2

Review of Literature

Traditional medicine has been using plants for medicinal purposes since antiquity (Ojha *et al.,* 2020, Rajeswara Rao, 2016). It includes all the knowledge, skills, and practices derived from theories, beliefs, and experiences shared by many different cultures, which may or may not be explainable, and are used to preserve health, as well to treat mental and physical illnesses. In consequence, most modern medicines are derived from medicinal plants, which makes human medicine highly dependent on plant biodiversity (Kiguba *et al.,* 2016). It is estimated that the use of medicinal plants has increased worldwide over the last few centuries, particularly in developing countries (Tabuti *et al.,* 2012). As well as its perceived safety and efficacy, medicinal plants are available and accepted by a wide variety of cultures (Chinsembu and Hedimbi, 2010). Worldwide, medicinal plant use is estimated to be between 50 and 95% (Pan *et al.,* 2019, Wanjohi *et al.,* 2020). A high demand for herbal medicine, as well as other factors such as climate change, has led to an estimated 15,000 medicinal plant species being threatened with extinction (World Health Organization, 2004, Phondani *et al.,* 2016). There are already over 20% of medicinal plant species are nearly extinct (Ross, 2005).

The herb *W. somnifera,* commonly known as Ashwagandha, is considered to be *a* Rasayana herb (Rejuvenator) in Ayurveda. It is one of the most popular traditional medicinal plants in India with immense therapeutic potential even in modern medicine. Many of the Ayurvedic formulations against various diseases contain *W. somnifera* roots as major ingredient. The plant is accumulated with plenty of chemical constituents such as alkaloids, flavonoids, steroids, and other important phytochemicals (Williamson, 2002). A major therapeutic benefit of this plant can be attributed to its key secondary metabolites, withanolides, a group of steroidal lactone compounds that exhibit an array of biological activities (Singh *et al.,* 2010, Vinod and Senthil, 2021).

As a result of increased exploitation for medicinal purposes and extraction of withanolides, this plant is on the verge of extinction. These methods of cultivation are also very time-consuming and labor-intensive. As a result, international markets have seen an increase in demand for field grown roots. The conventional propagation of this plant needs to be adapted in order to meet this growing demand. In addition, the quality of raw materials obtained from field grown plants is highly affected by genotypic and environmental variations (Praveen and murthy, 2010). To propagate medicinally important plants with reproducible phytochemical compositions, *in vitro* cultivation plays a major role. The present study focus on developing *in vitro* shoots as an alternative to traditionally used field grown roots for therapeutic purposes.

The literature related to the present study entitled **'A study on Neuroprotective potential of *in vitro* and field tissues of *Withania somnifera* using *Caenorhabditis elegans* model'** Is reviewed under the following headings in this chapter.

2.1 *W. somnifera* – a versatile medicinal plant

2.1.1. Historical background of *W. somnifera*

2.1.2. Morphology, Taxonomy and Ethnography of *W. somnifera*

2.1.3. Traditional formulations and health benefits in Ayurvedic perspective

2.1.4. Therapeutic uses of *W. somnifera* as per classical Ayurvedic text

2.2. Phytochemistry of *W. somnifera*

2.2.1. Chemistry of *W. somnifera*

2.2.2. Withanolide A and Withaferin A – Major Bioactive

2.2.3 Mechanisum of action Withanolides

2.3. Analytical approaches towards identification and quantification of withanolides

2.3.1. High-performance liquid chromatography (HPLC)

2.3.2. Gas chromatography- Mass spectrometry (GC-MS)

2.3.3. High-Performance Thin Layer Chromatography (HPTLC)

2.4. Therapeutic potential of *W. somnifera*

2.1 *W. somnifera* – a versatile medicinal plant

A medicinal plant of great significance, *W. somnifera* (L.) Dunal is widely used in India and worldwide. The species was ranked 85th in Asian countries and medicinal plant species traded internationally per surge in research publications of over 1767 within the past decade (VanWyk, 2015). There are approximately 3000 species in the Solanaceae family, which consists of 84 genera (Mirjalili *et al.,* 2009). Species of *Withania* are shrubs, subshrubs, or woody herbs. There are millennia of uses of *W. somnifera* as an essential plant in Ayurvedic medicine in the Mediterranean region, with its ecological and economic importance.

Since more than 2500 years ago, it has been an important herb used in traditional medicine (Bhattacharya and Muruganandam, 2003). Different parts of the plant have been employed in Ayurvedic preparations for treating a variety of ailments (Kulkarni and Dhir, 2008). However, roots of the plant can be used commonly to promote longevity, revitalize the body, arrests the aging process, and strengthen defense against infectious diseases (Bhattacharya and Muruganandam, 2003). Additionally, *W. somnifera* is a major ingredient in many Ayurvedic formulations currently available in India and elsewhere. In Ayurveda, *W. somnifera* is regarded as analgesic in a variety of musculoskeletal disorders (arthritis and rheumatism), in certain forms of hypertension, to stimulate sexual impulses and to increase sperm count, as a vaginitis treatment and for breast development during pregnancy (Mishra *et al.,* 2000, Puri, 2002).

For centuries, *W. somnifera* has been considered a nerve tonic, aphrodisiac, adaptogen, antirheumatic agent, astringent, and memory enhancer for people of all sexes and any stages of life (Bhattacharya and Muruganandam, 2003, Puri, 2002). The multiple biological properties of *W. somnifera* have been described in many pharmacological studies, and the results of these studies indicate that it may also be effective in treating bronchitis, asthma, ulcers, cancer, emaciation, insomnia, and senile dementia (Kulkarni and Dhir, 2008). Besides antidiabetic,immunomodulatory, hemopoietic, neurological inflammatory disorders, Parkinson's disease, and other multipurpose medicinal uses, *W. somnifera* has been proven to have clinical and preclinical trials.

2.1.1. Historical background of Ashwagandha

Traditionally, *W. somnifera* is known as Ashwa and Gandha. In Sanskrit, ‘Ashwa’ means horse and ‘gandha’ means smell. Hence, Ashwagandha means "smell of a horse" and means that it imparts the vigor and strength of a stallion. As the Latin term *somnifera* implies, the herb induces sleep or has sedative properties. After recovering from illness, Indian traditional medical practices used this herb to boost overall health and nourish immunity (Choudhary *et al.,*1995). Among the foremost medicinal herbs in Ayurveda material medica, *W somnifera* was incorporated into Balya and Brimhana-gana by Acharya Charaka *(*Nishteswar *et al., 2013).* It has been proven for to treat a wide range of diseases with *W. somnifera* in modern perspectives.

There are four specific properties of a drug according to the Ayurvedic medical system, namely 'rasa, guna, veerya, and vipaka'. A drug's rasa is its taste, and its guna is its properties and effects on the body after consumption. The term veerya refers to the potency of the drug, meaning whether it has a catabolic or anabolic effect on the body. The term vipaka refers to its post-digestive effects. In light of these factors, the mechanism of a drug inside a biological system can be understood as doshas (bio forces that control the body of an organism, whose balance and imbalance regulate health and ill health) (Kavya *et al.,* 2014). In terms of pharmacodynamics, *W. somnifera* has rasa-tikta (bitterness), kashaya (astringency), and kath (pungentness) in taste, guna (qualities) srigdha (oiliness, untudiness) –laghu (light for digestion), veerya – ushna (hot in potency) and - vipaka-kathu (pungent taste conversion after digestion) as well as rasayana (rejuviability), balya (improve strength) and vajeekarara (stimulation) (Joshi *et al.,* 2017).

Classical Ayurveda highlighted this herb *W. somnifera* as Balaprada and balya (that improves strength and immunity), vajikari (useful in premature ejaculation in some case of erectile dysfunction), act as vrushya (aphrodisiac), improve life expectancy, anti-aging, elixir (rasayani), improve body nourishment (pushtiprada), useful in cough and cold (kasam hanti), useful in vata disorder, anilam hanti (neurological and neuromuscular disorder like paralysis), vranam hanti (useful for wound healing), shophahara (bring down inflammation), kanduhara (relives itching, useful in pruritis), vishahara (anti-toxic, useful in detox programs), shivitrahara (useful in leucoderma on internal usage and external application), krumihara (useful in internal worm infection), shwasahara (useful in asthma and chronic respiratory disease, kshatahara (useful in injury healing), kshayahara (useful in treating muscle wasting, emaciation, post tubular treatment and to improve muscle mass and strength), atishukrala (improve semen quality and quantity), ama (helps to clear impurities).

2.1.1.a. Interpretation of *W. somnifera* as per Ayurveda

W. somnifera, known by its common name *'hayahwaya' (*providing horse potency and having a smell similar to that of a house*).* Ashwagandha, hayagandha – (root, part used, also emits horses smell), ashwavarohaka, vrisha,- (spermatogenic and aphrodisiac effect), balada,balya- (promote strength), elaparni- (leaves having shape of ela (cardamom plant), gandhapatri- (having smell like that of horse), gokarna- (herb with leaves resembling shape of cow’s ear), hayapriya- (favorite of horses, hayahvaya- provides horse potency), kaamaroopini- (increases libido), kancuka- (retains semen), kushtagandhini- (has smell of plant kushta), marutaghni- (useful in vatika disorders), pita- (having yellow colour), putrada- (provides male progeny), pushtida- (it is nourishing), thuragi- (it has smell of hoarse)(Sharma, 2000).

2.1.1.b. Categorization of *W. somnifera* as per classical Ayuvedic text

In Chaaka Samhita, Acharya Charaka included *W. somnifera* under balyadasaimani group and brimhaneeya group in 4th chapter of Suthra sthana as Balya, brimhana, madhuraskandha, virechanopaga (Agnivesha, 1994). In Susrutha samhita it is mentioned as urdhwabhagahara (Susruta, 2007). Nighantus categorized it as *W. somnifera* and *W. ashwagandha*. It is mentioned in all most all Nighantus such as Bhavaprakasha Nighantu, Madanapala Nighantu, Dhanwanthari Nighantu, Kaiyyadeva Nighantu and Raja Nighantu. Accoding to Bhavapakasha Nighantu it is mentioned under guduchyadi varga (Bhavaprakasanighantu, 2011), Madanapala Nighantu referred it as abhayaadi varga (Madanapala Nighantu, 2009). In Dhanwanthari Nighantu and Shodhala Nighantu it is guduchyadi varga (Kamat, 2002), Kaiyyadeva Nighantu it is ‘oushadi varga (Kaiyadeva, 2012) and Raja Nighantu mentioned it as shatahvadi varga (Burkill, 1985).

2.1.2. Morphology, Taxonomy and Ethnography of *W. somnifera*

An evergreen shrub with a woody, tomentose appearance about 30-150 cm high, *W. somnifera* is erect and small. Leaves are asymmetrical, simple, petiolate leaves with a length of 4-10 cm and a width of 2-7 cm are found in this plant. Monoceous or bisexual flowers appear in clusters of 4-25 flowers in yellow to green colors. Throughout the year, flowers are produced, with March - July being the peak months. The fruit is a small round orange-red fruit surrounded by a brownish, papery, turgid calyx and about 5-8mm in diameter. There are many seeds in fruits. Approximately 2.5mm in diameter, the seeds are kidney-shaped and pale brown in color (Mirjalili *et al.,* 2009). Roots are stout, fleshy, cylindrical, 1-2 cm thick, straight, and unbranched. Fibrous secondary roots grow from roots. The crown consists of 2-6 thickened stem bases with nodes prominent on the side from which the petiole emerges. In addition to being short and uneven, it has an odor, bitter taste, and acrid (Khare, 2008).

2.1: Taxonomic Classification of *W. somnifera*

Taxonomic classification			**Vernacular names**		
Kingdom	:	Plantae	Sanskrit	:	Asvagandha
Division	:	Angiospermae	English	:	Winter cherry
Class	:	Dicotyledoneae	Hindi	:	Asgandh
Order	:	Tubiflorae	Tamil	:	Amukira
Family	:	Solanaceae	Malayalam	:	Amukkuram
Genus	:	Withania	Kannada	:	Vemaddinagaddi
Species	:	Somnifera	Telugu	:	Vajigandha

(Gaurav *et al*., 2015)

Figure 2.1: Field grown shoot and root of *W. somnifera*

It is commonly known as Tarkukai in the Marakwet community of Kenya, Karamanta in the northern part of Nigeria, Zafua in Mali, and Winter Cherry in South Africa (Burkill, 1985). Throughout Africa, the Middle East, India, Sri Lanka, and the Canary Islands, *W. somnifera* has a wide distribution area (Schmelzer *et al.,* 2008). In dry and humid regions, it is most abundant (Gaurav *et al.,* 2015). The species of *W. somnifera* is found in northern, southern, and eastern-region countries of Africa and a few countries in the western region (Gaurav *et al.,* 2015). Among the north African countries, *W. somnifera* can be found in Morocco, Algeria, Tunisia, Libya, Egypt, and Sudan, but not in Western Sahara. It is found in all countries in the southern part of the African continent. It has also been reported in the western parts of Africa, including Chad, Cape Verde, Mali, Liberia, and Nigeria. Several countries are located in the eastern part of the continent, including Ethiopia, Tanzania, Angola, Zambia, Mozambique, and Eritrea. This small woody shrub, which can be found all over South East Asia, including India, Bangladesh, Sri Lanka, Nepal, and Pakistan, as well as in other parts of Australia, Africa, and North America (Hepper, 1991, Ilayperuma *et al.,* 2002, Beattie, 2008, Mirjalili *et al.,* 2009). In India, it is commonly found in Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat, and Rajasthan (Kulkarni and Dhir, 2008).

2.1.3. Traditional formulations and health benefits in Ayurvedic perspective

W. somnifera it is used as single herbal as well as polyherbal formulation and the roots are the major constituent of over 200 Ayurvedic formulations (Singh *et al.,* 2011). Among vast variety of rasayana herbs *W. somnifera* hold prominent position known as “Sattvic Kapha Rasayana” herb (Changhadi, 1938) and is also recognized as the Queen of Indian herbs (Bhaskar *et al.,* 2018). *W. somnifera* is major ingredient in Awagandhadichurna, Aswagandha-rasayana, Aswgandha-ghrita, Ashwagandha-rishta, Aswagandha- taila, Madhyamanarayana-taila, Brihat Ashvagandha-ghrita, Brihachchagaladya-ghrita, Saraswata-churna, Pramehamihihira-taila (Anonymous, 2018). Nagabala-ghrita, Aswgandha rishata, Aswgandha-taila, Madhusnuhi-rasayana. Aswgandha churna, Brihat Ashwagandha-ghrita, Chyawanprash, Balaaswgandhadithailam, Manasamitravada, Brahmivati and Ajaaswgandha dilehyam are other common *W. somnifera* formulations mentioned in traditional textbooks.

Table: 2.2: *W. somnifera* formulations mentioned Classical Ayurvedic Books

Withania somnifera reference from classical Ayurvedic texts
Charaka Samhita (1000 BC- 4th Century AD)

Sl No	Formulation	Indication	Charaka Samhita reference
1	*Agurwadi Taila*	*Jwara*	C.Ci. 3/266
2	*Amrta Ghrita*	*Antidote, Udara*	C.Ci. 23/244
3	*Brmhaniyamahakasaya*	-	C.S.Su 4/2
4	*Bilva Taila*	*Vata roga*	C.Si. 4/4
5	*Basti*	*Vata roga*	C.Si. 12/2
6	*Baladya Yapana*	*Vata roga*	C.Si. 12/6
7	*Balya*	*Mahakasaya*	C.S.Su 4/7
8	*Dhuma*	*Arsa*	C.Ci. 14/51
9	*Ghrita*	*Fumigation*	C.Ci. 14/5
10	*Ingudi Tvagadi Dhuma*	*Kasa*	C.Ci. 18/75
11	*Errand Basti*	*Asmari*	C.Si. 3/39
12	*Lehya*	Hiccup	C.Ci. 17/117
13	*Lepa*	*Rajayaksma*	C.Ci. 8/175
14	*Lepa*	*Udara Roga*	C.Ci. 13/108
15	*Lepa*	*Granthi visarpa*	C.Ci. 21/ ½ 123
16	*Lepa*	*Antidote unmada*	C.Ci. 23/70
17	*Lepa*	*Pilla Kanda*	C.Ci. 23/80
18	*Lepa*	*Urustambha ,thic Utsadana*	C.Ci. 27/50
19	*Lepa*	*Vataroga*	C.Ci. 29/73
20	*Madhura Skandha*	-	C.S.Vi. 8/140-146
21	*Mulasava*	*Dipan , Pachan*	C.S.Su 25/49
22	*Kusthadilepa*	*Kustha*	C.S.Su 3/7-8
24	*Rasana Taila*	*Vataroga*	C.Ci. 28/166
25	*Taila*	*Sirah kampa*	C.Si. 9/87
26	*Vajikarana Ghrita*	*Bajikarana*	C.Ci. 2-1/34
27	*Vrsamuladi Taila*	BoneFracture, Osteoporosis	C.Ci. 28/170

Susruta Samhita (100 BC- 4th Century AD)

Sl no	Formulations	Indications	Susruta Samhita reference
1	*Agada (antidote)*	Poison of *visvambhara*	S.Ka. 8/5
2	*Ami pama*	*Urdhvabhaga Dosahara*	S.Su. 39/3
3	*Anupana (with mamsa rasa)*	*Balavardhaka pustikaraka*	S.Su. 46/432-2
4	*Bala Taila*	*vatavyadhi*	S.Ci. 15/33
5	*Bhutikadi*	*Vataroga*	S.Ci 37/20
6	*Basti*	*Guda Rakta Srava*	S.U. 45/40
7	*Churna*	*Atikrsa*	S.S.Su. 14/40
8	*Citrakadi Taila*	*Vataroga*	S.Ci 37/16
9	*Churna*	*Yaksma*	S.U. 41/41
10	*Churna*	*Coupha*	S.U. 41/43
11	*Dasamuladi Ghrita*	Complication	S.U. 41/49
12	*Dhum*	*Karnaroga*	S.U. 21/7
13	*Kalka and Taila*	*Karnapali vriddhi*	S.Su. 16/22
14	*Kalka*	*Vrana ropaka*	S.Su. 36/24
15	*Kalka*	*Vatarakta*	S.Ci. 5/10
16	*Kwatha*	*Revatigraha*	S.U. 31/3
17	*Lepa*	*Karnapali vriddhi*	S.Su. 16/20
18	*Lepa*	*Kaphaja sopha*	S.Su. 36/6
19	*Lepa*	*Vrana ropaka*	S.Su. 36/31
20	*Lepa*	*Kaphajavisarpa*	S.Ci. 17/14
21	*Lepa*	*Paripotaka in karnapali Roga*	S.Ci. 25/14
22	*Lepa*	*Yaksma*	S.U. 41/42
23	*Phala Ghrita*	Pregnancy to infertile women	S.U. 62/27
24	*Sampakadi Basti*	Duodenal *roga,* increase blood	S.Ci 38/43
25	*Taila*	*Aroga Timira*	S.U. 17/34
26	*Taila*	*Rukhamandika roga*	S.U. 35/4
27	*Vacadi Taila*	*Vataroga*	S.Ci 37/12

Astanga Hirdaya (7th Century AD)

Sl no	Formulations	Indications	Susruta Samhita reference
1	*Anuvasana Vasti*	*Vataja Roga*	A.H.Ka. 4/54
2	*Bala Taila*	*Grbha Vyapada*	A.H.Sa. 2/50
3	*Basti*	*Duodenal roga*	A.H.Ka. 4/7
4	*Churna*	*Unmantha*	A.H.U. 18/45
5	*Dhupana*	*Arsa*	A.H.Ci. 8/19
6	*Ghrits*	*Balapustikara,kasa ojahksaya*	A.H.Ci 3/122-123

7	*Ghrits*	*Sosanasak*	A.H.Ci. 5/25
8	*Ghrita*	*Gulma, Apasmara*	A.H.Ci. 14/14
9	*Ghrita*	Strength	A.H.U. 3/53
10	*Kalka*	Oedema	A.H.Ci. 17/37
11	*Kwatha*	Cure emaciation	A.H.U. 2/51
12	*Kwatha*	*Karnaroga*	A.H.U. 18/56
13	*Laksadi Taila*	*Unamada,apasmara*	A.H.U. 2/52
14	*Laksadi Taila*	Fever, strength	A.H.U. 2/52
15	*Lehya*	Cure emaciation	A.H.U. 2/49
16	*Lepa*	*Palisoas*	A.H.U. 18/39
17	*Lepa*	*Kustha, Kandu, Pidika*	A.H.Ci.
18	*Lepa*	*Pusti, Varna,Balaprada*	A.H.Ci. 5/79
19	*Sukumarka Taila*	*Rasayana, Vataja, Roga*	A.H.Ci.13/41
20	*Syrup*	*Svasa- Hidhma*	A.H.Ci. 4/39
21	*Vata hara Basti*	*Vataroga*	A.H.Ka

Cikitsa kalika(10th centu AD)

Sl no	Formulations	Indications	Cikitsa kalika reference
1.	*Bala Taila*	*Vataja Vyadhi*	30/283
2.	*Bala Taila*	*Vataja Vyadhi*	30/290-291
3.	*Dasanga Taila*	*Vataja Vyadhi*	30/295-297
4.	*Laksadi Taila*	*Jwaracikatsa*	1/120
5.	*Mahatprasarini taila*	*Vataja Vyadhi*	30/303-304
6.	*Phlaghrita*	*Grahabadha, Vatavyadhi, Bandhyatva etc*	367
7.	*Prasarini Taila*	*Vataja Vyadhi*	30/298-302
8.	*Prthusatavari Taila*	*Vataja Vyadhi*	30/286-287

Cakradatta (11th Century AD)

Sl no	Formulations	Indications	Susruta Samhita reference
1.	*Ashwagandhadi Kasaya*	*Rajayaksma*	10/9
2.	*Ashwagandha Ghrita*	*ViryaVardhaka, Mamsavardhaka*	22/90
3.	*Churna*	*Urah ksata*	10/93-95
4.	*Kamdeva Ghrita*	*Raktapitta*	9/53-63
5.	*Krsnadileha*	*Rajayaksma*	10/14
6.	*Masabaladi kwatha*	*Vatavyadhi*	22/23-24
7.	*Mahabala Taila*	*Vatavyadhi*	22/101-110
8.	*Nagabala Ghrita*	*Rajayaksma*	10/78-82
9.	*Narayana Taila*	*Vatavyadhi*	22/120-130
10.	*Srnga-Arjunadya churna*	*Rajayaksma*	10/26
11.	*Trayodasanga Guggulu*	*Gradhrasi*	22/69-73

Sarngadhara samhita (13th Century AD)

Sl no	Formulations	Indications	Sarngadhara samhita reference
1.	*Ashwagandha churna*	*Vajikarana*	6/157-158
2.	*Baladya Taila*	*Vatavyadhi*	9/117-118
3.	*DhatturadiTaila*	*-do-*	9/200-210
4.	*Kamdeva Ghrita*	*Rakta-Pitta*	9/27-37
5.	*Kandarpa Sundara Rasa*	*Vajikarana*	12/268-274
6.	*Laksadi Taila*	*Visama Jwara*	9/94-98
7.	*Lepa*	*Stanyavrddhi*	11/112-113
8.	*Lepa*	*Linga Vrddhi*	11/115
9.	*Madadi Nasya*	*Pakdaghata*	8/36-37
10.	*Madankamadeva*	*Vajikarana*	12/259-266
11.	*Maharanadi Kwatha*	*Sarva-Vartaroga*	2/20-96
12.	*Mahasalvan Sweda*	*Vanaja Roga*	2/23-27
13.	*Narahyana Taila*	*Vataroga*	9/101-106
14.	*Satavari Taila*	*Vatajaroga*	9/133-141

Bhaisajya Ratnavali (18th century AD)

Sl no	Formulations	Indications	Bhaisajya Ratnavali reference
1.	*Adigyapakwa Taila*	*Khalitya Roga*	83/3,4
2.	*Amritaprash Ghrita*	*Rasayana*	74/299
3.	*Ashwagandha Taila*	*Rasayana*	78/355
4.	*Chandanadi Kwatha*	*Mastiska Roga*	101/2
5.	*Goksuradi Modaka*	*Rasayana*	74/230
6.	*Godhumadia Ghrita*	*Rasayana*	74/279
7.	*Jayantivati*	*Jwara*	5/536
8.	*Jwarabhairava Rasa*	*Jwara*	5/1375
9.	Kamadeva ghrita	*Raktapitta*	13/145
10.	Madanakamadeva	*Dhwajabhanga*	92/20
11.	Shalimali Ghrita	*Khalitya Roga*	88/32
12.	Sindukadi Dhupa	*Arsha*	9/153
13.	Suryavallavha taila	*Sanyu Roga*	82/20
14.	Yaminyadi Churan	*Gadaroga*	78/7

(Re produced by Santhanu *et al.,* 2021)

2.1.4 Therapeutic uses of *W. somnifera* as per classical Ayurvedic text

W. somnifera is a well-documented medicinal herb in ancient Ayurvedic texts but there is no direct reference in Vedas. In Vedas it is represented that 'rock like smell' and a word 'Aswasya Varah' mentioned in the visha chikitsa (treatment for poison). Ashwawal and Ashwawar both words were also used in Yajurveda and Atharvaveda. Ashwawati is described as Shrivardhaka and Rasayana in Rigveda, Yajurveda, and Atharvaveda. According to Charak Samhita the herb is used for treatment of Kandu (itching), kustha (skin disorder, sotha (inflammation), sheetajwaran (fever), rajayakshma (pulmonary tuberculosis), udarroga (abdominal disorders), arsha (piles), hikka (hiccough), shwas (ashthma), kaas (cough), granthivisarpa (erysipelas), visha (poison), urustambha (spasticity of thighs), vatavyadhi (neurological disorders), vatarakta (gout) kaphaavritavatavikara, vatavikar (neurological disorders), anantavata (trigeminal neuralgia) (Charaka Samhita, 2009). As per Sushruta Samhita it is used to treat karshyaroga (emaciation), karnapalivardhan (expansion of ear pinna), kaphajsopha (inflammation), vranaropana (woundhealing), vrana (wound), vamankarma (emesis), anupanarth (adjuvants), kaphajvatarkta (gout), sutika roga (puerperal diseases), visarpa (erysipelas), paripotakaroga (inflammation of the lobe of the ear), palivardhnarth (ear lobule elongation), anuvasanbasti (enema prepared by medicated oil), niruhabasti (decoction enema), shosaroga (emaciation), shosaroga (emaciation) and unmada (insanity) (Sushruta Samhita, 2012). Ashtanga Hridaya described the plant is useful for sutikarog (puerperal diseases), unmada (insanity), kasa(cough), rajayakshma (pulmonary tuberculosis), vatavyadhi (neurological disorders), gulma (abdominal lump), unmada(insanity), apasmara (epilepsy), balashosa (marasmus), balaamaya (child disorders), karnaroga (ear diseases), vranaropana (wound healing), Medhya (nootropic)) and vrishya (aphrodisiac). According to Bhel samhita it is effective for the treatment of krimi (worms), kustha (skin disorder), anupanarth (adjuvant), yakshma (pulmonary tuberculosis), hridroga (cardiac disorders), adhyavata (gout), urustambha (spasticity in thighs) and vatarogas (neurological disorders). As per Harita samhita it is mentioned for vishavikara (poisonous disorder), unmada (insanity), apasmara (epilepsy), vatavyadhi (neurological disorders) and apasmara(epilepsy). Chakradatta referred the use of plant for kshaya (emaciation), kshaya (emaciation), vatavyadhi (neurological disorder) , vatavyadhi (neurological disorder), udararoga(abdominal disorder), krimi(worm), sotha(edema), yonivyapada (vaginal disorders), balaroga(child disorders), balashosa (marasmus) and karnapalivardhana (elongation of ear lobule) (Chakradatta, 2015).

2.2. Phytochemistry of *W. somnifera*

2.2.1 Chemistry of *W. somnifera*

The phytochemical constituents of *W. somnifera* root, leaves, stem, and fruit extract have been extensively examined and characterized using chromatography and spectroscopy (Mirjalili *et al.,* 2009). The presence of several biochemically active components steroidal lactones, β-sitosterol, scopoletin, sitoindosides, somniferiene, somniferinine, pseudotropine, anaferine, anahygrine, cysteine, chlorogenic acid, cuscohygrine, withanine, withanolides, withananine, tropanol, 6,7β-Epoxywithanon and 14-α-hydroxywithanone were examined from *W. somnifera* tissues (Naz and Choudhary 2003, Saleem *et al.,* 2020). Several biological activities of these extract constituents have been attributed to the plant's ethnomedicinal usage and pharmacological effectiveness (Aye *et al.,* 2019, Nile *et al.,* 2019). The composition of the phytochemical extracts of *W. somnifera* whole plant and its different parts is as follows: -

Whole plant extracts of *W. somnifera* contain phytochemicals such as anaferine, anahygrine, choline, cuscohygrine, pseudotropine, dl-isopelletierine, and tropine (Saleem *et al.*, 2020). Additionally, the plant's methanolic extracts contain starch, acylsteryl glucosides, iron, ducitol, hantreacotane, withaniol, and amino acids such as alanine, aspartic acid, cysteine, tyrosine, glutamic acid, glycine, proline, and tryptophan (Alam *et al.,* 2011). Further the whole plant contains withanone and tubacapsenolide F (Saleem *et al.,* 2020), while similar extracts with equimolar ratios of water and methanol constitute chlorinated withanolide and 6α-chloro-5β,17α-dihydroxywithaferin A along with nine withanolides, namely 6α-chloro-5β-hydroxywithaferin A, (22R)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith24-enolide, 2,3-dihydrowithaferin A, withanone, withanoside IV, withaferin A, 2,3-didehydrosomniferícin, 3-methoxy-2,3-dihydrowithaferin A, and withanoside X (Saleem *et al.,* 2020). An ethanol extract of the whole plant contains isosominolide, sominone, and withasomniferin A (Misra *et al.,* 2008).

The root extract of *W. somnifera* contains pyrazole alkaloid withanolide A (WA) and withasomnine using alcohol extractants (Baek *et al.,* 2019). A methanolic extract of the plant root contains withanosides I-VII (Mirjalili *et al.,* 2009) whereas three withanolides are found in the benzene and ethyl acetate extracts of similar roots (Taha *et al.,* 2012). Petroleum ether and acetone are the two major solvents used to extract the roots of this plant, which contain the phytochemicals -sitosterol and d-glycoside (Saleem *et al.,* 2020). The butanol extracts of *W. somnifera* roots contain physagulin, withanoside IV, and withanoside VI (Mathur *et al.,* 2006, Chatterjee *et al.,* 2010).

Phytochemically, the plant leaves extracted with methanol contain ashwagandhine, cuscohygrine, dl-isopelletierine, somniferine, tisopelletierine, 3-tigloyloxtropine, 3-tropyltigloate, 3-tropyltigloate, hygrine, hentriacontane, mesoanaferine, visamine, withanine, withananine, and pseudowithanine (Siddique *et al.,* 2014). An alcohol extract of the plant leaves contains withanolide D, E (Lavie *et al.,* 1968, 1972), withanolides F–M (Glotter *et al.,* 1973, 1977), withanolides N, O (Ganzera et al. 2003), and withanolide P. Leaves of the plant extracted with ethanol contain (5R,6S, 7S,8S, 9S,10R, 13S,14S,17S, 20R,22R)-6,7α-epoxy5,17-α,27 trihydroxy -1-oxo-22R-witha-2,24-dienolide (Ben *et al.,* 2018). There are several dragendorf-positive alkaloids found in methanolic extract of the plant roots, including anaferine, anahygrine, choline, pseudotropine, cuscohygrine, isopelletierine, dlisopelletierine-3-tropyltigloate, hentriacontane, hygrine, mesoanaferine, somniferine, 3-tigloyloxtropine, visamine, withanine, withanine, and withasomine as well as ashwagandhine, pyrazole derivatives, and pseudowithanine (Saleem *et al.,* 2020).

The ethanolic extract of the stem bark of *W. somnifera* contains a wide variety of withanolides including somniferanolide, somniwithanolide, somniferawithanolide, withasomnilide, and withasomniferanolide (Siriwardane *et al.,* 2013). Fresh berries of *W. somnifera* contain elaidic acid, linoleic acid, oleic acid, palmitic acid, and tetracosanoic acid in the oil extracts (Sidhu *et al.,* 2011). The methanolic extract of the plant fruits contains all versions of withanamides A-I (Bhatia *et al.,* 2013, Adhikari *et al.,* 2020).

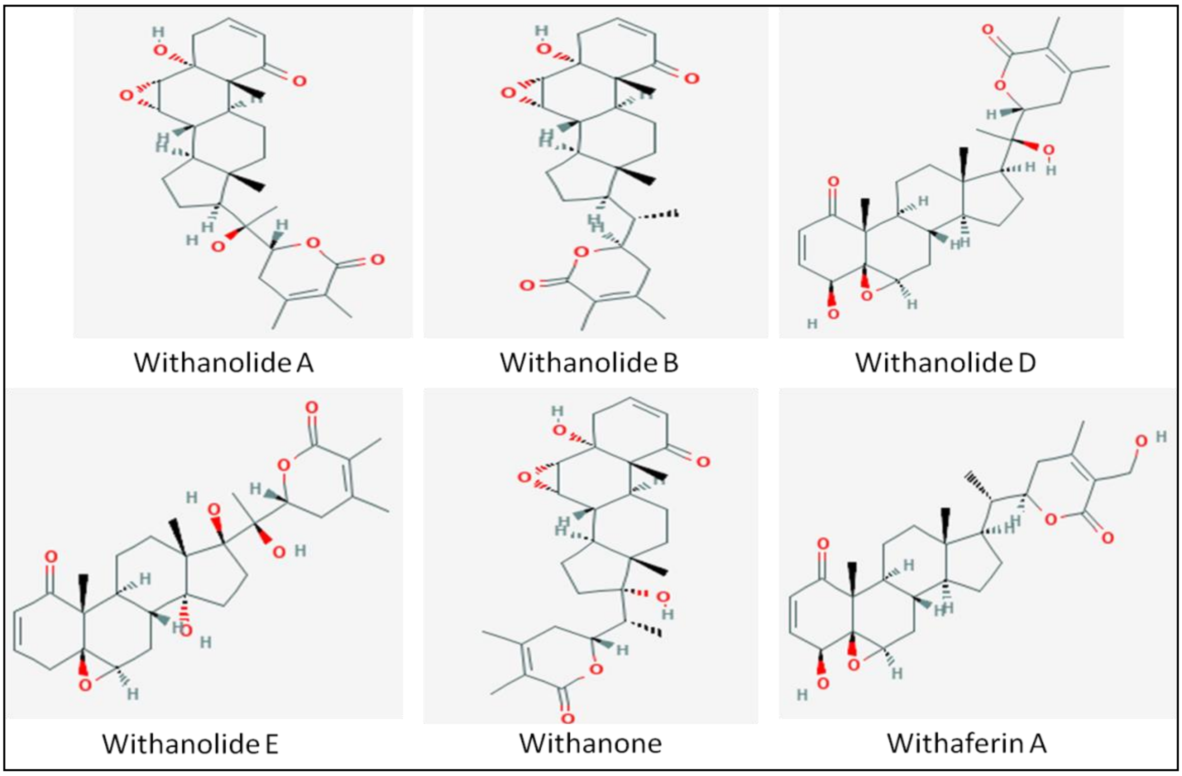
Table: 2.3: Phytoconstituents of *W. somnifera* and the extraction solvents

Plant part	Extract used	Phytoconstituents	Reference
Whole plant	Alcoholic extract	Anaferne, anahygrine, choline, cusohygrine, pseudotropine, dl-isopelletierine, tropine	Saleem *et al*., 2020
	Methanolic extract	Starch, iron, withaniol, acylsteryl glucosides, ducitol, and amino acids like tyrosine, glycine, proline, tryptophan, aspartic acid, glutamic acid, alanine and cysteine	Mirjalili *et al*., 2009, Alam *et al*., 2011
	Aqueous extract	Withanone, tubacapsenolide F	Saleem *et al*., 2020
	Methanol: water (1:1)	Withaferin A, 2,3 dihydrowithaferin A, 6α-chloro-5β-hydroxywithaferin A, ,3-didehydrosomniferícin, withanoside X, (22R)-5β-formyl-6β, 27-dihydroxy-1-oxo-4-norwith-24-enolide	Saleem *et al*., 2020
	Ethanol	Isosominolide, withasomniferin A, sominone	Misra *et al*., 2008
Root	Alcoholic extract	WithanolideA,withasomnine, pyrazole alkaloid	Baek *et al*., 2020
	Methanolic extract	Withanosides I-VII, anaferine,	Mirjalili *et al*., 2009
	Benzene and ethyl acetate extracts	Withanolide A, withanolide B, withanolide C	Kim *et al*., 2019
	Petroleum ether and acetone	β-sitosterol, *d*-glycoside	Saleem *et al*., 2020
	Butanol	Physagulin, withanoside IV , withanoside VI	Chatterjee *et al*., 2010
Leaves	Methanol	Cuscohygrine, somniferine, ashwagandhine, dl- isopelletierne, tisopelletieine, hygrine, visamine, withanone. Hygrine, mesoanaferine, withanone, pseudowithanine	Siddique *et al*., 2014

Plant part	Extract used	Phytoconstituents	Reference
	Alcoholic extract	Withanolide N, withanolide O	Ganzera *et al*., 2003
	Ethanol	(5R,6S,7S,8S,9S,10R,13S,14S,17S, 20R, 22R)-6,7α-epoxy-5,17- α,27-trihydroxy-1-oxo-22R-witha-2,24-dienolide	Ben *et al*., 2018
Stem	Ethanolic extract	Somniferanolide, somniferawithanolide, somniwithanolide, withasomnilide,	Siriwardane *et al*., 2013,
Fresh berries	Oil extract	Linoleic acid, oleic acid, palmitic acid, elaidic acid, tetracosanoic acid	Adhikari *et al*., 2020

Withanolides are steroidal lactone compounds that have been reported in *W. somnifera* as having medicinal properties. Approximately 40 withanolides have been elucidated so far, all of which contain 22-hydroxy ergostane-26-oic acid 26, 22-lactone as their backbone structure (Dhar *et al.,* 2015). Plate 2.1 illustrates the structures of the key withanolides of *W. somnifera.*

Plate 2.1: Key withanolides of *W. somnifera*



2.2.2. Withanolide A and Withaferin A –Major Bioactive

A withanolide is a natural compound containing 28 carbons and having a 6-member lactone ring structure formed by oxidizing the carbons C22 and C26. Withaferin A (WFA) (indicated by its structure as (27 Dihydroxy-1 Oxo-5, 6 Epoxy-witha-2-24-dienolide)) belongs to a group of substances regarded as extremely bioactive. There are two epoxy side chains (C5 and C6) and two hydroxyl groups (C4 and C27) in it. Due to its broad range of bioactivities, withanolide is the most potent withanolide and its molecular mechanisms must be clearly understood. Thus, numerous studies have been conducted to characterize the target proteins of withanolides and to explore their intracellular effects (Vanden Berghe *et al.,* 2012). To produce WFA, dihydrowithaferin A undergoes several dehydrogenations and reductions (Lavie *et al.,* 1968). Due to the slightly toxic nature of WFA, many different analogs were synthesized and tested for their bioactivity. WFA diacetate and dehydro-withaferin A were found to be the most potent inhibitors of the incorporation of thymidine, uridine, and L-valine into nucleic acids in the investigation of their cytotoxic activity. WFA also possesses cytotoxic properties due to its double bond, C2-C3. The removal of this double bond resulted in a decrease in the cytotoxic activity of withanolides. Furthermore, the addition of a carbonyl group to C4 increased its activity. However, the activity did not change when the hydroxyl group was removed at C27 or when the double bonds between C24 and C25 were disrupted (Fuska *et al.,* 1984). Additionally, the limited solubility of withaferin related compounds can be overcome by RAFT (Reversible Addition Fragmentation chain Transfer) polymerization of the OH group at C27 (Van Herck *et al.,* 2019). As well as the cytotoxicity of the compound, the heat shock inducing activity was also highly influenced by the modifications to the scaffold, indicating that the structure of the ring A plays an important role in the biological activity (Wijeratne *et al.,* 2014). In comparison with the parent molecule, the 3-azido analogue of WFA demonstrated a 35 fold increase in activity. According to Yousuf *et al.,* (2011), this compound is more toxic than its parent compound. There is another derivative of WFA known as 2, 3-dihydro-3-methyltryptophan (pWi-A) that has been reported to have protective effects on cells under stress, but it is lacking these protective properties compared to WFA (Chaudhary *et al.,* 2019).

Additionally, it was found that WFA derivatives are highly efficient when compared to their corresponding derivatives of withanolide D.

The Structure Activity Relationship (SAR) study of WA exposed that the epoxy group at C5 was responsible for the binding of withanolide to Hsp 90 and inhibit its chaperone activity. Heat shock protein 90 (HSP 90) is another molecular chaperone compound that serves as a target for the anti-cancer drugs. The docking efficiency of WA to disrupt the Hsp 90- Cdc37 (chaperone-cochaperone complex) supports the anti-cancer activity of WA (Grover *et al*., 2011). Further the hydroxyl group at C4 of ring A enhances the inhibition activity on Hsp 90. It also distrupts its interaction with Cdc37 (Hsp 90- Cdc 37) thus enhancing the anti-proliferative activity of WA (Gu *et al*., 2014). The SAR and ADMET(Absorption, Distribution, Metabolism, Excretion and Toxicity) studies on WA incorporated with silicon in addition to carbonyl group at C4 was found to enhance the activity on human epithelial ovarian carcinoma thus proving it as an essential candidate in ovarian cancer studies (Perestelo *et al*., 2019).The anti-tumour and effects of WA was studied in Ehrlich ascites carcinoma in swiss mice and reported to be effective against tumour with a LD 50 value of approximately 80mg/ kg (Sharada *et al*., 1996). The effect of WA on several malignant cell lines has been assessed and it has proved to have anti-proliferative effect against acute lymphoblastic leukemia (ALL) and human myelogenous leukemia. Mandal and his coworkers (2008) explored the effect of WA on p38MAPK signaling pathways in apoptosis of leukemic cell lines and primary cells of patients with ALL (Mandal *et al*., 2008)

2.2.3. Mechanism of action Withanolides

Various mechanisms have been proposed as explanations for the multi-therapeutic effects of withanolides, particularly WFA, such as their antiangiogenic, antimetastatic, proapoptotic, and radiosensitizing properties (Mishra *et al.,* 2000, Vanden Berghe *et al.,* 2012). Several breast cancer cell lines, including ER-positive T-47D, MCF-7, MCF-7/BUS cells, and triple negative MDA-MB-231, Sk-Br-3 cells, were found to be inhibited and reduced in proliferation by WA. (Jayaprakasam *et al.,* 2003). WA also inhibited growth in a mammary tumor virus-neu transgenic mouse model in an identical manner.

Although the exact mechanism underlying WA's anticancer activity is unknown, studies have reported that WA inhibits Hsp90 and induces ROS levels via FOXO3a and BIM dependence (Widodo *et al.,* 2010). WA is reported to promote vimentin disassembly in most metastatic breast cancer models (Thaiparambil *et al*., 2011). According to studies in silico, withanone binds to the TPX2-Aurora A complex and disrupts the spindle apparatus, causing anticancer activity (Grover *et al.,* 2012). As well as activating tumour suppressor proteins and ROS pathways, withanone also kills human cancer cells in a selective manner *(Widodo et al., 2010).*

Numerous studies have examined the antiproliferative properties of WA in different cell lines. As well as showing potent anticancer activity, WFA inhibits the PI3/Akt pathway in lung cancer cell lines (Cai *et al.,* 2014, Kyakulaga *et al.,* 2018). Studies on breast cancer models have demonstrated that WA has anti-tumor properties by phosphorylating Jak2 (Lee *et al.,* 2010), activating STAT-3 (Mulabagal *et al.,* 2009) and upregulating NOTCH-2 and NOTCH-4 (Lee *et al.,* 2012). *In prostate cancer, W somnifera* root extracts suppress lipogenesis by suppressing *p-Akt and c-Myc levels, indicating a new mechanism of antitumor activity.* By stimulating ROS production and cell cycle arrest, WA also reduced the cell death caused by ionizing radiation (Yang *et al.,* 2011, Okamoto *et al.,* 2016). The anticancer activity of WA and the other withanolides has been attributed to the regulation of ROS levels as the underlying mechanism. ROS accumulation induced by WA has led to mitochondria-mediated cell death, downregulation of Akt, stimulation of autophagy, upregulation of JNK, inhibition of oxidative phosphorylation, cell cycle arrest, and apoptosis (Singh *et al.,* 2015).

2.3. Analytical approaches towards identification and quantification of withanolides

In Indian ethnomedicine, whole plants or plant parts are used. The bioactive molecules found in plants serve as the basis for the synthesis of pharmaceutical drugs. Consequently, the isolation of active principles has become necessary, which requires selection of an appropriate extraction procedure. Using traditional soxhlet extraction, ultrasound assisted extraction, and microwave assisted extraction methods for different time intervals, an examination of the effects of ethanol, water, and ethanol-water extraction was conducted. Based on different extraction methods and different solvents used (Dhanani *et al.,* 2017), total phenolics and total withanolides, as well as DPPH and ABTS activity, varied. As a result of advances in the field of metabolomics, it is increasingly possible to identify changes in the metabolic profile of plants as a result of environmental factors. In the process of drug discovery, phytochemical analysis of plant-based formulations is of utmost importance.

2.3.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a versatile, robust, and widely used technique for characterizing and isolating natural products. This technique is frequently used to characterize and quantify secondary metabolites in plant extracts, especially phenol compounds, steroids, flavonoids, and alkaloids (Boligon *et al.,* 2012). Numerous studies have reported the use of HPLC to quantify WFA and WA from extracts of *W. somnifera* (Ganzera *et al.,* 2003, *Chaurasiya et al.,* 2008). Using the defatted leaf and root powders of *W. somnifera*, Keesara and Jat (2017) extracted WA using methanol. HPLC analysis confirmed that the WFA obtained by this method was 90% pure. Using chloroform and methanol as the mobile phases, the compound was identified as WFA by TLC. There was a similar peak to the standard peak at Rf value 0.65 for WFA. It has been developed and validated a modified HPLC-DAD method for quantification of withanolides including WFA and its fingerprinting analysis (Patil *et al.,* 2010). Using reverse phase preparative HPLC, the presence of WFA in *W. somnifera* butanol fractions has been identified (Pramanick *et al.,* 2008). RP-HPLC has been developed for the separation and quantification of three isomeric WA, WFA, and WTN using methanol as the mobile phase (Malik *et al.,* 2017).

Using this chromatographic technique, a mixture of compounds can be separated and identified, quantified, and purified. As a result of the high resolving power of HPLC, it is well suited for both analytical and preparative analyses of multicomponent samples (Martin and Guiochon, 2005). Although HPLC has all these advantages, its use is limited due to the requirement for high pressure to run the sample. Its limitations include sample cleanup after each run, a high running cost, a high level of maintenance, an increased duration for equilibration of the column, and a limited choice of detectors.

2.3.2. Gas Chromatography- Mass Spectrometry (GC-MS)

In the field of metabolomics, GC-MS is regarded as one of the most efficient and reproducible platforms. To identify various compounds in a test sample, Gas chromatography and mass spectrometry techniques are combined (Beale *et al.,* 2018). Due to its large number of libraries, including both commercial and in-house metabolite data, this technique is robust and selective (Beale *et al.,* 2018).

Phyochemical profiling of whole plant powder of *W. somnifera* has been successfully performed using this technique. GC-MS analysis of methanolic extracts of *W. somnifera* revealed the presence of 12 major compounds, including phytol, n-hexadecanoic acid, and oleic acid (Beale *et al.,* 2018). As a result of GC-MS combined with NMR analysis, several withanolides have been identified, such as WFA, withanolide D, withanoside IV or VI, withanolide sulfoxide, etc. (Trivedi *et al.,* 2017). The technique has been used effectively in the comparative metabolic profiling of field grown and *in vitro* developed shoots and roots of *W. somnifera* (Thirugnanasambantham *et al.,* 2015, Senthil *et al.,* 2015). However, GC–MS has two major limitations: it can only analyze a small number of volatile and thermally stable compounds. Additionally, EI mass spectra often show the absence or weakness of molecular ions, lowering the confidence level in sample identification via the library and making it impossible to identify the majority of compounds that are not in the library (Amirav *et al.,* 2020).

2.3.3 High-Performance Thin Layer Chromatography (HPTLC)

HPTLC is one of the most sophisticated instrumental techniques capable of processing the full capabilities of thin layer chromatography (TLC). Several advantages, including automation, scanning, full optimization, selective detection, minimal sample requirements, hyphenation, and so forth, make it an ideal analytical tool for the separation of complex pharmaceutical mixtures. Due to its flexibility, reliability, and cost-effectiveness, HPTLC is considered to be the best alternative to GC and HPLC (Bairy, 2015). Analyzing quantitatively and qualitatively is equally possible with this powerful analytical method. Compounds are separated either by adsorption or partition, or by both, depending on the nature of the adsorbents used on the plates and the solvent system used for the development.

There has been developed a sensitive, specific, robust, and validated densitometric HPTLC method for determining WA from *W. somnifera* (Srivastava *et al.,* 2008). A method for estimating WA using Si60 F254 plates and Toluene: ethyl acetate: formic acid (5:5:1) as the mobile phase has been validated for its repeatability and accuracy (Sharma *et al.,* 2007). The technique is effectively used to quantify withanolides. It has been established that Toluene: ethyl acetate: formic acid (5:5:1) is the best mobile phase for achieving maximum separation of withanolides in the plant extract (Preethi *et al.*, 2014). Using Scanner III (CAMAG, Switzerland) in the reflectance-absorbance mode, the standardization of wavelength for the quantitative scanning of WA was found to be accurate at 223nm (Senthil *et al.,* 2015). For the determination of WFA in mice plasma, a rapid high performance liquid chromatography-mass spectrometry technique has been developed and validated (Patil *et al.,* 2013).

Despite the fact that HPTLC is characterized by a minimum concentration requirement of samples, less chance of handling errors, higher accuracy and sensitivity, it also has a number of disadvantages. It is necessary to provide a larger space for HPTLC instrumentation and to have strict operating conditions and technically skilled personnel (Bairy, 2015).

2.4. Therapeutic potential of *W. somnifera*

Ashwagandha is an effective adaptogenic drug that is being used to combat the complications associated with stress. Numerous preclinical studies have been conducted in recent years in order to facilitate the validation of the therapeutic potential of *W. somnifera* and its phytoconstituents. Studies such as these have contributed to a better understanding of the mechanisms involved in the mode of action of the most important pharmaceutically active substances. Studying natural ways to overcome stress has become increasingly important since stress is a leading cause of many health problems. Stress-induced hepatotoxicity and gastric ulcers have been prevented by WA. Studies have been conducted to investigate the multifaceted properties of WA, and the results indicated its significant pharmacological properties (Shah *et al.,* 2021).

2.2. Different pharmacological potential of *W. somnifera*

(Mandlik and Namdeo, 2021)

2.4.1. Adaptogenic potential of *W. somnifera*

Several parts of the plant *W. somnifera* have been shown to be pharmacognostic and to scavenge free radicals (Mandal *et al.,* 2008). Sitoindosides VII-X and WFA, the active principles of *W. somnifera*, showed similar anti-oxidant activity as the standard drug deprenyl (Bhattacharya *et al.,* 1997). It has also been found that *W. somnifera* contains glycowithanolides that have the ability to reverse the effects of chronic stress. In rats, the oral administration of glycowithanolides one hr before the induction of foot shock stress restored the levels of SOD and LPO to normal (Bhattacharya *et al.,* 2001, Palash *et al.,* 2010). The effects of WFA on oxidative stress have been demonstrated against DEN-induced hepatocellular carcinoma in rats (Murugan *et al.,* 2015). There was a significant decrease in the levels of reactive oxygen species in rats treated with WFA. A major biologically active steroid found in *W. somnifera*, WFA, is responsible for the anti-inflammatory activity of this plant (Patel and Savjani, 2015). Raw 264.7 cells were found to be inhibited by WFA in the expression of both the protein and mRNA of nitric oxide synthase (iNOS). A study conducted by Oh *et al.,* (2008) examined the mechanism by which WFA inhibits iNOS gene expression. The researchers suggested that WFA inhibits inflammation by inhibiting both NO production and iNOS expression, as well as by blocking Akt and downregulating NF-κB activity.

2.4.2. Anti-microbial activity

W. somnifera leaf, stem, and root powders have been found to be effective against fungal (*Fusarium oxysporum* and *Radicis lycopersici*) and bacterial (*Escherichia coli*) species (Kumari and Gupta, 2015, Nefzi and Ben Abdallah, 2016). Cervical cancer is one of the most common cancers that kill women worldwide, caused by human papillomavirus (HPV). Through its interaction with E6, HPV 18 inactivates the tumor suppressor p53 (tumor suppressor protein). The interaction between WFA and the oncoprotein was studied since WA has traditionally been used to treat various cancers. Docking results showed that WA interacts with residues 108-117 of the p53 binding site on E6 oncoprotein, reversing the normal function of p53 (Singh *et al.,* 2015). In another simulation study, the terminal hydroxyl groups of WFA were shown to bind to DNA polymerase sites on the Herpes simplex virus DNA polymerase. The inhibitory activity of WFA is due to its reaction with the SH group of enzymes and metabolites required by the organisms. Moreover, glutathione in the media inhibits this activity of WA (Budhiraja *et al.,* 2000). It was predicted that WA would bind to the amino acid residues Gln 617, Gln 618, Asn 815 and Tyr 818, which are essential for enzyme function. It is therefore possible to use WA as a potent anti-herpetic drug (Grover *et al.,* 2011).

Associated with SARS-CoV-2 virus, COVID 19 is a transmissible severe acute respiratory syndrome. As a result of molecular dynamic simulation, nearly forty chemical constituents of *W. somnifera* have been examined for their activity against the main protease protein of SARS-CoV-2 (Tripathi *et al.,* 2021) containing several antiviral compounds and active phytoconstituents approved by the FDA (Chandel *et al.,* 2020). There was a strong interaction between WA and the main protease, spike glycoprotein, and RNA dependent RNA polymerase of SARS-CoV-2, with binding energies of -11.242 kcal/mol, -9.631 kcal/mol, and -9.27 kcal/mol, respectively (Pandit, 2020). There is evidence that WA interacts with eleven residues of the main protease Mpro: Thr 24, Thr 25, His 41, Cys 44, Ser 46, Met 49, Phe 140, Leu 141, Asn 142, His 164 and Glu 166. It has also been reported that WA of *W. somnifera* targets and represses TMPRSS2 protein, which is a gateway for viruses to enter host cells, as well as the cellular receptor GRP78 protein, which facilitates viral entry into cells (Sudeep, 2020, Wadhwa *et al.,* 2020). People with cancer or other comorbid conditions were found to be more susceptible to this infectious disease. Upon administration of WA, it is found to bind the viral protein and inhibit the host ACE2 receptor, resulting in an increase in the spread of the disease. (Straughn and Kakar, 2020).

2.4.3. Cardioprotective activity

Globally, cardiac-related issues have become a leading cause of death. A therapeutic dose of *W. somnifera* root extracts has been shown to improve cardiovascular endurance in healthy athletes, thus improving quality of life (Choudhary *et al.,* 2015, Perez-Gomez *et al.,* 2020). Regular administration of root powder of *W. somnifera* with milk has been shown to reduce systolic blood pressure, thereby reducing hypertension (Kushwaha *et al.,* 2012). *W. somnifera* extracts have shown significant cardioprotective effects as evidenced by histopathological evaluations of the myocardium (Mohanty *et al.,* 2004, Mohanty *et al.,* 2008). It has been reported that *W. somnifera* has similar therapeutic properties in suppressing mycocardial infarction and ischemia reperfusion injury. It has also been demonstrated that WA's anti-inflammatory properties, along with its ability to bind to the SARS-CoV 2 viral spike protein, are beneficial in controlling COVID-induced cardiovascular pathologies (Afewerky *et al.,* 2021).

2.4.4. Anti-diabetic activity

Withanolids increases the viability of HUVEC cells by counteracting the oxidative stress caused by palmitic acid. By suppressing oxidative stress and inflammation, the cells are protected from endothelial insulin resistance (Batumalaie *et al.,* 2016). Exposure to palmitic acid inhibited insulin-mediated

IRS-1 tyrosine phosphorylation in the presence of insulin, resulting in deterioration of insulin downstream. Pre-treatment of the cells with WFA mitigated this inhibitory effect. Based on the results of Jonathan *et al.,* (2015), WFA improved the efficiency of glucose uptake by the skeletal myotubes over other withanolides. This proves that the plant has anti-diabetic properties. Additionally, he reported that leaf extracts were more effective at uptake of glucose than root extracts. A further indication that WFA plays a significant role in glucose uptake was shown by the fact that methyl salicylate and chitosan elicited an increase in WA in the tissues compared to those that were not elicited (75% and 69% respectively).

2.4.5. Hepatoprotective activity

In an oxidative stress model induced by bromobenzene, *W. somnifera* root extracts reverted liver enzyme markers to normal (Vedi *et al.,* 2014). Several studies have shown that WFA can prevent as well as cure liver injury, thereby reducing liver inflammation and fibrosis in mice with nonalcoholic steatohepatitis (Patel *et al.,* 2019). In their study of the effect of WFA (40 mg/kg) on acetaminophen induced liver injury in mice, Jadeja *et al.,* (2018) reported a reduction in hepatocytic necrosis and intrahepatic hemorrhage after treatment with WFA. Furthermore, the treated mice showed a reduction in JNK (c-Jun N-terminal kinase pathway) activation, mitochondrial Bax (BCl2 associated X protein) translocation, and nitrotyrosine generation, all of which are induced by acetaminophen in untreated mice. Apart from NF-kB activation and vimentin inhibition, WFA has also been shown to change the expression of LOXL2/ Snail1, which reverses liver fibrosis (Sayed *et al.,* 2019). In addition, WA was found to inhibit hepatocellular carcinoma growth by inhibiting autophagy (Siddharth *et al*., 2019).

2.4.6. Anti-leishmanial activity

The endemic disease of leishmaniasis has become more problematic as a few species, such as L donovani, become resistant to available drugs. As a result, it is difficult to treat, and new drugs are needed to target different sites of the protozoan and cure the disease. Molecular dynamic studies found that WFA inhibits leishmanial protein kinase C, which anti-leishmanial drugs target (Grover *et al.,* 2012). One other enzyme that can be targeted for developing an effective antileishmanial drug is Pteridine reductase 1 (PTR1). As shown in Chandrasekaran *et al.,* 2016, molecular docking studies of WA against PTR 1 showed the lowest binding energy of -6.73kJ/mol. Because PTR1 and DHFR-TS have structural similarities, the inhibitor that targets PTR1 can also target DHFR-TS. Vadloori *et al.,* (2018) studied molecular dynamics against Dihydrofolate reductase-thymidylate synthase (DHFR-TS) and found two binding sites of WFA, one on DHFR and one on TS domains, both 40Å apart.

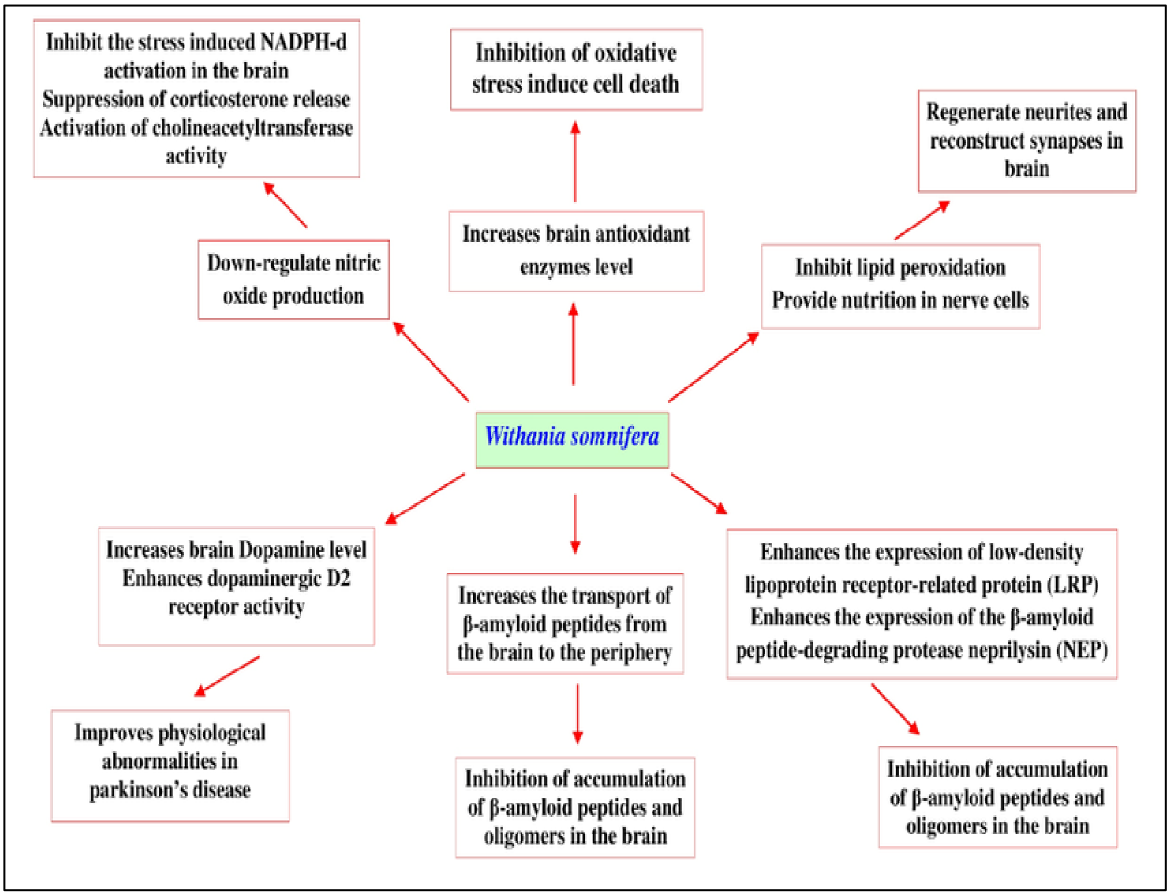
2.5. Pharmacology of *W. somnifera* with special reference to Neuroprotective activity

2.5.1. *W. somnifera* in brain disorders and Neuroprotective mechanisms

Over the past few decades, as life expectancy has increased, the incidence and prevalence of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases have increased substantially (Lopez and Kuller 2019). Because of this, neurodegenerative diseases in the aging baby boom generation should be given priority attention from a public health perspective. There have been several preliminary studies, preclinical studies, and clinical trials conducted to address this concern. As a result of the pathophysiology of neurodegenerative diseases, there is currently no effective pharmaceutical approach for their prevention or treatment.

In spite of the need for further research, ongoing pharmaceutical investigation efforts for neurodegenerative diseases indicate that medicinal plant extracts, such as *W. somnifera*, may have a greater impact on treatment efficacy and access than synthetic approaches (Pohl and Kong 2018) in terms of both treatment efficiency and accessibility. As a medicinal plant, *W. somnifera* has a long history of being used to treat various neurodegenerative conditions (VenMurthy *et al.,* 2010). There is a slow and progressive deterioration of the structure and function of the central nervous system in neurodegenerative diseases (Dugger and Dickson, 2017). *W. somnifera* has gained particular attention over the past decade in the context of treating neurodegenerative disorders, including memory loss (Uddin *et al.*, 2019), bipolar disorder (Chengappa *et al.,* 2013), and locomotor defects (Manjunath and Muralidhara, 2015). *W. somnifera* extracts have been shown to possess neuroprotective properties against several neurodegenerative diseases by restoring mitochondrial function simultaneously with the mitigation of oxidative stress, inflammation, and apoptosis (Dar *et al.,* 2017, Birla *et al.,* 2019, Gupta and Kaur, 2019).

Figure 2.3: Neuroprotective mechanisms of *W. somnifera*



(Afewerky *et al*. 2021)

2.5.1. Role of *W. somnifera* against Alzheimer's diseases

The most common form of neurodegenerative disease is Alzheimer's disease (AD), which results in progressive impairment of higher cognitive function, memory, and interpersonal skills (SoriaLopez *et al.,* 2019). There is still a lack of understanding regarding the pathogenesis of AD. Age, however, is considered to be the primary risk factor for AD, and Accumulations of extracellular Amyloid-beta (A-) and Intracellular Neurofibrillary Tangles (NFT) are the primary pathological hallmarks of the disease (Patel and Bihani, 2018). Neuronal calcium dyshomeostasis, mitochondrial dysfunction, oxidative stress, inflammation, and apoptosis were identified as the principal contributing factors to the pathology and pathophysiology of AD in several research studies (Yan et al. 2018a, Mahaman *et al*., 2019). In particular, the root extract of *W. somnifera* has been shown to significantly reduce the formation of reactive oxygen species in N-SH neurons (Singh and Ramassamy, 2017) and to reverse cognitive impairment in animal models of Alzheimer's disease by ameliorating dendritic, axonal, and synaptic integrity (Uddin *et al.,* 2019). The root extracts of *W. somnifera* have also shown promising results in several preclinical studies of AD, including a pilot study in adults with mild cognitive impairment (Choudhary *et al.,* 2017). The protective mechanisms of *W. somnifera* root extract against AD pathology presumably involves binding of the extract–biochemically active constituents to the active motif of Aβ, thereby preclude Aβ fibril formation. In order to disseminate plant extracts for the AD pharmacological approach, multidisciplinary extensive preliminary research is required.

2.5.3. Role of *W. somnifera* against Parkinson's disease

It is the second most prevalent progressive neurodegenerative disease of aging after Alzheimer's disease (Marino *et al.,* 2020). PD is characterized by a degeneration of the dopaminergic neurons in the substantia nigra and in the midbrain and the accumulation of neuronal Lewy bodies in the substantia nigra (Marino *et al.,* 2020). There is evidence that environmental toxins, such as certain pesticides, that induce mitochondrial dysfunction are major contributors to Parkinson's disease (Wirdefeldt *et al.,* 2011, Bragoszewski *et al.,* 2017). The underlying mechanisms of PD pathogenesis remain unknown, and there is no definitive treatment for PD. Interestingly, *W. somnifera* extracts have been shown to reverse PD-pathology, including striatal dopamine levels (Manjunath and Muralidhara, 2013), and improve locomotor defects in the Drosophila model of PD (Manjunath and Muralidhara, 2015). Furthermore, studies conducted in several Parkinsonian models indicated that *W. somnifera* extracts significantly reversed the Parkinsonian phenotype (Surathi *et al.,* 2016). Despite the need for further research to determine the protective mechanisms of *W. somnifera* extract against Parkinson's disease, its distinct antioxidant and anti-inflammatory properties may explain how it corrects mitochondrial aberrations and dopamine levels in striatum by correcting mitochondrial aberrations.

2.5.4. Role of *W. somnifera* against Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that causes progressive loss of locomotor coordination and cognitive abilities (Jimenez Sanchez *et al.,* 2017). It is known that an inherited huntingtin gene on chromosome four causes nerve cell damage in the HD brain (Nance, 2017) through mechanisms that are not completely understood. The root extract of *W. somnifera* has been shown to enhance biochemical parameters, including antioxidant enzymes, which are responsible for protecting basal ganglia tissue from huntingtin protein-induced lesions and the behavioral outcomes of HD animal models (Kumar and Kumar, 2009).

In addition, reports of the pharmacological effects of *W. somnifera* extracts have suggested potential roles for these extracts in decreasing lipid peroxidation and choreiform movements in an animal model of HD (Dar *et al.,* 2015). As *W. somnifera* root extracts possess GABAergic and antioxidant functions, they are suitable neuroprotective candidates against HD, however, further research is required to elucidate the mechanisms at play.

2.6. Biotechnological approaches towards development of medicinal plants

2.6.1. Micropropagation of *Withania* species

Studies on the exploration of genetic and chemotypic variations, identification and characterization of important genes, and the understanding of secondary metabolite production and modulation have gained significant momentum in response to the increased recognition of therapeutic benefits and the challenges associated with improving *W. somnifera*. In the therapeutic industry, the increasing demand for bioactive withanolides is mostly met by field grown plants that are uprooted completely in order to meet the demands (Mir *et al.,* 2014). As well, the longer gestation period between planting and harvesting in the wild of 4-5 years, as well as the effects of genotypic and environmental fluxes on the quality and quantity of metabolic constituents (Banerjee *et al.,* 1994), limit the application of plants grown in natural habitat as pharmaceutical preparations. Moreover, withanolides are complex molecules characterized by stereochemical ring closure, multiple chiral centers, high energy epoxy rings and rigid trans-lactone groups that make their synthesis economically unviable. As a result, plant tissue culture becomes an attractive method for enhancing the commercial production of withanolides. For the commercial propagation of endangered medicinal plant species, tissue culture has emerged as the most effective approach (Baskaran *et al.,* 2013).

2.6.2. Plant tissue culture of *W. somnifera*

As *W. somnifera* seeds exhibit poor viability under field conditions, alternative culture methods have been sought under optimized conditions (Khanna *et al.,* 2013). It was then determined that various nutrient media, temperatures, and light conditions could be optimally optimized in vitro. Apparently, soaking seeds increases germination chances by softening their coats (Vashistha *et al.,* 2010). Furthermore, germination rates were increased under a 16/8 hr photoperiod at 25°C and 3000lux light intensity (Khanna *et al.,* 2013). It has been shown that all plant materials, including seeds, embryos, seedlings, cotyledons, petioles, leaves, stems, shoot tips, roots, nodes, and inter-nodes, are capable of induced callus and adventitious shoot/root formation, differentiation, regeneration, flowering, and fruit production (Singh *et al.,* 2017).

Nevertheless, the ultimate objective of these studies is to identify a method that can be used to continuously produce better plant material of *W. somnifera* for therapeutic purposes. *In vitro* culture techniques can increase the production of plants in terms of both biomass and withanolide accumulation when compared to traditional methods of extraction of valuable products from plants (Mir *et al.,* 2014). Due to its active growth and metabolism in a short period of time, *in vitro* cultures are also effective experimental models for the study of secondary metabolite production (Sivanandhan *et al.,* 2011). In addition, this technique could be utilized to produce disease-free healthy plants for drug preparations as well as a continuous and standardized production of metabolites throughout the year (Gawde and Paratkar, 2012).

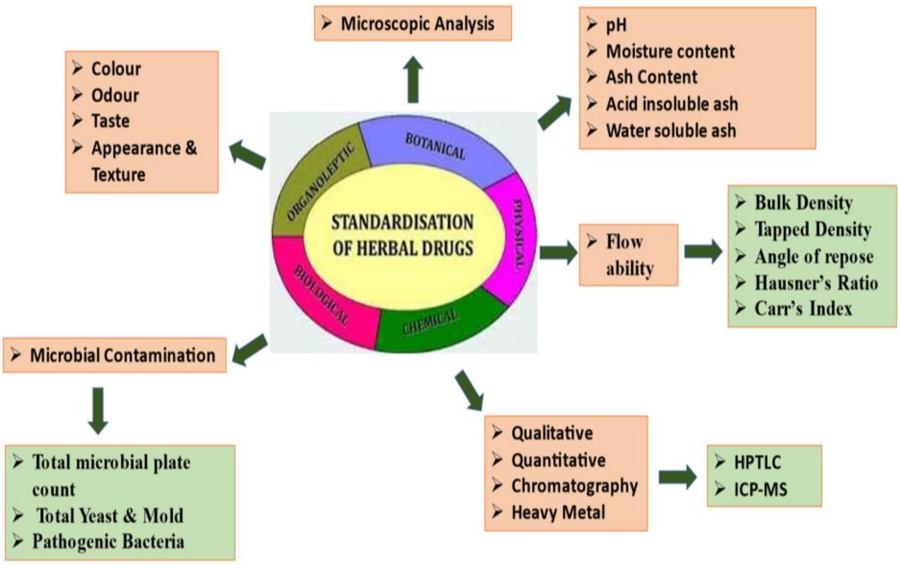
Withanolide content and biomass in plants are also dependent on the growth stage, with maximum accumulation occurring at the exponential phase (7-28 days). There was an increase in the amount of withanolides in 28 days old plants in suspension (Sivanandhan *et al.,* 2014). There has been a report that *in vitro* plants accumulate more WFA than field grown plants. There was a

1.14-fold increase in WFA content in *in vitro* leaves of *W. somnifera* compared to the leaves grown in the field (Sharada *et al.,* 1996). According to Senthil *et al.,* (2015) a maximum WFA accumulation was reported in 45-day-old *in vitro* cultured leaves, which decreased gradually following extended culture. WA accumulation in root cultures also exhibited a similar synchronization with culture age. Plant growth regulators are significant factors that influence the growth and metabolite production of plant cells. As a result of any change in the concentration or ratio of auxin and cytokinin, the growth pattern and metabolite accumulation would be significantly altered (Rao and Ravishankar, 2002). Among the various concentrations and combinations of plant growth regulators studied (BAP and Kn), MS medium supplemented with 4.44μM BAP showed a 1.86-fold increase in WA content in comparison to control (Murugesan *et al.,* 2017). The results of similar studies indicate that media supplemented with 5.0 mM BAP and 1.0 mM Kinetin (Kn) caused the highest levels of WFA accumulation (13.4±1.15 mg/g of DW). Furthermore, this study demonstrated that *in vitro* and *ex vitro* shoots contained higher levels of WFA than field grown shoots, while root tissues contained only a trace amount of WA. The WFA content increased steadily from the first to the fifth week of culture (*Mir et al.,* 2015). In spite of the fact that various researchers have described various methods for inducing callus tissue and withanolide production from W. *somnifera*, the callus failed to synthesis withanolides. However, multiple shoot cultures and transformed roots were capable of producing withanolides. According to Chakraborty *et al.,* (2013), the presence or absence of withanolides in a given tissue type depends on the plant growth regulators used. In his study on withanolide quantification in *in vitro* induced callus using different plant growth regulators, it was surprising to note that callus obtained from culture media containing 2,4 D and Kinetin did not contain WA and WFA, whereas callus obtained from media containing IBA and BAP did contain both compounds. Thus, revealing the role of plant growth regulators in the accumulation of metabolites *in vitro*.

2.6.3. Quality control of Ayurvedic and herbal medicines

In herbal formulations, phytochemical constituents vary according to climate, soil composition, and geographical location; all of these factors contribute to obstacles in achieving standardization. Due to the increase in deforestation areas, herbal drugs have gradually become adulterated and substituted for other drugs. There is a risk of harming the safety and efficacy of a drug as a result of this adulteration and substitution. Unavailability of genuine herbal medicines is primarily due to adulteration, substitution, and a lack of skilled personnel. It is necessary to ensure the quality of medicinal herbal products by utilizing advanced quality control techniques and appropriate standards. It is through standardization that herbs are identified, quality, and purity are assured, as well as herbal products are confirmed as safe and effective. The preliminary identification, physical properties, chemical properties, and biological properties of herbs contribute to their purity. Herbal products are distinguished by their purity as well as their quality.

Figure 2.4: Quality control of Ayurvedic and herbal medicines



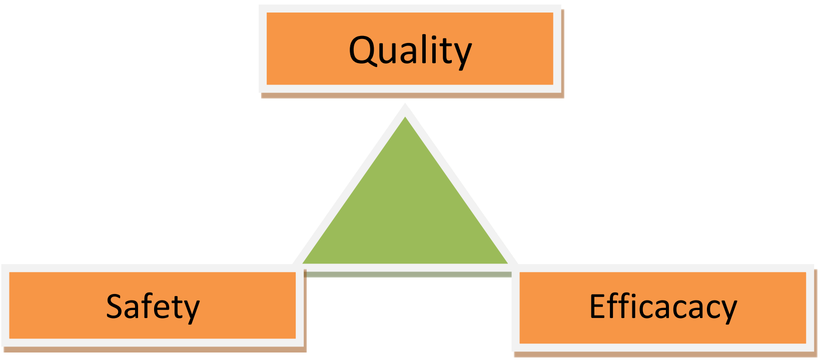
*Different steps such as Botaniacal, Physical, Chemical, Biological, and Organoleptic characteristics of quality control of a herbal medicine

To maintain the quality of natural herbs and products, quality control is of utmost importance. When quality control involves the identification of substances, adulterants, and substitutes, as well as the testing of active chemical constituents of greater importance to the herb, these aspects are known as pharmacopeial quality control. As a result of standardization, herbs are evaluated against prescribed or set standards and parameters in order to determine their qualitative and quantitative values. For the standardization of herbal drugs with current and future trends, the WHO has established guidelines for standardization methods and procedures based on the different important evaluation parameters such as organoleptic properties, ash values, moisture contents, microorganism contamination, and chromatographic and spectroscopic analysis(Kamble *et al.,* 2018, Nazim *et al.,* 2018, Marchese *et al.,* 2017).

For Ayurveda and traditional herbs to gain global acceptance, modern analytical techniques are essential. A complete and accurate pharmacognostical assessment can provide scientific fundamentals of traditional herbs and ayurvedic products. In order to authenticate and standardize, organoleptic tests, physicochemical studies, and pharmacognostical schemes are essential (Deogade and Prasad, 2019). Identifying adulterants and authenticating genuine herbs can be made easier with data from microscopic and macroscopic studies. Also, it will be useful to confirm the parameters for the standardization and identification of secondary metabolites (alkaloids, tannins, glycosides, saponins, flavonoids)(Liu *et al.,* 2018, Steinhoff *et al.,* 2019)

In accordance with the process for formulation of standard setting of herbal drugs in the pharmacopeia and other standard texts, the microscopic investigation (qualitative and quantitative), macroscopic (shape and markings), identifications (adulterants and genuine drug), physicochemical parameters (moisture content, acid insoluble ash, water soluble ash), pharmacognostical scheme, and other parameters reported for the first time can play a role of significant tool for authentication of herbs in future studies (Rashid *et al.,* 2018, Zhang *et al.,* 2012, Fernandes *et al.,* 2010).

Figure 2.5: Three pillars of herbal drug development



2.6. *C. elegans* a perfect organism for ageing study

For aging research, *C. elegans* is an excellent model organism. With its easy maintenance in the laboratory, transparent body for anatomical observations, high genetic homology (60–80%) to humans, complete genome sequence, conserved biological molecular responses, high fertility rate (250 eggs/worm within several days), and the availability of molecular biology tools (such as transgenic, gene knockouts, and RNAi knockdowns), *C. elegans* makes an ideal model to study aging mutations (Matsunami, 2018). As a result of their small size and short lifespan, they are also an excellent candidate for high-throughput anti-aging drug screening (Choi *et al.,* 2015). Moreover, experiments with *C. elegans* are ethically sound. The advantages of *C. elegans* have led to many breakthrough discoveries in aging research (David *et al.,* 2010).