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Appendices

Appendix I

Preparation of stock solutions for MS (Murashige & Skoog) medium

(Murashige and Skoog, 1962)

Stock solutions	Ingredients	Composition in media (mg/ L)	Stock Solution (w/v) (g)	Volume in media
MS Macro I (10 X) 1000ml	NH_4NO_3 KNO_3 $MgSO_4.7H_2O$ KH_2PO4	1650 1900 370.6 170	16.5 19 3.7 1.7	100ml
MS Macro II (10 X) 1000ml	$CaCl_2.2H_2O$	439.8	4.398	100ml
Fe-Na EDTA (1000 X) 100ml	Fe-Na EDTA	36.7	36.7	1ml
Micro Nutrients (1000 X) 100ml	$NaMoO_4.7H_2O$ $CuSO_4.5H_20$ $CoCl_2.2H_2O$ $MnSO_4.4\ H_2O$ $ZnSO_4.4H_2O$ H_3BO_3	0.25 0.025 0.025 13.2 8.6 6.2	0.025 0.0025 0.0025 1.32 0.86 0.62	1ml
KI (1000X) 100ml	KI	0.83	100ml	1ml
MS Vitamins (1000 X) 100 ml	Nicotinic Acid Pyridoxine HCl Thiamine HCl	0.5 0.5 0.1	0.05 0.05 0.01	1ml
Myo-Inositol				0.1g
Glycine 5ml	Glycine	2	0.2	1ml

Preparation of MS medium

1. To make 1 L of MS medium, the required volume of each stock solution, 100 mL macronutrients I & II, 1 mL micronutrients, 1 mL iron source, 1 mL potassium iodide and 1 mL vitamins were added into a 2 L beaker containing 200-250 mL of distilled water and stirred magnetically.

2. Then, 15 g sucrose, 0.1 g myo-inositol and 1 mL of glycine (0.01 g glycine in 5 mL of distilled water) were added and stirred to achieve complete dissolution. Growth regulators i.e. auxins 0r cytokinins were added as per requirement.
3. The volume was made up to approximately 450 mL (950 mL for suspension media) with distilled water. The pH was adjusted to 5.70±0.1 with 1 N NaOH and/or HCl.
4. The whole of the content was transferred to a 500 mL (1 L for suspension) measuring cylinder and the volume was made up to the mark with distilled water.
5. For MS agar medium, 8 g of agar was added to 500 mL distilled water and stirred for complete mixing. Agar was dissolved by heating the content in a microwave oven.
6. The medium and agar solution were mixed well.
7. The medium was transferred into Schott bottle (suspension media) and/or the medium was distributed (approximately 25-30 mL) to each sterile bottle (solid media). Bottles were labelled before autoclaving.
8. The culture medium was autoclaved for 15-20 min at 121℃ and 15 psi.

Appendix II

Estimation of total phenolic content

The total phenolic content (TPC**)** was estimated by the modified Folin-Ciocalteu method (Hithamani and Srinivasan, 2014**).** The methanolic extracts of all the samples were dissolved in DMSO. 0.5 mL aliquot of each sample (1 mg/mL concentration) was diluted with 4.5 mL of distilled water and treated with 0.5 mL of Folin-Ciocalteu's reagent for 2 min at room temperature. After 2 min, 1.5 mL of 20% (v/w) sodium carbonate solution was added. The mixture was allowed to react in the dark for 1.5 hrs, and the absorbance at 765 nm was subsequently measured using microplate spectrophotometer (xMark, Biorad, Berkeley, CA). A calibration curve for gallic acid was prepared in the concentration range of 100-500 µg/mL. The results are expressed as milligrams of gallic acid equivalent per gram of the dried extract.

Appendix III

Estimation of total flavonoid content

The total flavonoid content (TFC) was determined as per the method described previously (Lahouar *et al*., 2014). All the extracted samples were dissolved in DMSO. Briefly, 0.5 mL of each sample (1 mg/mL concentration) was mixed with 0.3 mL of 5% sodium nitrite. The mixture was allowed to stand at room temperature for 5 min and then 0.3 mL of 10% aluminum chloride was added and incubated at 25°C for 5 min. Finally, 2 mL of 1 M sodium hydroxide was added and the total volume of the mixture was adjusted to 5 mL by adding 1.9 mL of deionized water. The solution was mixed well again and the absorbance was measured spectrophotometrically at 510 nm. Quercetin was used as the standard for the calibration curve (10–100 µg/mL). The results are expressed as milligrams of quercetin equivalent per weight of the dried extract.

Appendix IV

Estimation of total steroid content

Total steroid content (TSC) was determined as per the method described by Naik and Mishra (2015). Briefly, 0.5 mL of each sample (1 mg/mL concentration) was mixed with 2 mL of Libermann Burchard reagent along with an equal volume of chloroform. The tubes were covered with carbon paper and incubated in dark for 30 min. The intensity of green color developed was measured spectrophotometrically at 640 nm. Cholesterol was used as the standard for the calibration curve (10-100 µg/mL). The results were expressed as milligrams of cholesterol equivalent per weight of the dried extract.

Appendix V

Estimation of total alkaloid content

Total alkaloid content (TAC) was determined as per the method described by Naik and Mishra (2015). Each extract (50 mg) was dissolved in 100 mL of 2N HCl and then filtered. The mixture was shaken and the content was extracted with 2 mL of chloroform by vigorous shaking. Then 0.5 mL of phosphate buffer (pH 4.7) and 0. 5 mL of BCG solution were added and the mixture was shaken vigorously. The absorbance of the content in chloroform was measured spectrometricaly at a wavelength of 470 nm against the blank. The results are expressed as milligrams of caffeine equivalent per weight of the dried extract.

Appendix –VI

Preparation of Worms by Alkaline hypochlorite method

(Sulston and Brenner, 1974)

Reagents

M9 Buffer (1L)

- 5.8 g Na2HPO4.7H2O
- 3.0 g KH2PO4
- 5.0 g NaCl
- 0.25 g MgSO4.7H2O

Dd H2O to 1 L.

Alkaline hypochlorite solution (3 mL)

* Made up fresh each time (1: 2 ratio)

- 10 N NaOH
- Sodium hypochlorite

Procedure

1. The chunked worms were seeded onto a 10 cm NGM plate. The worms were allowed to grow 2-3 days so that there are lots of eggs and gravid adults on the plate.
2. Approximately 15 mL distilled water was poured onto the plate.
3. After 10-15 min, the worms were transferred in to a 15 mL centrifuge tube and centrifuged at 1300 ×g for 1 min.
4. The supernatant was discarded without disturbing the worm pellet.
5. Then, 1.5 mL 10 N sodium hydroxide and sodium hypochlorite (1:2 ratio followed by vortex continuously for 5 min) were added.
6. After ensuring the dissolution of most of the bodies, the tube was centrifuged at 1300 ×g for 1 min.
7. The supernatant was discarded, the pellet was resuspended in 15 mL M9 buffer, mixed well and centrifuged again at 1300 ×g for 1 min. The process was repeated 3-4 times
8. Then, about 7.0 mL of fresh M9 buffer was added and agitated to resuspend the pellet
9. The egg was let to hatch overnight with gentle rocking. The larvae were halted at the L1 stage. The liquid was distributed onto the seeded plates or into liquid culture.

Appendix - VII

Preparation of NGM Petri plates

(Caldicott *et al.,* 1996)

C. elegans was maintained in the laboratory on Nematode Growth Medium (NGM) agar (Brenner, 1974), which has been aseptically poured into larger Petri plates (100 mm diameter) using a Wheaton Unispense liquid dispenser (Wheaton Science Products). A constant amount of agar in the plates reduces the need for refocusing the microscope when switching from one plate to another. The drug was added to the NGM solution just prior to pouring.

Equipment and Reagents

- NaCl
- Agar
- Peptone
- 5 mg/mL cholesterol in ethanol (not autoclaved)
- 1 M KPO4 buffer pH 6.0 (108.3 g KH2PO4, 35.6 g K2HPO4, H2O to 1 litre)
- 1M MgSO4
- Petri plates
- Peristaltic pump

Methods

1. 3 g NaCl, 17 g agar, and 2.5 g peptone were mixed in a 2 L Erlenmeyer flask containing 975 mL of H2O. The mouth of the flask was covered with an aluminium foil and autoclaved for 50 min.
2. The flask was kept at 55°C in a water bath for 15 min.
3. 1 mL of 1 M CaCl2, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M MgSO4 and 25 mL of 1M KPO4 buffer were added to the flask and swirled to mix well.
4. The NGM solution was dispensed into Petri plates with the help of a peristaltic pump under sterile conditions. The plates were filled with 2/3 of agar.
5. The plates were left at room temperature for 2-3 days before use to allow for detection of contaminants, and to allow excess moisture to evaporate.

Seeding NGM plates

Using sterile technique, 0.1 mL of E. coli OP50 liquid culture was applied to the NGM plates using a pipette. The drop was spread using a glass rod. Spreading will create a larger lawn, which can aid in visualizing the worms. The worms tend to spend most of the time in the bacteria. The E. coli OP50 lawn was allowed to grow overnight at room temperature. The seeded plates were stored in an air-tight container for further use.

Appendix - VIII

Preparation of growth media

(Byerly *et al.,* 1976)

A starter culture of E. coli OP50 was recovered from worm plates. The starter culture was used to isolate single colonies on a streak plate of a rich medium containing LB agar (10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, 15 g agar, H2O to 1L, pH 7.5). A single colony from the streak plate was aseptically inoculated in LB broth (10 g Bactotryptone, 5 g Bacto-yeast, 5 g NaCl, H2O to 1 L, pH to 7.0 using 1 M NaOH). The inoculated cultures were allowed to grow overnight at 37°C. The E. coli OP50 streak plate and liquid culture were stored at 4°C for further use.

Appendix – IX

Age synchronization in *C. elegans*

Reagents

• M9 Buffer] 3 g KH_2PO_4, 6 g Na_2HPO_4 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilize by autoclaving

• axenized C. elegans eggs from 4-8 100 mm plates

• concentrated *E. coli* OP50

Methods

1. Aseptically transfer the axenized eggs to 250 ml M9 Buffer in a 1-2 litre flask and allow to incubate overnight at 20°C using fairly vigorous shaking to obtained starved L1 animals.
2. Put the flask on ice for 15 minutes to allow the worms to settle
3. Aspirate most of the liquid from the flask (to remove any dauer pheromone accumulated during starvation)
4. Trasfer the remaining liquid to a 50 ml sterile conical centrifuge tube and spin for at least 2 min at 1150 x g to pellet the worms.
5. Aspirate the remaining liquid.
6. Transfer the worms to 250 ml of S Basal inoculated with concentrated *E. coli* OP50 in a 1-2 litre flask.
7. Moniter by checking a drop of the culture under the microscope. Add more food as necessary. At 20°C mid-L1 larvae can be harvested after approximately 8 hours, mid-L2 larvae at approximately 18 hours, mid-L3 larvae at approximately 25 hours and mid-L4 larvae at approximately 37 hours (Sulston and Hodgkin, 1988).

If only a small number of synchronized animals are needed, the eggs can be added to a thin layer of M9 Buffer in a 60 mm petri plate and allowed to grow overnight. The starved L1 animals can then be placed on seeded NGM petri plates, and synchronous growth will begin with the reintroduction of food. Animals should be monitored using the microscope and harvested at the desired stages. A population of dauer animals can be obtained by adding dauer-inducing pheromone to the liquid culture (Vanfleteren, 1980). Dauers can also be

obtained by allowing a liquid culture to grow for several days after the culture is cleared of bacteria. The dauers can be separated from other stages by treating with 1% SDS for 30 min in a rotating tube and then washing once with H2 O and once with M9 Buffer

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Appendix L2

(Item No 5 of Check List) Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	Thraputic potential of Withania somnifera (Linn) Dunal (Aswagandha) in historical perspective and Pharmacological evidences	Annals of Ayurvedic medicine	10 (2): 135-147 (2021)	UGC CARE
2	Comparative Cytotoxicity of invitro and field grown Shoots of Withania Somnifera in Caenorhabditis elegans model.	Current Botany	13: 70-75 2022.	UGC-CARE

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : [illegible]

Supervisor : [illegible] 26/10/22

Checked By:

HoD/Dean of Respective School

The scholar Ms Krishnapriya, C has published her article in "Annals of Ayurvedic Medicine" in Vol 10(2) Pg. 135-147 (2021) and "Current Botany" Vol.13, Pg. 70-75 2022. These two journals are indexed in Group I UGC care list and active as of today 26.10.22 [illegible]

Review Article

Therapeutic potential of *Withania somnifera* (Linn) Dunal (Ashwagandha) in historical perspective and pharmacological evidence

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Abstract

Withania somnifera widely known as *Aswagandha,* highly acclaimed medicine in ancient medical systems such as Ayurveda, Unani, Siddha and Chinese traditional medicine. The pharmacological importance of *Withania somnifera* is described in the different classical Ayuvedic narrative such as Charaka Samhita, Sushruta Samhita, Astangahridaya, Chakaradatta and various Nighantus. The plant used for the treatment of various ailments and works as nonspecific basis for better health and finer longevity. Nowadays it is scientifically proved that the plant possesses antioxidant activity, protect from anxiety and depression, nootropic effect, cardiovascular protection, anti-bacterial activity, anti-inflammatory properties, aphrodisiac, anti-venom, anti-fungal, anti-viral, sedative effects, hypoglycemic, thyro-protective, anti-diabetic and different cancer. This study aims to review and relist the various therapeutic applications of this plant and its importance in Ayurveda as per ancient classical texts.

Keywords: Aswagandha, *Withania somnifera*. Indian Ginseng

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Introduction

Withania somnifera (Linn.) Dunal ordinarily known as Indian Ginseng and Indian winter cherry is a rejuvenating herb and a celebrated medicinal plant in classical medical practice for more than 3000 years. In Sanskrit, 'Ashwa' means horse and 'gandha' means smell. Therefore Ashwagandha' means "smell of a horse" and thus indicate that the herb imparts the vigor and strength of a stallion. The Latin meaning of *somnifera* is 'sleep-inducing'; indicate sedative property of the herb. In Indian traditional medical practices used this herb to boost the overall health and nourish the immunity of a person after a recovery from an illness [1]. *Withania somnifera* is one among the foremost medicinal herb in Ayurveda material medica and the Acharya Charaka incorporated the plant in *Balya* and *Brimhana-gana* [2]. From its ancient use to its modern perspective, it has been proven to be effective and safe for wide range of disease condition.

Vernacular names of *Withania somnifera*

English: Winter Cherry; **Sanskrit**: Ashwagandha; **Tamil**: Amukkura, Amukkuram-kilangu; **Hindi:** Punir, asgandh; **Malayalam**: Amukkuram; **Bengali**: Ashvagandha; **Gujrati:** Ghodakun, Ghoda, Asoda, Asan; **Telgu**: Pulivendram, Panneru-gadda, panneru, **Kannada** : Viremaddlinagadde, Pannaeru, aswagandhi, Kiremallinagida; **Konkani** : Fatarfoda; **Punjabi**: Asgand, isgand; **Marati**: Asgund, Asvagandha ; **Rajasthani**: Chirpotan

Distribution and Botanical illustration of *Withania somnifera*

This plant is an evergreen, small woody and tormentors shrub grown both in humid and dried areas mainly the tropical region of Asia, South Africa, Africa, Egypt, Congo, Jordan and Morocco [3]. It is commonly cultivated in Europe, South Australia and in India for medical and industrial purpose. In India it is cultivated in sub-tropical regions of Haryana, Gujarat, Madhya Pradesh, Maharashtra, Punjab, Rajasthan, and Uttar Pradesh [4]. The plant height is almost 30-150 cm, covered with white to brown wooly pubescence. The leaves are oval shaped

having almost 10 cm length and 2.5-5 cm wide, margins are arranged in an alternate manner. The flowers are usually yellow to green in colour, contains axillary fascicles, giving rise to bright red-coloured berries. The fruits are orange to red, contains plenty of seeds.

Phytochemistry of *Withania somnifera*

The phytochemicals profile of Withania somnifera always fascinating to researches because of its wide range of therapeutic applications. The roots enrich with amino acids, alkaloids, dulcitol, glycosides hentriacontane, starch, steroids, reducing sugar, volatile oil, and withaniol [5]. Anahygrine, anaferine, cuscohygrine, Isopelletierine, pseudotropine, pseudo-withanine, tropine, somnine, somniferine, somniferinine, withananinine withananine are the basic alkaloids which isolated from roots. In addition, visamine, withasomnine and withanine are also extracted from roots of the plant. Alanine, aspartic acid, cysteine, glycine, glutamic acid, tyrosine and tryptophan are the amino acids extracted from roots [6]. The leaves of the plants contain 12 variants of withanolides and 5 unidentified alkaloids. Withaferin A is the main withanolide and in addition to that presence of free amino acids, condensed tannins, chlorogenic acid, flavonoids, glucose, and glycosides also present in leaves. As well the green berries contain flavonoids, condensed tannis and proteolytic enzymes. Furthermore, the shoots specifically tender shoots are ample source of crude protein, calcium, flavonoids, phosphorous, condensed tannins and scopoletin (a coumarone) [7].

Pharmacological properties according to classical Ayurvedic texts and Indications

In Ayurvedic medical system a drug has four key properties, namely 'rasa, guna, veerya, and vipaka'. Rasa constitute the taste of the drug, Guna means the properties and effects it has on the body following consumption. Veerya represents potency of the drug i.e., whether it has a catabolic or anabolic effect on the body and vipaka means post digestive effect on metabolism. Based up on these factors, mechanism of a drug inside a biological system can be understood in terms of doshas (bio forces that control the body of an organism, whose balance and imbalance regulates health and ill health) [8]. The pharmacodynamics of Withania somnifera is rasa- tikta (bitter), kashaya (astringent) and kath (pungent) in taste, guna(qualities) srigdha (oiliness, untuousness) –laghu (light for digestion), veerya – ushna (hot in potency) and - vipaka- kathu (undergoes pungent taste conversion after digestion) and it exhibits karma – rasayana (rejuvinator), balya (improve strength) and vajeekarara (sex-stimulation)[9]. Classical Ayurveda highlighted this herb Withania *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). *Withania somnifera* also advisable for *apasmara* (epilepsy), *arsha* (piles), *arbuda* (tumour), *asthibhanga* (bone fracture), *bhagandara* (fistula-in-ano), *gridhrasi* (sciatica), *guhya-vrana* (ulcer in genitalia), *gandamala* (cervical lymphadenitis), *hanugraha* (lockjaw), *hrudgraha* (cardiac failure), *karshya* (emaciation), *kilasa* (vitiligo), *katigraha* (stiffness in lumbo-sacral region), *kushtha* (diseases of skin), *murchha* (syncope), *janustabdhata* (stiffness of the knee), *pramehapidika* (diabetic carbuncle), *shosha* (cachexia), *unmada* (mania/psychosis), *vatarakta* (gout), *yonidosha* (disorders of female genital tract) and *vidradhi* (abscess)[10].

In modern science through scientific proof the herb works on a nonspecific basis and possesses antioxidant activity, anti-inflammatory activity, antibacterial activity, antidiabetic activity, aphrodisiac, liver tonic and effective to reduce obesity. Moreover, the animal experiment and clinical evaluation supported it is effective to treat against

certain mental stress such as anxiety, cognitive and neuron disorders and acts as an adjunct for the cancer patient treating with radiation therapy as well as chemotherapy [11,12]. The herbal medicine also used as skin ointment and to improve reproductive fertility in Japan. In addition, the United States has taken patent on *Withania somnifera* formulation that helps to relives pain associated with arthritis. Moreover, the Aswgandha thailam (oil formulation of *Withania somnifera*) combined with Almond oil and Rose water act as an excellent skin toner[13].

The root of *Withania somnifera* is well a popular antibacterial and antifungal agent. It also used as overall tonic for stabilizing the health and as medicine to control fever [14]. It helps to manage body weight, expand life span, and maintains genuine nourishment of tissues, specifically bones and muscle tissues [15]. One of the exceptional hallmarks of this medicinal plant is enhancing the Ojas. Ojas is the most ultrafine and purified level of the physical body and is the ultimate result of healthy nutritional diet which is correctly digested. The ojas is accountable for fine immune system, lustrous complexion, healthy mental health, physical strength [16]. As well it is capable to treat various neuron degenerative disorders such as Parkinson's disease and Alzheimer's disease [17], emerging evidence suggests that it possess anti-cancer activity [18], anti-tumor, anti-inflammatory, antioxidant and rejuvenating properties [19, 20].

Ayurvedic formulations of *Withania somnifera*

Withania somnifera it is used as single herbal as well as polyherbal formulation and the roots are the major constituent of over 200 Ayurvedic formulations [21]. Among vast variety of rasayana herbs *Withania somnifera* hold prominent position known as "Sattvic Kapha Rasayana" herb [22] and is also recognized as the Queen of Indian herbs [23].

Withania somnifera is major ingredient in Awagandhadi-churna, Aswagandha-rasayana, Aswgandha-ghrita, Ashwagandha-rishta, Aswagandha- taila, Madhyamanarayana-taila, Brihat Ashvagandha-ghrita, Brihachchagaladya-ghrita, Saraswata-churna, Pramehamihihira-taila [24]. Nagabala-ghrita, Aswgandha rishata, Aswgandha-taila, Madhusnuhi-rasayana [25]. Aswgandha churna, Brihat Ashwagandha-ghrita, Chyawanprash, Balaaswgandhadithailam, Manasamitravada, Brahmivati and Ajaaswgandha dilehyam are other common *Withania somnifera* formulations mentioned in traditional textbooks.

***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 Century 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

Sarangadhara 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

Interpretation of *Withania somnifera* as per Ayurveda.

Withania somnifera known by its name '*hayahwaya-*' (providing horse potency and it has smell like those of house). *Ashwagandha*, *hayagandha* – (root, part used, also emits horse`s smell), *ashwavarohaka*, *vrisha*,- (spermatogenic and aphrodisiac effect of a dravya), *balada,balya-* (promote strength), *elaparni-* (leaves having shape of *ela) 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)[26].

Categorization of *Withania somnifera* as per classical Ayuvedic text

In Chaaka Samhita, Acharya Charaka included *Withania somnifera* under *balyadasaimani* group and *brimhaneeya* group in 4th chapter of *Suthra sthana* as *Balya, brimhana, madhuraskandha*, *virechanopaga* [27]. In Susrutha samhita it is mentioned as *urdhwabhagahara* [28]. Nighantus categorized it as *Withania somnifera* and *Withania 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* [29], Madanapala Nighantu referred it as *abhayaadi varga* [30]. In Dhanwanthari Nighantu and Shodhala Nighantu it is *guduchyadi varga*[31], Kaiyyadeva Nighantu it is '*oushadi varga* [32] and Raja Nighantu mentioned it as *shatahvadi varga* [33].

Therapeutic uses of *Withania somnifera* as per classical Ayurvedic texts

Withania somnifera is a well-documented medicinal herb in ancient Ayuvedic 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) [34].

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) [35]. 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) [36].As per Harita samhita it is mentioned for

vishavikara(poisonous disorder), *unmada*(insanity), *apasmara*(epilepsy), *vatavyadhi* (neurological disorders) and *apasmara*(epilepsy) [37]. 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) [38].

Clinical trials and modern approach in *Withania somnifera*

There are plenty of clinical research which have been proven *Withania somnifera* is safe and effective for several medical conditions. Meta-analysis of clinical trials carried out that, the *Withania somnifera* supplementation enhances the volume of semen, sperm count, and motility rate of sperm in oligospermic males [39] and concentrated *Withania* somnifera root extract helps in improving female sexual function [40]. *Withania somnifera* roots shows increase the overall muscle mass and strength which helps is high resistance in physical workout [41]. It is also reported that the aqueous extract of *Withania somnifera* significantly increased pain threshold force against mechanical pain in healthy human subjects [42]. A double-blind placebo control study on HIV patients with pulmonary tuberculosis the root extract of *Withania somnifera* administration behave as an adjuvant in conjunction with anti-TB drugs to DOTS with higher CD4 & CD8 count [43,44]. In an open-label preliminary feasibility analysis in cancer patients supplement of powder of *Withania somnifera* prevented loss in lean muscle mass, reduce fatigue, and curtail weight loss[45] .Similarly, a randomized, placebo-controlled, double-blind analysis of extract of *Withania somnifera* suggested a promising result in the depression treatment and anxiety [46].Further another study analyzed supplementations of the plant shown to be a ameliorative in post chemotherapy fatigue condition [47]. Furthermore, treatment with standardized *Withania somnifera* both roots and leaves extract significantly improved salivary dehydroepiandrosterone sulfate and testosterone level, but not cortisol and estradiol, in healthy individuals [48].

The *Withania somnifera* was shown to be beneficial in treating many neurodegenerative diseases and increases the longevity in *Drosophila* Alzheimer's disease model [49]. It is also investigated that the plant possesses significant role in ameliorating Parkinson's disease by oppose the oxidative damage and regulate the level of apoptotic proteins Bcl-2 and Bax [50]. The shirodhara procedure (Ayurveda therapy that involves gently pouring liquids over the forehead) with ashwagandha taila (*Withania somnifera* processed in sesame oil) is highly effective in management stress induced insomnia and associated symptoms [51]. It is reported that the extract of *Withania somnifera* attenuate {\alpha}-MSH-stimulated melanin synthesis by modulating MITF expression and that they may be a useful therapeutic agent for treating hyperpigmentation and suitable ingredient of whitening cosmetics [52].

New aspects of *Withania somnifera* to control COVID-19

The COVID-19 pandemic significantly becomes a health warning to the entire globe. Numerous studies, although some *in vitro* and *in vivo* studies, showed the bioactive components from *Withania somnifera* avenge against COVID-19 pandemic. It is reported that Withaferin A, alone or in combination with other drugs, like hydroxychloroquine, dexamethasone is capable to bind with the S-protein of SARS-CoV-2, thereby inhibits the spread of the infection [53]. In another report Withanone from *Withania somnifera* may restrict the entry of Coronavirus by disrupting the interactions between viral S-protein receptor binding domain and ACE2 receptor of host cell [54]. The Withanoside V and Somniferine also inhibit Mpro (Main protease) of SARS-CoV-2 [55]. It is also reported the multiple withanolides from *Withania somnifera*, such as withanolide-D, -G, -M, and -Q were boost immune system and inhibit the COVID infection [56]. So, the *Withania somnifera* may the primary option to control COVID-19 infectivity.

Discussion

The obtainable documentation by ancient Ayurvedic literature explained that the *Withania somnifera* is a potent regenerative herb and a best *rasayana.* In Ayurveda the whole plant is medically valuable, but roots are commonly used in formulations. Various literatures revealed that *Withania somnifera* contains plenty of pharmacologically valuable phytochemicals that attributes the pharmacological activity of the herb. The withanolides are the vital chemical constituents and around 138 withanolides are identified. Various clinical studies underlined its efficiency of this herb to treat against number of diseases. It acts as sedative, diuretic, generally respected for increasing energy anti-inflammatory, anti-stress agent endurance, acts as an-adaptogen that exerts a strong immunostimulatory. Ashwagandha is taken for treating ulcers, cold coughs, emaciation, epilepsy, diabetes, conjunctivitis, insomnia, senile dementia, leprosy, Parkinson's disease, nervous system disorders, epilepsy, rheumatism, arthritis, intestinal infections, bronchitis, asthma, a suppressant in HIV/AIDS patients. In Ayurveda the plant has been mentioned in *Brimhaniyamahakashaya, Balyamahakashaya* and *Madhuraskandha.* It is used as single herb as well as in combination with other herbs. While the *Withania somnifera* has been used successfully in Ayurvedic medical systems for centuries but still there is enough scope for much more scientific research and clinical trials for proving efficacy, mode of action, drug interactions and molding effects in combination with other herbs. Although the data from this review is quite promising regarding the reference of this plant from ayurvedic literature and its modern application.

Conclusion

Since the prehistoric period, herbal based medical system existed around the globe with a perfect and long recorded history. The herbs are considered as one of the most powerful ingredients in Ayurvedic formulations. Recently the Ayurveda and herbal based medicines commanded considerable attention owing to their prospective nutraceutical values. *Withania somnifera* one of the important plants which is well documented in Ayurvedic classical texts, has multiple health benefits. The plant possesses numerous pharmacological activities which is supported by various experimental and clinical studies. The vital phytochemicals attribute the medical properties that can directly and indirectly treat several illnesses that affect human. Diverse clinical and experimental studies are conducted on this herb to evince the scientific basements and documented medical reports in Ayurvedic texts. There must be well categorized evidence and research based classical documentation is essential to prove the medical significance of this plant to treat against various disease conditions.

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Fig. 1 : Ashwagandha Plant

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Comparative cytotoxicity of *in vitro* and field grown shoots of *Withania somnifera* in *Caenorhabditis elegans* model

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ABSTRACT

Indian ginseng, also known as *Withania somnifera* is a popular medicinal plant used as a domestic treatment for a number of age-related illnesses. The field grown *W. somnifera* roots are referred to as a *Rasayana* (Rejuvenator) medication in the traditional Ayurvedic medicine of India. It has been utilized as the main component in many formulations to help slow down the ageing process, manage stress, and be a remarkable neuroprotectant. The quantity and quality of traditionally grown plants, however, provide a considerable hurdle to their use in herbal-based products. The objective of this study was to determine the toxicity of shoots of *in vitro* developed *W. somnifera*, in *Caenorhabditis elegans* model and to compare the toxicological effect with that of plant shoots grown in the field. We found that biosafety is strictly concentration dependent. It was clear from the results that 250 µg/µL of *W. somnifera* shoot extract exhibited maximum viability for wild type animals.

KEYWORDS: *W. Somnifera*, toxicity, *C. elegans*, *in vitro* cultures

INTRODUCTION

Mother Earth is a plentiful supply of natural resources, especially therapeutic plants. It has been utilized for treating a wide range of illnesses and disorders in many people for many generations. According to the World Health Organization, a large portion of the population utilizes medicinal plants as their primary source for treating minor ailments (Dolatkhahi *et al.*, 2014). Despite accounting for the majority of pharmaceuticals, only 30% of human diseases can be successfully treated with allopathic medications. Ayurvedic medications made from plants are becoming more popular because synthetic medications not only worsen health but also place a heavier financial load on them (Pandey *et al.*, 2013). *W. somnifera* is a well-known species in Chinese, Siddha, Unani, and Ayurvedic medicine. The root of the plant is considered as a rejuvenator for at least 6000 years. There were several different *W. somnifera* compositions reported in traditional Ayurvedic literature. Across the world, people of all ages regularly utilize powder, infusions, decoctions, pastes, tablets, capsules, and syrups as medical preparations without experiencing any negative side effects, even during pregnancy (Archana & Namasivayam, 1999; Davis & Kuttan, 2002; Kumar *et al.*, 2005; Gupta & Rana, 2007).According to Sangwan *et al.*, (2004), the plant is used as a stimulant, aphrodisiac, anthelmintic, health tonic tonic, astringent, narcotic and diuretic. Emaciation, rheumatism, constipation, old age weakness, sleeplessness, leukoderma, goiter, and nervous system disorders are among the conditions it is frequently prescribed for in youngsters (Narendra *et al.*, 2004). When combined with other herbs, the plant's root is specifically used to treat snake venom. In addition, boils, flatulent colic, pimples, piles, and worm disturbance are treated with it (Misra, 2004). Additionally, the paste makes using its roots efficient for reducing joint swelling (Bhandari, 1970). Being a powerful antioxidant, it guards a biological system against harm caused by free radicals (Sangilimuthu *et al.*, 2011).

The active ingredients in this herb are steroidal lactones known as withanolides. The most important withanolides found in plant tissues are withaferin A, withanolide A, and withanone (Jayaprakasam & Nair, 2003; Ichikawa *et al.*, 2009; Praveen *et al.*, 2010). The type of plant tissue and the environment in which the plants were raised are two factors that can affect the synthesis of secondary metabolites. The commercial products produced from field grown *W. somnifera* vary from batch to batch because of the heterogeneity in pharmacologically active metabolites. These elements ultimately lead to a quality variation and commercial

exploitation of W. *somnifera* (Sangwan *et al.*, 2007). The estimated yearly requirement for dry W. *somnifera* powder in India for the synthesis of withanolide is 9127 tonnes however, only 5905 tonnes was the actual production. (Sharadha *et al.*, 2007). Field farming is also arduous and time-consuming and high chances of metal ion accumulation due to environmental pollution. These elements might cause a persistent global shortage of this plant (Sivanandhan *et al.*, 2012). As a result, the in vitro cultivation of medically significant plants, such as W. *somnifera*, may be used as a replacement for field cultivated plants in the production of high-yield plants. However, without scientific validation, the acceptance of this strategy cannot be justifiable. Preclinical safety considerations are crucial in the drug development process because they advance the candidacy of the molecule being tested. Although the active ingredients found in plants are considered natural, they still have certain harmful effects. Even in tiny amounts, some phytochemicals can cause toxicity in the formulation and stop it from moving on to higher-level trials. Therefore, utilizing a model system to examine the toxicity is essential. C. *elegans* is a perfect model system to evaluate the toxicity of test substances due to its simple anatomical structure and physiological resemblance to humans. The purpose of the study was to determine the toxicity of in vitro grown W. *somnifera* shoots, as well as to compare toxicity with that of plant shoots grown in the field.

MATERIALS AND METHODS

Maintenance of *In Vitro* Tissues

For the present study, the *in vitro* shoots of W. *somnifera* seeds of the "Jawahar Aswagandh 20" verity were bought from the Gujarat-based ICAR Directorate of Medicinal and Aromatic Plants Research. Seeds were surface sterilized in accordance with the procedure previously described by (Murthy *et al.*, 2008). The seeds were planted in Murashige and Skoog (MS) solid basal medium that also contained 2% sucrose, and they were then incubated at of 25±2°C in the dark. Under conventional tissue culture conditions, the shoots of *in vitro* plants produced from in vitro germinated seeds were kept on MS basal medium. The explants were grown at a constant and standardized temperature of 25±2°C for 16 hours in the light and 8 hours in the dark.

The nodal regions obtained from the 2 months old *in vitro* plantlets maintained on MS basal media were used as the explants for shoot multiplication. The nodal sections were carefully excised and 6-7 explants per bottle were inoculated onto MS basal medium fortified with 3% sucrose in 0.8% agar and various concentrations and combinations of 6-benzylaminopurine (BAP) and kinetin (Kin). The explants were cultured for 16 hrs in light and 8 hrs in dark and a constant temperature of 25±2°C was maintained throughout the culture period. After a period of 15, 30, and 45 days, the number of multiple shoots was recorded (Parameswari *et al.*, 2017).

Similarly, in suspension culture, shoot cultures of initial inoculums were transferred in 30 mL of MS liquid medium with the same concentrations, and combinations of BAP and Kin were used. The cultures were maintained at 25±2°C and observed regularly for contamination or for any other morphological changes. Each experiment had 4 replications. The culture was maintained on an orbital shaker under continuous agitation at 50 rpm; a photoperiod of 16 hrs was maintained for all experiments.

For mass production, the fresh and healthy shoot culture maintained in a suspension culture medium contained with 3% sucrose, 0.8% agar, BAP, and Kin were separated aseptically transferred to a bubble column bioreactor (Biopia, Korea) containing one litter of liquid MS media with 3% sucrose, BAP and Kin. The culture is maintained under aseptic aeration (aeration rate 0.1 vvm) by using a mini aeration system (Biopia, Republic of Korea) through a membrane filter (0.45 µm). The fresh culture medium was refilled into the bioreactor once every 15 days and the temperature were fixed at 25±2°C throughout the cultivation time.

The field grown W. *somnifera* shoots, were procured from the Directorate of medical and aromatic plants research, Gujarat. The raw materials such as *in vitro* and field-grown shoots of W. *somnifera* were cleaned in flowing tap water by sorting out tissue culture media, soil, or any other minute particle or media components and wiped the water using a cotton cloth.

Maintenance of *Caenorhabditis elegans*

The C. *elegans* (N2) were supplied by the CGC (Caenorhabditis Genetics Center), which is funded by the NIH National Center for Research Resources (NCRR) and NCBS (National Centre for Biological Sciences, Bangalore, India). Based on the standard procedures were followed by strain preservation, growth, and manipulation of worms (Brenner, 1974; Stiernagle, 2006). In particular, the worms were grown at 20°C on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 strain (live bacteria) and were used as a nutrient source.

Preparation of Plant Extract

The *in vitro* grown leaf tissues were collected from the bioreactor. Both *in vitro* and field-grown plant shoots were shade dried and pulverized. Using an electric mixer/grinder, grind the tissues properly. For extraction, One gram of this powder was used. Methanol (HiMedia) was used as an extraction solvent. Throughout extraction, a sample-to-solvent ratio of 1:50 was kept. Four times the extraction was done. The extract was sonicated for 20 minutes and shaken for 2 hours at 100 rpm and Whatman No. 1 filter paper was used for the filtering. The fractions were then combined, filtered, and dried using a rotary vacuum evaporator operating at 125 revolutions per minute in a water bath at 40 degrees Celsius. The residue was kept at -20°C until it was dissolved in 10 mL of HPLC-grade methanol.

Assay using *C. elegans* as a Model Organism

All strains of C. *elegans* were maintained and propagated onto NGM agar plates carrying a lawn of *Escherichia coli OP50*

(uracil auxotroph) as the food source at 20 °C according to the standardized protocols (Brenner,1974). To obtain synchronized cultures, gravid hermaphrodites were lysed in 5 M sodium hydroxide +5% household bleach and the gathered eggs were incubated overnight at 20 °C in M9 buffer to favor hatching. The retrieved eggs were then kept at 20°C in M9 buffer to promote hatching. *E. Coli* OP50 was cultivated overnight at 37°C in Luria-Bertani (LB) broth (Fabian & Johnson,1994).

To each well of 96 well plates containing 150 µL NGM agar, various pharmacological doses (100–250–500–1000–2500–5000–µg) of invitro shoot extracts and field-grown shoot extracts of *W. somnifera* were added. The plate was then seeded with 20 µl of *E. coli* (OP 50). The Bacterial culture was allowed to grow at room temperature for 2 days. After preparing the plates, five synchronized L1 wild-type worms were seeded on each well plate and incubated at 20°C, and each well was photographed on day 6 by using a Trinocular compound microscope (Motic 1000; 1.3 M pixel). The worms were checked for inactivity after exposure. Nematodes were considered as dead if they fail to respond to gentle physical prodding using a metal loop. Three separate studies, each carried out in triplicate.

Statistical Analysis

All the analyses were performed in triplicates (n=3) and the values were represented as Mean±SE (standard error) of six replications. Two-way ANOVA with Duncan's multiple range test (DMRT) was performed to check the statistical difference among the treatment group at 5% level ($p<0.05$) in SPSS.

RESULTS AND DISCUSSION

Cytotoxicity Assay of *In Vitro* and Field-grown Shoots of *W. somnifera*

An organism's development is directly related to the type of environment it is exposed to. This fact can be used to learn more about an organism's biology. Any changes to the ecological conditions are likely to have an impact on the biology of the organism. One of the most extensively used models in biological research is the non-parasitic nematode *C. elegans*, which ages similarly to higher mammals like humans in terms of behavior and physiological changes (Kirkwood, 2011). The animal's ability to move is hampered as it ages, and it develops sarcopenia, loses its reproductive ability and suffers severe muscle tissue loss. This results from the accumulation of lipofuscin and oxidized proteins (Klass, 1977; Johnson, 2003).

Because *C. elegans* is a translucent organism, morphological and developmental changes within a single worm can be seen. It is a desirable alternative because wild animals typically have a storage of 10-15 eggs in the uterus and can reproduce in two to three days with a lifespan of about three weeks (Fielenbach & Antebi, 2008). At the genomic level, it shares 80% of our genetic makeup (Braeckman & Vanfleteren, 2007; Bell *et al.*, 2009). It is simple to keep an eye on these nematodes throughout experimental treatments due to their ease of culture and short lifespan. This model organism has proven to be very beneficial in research on pharmacological and gene interactions (Rand *et al.*, 1995).

In particular, pharmaceutical therapies using phytochemicals and antioxidant supplements are known to lengthen longevity or delay physiological ageing in *C. elegans* (Collins *et al.*, 2006; Lucanic *et al.*, 2013) toxicity characterization with this method saves money and minimizes the use of animals, and because animals are transparent, high-quality microscopic photographs may be taken (Flecknell, 2002). Toxicity studies have been conducted by Himri *et al.* (2013) to test tartrazine and sulphanilic acid at different concentrations (0.5 mM to 3.0 mM) on worm *C. elegans* and assess the nematode growth and development. Yang *et al.* (2015) studied the toxicity of AFB1 and T2 mycotoxin using *C. elegans* as a model organism.

In view of this, we planned to test the toxicity of *W. somnifera* shoot extracts at different concentrations on *C. elegans*. The investigation was carried out to determine the viability of animals treated with various concentrations of *W. somnifera* shoot extract grown in the field and *in vitro* Different quantities of plant adaptogens, which reduce animal survivability to 50%, were used to test the cytotoxic property. The data shown in Table 1 and Figure 1 unmistakably show that extracts of *in vitro* samples (IC50= 2.88 mg/L) have much higher animal viability than field-grown samples (IC50=1.37 mg/L). This might be due to the poisonous chemicals and plant-to-plant chemical diversity seen in the natural population of *W. somnifera*. The number and quality of phytoconstituents in natural plants can also change based on the time of year, the climate, and illnesses and these might also factor for toxicity.

The full assessment of nematode growth and development of the wild population at various doses of plant extract is shown in Figures 2 and 3. In the laboratory, *C. elgans* are easily grown on agar plates, with *E. coli* (OP50) as a food source. Visual inspection of the bacterial turbidity on top of the agar demonstrated that the growth of *E. coli* (OP50) was unaffected by the various doses of plant extract employed in the toxicity assay. The analysis's findings showed that worms can be cultivated successfully on 96-well plates using a solid medium.

Table 1: Influence of *W. somnifera* shoot samples on the percentage viability of *C. elegans*

Concentrations of shoot/leaf extracts (mg/µl)	(%) of Viability	
	In vitro shoot sample	Field grown shoot sample
0 100[a]	0 100[a]	0 100[a]
100[a]	100[a]	100[a]
0.1 90.38[b]	0.1 90.38[b]	0.1 90.38[b]
73.08[b]	73.08[b]	73.08[b]
0.25 88.46[c]	0.25 88.46[c]	0.25 88.46[c]
61.54[c]	61.54[c]	61.54[c]
0.5 78.85[d]	0.5 78.85[d]	0.5 78.85[d]
53.85[d]	53.85[d]	53.85[d]

Data represent the Mean±SE (standard error) of six replications. Values followed by different letters within a column are significant at $p<0.05$

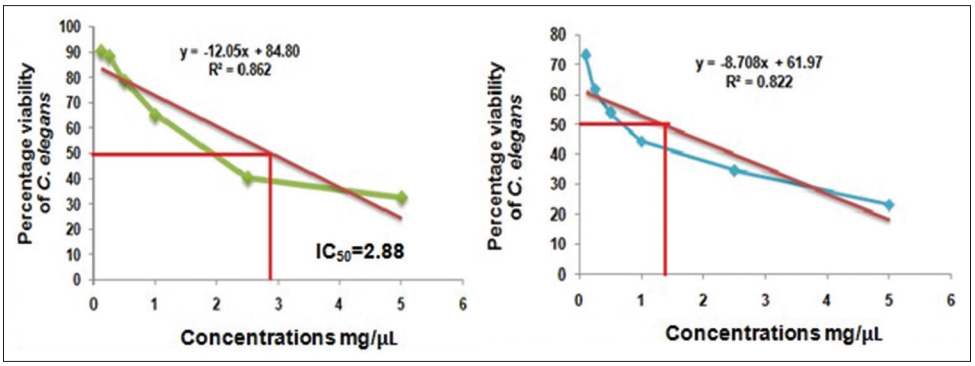

Figure 1: Influence of *W. somnifera* shoot/leaf samples on the percentage viability of *C. elegans*

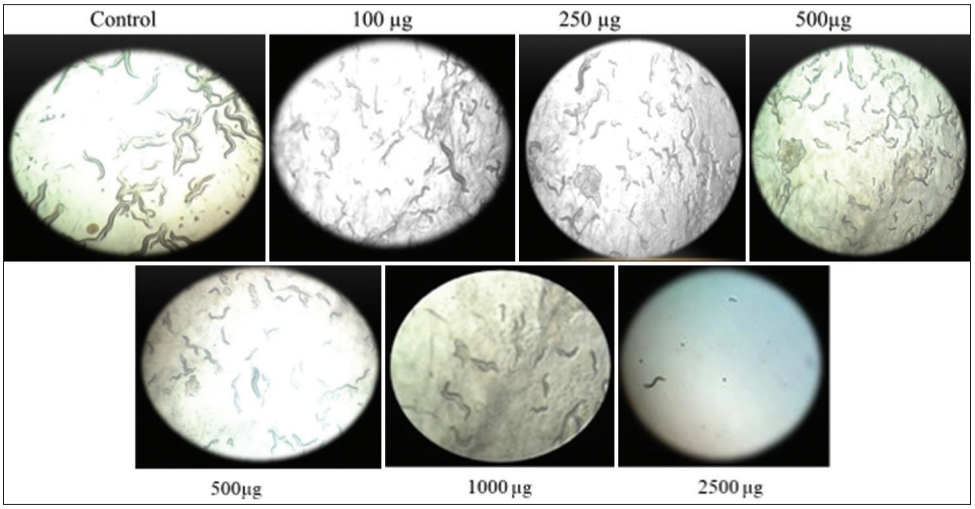

Figure 2: The plates indicate the toxicity effects of *in vitro* cultivated shoot samples of *W. somnifera* at different concentrations that affect the growth of *C. elegans*

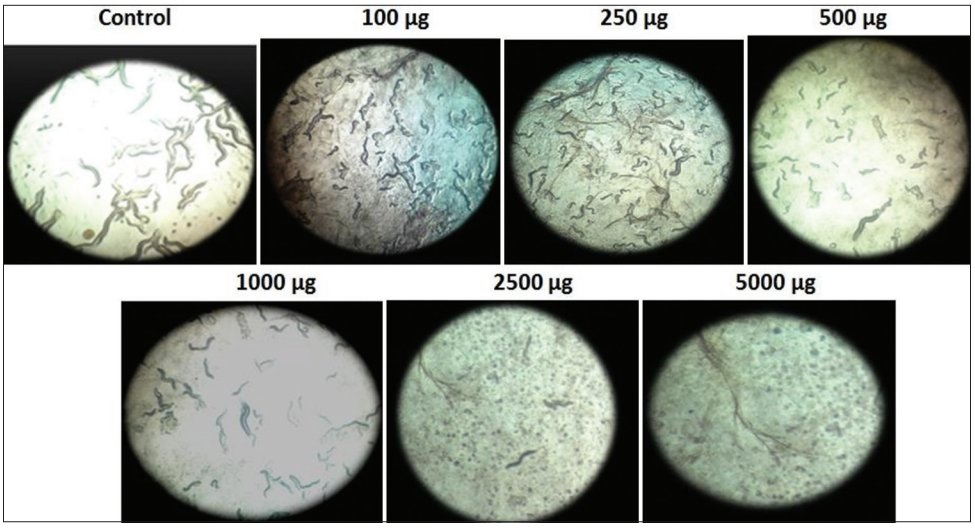

Figure 3: The plates indicate the toxicity effects of field grown shoot samples of *W. somnifera* at different concentrations that affect the growth of *C. elegans*

First, we attempted to determine the maximum number of worms that can develop in a single well without depleting the available nutrients before the end of the reproduction cycle (six days).

In order to determine cytotoxicity by plate-based assay, the worms were exposed to different concentrations (0-5.0 mg/µL) of *in vitro* and *in vivo* shoot extract of W. *somnifera*. Among the different concentrations, a higher concentration of plant extract

above 1.0 mg/µL caused premature mortality of the worms. Exposure to concentrations between 0.1 mg/µL to 0.5 mg/µL showed a beneficial effect on an increasing developmental delay with associated delay in the reduction of nutrients. One common type of toxicant-induced detrimental effect on worms may be the development of aberrant valva. According to Jiang *et al.* (2017), exposure to doses of 0.2 mg of Impatiens Balsamina stem extract had an impact on *C. elegans* survival, movement, growth, and development. Using *C. elegans*, Xiong *et al.* (2017) performed a plate-based assay for the assessment of toxicity. On 24-well plates, the worms were cultivated in various boric acid concentrations (1.2-18.0 mM). The findings demonstrated that greater concentrations (12 mM and beyond) resulted in acute toxicity and also inhibited the formation of offspring.

CONCLUSION

The biosafety of *in vitro* and field grown shoots or leaves of *W. somnifera* extract was determined using *C. elegans* as a model organism. The *C. elegans* worms were grown in media containing *W. somnifera* extract and were observed for growth and any morphological variations. The percentage of viability was found to be concentration dependent. The results clearly indicated that 250 µg/µL of *W. somnifera* shoot/leaf extract showed maximum viability of wild type worms. So, an exposure concentration of 250 µg/µL is recommended for further studies and drug development in the future.

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PLAGIARISM CHECK REPORT (THESES)

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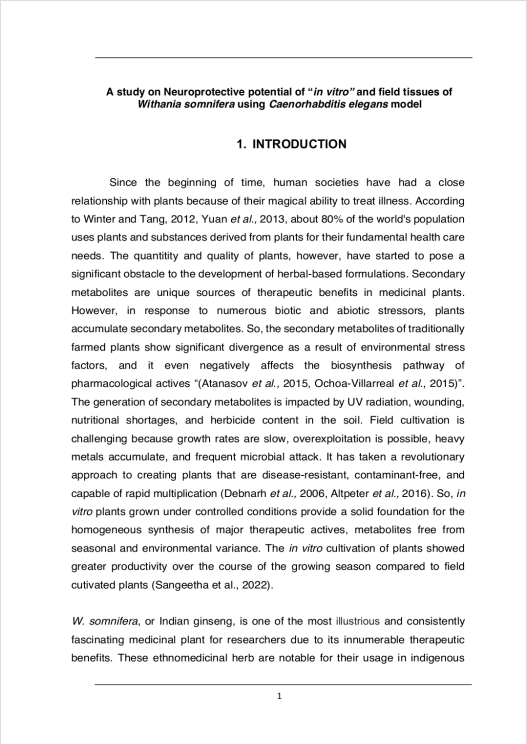

A study on Neuroprotective potential of "*in vitro*" and field tissues of *Withania somnifera* using *Caenorhabditis elegans* model

1. INTRODUCTION

Since the beginning of time, human societies have had a close relationship with plants because of their magical ability to treat illness. According to Winter and Tang, 2012, Yuan *et al.,* 2013, about 80% of the world's population uses plants and substances derived from plants for their fundamental health care needs. The quantitity and quality of plants, however, have started to pose a significant obstacle to the development of herbal-based formulations. Secondary metabolites are unique sources of therapeutic benefits in medicinal plants. However, in response to numerous biotic and abiotic stressors, plants accumulate secondary metabolites. So, the secondary metabolites of traditionally farmed plants show significant divergence as a result of environmental stress factors, and it even negatively affects the biosynthesis pathway of pharmacological actives "(Atanasov *et al.*, 2015, Ochoa-Villarreal *et al.*, 2015)". The generation of secondary metabolites is impacted by UV radiation, wounding, nutritional shortages, and herbicide content in the soil. Field cultivation is challenging because growth rates are slow, overexploitation is possible, heavy metals accumulate, and frequent microbial attack. It has taken a revolutionary approach to creating plants that are disease-resistant, contaminant-free, and capable of rapid multiplication (Debnarh *et al.*, 2006, Altpeter *et al.*, 2016). So, *in vitro* plants grown under controlled conditions provide a solid foundation for the homogeneous synthesis of major therapeutic actives, metabolites free from seasonal and environmental variance. The *in vitro* cultivation of plants showed greater productivity over the course of the growing season compared to field cutivated plants (Sangeetha et al., 2022).

W. somnifera, or Indian ginseng, is one of the most illustrious and consistently fascinating medicinal plant for researchers due to its innumerable therapeutic benefits. These ethnomedicinal herb are notable for their usage in indigenous

1

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by Krishna Priya C

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