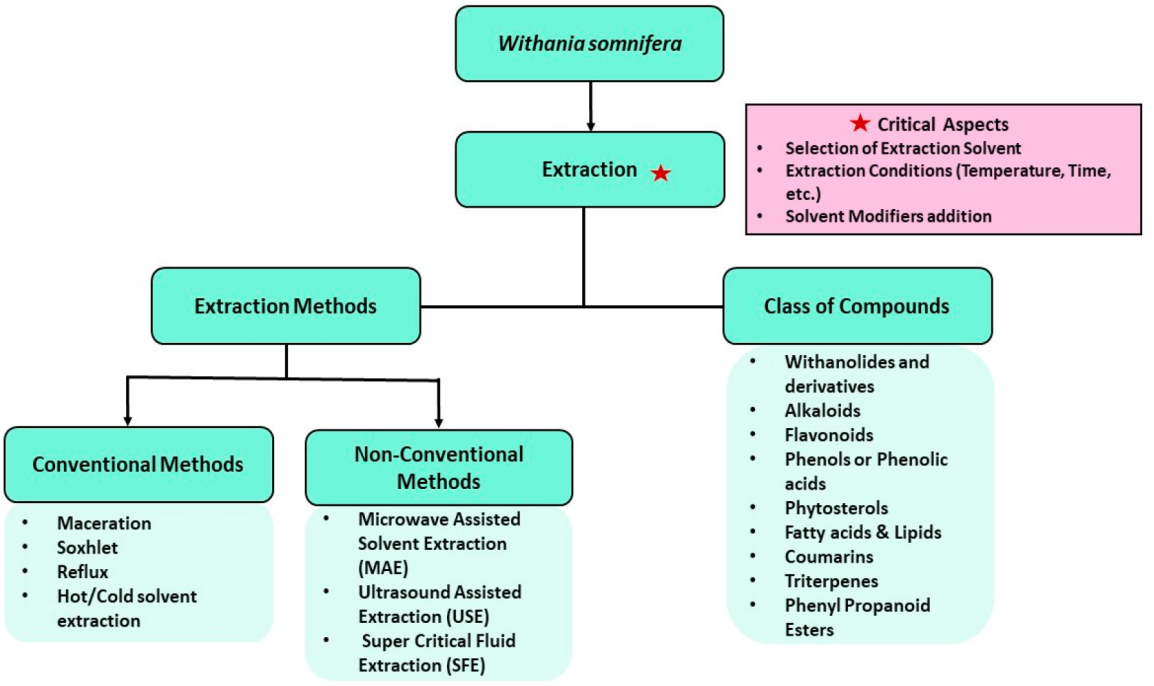

Figure 8. Extraction techniques and class of compounds found in *Withania somnifera*.

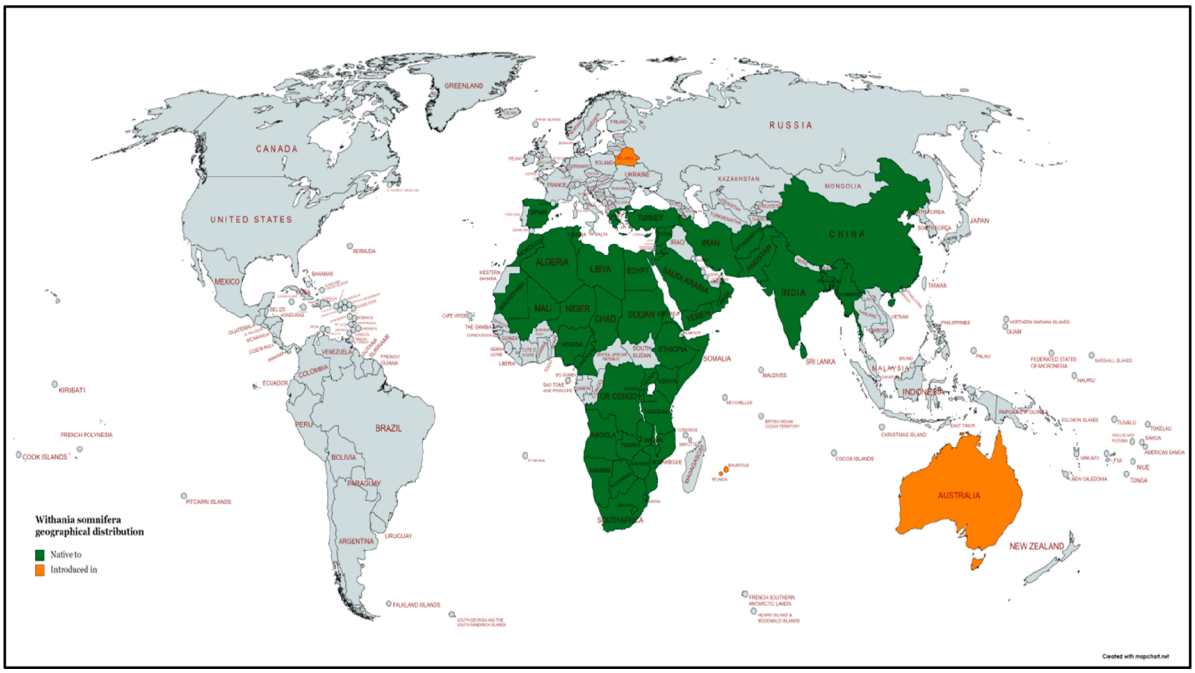

Figure 9. Distribution of *Withania somnifera* in the world.[115] Created with mapchart.net.

The WS plants are upright, branching shrubs that grow up to one meter tall. Simple petiolate, ovate, exstipulate, whole, acute, and glabrous describe the leaves [Figure 10]. Flowers are straightforward, short-pedicelled, gamosepalous, and persistent and have five sharply lobbed sepals. Gamopetalous corolla has five spreading or recurved lobes that are acutely pubescent and greenish-yellow; epipetalous stamens have petals at the base. Slender filaments are inherent and have round anthers. A tiny, bulging ovary called the gynoecium syncarpous is surrounded by a long, thin style.[117−120] Roots have more useful chemical components in WS than other parts [Figure 10]. India's production is substantially lower (1500 tons), but the domestic market is promising. The roots can be harvested 180 to 210 days from the seeding date. The entire plant is pulled up, and the aerial part's roots are removed. The maximum yield for cultivation is often 3 to 5 quintals per hectare.

WS thrives in arid and subtropical climates. With its vast biochemicals, this plant is tough and drought-resilient. Because of this, it continues to enjoy a monopoly in many regions of India, especially in Madhya Pradesh.[117] It grows in dry parts in subtropical regions like Rajasthan (Nagour), Punjab, Haryana,

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Figure 10. (a) Whole plant of *Withania somnifera* (L.) Dunal, (b) *Withania somnifera* (L.) Dunal aerial parts, and (c) *Withania somnifera* (L.) Dunal roots.

Uttar Pradesh, Gujarat, Maharashtra, and Madhya Pradesh.[114,121−123]

As the plant has global demand, it has been brought under commercial cultivation, and few varieties have been released. Pure line selection was used to create these variants from the local cultivars. Since species have been cultivated for over a century, systematic breeding techniques have become crucial.[124,125] Berries with diverse colors are now available with both annual and perennial growth habits since both natural (perennial) and farmed (annual) types are easily crossable.[126]

8. PHARMACOPEIAL METHODS

Currently, multiple pharmacopeial methods are available to standardize the WS extracts. These include Indian Pharmacopoeia (IP), which provides analytical methods for standardized root extracts based on withanolide glycosides and withanolide aglycone contents.[127] The United States Pharmacopeia (USP) also suggests an HPLC method for standardizing WS extracts. The USP also provides an HPTLC method to identify withanolide A and β-sitosterol in WS raw material and extracts. This pharmacopeial monograph also suggests a limit of flavanol glycosides from WS aerial parts using the HPLC method for evaluating the assay of glycoside content in the extract.[128] American Herbal Pharmacopoeia (AHP) also gives an HPTLC method to analyze β-sitosterol in WS extracts.[129] British Pharmacopeial (BP) methods are used for microscopic analysis for identification purposes.[130] Ayurvedic Pharmacopoeia of India (API) offers a gravimetric method for the total alkaloids and total withanolides.[131] The AOAC method quantitatively calculates withanolide glycosides and withanolide aglycones from WS roots.[132] These pharmacopeial methods have analyzed withanolide glycosides and withanolide aglycone content in WS samples.

9. FOOD APPLICATIONS

Herbs were used as a food source in numerous cultures throughout history, including India, Rome, Persia, Mesopotamia, Greece, and Arabian kingdoms.[133] Due to consumer preference for health-promoting substances, the market for functional foods is expanding exponentially, and the well-established relationship between food and bioactives explores the vast potential of novel and inventive foods to preserve or improve health, thereby increasing the desire for research on advanced products with therapeutic effects.

As a result, several investigations have been carried out to elucidate efficient extraction methodologies for enriching bioactive compounds of interest and determining their impacts on human health. The data on raw plant components are being collected as part of the standardization of herbal medicinal products and functional foods based on specifications. A medico-botanical survey, identification, botanical authenticity, and macroscopic evaluation are also conducted. As the global demand for medical plants and herbal products rises, the necessity for quality control measures has become increasingly crucial. Internal control, final goods, and batch-to-batch variation of end products are among the elements of the production process that should be subjected to quality control for products derived from herbs. Numerous governments and regulatory organizations are presently developing recommendations to enhance the quality of herbs and extracts, medicines, and food products developed from them.

While extracts from various plants have been studied and reported to improve food storage quality due to their antioxidant and antimicrobial properties, using WS extracts as a biopreservative to improve food storage stability has received little attention. WS is expected to improve the storage stability of fat-rich food products, such as cheese, due to its strong antioxidant and antimicrobial properties. During 4 weeks of refrigerated storage, the WS fruit extract (0.5%) significantly improved the cheese samples' lipid stability and microbial quality. The extract also improved the cheese's sensory quality near the end of the storage period by slowing the rate of lipid and microbiological deterioration. The feeding trial results showed that the addition of the extract increased the functional value of the product. The cheese-based diet significantly reduced the oxidative stress caused by CCl_4 in Wistar rats. The extract-based diet affected body weight, endogenous enzymes, ALT and AST, liver marker enzymes, and lipid peroxidation. WS extract improves other foods' functionality and storage quality.[134]

Although the potential medicinal uses of WS have been thoroughly researched, its food applications are still under exploration. Its roots and leaves are both high in nutritional fiber. The included WS leaves and roots can be used to make extrudates, juices and beverages, sweet products, cereal and bakery goods, dairy goods, and other goods. Many therapeutic substances have been shown to exhibit a wide range of biological behaviors. These substances have the potential to have a significant impact on the nutritional system.[7]

The effects of WS root powder (WSRP) on the composition and physicochemical, physical, functional, and sensory properties of cow milk were investigated. Skimmed and standardized (3% fat) milk were fortified separately with WSRP at 0, 0.5, 1.0, 1.5, and 2%, thoroughly mixed, and pasteurized (63 °C for 30 min), yielding ten treatments. Except for the RCT and color parameters, adding WSRP to skimmed and 3% fat-standardized milk did not significantly change the tested compositional,

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ACS Omega XXXX, XXX, XXX−XXX

Table 6. Food Applications of *Withania somnifera*[a]

sr. no.	plant part	food application	extraction methodology	significance	ref
1	fruits	shelf life improvement of cheese	cold maceration	prevents microbiological deuteriation of cheese	131
2	roots	milk fortification	NA	for creation of functional fermented dairy products	133
3	roots and leaves	food adjuvant in ghee products	decoction for aqueous extract and percolation for ethanolic extract	enhances stability and antioxidant capabilities of ghee product	132, 136
4	roots and leaves	pills or powder, kombucha, ghee, honey	Soxhlet extraction	nutritious snacks fortified with ashwagandha retains antioxidant property	134
5	roots	Indian flatbreads (chappati, naan, and thepla)	NA	an effective ingredient in making low glycemic food product	137

[a]NA: Not available.

physicochemical, physical, and functional properties. It demonstrates that WSRP fortification has no negative impact on culture microorganisms, implying that it could be used to produce fermented dairy products. Sensory scores fell as WSRP levels increased in both milk types. The sedimentation of WSRP in fortified milk had a greater impact on the sensory scores. According to the results of the tests, WSRP can be used to fortify milk and create functional fermented dairy products, such as cheese and yogurt. As a result, the information gathered would be critical for developing new items for the rapidly expanding category of functional dairy goods.[135] [Table 6].

The nutritional value of WS-fortified beverage blends could be used in functional fruit drinks and juices, and the produced stored product was stable and suitable for 90 days at room temperature. Several concentrations of ashwagandha powder, which is present in many goods, are studied in bakery goods and cereals, ranging from 5% in traditional Indian foods items such as Namakpara, Pap Chakal, and Muruk to up to 10% in Missy Roti and Chutney powders. Biscuits are high in protein, fiber, energy, and minerals, and they can contain up to 5% WSRP, increasing their therapeutic properties. The blood sugar level is reduced by incorporating 2% WSRP into bread and other baked goods, such as Thepla. Ashwagandha powder in dairy products containing ghee (fat), such as vidarikand (*Pueraria tuberosa*) and shatavari (*Asparagus racemosus*), has more potent antioxidant capabilities than ghee made traditionally. Shrikhand candies, for example, have a shelf life of up to 52 days in the refrigerator and contain 0.5−0.6% ashwagandha powder, which is said to promote stability[7] [Table 6].

WS had already conventionally been accessible as either a supplement in the form of pills or powder.[136] It is now found in a wide range of products, which include kombucha, ghee, honey, etc. WS is found in baked products, juices, and beverages, as well as sweets (candies/snacks) and milk products marketed as "Functional Foods" or "Nutraceuticals".[136,137] Adding different bioactive enriched extracts of WS into meals can serve various purposes, such as amazing antioxidant and human health advantages.[138] The cereal-legume-based Ashwagandha root powder with sweet Ladoo was developed. The sensory properties and nutrient profile, including mineral content, crude fat, crude fiber, and total dietary fiber, were studied.[136] The WS root powder was incorporated into baked goods like flatbread, and a reduced glycemic index and reducing blood sugar levels were reported.[139]

10. PHARMACOLOGICAL RELEVANCE OF *WITHANIA SOMNIFERA*

WS is well-known for a wide spectrum of biological activities, such as in animals treated with cyclophosphamide to reduce DTH, and extracts of WS were found to be effective. A significant increase in platelets, WBC, and hemagglutinating, hemolytic antibody responses was also observed, indicating activity against cyclophosphamide-induced myelosuppression and immunosuppression.[140] Pretreatment of C6 cells with 0.1% WS extract provided cytoprotection against lead toxicity (25−400 M).[141] Furthermore, a 20 mg/dose/animal dose of 70% methanolic root extract increased WBC count, circulating antibody titer, antibody-forming cells, spleen and thymus weight stimulation, and macrophage phagocytosis. In Ehrlich ascites tumor (EAT)-bearing mice, WS (20, 50, and 100 mg/kg) had dose-dependent immunomodulatory activity.[142]

The plant showed some promising activity in diabetes. In rats with noninsulin-dependent diabetes mellitus (NIDDM), WS treatment improved insulin sensitivity significantly. Blood glucose, HbA1c, and insulin levels were found to be reduced by aqueous extracts of WS (200 and 400 mg/kg for 5 days). Furthermore, withaferin A from WS was able to inhibit inflammatory responses caused by cytokine-induced damage to the islets of Langerhans *in vitro* after transplantation and has antiglycating properties.[143] In the leukemic murine mouse model, withanolide D reduces antiapoptotic genes (TERT, Bcl-2, and Puma).[144] Furthermore, the crude water extract (0.5%) of WS influenced a signaling pathway involving pro-apoptotic and tumor-promoting proteins, aiding tumor growth suppression. Thus, inhibiting proteins involved in cell survival [(Nuclear factor-kappa B (NFB), Phospho-Akt (p-Akt), B-cell lymphoma extra-large (Bcl-xl), and Heat shock protein 70 (HSP70)] exhibited a pleiotropic antiglioma phenomenon in both *in vitro* and *in vivo* systems. With an IC50 dose of 10 μg/mL, the methanolic root extract promotes cell cycle arrest in the G2/M phase and attenuation of proliferative, metastatic, and angiogenic signals mediated by interleukin-8 and cyclo-oxygenase-2 expression in prostate cancer PC3 cells.[145] Withaferin A derivative 2,3-dihydro-3-methoxy withaferin A has been shown to dose-dependently enhance the circadian rhythm in sarcoma 180 cancer cells.[146]

In female Balb/c mouse models, oral administration of WS root extract powder (500 and 1000 mg/kg body weight) was found to be effective in reducing pro-inflammatory cytokines (IL-6), tumor necrosis factor (TNF), reactive oxygen species (ROS), and nitric oxide (NO) and in reducing systemic lupus erythematosus-like symptoms (nephritis, proteinuria, autoantibody production).[147] Withaferin A suppressed p38 phosphor-

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ACS Omega XXXX, XXX, XXX−XXX

ylation by energizing PMA, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2.[148] Furthermore, withaferin A (3 M/ml *in vitro*) inhibited IL-8 and NFB in cystic fibrosis cellular models (HEK293).[149]

Despite the lack of a scientific explanation, WS has been used to treat infections for centuries. The plant's methanolic leaves extract (2 mg/mL, 100 μL per well in an agar well diffusion assay) showed remarkable antimicrobial activity against Gram-positive isolates of MRSA and Enterococcus sp. isolated from pus samples.[150] It also showed antimicrobial potential against Gram-negative bacteria.[151] A methanolic extract of WS was tested *in vitro* (0.125−2 mg/mL) and inhibited acid production, tolerance, and biofilm production of oral microflora like *Streptococcus sobrinus* and *Streptococcus mutans* at sub-minimal inhibitory concentrations (MICs).[68] Numerous studies have thoroughly examined the neuroprotective abilities of WS, and findings from preclinical studies and clinical trials have validated this claim. In test animals, the leaves extract's withanone can protect glial and neuronal cells from scopolamine's toxicity.[152,153] WS extracts significantly reduced several neuronal cell markers [microtubule-associated protein 2 (MAP-2), neurofilament (NF)-H, growth associated protein 43 (GAP-43), and postsynaptic density protein 95 (PSD-95)], glial cell markers [glial fibrillary acidic protein (GFAP)], and DNA oxidative stress markers.[154] The number of degenerating cells in the carbonic anhydrase (CA)2 and CA3 of the rat's hippocampus subjected to stress was significantly reduced by a root powder extract of WS (20 mg/kg body weight for 30 days).[155]

Parkinson's disease (PD) is a progressive neurodegenerative disease caused by the destruction of nigral dopaminergic neurons in the striatum, which results in dopamine depletion in the striatum, oxidative stress, and mitochondrial dysfunction. Resting tremors, postural abnormalities (stooped posture), bradykinesia, festinating gait, and akinesia are all symptoms of Parkinson's disease.[156] Oral administration of ethanolic root extract of WS resulted in increased striatum dopamine levels, which reduced the effects of 6-hydroxy dopamine-induced Parkinsonism, demonstrating that the ethanolic extract of WS has potent antioxidative properties. 6-Hydroxydopamine (6-OHDA) is a popular alternative for eliciting PD symptoms. When 6-OHDA is injected stereotactically into the striatum of the brain or the substantia nigra tract, catecholaminergic neurons undergo specific degeneration. 6-OHDA-induced dopaminergic toxicity causes oxidative stress by generating hydrogen peroxide (H_2O_2) and hydroxyl radicals, which lower GSH content and SOD activity, increasing malondialdehyde levels in the striatum (MDA).[157]

In 1872, George Huntington presented an elaborate and detailed description of the clinical manifestations of Huntington's disease (HD), a rare, progressive neurodegenerative disorder. The function of the mitochondrial enzyme complexes I, II, and III could also be restored by the root extract, whereas 3-NP treatment impaired the activity of SDH, isocitrate dehydrogenase, ketoglutarate dehydrogenase, and malate dehydrogenase.[158] WS has been proven to have antifungal effects against several pathogenic fungus taxa. For the first time, researchers investigated the antifungal activity of WS water extract (WSWE) against an itraconazole-resistant strain of *S. globose* with an IC50 of 1.40 mg/mL; the treatment with WS water extract reduced the growth of the *S. globosa* yeast form in a dose-dependent way.[159] Pharmacokinetic studies provide crucial information about how the body absorbs, metabolizes, and eliminates compounds from herbal extracts. This information is necessary for determining the optimal dose, duration of treatment, and potential drug interactions. For example, the pharmacokinetic parameters of withaferin A and withanolide A were investigated through a study in which an aqueous extract of WS was orally administered to Swiss albino mice at a dose of 1000 mg/kg of body weight. The study employed the HPLC-MS/MS method to evaluate the bioactive compounds. The results showed that the maximum plasma concentrations of withaferin A and withanolide A were 16.69 ± 4.02 and 26.59 ± 4.47 ng/mL, respectively, with 10 and 20 Tmax. These findings suggest a rapid absorption of the bioactive compounds.[160]

Another significant benefit of pharmacokinetic studies for herbal extracts is that they can help standardize their production. There can be significant variations in the composition of herbal extracts depending on the extraction method, plant source, and processing conditions. Standardizing the production of herbal extracts can ensure consistency in their quality and efficacy.[161] Essential trace elements (ETEs) are essential for cell functions, acting as cofactors for enzymes and stabilizing protein structures. The study aimed to develop ASH-FMB [(Ashwagandha- fusion (F), micronization (M), bioligation (B)], a novel herbal extract of Ashwagandha fused with ETEs using FMB technology. ASH-FMB was evaluated for its antioxidant, anti-inflammatory, and immunomodulatory activities, and ETEs and bioactive compounds were identified through atomic absorption spectrometry and HPLC. FMB technology replenished iron, zinc, and calcium, and phytochemical analysis identified withanoside IV and withanolide A. ASH-FMB demonstrated higher antioxidant and anti-inflammatory activities than ASH, significantly stimulated spleen cell proliferation, and showed similar results in pinocytic activation. The study suggests that FMB techniques enhance the therapeutic value of standardized extracts.[162]

Several studies in various biological models have indeed been conducted to elaborate on the pharmacokinetic properties of WS. In male Sprague−Dawley rats, the validated UHPLC-MS/MS method was employed to calculate seven constituents instantaneously after oral administration of WS extract (500 mg/kg) (n = 6). At the first time point (i.e., 15 min) four compounds were quantified except for withanone, withanolide B, and withanoside V. Plasma peak concentrations (C_{max}) for withanoside IV, withaferin A, 12-deoxy-withastramonolide, and withanolide A were found to be 13.833 ± 3.727 ng/mL, 124.415 ± 64.932 ng/mL, 57.536 ± 7.523 ng/mL, and 7.283 ± 3.341 ng/mL, individually, with an observed T_{max} of 0.750 ± 0.000, 0.250 ± 0.000, and 0.291 ± 0.102. According to the findings, withanosides and withanolides were quickly absorbed into the stomach. Withaferin A and 12-deoxy-withastramonolide LLOQ values are below the C_{max}/20 ratio, denoting that the developed method has been responsive to determining the amount of these compounds in plasma.[163]

The overall relative oral bioavailability of withaferin A was 1.44 times higher than withanolide A.[160] Furthermore, withaferin A reaches peak plasma levels of up to 2 μM with a half-life of 1.36 h after a single 4 mg/kg dose in 7−8 week old female Balb/C mice, so although removal from plasma is quick (0.151 ng/mL/min), peak plasma levels are higher. Another study found that a single ingestion of 500 mg/kg in six healthy buffalo calves resulted in an implied peak plasma concentration of withaferin A.[164] Up to 3 h later, a mean plasma

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ACS Omega XXXX, XXX, XXX−XXX

concentration of 6.55 ± 0.12 μg/mL had been spotted. From 10 min to 3 h, the average therapeutic concentration of WS in the plasma of good-health buffalo calves was sustained. WS's mean elimination half-life ($t_{1/2}$) was found to be 0.92 ± 0.032 h, but a complete body clearance varies.[165] A single oral dose of 0.42 g/kg was given to fasted Albino rabbits (1.5−1.8 kg, either sex, n = 6) in a study. WS (obtained from two sources) was well absorbed, with a C_{max} of 18,317.8−21,360.7 ng/mL. The biological half-life ranged between 18.29 and 27.69 h.[166]

11. CONCLUSION

WS is known for its pharmacological significance and is widely assessed for its various phytochemical attributes. This Review attempts to capture and systematically segregate the reported compounds based on their functionalities. The Review also gives attention to the phytochemistry of compounds, the structural variation depending on substitutions on the parent skeleton, and the stereochemical explanation. The present review further explores the wide array of extraction methodologies and their impact on subsequent output, which has been studied previously.

It also attempts to signify the bioactive extractions in different plant parts, along with the impact of solvents and seasonal variations, by making its selection well evident for future targeted studies. The analytical and pharmacopeial perspectives reported on the herb were captured briefly. Their view briefly explores its diverse pharmacological landscape, along with its pharmacokinetic studies. The presence of withanolides and other phytoconstituents can explain the plant's numerous pharmacological and therapeutic actions. It also highlights the gaps and limitations of some extraction techniques and iterates the need for further standardizations and strict regulatory frameworks with profound safety and efficacy studies. This is imperative for its wider application and overall benefits to society.

Finally, it indicates the new trends in applications for WS in diversified platforms, specifically in foods. Tapping the enormous therapeutic potential of this herb and its different extracts through various delivery platforms required further exploration. This review will provide a supporting reference for any further work in the domain.

■ ASSOCIATED CONTENT

Data Availability Statement

The data presented in this study are available within the article, the associated Supporting materials, or on request from the corresponding author.

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Notes

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