3

Materials and Methods

As part of this study, we evaluated the comparative neuroprotective effectiveness of tissues from *W. somnifera* that had been developed *in vitro* compared with those that had been harvested from field. This study mainly focused on *in vitro* propagation of *W. somnifera* and prove its bioactivity as equivalent to the roots that grow in the field. As a whole, the study was divided into two broad phases. The first major phase emphasized comparative validation and analysis of four different tissues *ie., in vitro* shoots (IS), field grown shoots (FS), *in vitro* root (IR), and field grown roots (FR) in all physical, chemical, and biological aspects. Phase I, of the study, focused on the propagation of IS and IR separately in tissue culture medium and its mass cultivation. The next step of this phase involved the collection of authenticated field materials (FR and FS) and formulation powder using four variants *of W. somnifera* tissues. Standard pharmacogenetic and physicochemical analysis protocols were followed to validate the four powder formulations. A qualitative and quantitative analysis of the secondary metabolites in the formulated powders will be conducted in Phase II, followed by quantification of dominant secondary metabolites or withanolides in both *in vitro* and field grown *W. somnifera* tissues using a standardized HPTLC (High-performance thin-layer chromatography) procedure. Using ICP-MS (Inductively coupled plasma mass spectrometry) analysis of four powders the variation in metal ion accumulation pattern were studied. Additionally, this phase involves comparative *in vitro* antioxidant potentials of IS and IR with those of FR and FS. During phase III, comparative metabolite profiling will be performed using GC-MS (Gas Cromatography- Mass Spectroscopy) on both *in vitro* and field grown tissues of W. *somnifea.*

The second part of the study consists of a comparative evaluation of the bioactivity of field grown and *in vitro* tissues of *W. somnifera i.e.,* To demonstrate the *in vitro* culture system as efficiently bioactive as that of the FR root based on an animal model. This objective was achieved through phase IV of the study by utilizing *C. elegans* as the model organism. This phase involved the examination

of the toxicity effect of *W. somnifera* tissues in *C. elegans*. The study also highlighted whether the *W. somnifera* extracts effects on the natural progeny production in *C. elegans*. The oxidative stress resistance potential of *W. somnifera* extracts was also evaluated using wild type *C. elegans* worms. Life span extension and genetic screening to identify the specific genes that participate in life extension were carried out with mutant worms with mutations in specific genes that play a role in IIS signelling (insulin/insulin-like growth factor-1 signals), mETC (mitochondrial electron transport chain complexes) and DR (dietary restriction) pathways. The study also continued with the study of the reporter gene expression study of *W. somnifera* extracts treated transgenic *C. elegans* strains stably expressing sod-3::GFP, ctl-1,2,3::GFP, and gst-4::GFP. Based on transgenic *C. elegans* (NL5901), which expresses the human synuclein gene fused with a YFP (yellow fluorescent protein) in muscle cells, aggregation of synuclein protein was evaluated. To examine the neuroprotective effect of *W. somnifera* extract against 6-OHDA (6-hydroxydopamine- 25 mM) induced selective degeneration of DAergic neurons (dopaminergic neurons) BZ555 worms carrying GFP (Green Flurecent Protein) construct tagged in all eight DAergic neurons were utilized.

This study describes the materials used and the experimental methods employed under the following headings:

3.1 Materials

3.1.1. Plant material

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3.1.1b. *In vitro* shoot and root cultures of *W. somnifera*

3.1.1c. Field roots and shoots of *W. somnifera*

3.1.1d. *Caenorhabditis elegans* cultures

3.1.2. Chemicals

3.2 Methods

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3.2.5a. Assay for α-synuclein aggregation and neuroprotection

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3.2.5d. Assay for food-sensing behavior

3.2.6. Statistical analysis

3.1 Materials

3.1.1. Plant material

3.1.1a. Seed material ‘Jawahar 20’variety

Central Institute of Medicinal and Aromatic Plants (Lucknow) provided seeds of *W. somnifera* (Jawahar 20). A half strength MS (Murashige and Skoog, 1962) media supplemented with 2% sucrose was used to germinate the seeds that had been soaked overnight. Upon germinating the seedlings, they were transferred to a full-strength MS basal medium and cultured under

22°C and 16/8 hrs photoperiod. For shoot multiplication, fresh healthy nodes obtained from 60 day old MS grown *in vitro* plants were inoculated with 4.44M BAP (Benzylaminopurine) in MS basal media (Parameswari *et al.,* 2017). All studies were conducted using shoots from MS+BAP medium that are one month old.

3.1.1b. *In vitro* shoot and root cultures of *W. somnifera*

In vitro germinated seeds of *W. somnifera* were subcultured in basal MS media for two months, then subcultured periodically (15-20 days intervals). In order to facilitate the growth of secondary shoots, fresh and healthy nodal sections were used as explants. A healthy leaf from an aseptic plantlet two months old was used as an explant to induce adventitious roots (Wasnik *et al.,* (2009)).

3.1.1c. Field roots and shoots of *W. somnifera*

The dried roots and leaves of field grown plants of *W. somnifera* were obtained from the Indian Council of Agricultural Research (ICAR) -, Gujarat for all the studies.

3.1.1d. *Caenorhabditis elegans* cultures

The following *C. elegans* strains were used in this study: Bristol N2 (wild-type), CB1370 (*daf-2*(e1370), RB759 (*akt-1*(ok525)), VC345 (*sgk-1*(ok538)), GR1307 (*daf-16*(mgDf50)), EU1 (*skn-1*(zu67)), PS3551 (*hsf-1*(sy441)), DA1116 (*eat-2*(ad1116)), TK22 (*mev-1*(kn1), MQ887 (isp-1 [qm150]), CB4876 (*clk-1* (e2519)), RB665 (*dop-1*[ok398]), BZ873 (*dop-3*(ok295)), CF1553 (*muIs84*(sod-3::GFP)), CL2166 (*dvIs19*(gst-4::GFP)), NL5901 (*pkIs2386*(unc-54p::alpha-synuclein::YFP+unc-119)), and BZ555 (*egIs1*(dat-1::GFP)).

3.1.2. Chemicals

The chemicals that were used in plant tissue culture were nutrient salts and nutrients purchased from HiMedia. A mixture of silica gel plates 60 F254 (Merck) and HPLC grade methanol (Rankem) was used, as well as ethyl acetate (Rankem), formic acid (Qualigen), methanol (SRL), and toluene (Rankem). In addition to wthanolide A (WA), withanone (WTN), and withaferin A(WFA), purchased them from ChromaDex (USA) and Natural Remedies (Bangalore).

For ICP-MS Nitric acid (HNO_3), 67-70%, for trace metal analysis was procured from Fisher Chemicals, India. All samples and standards were prepared using Milli-Q water. All lab accessories used were of A grade and duly calibrated. Calibrated micropipettes were used with the range of 10µl to 1000µl. 5% (v/v) nitric acid and deionized water were used to clean all the containers (polypropylene). By successive dilutions of a high purity ICP multi-element calibration standard (10 g/L from 30 elemental ICP-MS standard, matrix: 2.5% (v/v) HNO_3, Inorganic Ventures), calibration standard solutions and internal standards were prepared.

As part of the GC-MS analysis, the chemicals used for derivatization were BSTFA (N,O bis(trimethylsilyl)trifluoroacetamide), and TMCS (trimethyl chlorosilane) from Sigma. 2-2-Chloronaphtahalene was used as a standard (Sigma). Other commonly used standards and chemicals used in quantitative estimations have also been purchased from Sigma. The chemicals and materials used in this study were of the highest quality available on the market.

For the cultivation and maintenance of *C. elegans*, LB Broth (Luria-Bertani Broth), NaOH, NaCl, agar, peptone, cholesterol, ethanol, $KH2PO_4$, $K2HPO_4$, and $MgSO_4$ were used (HiMedia), 5-hydroxy-1,4-naphthoquinone, Sodium azide (Sigma).

3.2 Methods

3.2.1. Powder preparation using *in vitro* and field grown tissues and validation of formulation.

3.2.1a. Media preparation

To propagate the plants, induce root growth, and prepare suspension culture, Murashige and Skoog (MS) medium were used (Murashige and Skoog, 1962). The composition of the medium for plant tissue culture is provided in (Appendix I). Milli-Q water was used to prepare the medium. The pH of the media was adjusted to 5.6-5.8 by adding 0.1 N NaOH or HCl. The gelling agent was agar-agar Type-I 0.8 %, which was added to the media prior to autoclaving.

Thereafter, the suspension medium was poured into culture bottles (30 ml each) and Erlenmeyer flasks (100 ml each). As part of the autoclaving process, the

medium was autoclaved for 20 mins at 15 psi and 121°C. Following this, it was transferred into plant tissue culture bottles (30 ml each) and suspension media into Erlenmeyer flasks without adding agar (100 ml per 250 ml flask).

3.2.1b. Preparation of explants and *in vitro* shoot and root cultivation

The nodal segment of plant which grown in MS (Murashige and Skoog,1962) basal medium were used for *in vitro* shoot multiplication. Explants were implanted onto MS baseline medium containing 3% sucrose in 0.8% agar and BAP after the nodal fragments had been removed. 25±2°C is the constant temperature that maintained for 16 hrs of light and 8 hrs of dark for the cultivation of explants. In this study, the number of multiple shoots were counted after a period of 15, 30 and 45 days. Suspension culture of 2.0 g shoot clusters was transferred to 30 ml of MS liquid medium containing BAP. As long as the cultures were maintained at 25±2°C, contamination and morphological changes were regularly evaluated. A four-fold replication of each experiment was conducted. A continuous agitation at 80 revolutions per minute was maintained for the duration of all suspension cultivations on an orbital shaker with a 16 hrs photoperiod. It was observed that the biomass had increased after 30 days and that several shoots had appeared.

Wasnik *et al.,* (2009) describe a procedure for induced adventitious roots from leaf explants. *W. somnifera* cultivar 'Jawahar 20' leaf explants were induced to form direct adventitious roots on MS medium with 30 g/L sucrose, 1 mg/L Indole-3-butyric acid (IBA), and 0.25 mg/L Indole-3-acetic acid (IAA). The inoculated explants were then cultured at 25±2°C and monitored for contamination or any other changes in morphology. For *in vitro* cultivation, a photoperiod of 16/8 hrs were maintained. Aseptically separated adventitious roots from MS media after one month. The roots were transferred into 50 ml aliquots of half strength half strength MS media at with a length of 2.0-4.0 cm and a fresh weight of 500 mg. An orbital shaker was used to maintain the cultures under continuous agitation at 90 rpm (Revolustion per minitus) and a 16 hrs photoperiod at 25±2° C. At 15th day intervals, the roots were subcultured in half-strength liquid MS media and adventitious roots were appeared.

3.2.1c. Culture of shoots and roots in bioreactor

After removing the roots from the suspension media, sterile water was used to rinse them once or twice. RFW (Root fresh weight) was determined after surface water had been removed. Roots were introduced into the root tips of bubble column bioreactors (Biopia, Korea) with MS suspension media initially containing 1.0 mg IBA and 0.25 mg IAA. In order to achieve mass production, fresh and healthy shoot clusters were separated and transferred aseptically into bubble column bioreactors (Biopia, Korea). Both cultures were maintained under aseptic aeration (0.1 vvm) using a mini-aeration system (Biopia, Korea). For each 15-day cycle, the bioreactor was refilled with fresh culture medium, and the temperature was kept at 25°C. A 45-day cultivation period was used to harvest *in vitro* plants.

3.2.1e. Preparation, pharmacogenetic and physicochemical validation of *in vitro* and field grown tissue powder

According to the protocol described by the Ayurvedic Formulary of India (AFI) and Ayurvedic Pharmacopia of India (API), the powder was formulated. Shade dried tissues of *W. somnifera* were ground with an electric mixer/ grinder and sieved through #80 meshes (Anonymous *et al.,* 2007, Sangeeta *et al.,* 2017). The foreign matter was evaluated by spreading out 10 grams of each sample thinly on white paper. A total of foreign substances was calculated using the number of detectable and probable foreign matters and other contaminants (Zhang *et al.,* 2012). Following the method previously described (Patra *et al.,* 2009), organoleptic or sensory characteristics of all formulations were evaluated, including colour, odour, taste, overall appearance, and texture. Following standardized pharmaceutical procedures, different physicochemical evaluation of powders was conducted. Total Ash, Water soluble ash, Acid insoluble ash, moisture percentage, and pH range were monitored for each powder sample (Vikas *et al.,* 2020, Lohar *et al.,* 2007, Satheesh *et al.,* 2011). Using a standard protocol, physical parameters including Tap density, Bulk density, Angle of repose, Hausner's ratio, and Carr's indexwere also examined for each powder sample.

3.2.2. Secondary metabolite quantification

3.2.2a. Secondary metabolite extraction

The secondary metabolites of *W. somnifera* tissues were evaluated using shade dried powder of one gram of IS, IR, FR and FS. In order to extract thesamples, the extraction solvent used was methanol. Extraction was performed in a ratio of 1:50 sample to solvent. The extraction was repeated four times. After being sonicated for 20 mins and shaken at 100 rpm for 2 hrs, the extract was filtered using Whatman No. 1 filter paper. The fractions were combined, filtered, and dried in a rotary vacuum evaporator operating at 125 rpm at 40°C. The residue was diluted in 10 ml of HPLC grade (High-performance liquid chromatography) methanol and stored at -20°C for further analysis. A total amount of steroids, alkaloids, phenols and flavonoids, as well as major withanolides were estimated from this extract.

3.2.2b. Quantitative analysis of essential phytoconstituents

All powdered samples of *W. somnifera* roots, shoots, and field-grown material were analyzed for phenols, flavonoids, steroids, and alkaloids. As part of the phytochemical evaluation, the total phenolic content was assessed (Hithamani and Srinivasan, 2014) (Appendix - II), the total flavonoid content was assessed (Lahouar *et al.,* 2014) (Appendix - III), the total steroid content was assessed (Naik and Mishra, 2015) (Appendix - IV), and the total alkaloid content was assessed (Naik and Mishra, 2015) (Appendix - V). Samples are analyzed by measuring each phytoconstituent's concentration in mg/g.

3.2.2c. Determination of free radical scavenging activity

Based on the method of Vaz *et al.,* (2011) with minor modifications, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured. 20 µL each extract was introduced into a 96-well plate, and along with 180 µL of DPPH solution (100 mM) was added after 30 min of incubation in room temparature. Using a microplate spectrophotometer (xMark, Biorad, Berkeley, CA), The absorbance was measured at 517 nm. As a blank, DMSO (Dimethyl sulfoxide) was used. The following formula was used to calculate antioxidant activity:

(Inhibition rate, %) = (blank OD - sample OD)/blank OD x 100

3.2.2d. Quantification of major withanolides using HPTLC

Standard Withanolide A (WA) and Withanone (WTN) were obtained from Chromodex (USA). The withaferin A (WFA) was obtained from Natural remedies (Bangalore). Standard stock solutions were prepared using HPLC methanol at a concentration of 1 mg/ml and stored at 4°C. To prepare the working standards, the stock was diluted to a final concentration of 0.5 mg/ml before use. In this study, HPTLC quantifications were carried out in a solvent system containing toluene, ethyl acetate, and formic acid in a ratio of 5:5:1 (v/v). The procedure was conducted on silica-precoated aluminum plates with a thickness of 0.2mm. For quantification, the standards withanolide-A, withanone, and withaferin-A were applied at concentrations ranging from 0.5 to 2.0 g per band. HPLC methanol samples were spotted onto the plates along with the standards as 6 mm bands marked 8 mm from the bottom. Using a semi-automatic sample applicator, the CAMAG Linomat V (Switzerland), operated with a Hamilton syringe of 100 ml, was employed to apply the samples under a nitrogen stream (rate of spraying 150 nLs-1). In a presaturated twin trough chamber, loaded plates were developed to a distance of 80mm, air dried, and visualized at 254 and 366 nm. A densitometric analysis was conducted with a Camag TLC scanner III controlled by CAMAG CATS 4 integration software utilizing a Savitsky-Golay7 filter in the reflectance-absorption mode, at a scanning speed of 20 mm per second for WFA, 234 millimeters per second for WA, and 231 mm per second for WTN. A comparison was made between the Rf values and peak areas of the samples and the standards. Using the anisaldehyde sulphuric acid reagent, derivatize the plates, the plates were placed in a hot air oven at 110°C for approximately 10 mins to detect spots (Preethi *et al.,* 2014).

3.2.2e. Trace elemental fingerprinting of *in vitro* and field cultivated *W. somnifera*

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed for the evaluate the variability of major elements and trace elements in the *Withania powders*. Concentrations of elements such as P, K, Ca, Mg, B, Cu, Fe, Mn, Mo, Zn, Cd, Ni, Pb, Hg, Cr, Co, As determined using ICP-MS (Thermo Fisher iCAP RQ ICPMS). Sample decomposition was the first step in the multi-elemental analysis by ICP-MS. Quantitative determination of the elements B,

Mg, P, K, Ca, Mn, Fe, Cu, Zn, Mo, Cr, Co, Ni, As, Cd, Hg, and Pb in samples was performed using an inductively coupled plasma mass spectrometer, ICPMS iCAP-RQ, Germany), equipped with a TELEDYNE, ASX -560 Autosampler and collision-reaction interface iCRI working in Ar and He modes. The optimal conditions were as follows: RF power of 1500W, plasma gas flow rate of 14L/min, auxiliary gas flow rate of 0.8 L/min, nebulizer gas flow rate of 1.01 L/min, Pirani pressure of 1.81 mbar, Penning pressure of 3.05x10-7mbar & 3 number of replicates.

A constant weight was obtained by oven drying the samples at 60 °C for 12 hours before chemical analysis. Stainless-steel grinders were used to grind the dried samples into small particles which is enough to pass through a mesh of 0.5 mm, which was kept dry for analysis. A mixture of the contents of five different batches was prepared. The first step involved the preparation of 0.2 gm each sample along with 6.0 ml of concentrated ultra-pure HNO3 in a tightly closed PTFE vessel, which was digested separately in a microwave digester (AntonPaar multiwave GO, Austria). The digestion was carried out in three steps with constant microwave power, with the program set to increase the temperature to 160°C in 10 mins and hold at that temperature for 5 mins, followed by cooling down the digester at room temperature and diluting the digested samples with Mili Q water to a concentration of 50 ml. Additionally, blank samples were prepared in accordance with the analytical methodology outlined above. ICP-MS was used to analyze these solutions after appropriate dilution, using external standards for calibration, and considering five points on the curve and one for quality control.

3.2.3. Metabolic profiling of *in vitro* and field grown of *W. somnifera*

3.2.3a. Total metabolite profiling using GC-MS

The methanolic extracts (100 µl) were vacuum evaporated and the dried residues were incubated at 37^0C thick Rxi with 50 µl of methoxylamine hydrochloride (20 mg/ml pyridine) for 2 hrs. As a result, acidic protons were formed that were derivatized at 37^0C for 30 min using 50 µl of MSTFA (N-methyl N-trimethyl silyl trifluoroacetamide) to identify metabolites. Gas chromatography was carried out using a 0.25 µm 5 silicon MS column with a diameter of 0.25mm and a length of 30m. An ion source was used to inject the sample at a

temperature of 250^{0}C with a flow rate of 1 ml per min of helium. A solvent delay time of 8 minutes was followed by a temperature increase of 6.5^{0}C min-1 up to 300^{0}C with a 5mins hold time. After tuning the system according to the manual, each 1µl aliquot of the derivatized extract was injected into the GC/MS instrument (Shimadzu 2010 plus) with a split ratio of 1:10. Helium was used as the reference gas. To achieve a maximum resolution of Rfwhm=2800 at 614 m/z, A manual improvement was made to the high mass resolution. Observations were made between 45 m/z and 650 m/z with a scanning speed of 0.5 seconds per scan. Data obtained from the GC-MS were interpreted using AMDIS software (version 2.62). In order to confirm the peaks obtained, the peaks were compared to the two spectral libraries NIST 05 (https://chemdata.nist.gov) and GOLM metabolome database (gmd.mpimp-golm.mpg.de). PCA (Principal component analysis) was performed on the relative amounts and percentages of the metabolites.

3.2.4. Toxicity and Life span extension determination in *C. elegans*

3.2.4a. Worm strains, genetics, and maintenance

The *C. elegans* strains were maintained and propagated on nematode growth media (NGM) agar plate pre-inoculated with *E. coli* strain OP50 as food source at 20°C. The *C. elegans* cultivation and maintenance are given in (Appendix - VII, Appendix - VIII) and age synchronization in (Appendix – IX). Age synchronized worm populations were obtained by treating the gravid adult worms carrying eggs with alkaline hypochlorite solution (Appendix –VI), and the collected eggs were then incubated at 20°C in M9 buffer to favor the hatching. *E. coli* OP50 was cultured in the Luria-Bertani (LB) broth at 37°C overnight (Brenner, 1974, Stiernagle, 2006). The phenotypic and genotypic characteristics of all strains of *C. elegans* used in this study are presented in **Table 3.1.**

Table 3.1: Genotypic and phenotypic descriptions of all *C. elegans* strains used in this study

Genotype	Gene description	Phenotype
N2	-	Wild type
daf-2(*e1370*)	The gene encodes an insulin/IGF receptor tyrosine kinase ortholog in *C elegans.* A variety of biological processes are regulated by DAF-2	Long-lived, Temperature sensitive dauer constitutive
akt-1(*ok525)*	*This gene encodes an ortholog of the serine/threonine kinase Akt/PKB, which is downstream of the IIS pathway.*	Long lived
sgk-1(*ok538*)	The *sgk-1* gene encodes a protein serine/threonine kinase and phosphatidylinositol-3,4,5-triphosphate binding activity. Participates in a number of processes, including peptidyl-serine phosphorylation, vitellogenesis, and protein localization to the basolateral plasma membrane.	Decreased body size
daf-16(*mgDf50*)	*C. elegans* homolog of forkhead box O (FOXO) transcription factor is encoded by the daf-16 gene. Innate immunity, fat metabolism, longevity, and dauer formation are all controlled by this transcription factor.	Dauer defective. Short lived
skn-1(*zu67*)	An orthologous transcription factor to the mammalian Nrf is encoded by *skn-1*	Heterozygotes are Uncand segregate Unc, WT which give only dead eggs, and dead eggs. Maintain by picking Unc.
hsf-1(*sy441*)	Heat-shock transcription factor ortholog *hsf-1* regulates the transcription of genes induced by stress	Long-lived, thermotolerant. Small brood size.
eat-2(*ad1116*)	A cation-selective acetylcholine-gated channel is encoded by *eat-2.* Involved in several processes, including the regulation of eating behavior, feeding behavior, and gene expression.	Eat. Slow pumping. Long lived.
mev-1(*kn1*)	A mitochondrial respiratory chain complex II subunit encoded by *mev-1* is essential for the oxidative phosphorylation of succinate dehydrogenase	Stress and oxygen sensitive. Short life span
isp-1 (*qm150*)	Rieske iron sulphur protein (ISP) encoded by *isp-1* belongs to the mitochondrial complex	Low oxygen consumption, resistance

Genotype	Gene description	Phenotype
N2	-	Wild type
	III.	to stress, slow development, and long lived
clk-1 (*e2519*)	Demethoxyubiquinone (DMQ) hydroxylase is encoded by *clk-1* and is necessary for ubiquinone biosynthesis	Low oxygen consumption, resistance to stress, slow development, and long lived
dop-1(*ok398*)	Activates dopamine neurotransmitter receptors, coupled via Gs, epinephrine binding activity, and norepinephrine binding activity. Plays an important role in a variety of processes, including the adenylate cyclase-activating dopamine receptor signaling pathway, habituation, and mechanosensory perception	Defective in tap habituation. Defective in basal slowing and response to exogenous dopamine
dop-3(*ok295*)	Activates the dopamine neurotransmitter receptors via the Gi/Go pathway. Adenylate cyclase, which inhibits dopamine receptor signaling, male mating behavior, and response to odors are among the processes in which it is involved.	Defective in basal slowing and response to exogenous dopamine
muIs84(*sod-3::GFP*)	Promotes protein homodimerization and superoxide dismutase activity. Additionally, it is capable of removing superoxide radicals from the body. It is located in the mitochondrial respirasome. A number of structures are expressed, including the head, the intestine, the pharynx, the tail and the vulva. There have been several diseases linked to human orthologs of this gene, including Alzheimer's disease, artery disease (multiple), autoimmune disease (multiple), and hematologic cancer (multiple). The enzyme is an ortholog of the human superoxide dismutase 2 (SOD2).	Green expression in head, tail and around vulva. Many animals roll weakly or not at all, but still express GFP. Grows at all temperature.

3.2.4b. Test compounds, toxicity, reproduction, and growth

DMSO was used to dissolve the extracts of *W. somnifera*, and the final concentration of DMSO was maintained at 0.1 percent throughout the experiments. 1.0 ml aliquots of test solution containing varying pharmacological

doses of *W. somnifera* extracts (IR, FR, IS, FS; 0, 10, 20, 40, 80, 160, 320, and 640 g/ml) and heat-killed *E. coli* OP50 were used to check the toxicity of age-sorted N2 worms (n = 20–25 per replicate). During sterile tissue culture plates at 20°C, test compounds were administered to L4-stage worms for 24 hrs (acute exposure) or from L1-stage to adult worms (chronic exposure). Under a stereo zoom microscope, the worms were observed for inactivity following the exposure. When nematodes fail to respond to gentle physical prodding with a metal loop then they were considered dead. Three independent experiments, each conducted in triplicate, were conducted to assess the toxicity of the substance. Each treated and untreated worm was counted for the number of eggs and offspring produced. A body length measurement was performed on approximately randomly selected worms (30 no's) day-5 worms per treatment using the OptikaIsView image analyzing system (Optika, Italy).

3.2.4c. Lifespan determination assay

The age synchronized worms (10-15 worms per 60 mm plate) were aseptically transferred to NGM plates spotted without *W. somnifera* extracts and with extract at different pharmacological doses. During their reproductive age, the worms were transferred to fresh NGM treatment plates every day, and then every 3 to 4 days. During the course of the experiment, live and dead worms were counted each day. In the absence of pharyngeal pumping and worm failure to respond to gentle prodding with a metal worm picker, the worms were scored dead. Censoring occurred if the worms displayed extruded inner organs, died due to internal hatching, and crawled off the plate. As soon as the last worm was marked as dead or censored, the experiment was terminated. Under similar conditions, three independent biological trials were conducted for each concentration (Pickett *et al.,* 2013).

3.2.4d. Oxidative stress resistance

L4 Stage Worms (hermaphrodites have a tapered tail, and the developing vulva (white arrowhead) can be seen in the center of the ventral side) were age-synchronized and placed on NGM plates with and without *W. somnifera* extracts for 72 hrs at 20°C. After that, the worms were exposed to oxidative stress. Control and treated worms (40-50 worms per experiment) were moved to fresh

NGM plates containing 240 M 5-hydroxy-1,4-naphthoquinone, an intracellular ROS (reactive oxygen species) producer, for the assay for oxidative stress resistance. After 3 hrs of continuous exposure, the vitality of the worms was measured. Nematodes that do not showed response to gentle probing with a metal worm picker were judged dead (Devagi *et al.,*2018).

3.2.4e. Reporter gene expression assay

Starting from the L4 stage, transgenic worms stably expressing sod-3::GFFFP, ctl-1,2,3::GFP, and gst-4::GFP were treated with *W. somnifera* extract, according to lifespan assay results. A 72 hrs continuous exposure of the worms was followed by three washes with M9 buffer and anesthetized with 25 mM sodium azide. Using a fluorescence microscope (B41, Olympus, Japan), the immobilized live worms (20-25 per experiment) were mounted onto 3% agar padded microscopic glass slides. Daf-16::GFP localization was classified into cytosolic, intermediate, and nuclear patterns. In order to evaluate the expression of Skn-1b/c::GFP, the following categories were used: none-no evidence of nuclear accumulation; low-only a few nuclei showed positive expression; medium-half of the nuclei showed positive expression; high-all nuclei showed positive expression. Using ImageJ software (Optika, Italy), determined the mean pixel intensity of GFP signals in worms bearing the sod-3::GFP, hsp-16.2p::GFP, and gst-4::GFP transgenes.

3.3.5. Study for α-synuclein protein aggregation and Neuroprotection

3.3.5a. Assay for α-synuclein protein aggregation

A transgenic *C. elegans* strain (NL5901) expressing a yellow fluorescent protein (YFP) with the human synuclein gene was used to evaluate α-synuclein protein aggregation. A number of age-synchronized worms (20-25 worms per experiment) were raised on NGM plates containing the appropriate concentration of *W. somnifera* extract from the L1 stage. Using ImageJ, worms on day 6 of adulthood were assessed for synuclein aggregation under a fluorescent microscope.

3.3.5c. Quantification of lipid deposits using Nile Red staining

NL5901 *C. elegans* was stained with Nile Red as described previously (Shanmugam *et al*., 2017). Before staining, 0.5 mg of Nile Red was dissolved in 1 ml of acetone to prepare a stock solution. In the experiments, treated and untreated worms (n=25 to 30 individuals per experiment) were washed and resuspended in M9 buffer, and then fixed in 40% isopropanol solution for 3 min. A Nile Red/isopropanol solution of 150 mL was prepared by dissolving 6 mL of Nile Red in 1 ml of 40% isopropanol. Worms were then incubated for 30 minutes at room temperature in the dark and examined under a fluorescent microscope.

3.3.5b. Dopaminergic neurodegeneration assay

The present study used BZ555 worms carrying GFP constructs tagged in all 8 dopaminergic neurons in order to examine the neuroprotective effect of *W. somnifera* extract against 6-hydroxydopamine (6-OHDA; 25 mM)-induced selective degeneration of dopaminergic (DAergic) neurons. The worms were treated with either 6-OHDA or 6-OHDA/*W. somnifera* extract, as described in our previous article (Mohankumar *et al*.,2018). At the final of described treatments, the worms were imaged, and the GFP signal in the head region was quantified using ImageJ.

3.3.5d. Assay for food-sensing behavior

As part of this study, the food sensing behavior mediated by DAergic neurons were measured according to the Bargmann method (Bargmann *et al.*, 1993). An assay plate was prepared in a Petri dish with a diameter of 9 cm by spreading *E. coli* OP50 overnight in a ring with an inner diameter of 1~ cm and an outer diameter of ~8 cm on NGM agar. Nematodes, treated with either 6-OHDA or 6-OHDA/EISO or 6-OHDA/Santalol (n = 30–40 per treatment), were washed with M9 buffer and released onto an NGM agar plate sprayed with or without *E. coli* OP50 lawn. For each nematode, the body bends were measured for 1 min in the presence or absence of food and the slowing response was calculated as follows:

$$\text{Slowing rate} = (N_{\text{without food}} - N_{\text{with food}})/N_{\text{without food}}$$

where, the number N represents the total number of body bends in the presence or absence of bacterial food sources.

3.2.6. Statistical analysis

We conducted a one-way analysis of variance (ANOVA) to analyze differences between the control and treated groups, followed by a Bonferroni's posthoc test in SPSS 17 (IBM Corporation, USA). An analysis of the data was conducted using Microsoft Excel 2010 (Microsoft Corporation, USA). The Kaplan-Meier survival curves were plotted using MedCalc 14 (Ostend, Belgium) and analyzed using the log-rank (Mantel-Cox) test. In all cases, p-values greater than 0.05 were considered statistically significant.