4

Results and Discussion

Biotechnological approaches combined with plant tissue culture enable the industrial production of pharmaceuticals and nutraceuticals from *in vitro* developed plants. In the Ayurvedic system of medicine, *W. somnifera is* highly revered as a medicinal plant. *In vitro* propagation of these species is imperative due to the ever-expanding use of plant species both in traditional and modern medicine. *In vitro* botanicals, however, need comprehensive standardization using contemporary medical standards and clinical studies to be considered an effective medicament. Therefore, extensive research is required to develop *in vitro* cultures of *W. somnifera* and to ensure its bioactivity and biosafety. A medicated plant's pharmacology is primarily determined by its secondary metabolites. The external stress factors that plants are exposed to cause them to produce these secondary metabolites. A result of environmental stress factors is that secondary metabolite production in traditionally cultivated plants can fluctuate between batches due to overproduction or underproduction. This can lead to variation in pharmaceutical product manufacturing from batch to batch. The present study addresses this issue by demonstrating the efficacy of *in vitro W. somnifera* as an alternate to field-grown material and the possibility that *in vitro* shoots are equally bioactive as their field grown counterparts. Moreover, plant tissue culture offers tremendous efficiancy for the production of medicinally significant plants on a large scale, regardless of seasonal or geographical variations. Because of its widespread usage in traditional medicine and high global demand, *W. somnifera* represents one of the potential species that would necessitate *in vitro* production. Although there have been numerous reports on its successful *in vitro* propagation, it is necessary to fine-tune and optimize the existing protocols to obtain a cost-effective mass production technique that can meet the needs of subsequent experiments as well as industry.

It is the chemical constituents of a medicinal botanical that determine its pharmacology. For this reason that the quantification of phytoconstituents is mandatory in order to achieve official standardization. This study is designed to

compare the major pharmacological actives and total metabolome of *W. somnifera* grown *in vitro* and in the field grown. As a result of their metabolic contents, particularly secondary metabolites, *Withania* preparations are alleged to be poorly reproducible due to variety, tissue type, and sometimes growth conditions (Dhar *et al.,* 2006; Sangwan *et al.,* 2004). The purpose of the present study was to analyze the differences in metabolites among tissues through comprehensive metabolite profiling. Phytochemicals play a key role in determining the quality of crude drugs made from herbs. Accordingly, *Withania* tissues can be quantified for their withanolides content by using HPTLC technique (Mahadevan *et al.,* 2003, Bala *et al.,* 2004, *Sharma et al.,* 2007, Nayak *et al.*, 2009). Several studies have demonstrated positive results with the use of *W. somnifera* in traditional medicine including the treatment of neurological diseases (Durg *et al.,* 2015, Kuboyama *et al.,* 2014) in addition to neurodegenerative disorders (Ven Murthy *et al.,* 2010, Singh *et al.,* 2008) without significant side effects (Cooley *et al.,* 2009). As of now, there have been no reports published regarding the bioactivity of *in vitro* cultivated *W. somnifera*. The multidimensional animal model system *C. elegans* was used to investigate and compare the efficacy and safety profiles of *in vitro* and field-generated *W. somnifera*. Furthermore, the study also examined *in vitro* and field grown *W. somnifera* tissues in terms of lifespan extension and neuroprotection, as well as its underlying mechanisms.

This chapter presents and discusses the results of the study under the following headings:

4.1. Pharmacological validation *W. somnifera* powders

4.1.1. Development of *in vitro* root and shoot culture of *W. somnifera*

4.1.2. Comparative analytical standardization of *W. somnifera* powders developed using *in vitro* and field grown tissues

4.2. Comparative analysis of chemical constituents and metal ion profiling of *in vitro and field developed* of *W. somnifera*

4.2.1. Qualitative & quantitative evaluation of phytoconstituents

4.2.2. DPPH radical scavenging activity of methanolic extracts of *W. somnifera*

4.2.3. Assessment of withanolide accumulation pattern using HPTLC

4.2.4. Comparison of metal ion profiling of *Withania* tissues using ICP-MS

4.3. Comparative metabolic profiling of the *in vitro* developed shoots of *W. somnifera* with field grown tissues

4.3.1 Gas chromatography- Mass spectroscopy analysis (GC-MS)

4.3.2 Principal component analysis (PCA)

4.4. Comparison of Neuroprotective activity and among *in vitro* and field grown tissues in *C. elegans*.

4.4.1. Safety evaluation of *W. somnifera* extracts on *C. elegans*

4.4.2. Determination of lifespan extension potential of *W. somnifera* in wild type *C. elegans*

4.4.3. Study the rudimentary genetic mechanism of *W. somnifera* mediated lifespan extension in mutated *C. elegans*

4.3.4. Evaluation of stress resistance capability of *Withania* extracts in oxidative stress condition.

4.3.4. Neuroprotective efficacy of *W. somnifera* extracts

4.1 Pharmacological validation *W. somnifera* powders

Herbal medicine has been subjected to extensive and profound pharmacological validation. Currently, *in vitro* propagation of medicinal plants such as *W. somnifera* is a necessity for both conservation and the supply of quality stocks to the pharmaceutical industry. This strategy, however, would only be acceptable if herbal products were assessed and examined by sophisticated modern standardization techniques. The Government of India implemented GMP (Good Manufacturing Practices) in the production process of Ayurvedic medicines to ensure high-quality products for consumers on the domestic and international markets. There is a detailed description of these practices in the section on Quality Assurance of Ayurvedic, Siddha, Unani, and Homoeopathic Drugs.

In order to make sure that drugs are made with real raw materials, of high quality, and free of contamination, (a) The pharmaceutical industries should follow GMP (b) The manufacturing process is done as instructed to keep the standards high (c) Strict and adequate quality control procedures should follow

(d) The raw material for drug preparation should pass all ISO (International Organization for Standardization) standards (e) It accomplishes the intended goal. An effective health management program requires precise quality control analysis for all herbal products. Physico-chemical analyses provide objective parameters for setting standards for raw pharmaceuticals, intermediate processes, and materials, as well as finished products. In addition, the analysis aids in interpreting the pharmacokinetic and pharmacodynamic data of the drug. In accordance with the standard protocol of (API) Ayurvedic Pharmacopia of India, four *W somnifera* powders including Field Root (FR), *In vitro* Root (IR), Field Shoot (FS) and *In vitro* Root (IR) have been validated. Withanolide A (WA), Withaferin A (WFA), and Withanone (WTN), were quantified using sophisticated instruments. Based on previous studies on the assessment of biomass and withanolide content and maximum GI (Growth Index) were found in 45 days of *in vitro* cultivated plants (IS, IR) (Senthil *et al.,* 2015).

4.1.1. Development of *in vitro* root and shoot culture of *W. somnifera*

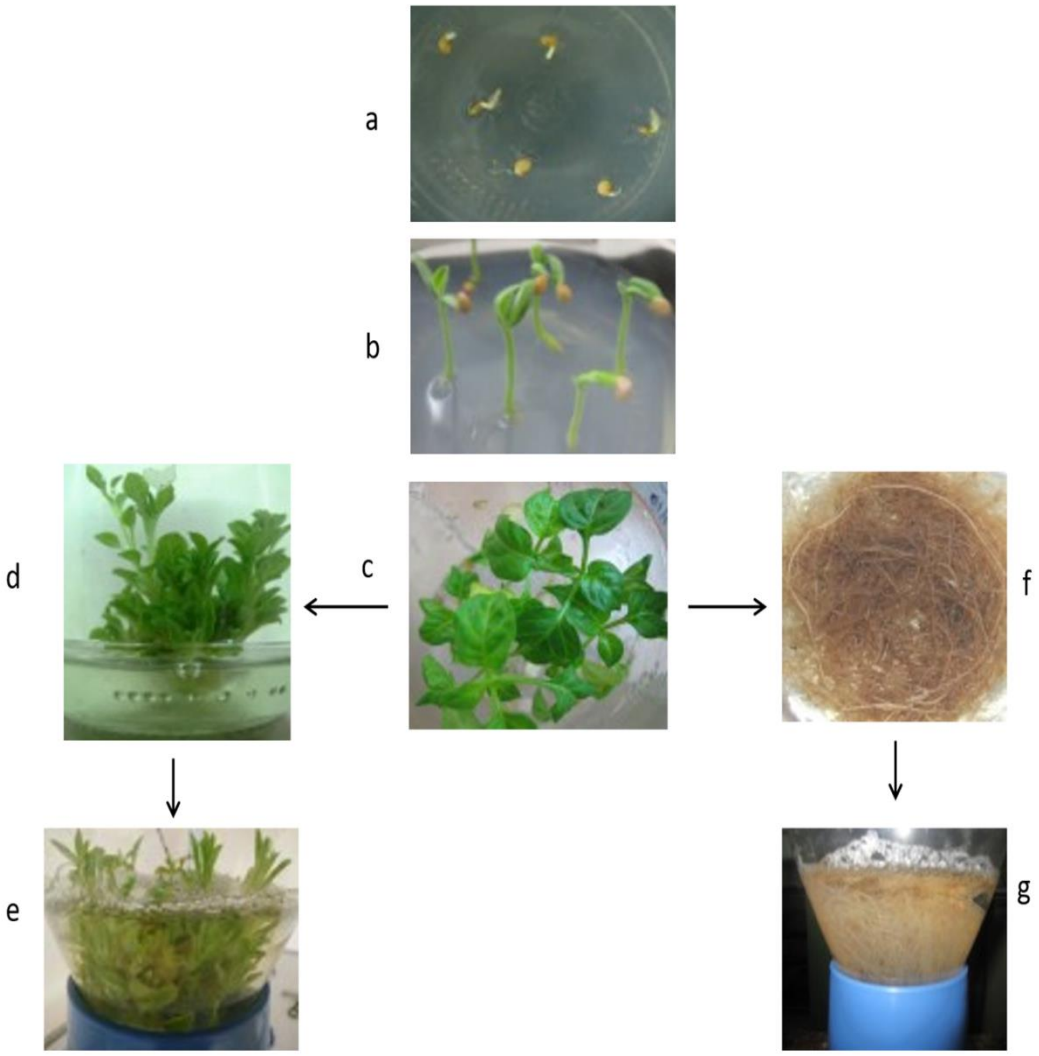
A great deal of success can be achieved through micropropagation when producing plant-based medicines. Aitken-Christie and Connett (1992) describe the use of juvenile explants from seedlings for micropropagation. Under controlled conditions, valuable and endangered medicinal plants can be produced *in vitro* under continuous and intensive efforts (Zibbu and Batra, 2010).

Axillary buds are most commonly stimulated for *in vitro* soot cultivation. In the present study two months old shoots grown in MS agar medium supplemented with BAP medium were used as explants. The plantlets were aseptically inoculated on full strength MS solid medium. So the proliferation of multiple heathy shoot buds begins after seven days in culture media supplemented with BAP (6-Benzylaminopurine). Our results supported the finding that BAP induced more proliferation of axillary shoots in *W. somnifera*. In many previous studies, solid medium was recommended for the multiplication of shoots in *W. somnifera* (Sen and Sharma, 1991, Kulkarni *et al.,* 2000, Furmanowa *et al.,* 2001). Nevertheless, in shoot culture, solid medium reduces withanolides production, biomass, and proliferation. Consequently, we followed liquid medium for shoot cultivation. The maximum number of proliferated shoots occurred in MS basal medium were transfred to liquid medium. The rapid

multiplication of shoots is a result of continuous shake culture conditions that provide adequate airation for tissues. The use of shake flask systems has been used in a number of studies to replicate shoots and produce secondary metabolites (Zobayed *et al.,* 2004, Karppinen *et al.,* 2006, Charchoglyan *et al.,* 2007, Liu *et al.,* 2007 and Kirakosyan *et al.,* 2008). 3% percent sucrose, 0.8% agar, and BAP, suspension medium was used to keep healthy bunches of shoots. Liquid cultivation accelerated the growth of healthy shoots in a shorter period of time. Aseptically transferred healthy shoot clusters from the liquid media were placed in a bubble column bioreactor with appropriate aeration, temperature, and light conditions. Approximately 45 days after starting the bioreactor, the shoots were harvested. Shoots were washed under running water after they were harvested.

It is also possible to grow adventitious roots of *W. somnifera* in suspension cultures. Due to their rapid growth, they can be examined for the presence of beneficial secondary metabolites. Flasks or bottles can be used to cultivate the plants. On MS medium supplemented with 30 g/L sucrose, 1 mg/L IBA (Indole-3-butyric acid), and 0.25 mg/L IAA (indole-3-acetic acid), direct adventitious roots of the *W. somnifera* cultivar 'Jawahar 20' were induced from leaf explants. In addition to regulating root development, auxin plays a vital role in adventitious root formation. It was discovered in 1941 by van der Lek that auxin is an effective root-inducing agent. Auxins such as IAA and IBA were first identified and used to stimulate the root growth of cuttings (Cooper, 1935). Eventually, another auxin, IBA, was discovered and was considered even more effective (Zimmerman and Wilcoxon, 1935). After being supplemented with auxins for 8-12 days, adventitious roots developed from the protuberances of the leaf explants. In an orbital shaker with constant agitation at 50 rpm and a 22±1°C photoperiod, root tips and branches were aseptically transplanted into aliquots of 50 ml full-strength liquid MS medium with IAA+IBA (Vinod *et al*., 2022). The root tips and branches of *in vitro* induced adventitious roots were excised and transferred to a bubble column bioreactor with MS suspension medium. This was done in accordance with the conditions for the formation of adventitious roots. The roots were harvested after 45 days of cultivation and were washed with running tap water.

Figure 4.1: The development of *in vitro* shoot and *in vitro*



root cultures of *W. somnifera*

*All experiments were performed in triplicates. a) *In vitro* seed germination, b) Development of shoots and leaf from the germinated seeds, c) *In vitro* shoot suspension culture (Initial stage), d). Multiple shoots in suspension (mature stage), e) Mass Cultivation of shoots in bioreactor f) Roots induction from a matured leaf, g) Mass Cultivation of roots in bioreactor.

4.1.2. Comparative analytical standardization of *W. somnifera* powders developed using *in vitro* and field grown tissues.

In order to be deemed safe and effective, a medicine must meet the same requirements regardless of whether it is synthetic or derived from plants (EMEA, 2005, WHO, 2002c, WHO, 1996a, WHO, 1988a, WHO, 1998, WHO, 1988b, WHO, 1992, WHO 1990). Herbal drugs are transformed into

phytopharmaceuticals through simple processes such as harvesting, drying, and storing (EMEA, 1998). Therefore, they are capable of variation. Variability arises from a variety of factors, including growth conditions, geography, and harvesting times.

Essentially, standardization is the process of defining attributes, constant parameters, and quantitative and qualitative values that guarantee the quality, safety, efficacy, and reproducibility of herbal medicines. As a result, technical standards are established and agreed upon. To prescribe a set of characteristics displayed by a particular herbal medicine, observation and experimentation are required. As such, standardization plays a crucial role in quality assurance and quality control.

An authority's primary responsibility is to ensure that consumers receive safe, effective, and pure medication. In pharmacopeia, formularies, and manufacturing operations, regulatory authorities adhere strictly to standards of quality prescribed by statutory standards of manufacturing practices. These procedures apply to all medications, whether they are included in modern or traditional medical systems. It has been described by the World Health Organization (WHO,1996a, WHO,1990) as the process of evaluating crude drugs from a physicochemical standpoint when standardization and quality control of herbals are concerned. The process involves selecting and handling crude materials, assessing the safety, effectiveness, and stability of finished products, documenting safety and risk based on experience, educating consumers about products, and promoting them. The current study compares *W. somnifera* tissues developed *in vitro* with traditional *Withania* formulations and suggests that it may be a viable alternative to field-grown plants for the production of pharmaceuticals. Even so, it is necessary to conduct a clinical trial

and to conduct scientific validation in order to justify this approach's acceptance. For plants developed *in vitro* for medicinal purposes to be validated through the use of contemporary scientific methods and evidence, systematic validation is required. The process of pharmacological standardization begins with harvesting the plants and concludes with the performance of extreme clinical trials. Therefore, the present study was carried out to standardize and compare the

qualitative and quantitative physicochemical parameters of powder formulations prepared from *W. somnifera* tissues grown *in vitro* and in the field.

4.1.2.a. Method of preparation of herbal powders using *W. somnifera* tissues

In the present study, powder formulations were prepared according to the standard procedure described in Ayurvedic Pharmacopeia of India (API). Each sample (FR, IR, FS, and IS) was cleaned, washed, and shade dried prior to quality analysis. The dried materials were ground using a mixer grinder and powdered individually. To achieve a homogeneous blend, all powders were separately passed through # 80 sieve.

A detailed assessment of major differences in formulations developed using IS, IR, FR and FS tissues was presented in this comparative study. The physicochemical and pharmacological characteristics of the powders have also been determined using standard guidelines, such as API, WHO, European Agency for Evaluation (EAE) of Medicinal Products, and United States Pharmacopeias (USP). Indigenous systems of medicine have used fine plant powders as drugs. An airtight container will preserve the potency of the powdered medication for one year. In this regard, the material should be cleaned, dried properly, pulverized, and sieved. Also, it is believed that it bears a great deal of resemblance to powder formulations used in the allopathic system of medicine. Due to the fact that it can easily be converted into tablets to fix the dose. Among the main characteristics of powdered medications is their small particle sizes. As the particle sizes become smaller, the rate of absorption and the bioavailability of the drug in the body will increase.

4.1.2.b. Examination of Foreign matter

For herbal drugs to be effective, they must be derived from only the stated part of a plant and must not contain any other parts of the same plant or any other material from different plants. In addition to visual contaminants such as sand and stones, toxic or harmful foreign matter, or residues of any chemicals, molds or insects should not be present. As well as insects, invisible microbial

contaminants can also synthasis toxic compounds when combined with herbal medicines (WHO, 2004, WHO 2003, WHO 2002a,c, EMEA, 2005). The presence of foreign matter can easily be detected using a macroscopic examination. The foreign matter was less than 0.5% (w/w) in FR, IR, FS, and IS, indicating their high quality and purity. According to API standards, herbal powders are permitted to contain 0.5–2% foreign matter. *In vitro* tissues will be free of biological and non-biological contaminants due to the sterile conditions throughout the cultivation period.

4.1.2.c. Organoleptic Inference

A comparative organoleptic evaluation of all formulations was presented in (**Table 4.1**) powder prepared from leaf samples appeared green, powder prepared from *in vitro* roots appeared brown, and powder prepared from field grown roots appeared off-white. As a scientific discipline, organoleptic evaluation is concerned with analyzing the characteristics of food or drugs as perceived by the senses of sight, smell, taste, and touch. Sensory evaluation provides the fastest and easiest method of ensuring the quality of plant-based medicines (Jarald, 2007, Anonymous, 2007). Any deviation from any of these parameters serves as a primary indicator of quality variation. A sensory investigation involves the observation of human senses, and the collection of information is organized according to scientific discipline, applying experimentation and statistical analysis to the senses. In quality assurance, sensory analysis is defined as a scientific discipline that evokes, measures, analyses, and interprets the sensory properties of foods and materials as they are perceived by the senses. This process assists in improving methods and processes for analyzing sensory data and conducting research. This method is widely used in the research, marketing, and development of food products in the food industry. Ancient people used their senses to evaluate quality in order to determine its quality.

Table 4.1: Organoleptic character evaluation - colour, odour, taste appearance, and texture of the formulations FR, IR, IS, and FS

Organoleptic parameters	FR	IR	FS	IS
Colour	Off White	Dark Brown	Dark Green	Green
Odour	Characteristic Odour	Characteristic Odour	Characteristic Odour	Characteristic Odour
Taste	Bitter and aromatic	Bitter and aromatic	Bitter and aromatic	Bitter and aromatic
Appearance and Texture	Powder	Powder	Fine Powder	Fine Powder

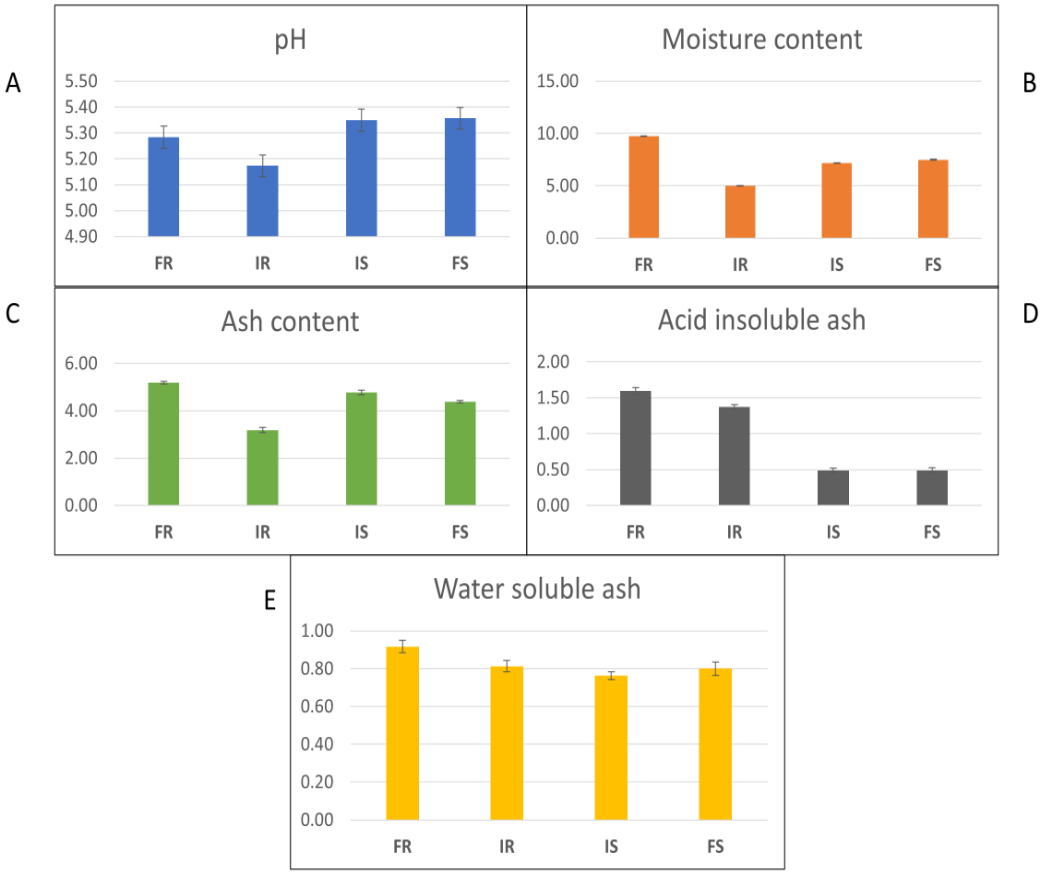
*****All experiments were performed in triplicates. The sensory characteristics of the four-variant herbal powder were evaluated through human senses. Samples- FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots

4.1.2.d. Physicochemical investigation

A standard protocol described by API was followed for determining physical characteristics. To ensure plant identity and purity, each monograph contains detailed descriptions of the botanical, macroscopic, and microscopic characteristics of each plant. The descriptions are accompanied by illustrations and photographs that provide visual documentation of accurately identified material. In accordance with API standards, all parameters are within the standard range. In the pH determination, acidity, and alkalinity of a formulation were assessed (Shivatare *et al.,* 2013). In order to avoid gastric irritation after the consumption of herbal medicine, it is necessary to determine the pH of the drug. In dried raw materials, *W. somnifera* powders showed slight acidic nature, possibly caused by acidic salts. Standardizing plant-based formulations is impossible without evaluating raw materials' moisture content, and it must be abolished as much as possible. In formulated products, phytochemicals will be fixed as a result of the drying process. Any hydrolytic and enzymatic activity will be examined to determine if there are any changes to the phytochemicals. Microbial contamination may also occur as a result of improper drying, which could lead to altered pharmacological activity. Both volatile matter and water are determined by the moisture percentage test. According to API, the moisture percentages of IR, IS, FR, and FS were within the permissible limit (≥12%) by

API. However, the FR contained a relatively high amount (9.75%) of moisture. It might be due to the higher thickness of field-grown roots and the soil moisture.In powdered formulations, the ash value indicates the amount of inorganic salts present. In FR, the ash content was significantly higher (6.19%). As a result, raw materials taken from the field are contaminated, substituted, and adulterated. Water-soluble and acid-insoluble ash values are also higher in FR (1.59%). Total ash value includes water-soluble ash, which is soluble in water. Siliceous materials, such as earth and sand, can cause acid-insoluble ash. A physicochemical analysis was carried out on each sample, with the results illustrated in **Fig:4.2** and the values shown in **Table:4.2.**

Figure 4.2: Comparative physicochemical analysis of the *W. somnifera* tissue powders



All experiments were performed in triplicates. A) pH, B) Moisture content, C) Ash content D) Acid insoluble ash, and E) water-soluble ash. The data presented in the figure are the mean ± standard deviation of three replicates obtained from three independent experiment. Samples-FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots.

Table 4.2 : Comparative physicochemical analysis of the formulations

Chemical properties	FR	IR	IS	FS	API Limit
Foreign matter	Nil	Nil	Nil	Nil	NMT 3%
pH	5.28±0.05	5.17±0.03	5.35±0.02	5.36±0.03	-
Moisture content	9.75 %±0.01	4.99%±0.03	7.16%±0.02	7.49%±0.04	NMT12.0%
Ash content	5.19%±0.05	3.19%±0.01	4.78%±0.09	4.38%±0.04	NMT 8.0%
Acid insoluble ash	1.59%±0.04	1.37%±0.03	0.48%±0.03	0.49%±0.03	NMT 3.0%
Water soluble ash	0.92%±0.03	0.81%±0.03	0.76%±0.02	0.80%±0.03	-

All experiments were performed in triplicates and the values are presented as mean ± SD (n=3). Limits mentioned as per values obtained from API; NA -Not available; NMT – Not more than; NLT – Not less than. Samples- FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots

4.1.2.e. Determination of Physical characteristics

It is essential to understand the flowability of plant-based powder formulations before mixing, packaging, and transporting them. Flowability was investigated through the evaluation of several parameters. It includes bulk density, tapped density, angle of repose, Carr's index or Carr's compressibility index, and Hausner's ratio. An untapped powder's bulk density is calculated by dividing its mass by its contribution to the interparticle void volume. Data regarding the consolidation of a powder is represented by its density. The arch strength of consolidated powder is significantly higher than that of less consolidated powder, so the powder may be more resistant to powder flow (Sharma *et al.,* 2013). Larger and denser particles tend to be free-flowing, while fine particles with a lower bulk density tend to be less. An angle of repose measurement is one of the most effective ways to measure the flowability of powder. As observed, the flowability of FR and IR was poor, with tapped densities of 0.548% and 0.542%, respectively, and bulk densities of 0.446% and 0.442%. In addition, Hausner's ratio (1.23 and 1.22) and Carr's index (18.61%, 18.75%) were high. In general, flowability is defined as an angle of

repose under 30°, while 30-45° indicates some cohesiveness, 45-55° indicates some cohesiveness of the powder, and values above 55° indicate very high cohesiveness and limited flowability (Geldart *et al.,* 2006). If the Hausner's ratio is less than 1.25, the powder is free flowing, while a value above 1.25 indicates a powder that is poorly flowable. The smaller the Carr's index, the better the flowability. According to these comparative results, FR and IR were showing some cohesiveness, while IS and FS were showing high flowability. As shown in (**Table 4.3**), the comparative evaluation of powder flow characteristics is represented along with a scale of flowability.

Table 4.3: Comparative evaluation of flow characteristics

Parameters	FR	IR	IS	FS
Bulk Density	0.446±0.01	0.442±0.02	0.441±0.02	0.448±0.01
Tapped Density	0.548±0.01	0.542±0.02	0.514±0.01	0.512±0.02
Angle of repose	36	38	30	30
Hausner's Ratio	1.23	1.22	1.16	1.14
Carr's Index	18.61%	18.75%	14.14%	12.5%

*All experiments were performed in triplicates and the values are presented as mean ± SD (n=3). Samples- FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots.

Table 4.4: Standard values of flow characteristics of powder formulations as per API

Flow Properties	Angle of Repose	Carr's Index (%)	Hausner Ratio
Excellent	25-30	<10	1.00 -1.11
Good	31-35	11-15	1.12 -1.18
Fair	36-40	16-20	1.19- 1.25
Possible	41-45	21-25	1.26- 1.34
Poor	45-46	26-31	1.35- 1.45
Very Poor	55-56	32-37	1.46- 1.59
Very very Poor	>66	>38	>1.6

4.1.2. Microbial screening of *W. somnifera* powders

Plant-based medications are being integrated into primary health care systems in developing countries; however, safety issues remain unresolved (WHO, 2002, WHO, 2005). The herbal medicines and other plant based heath supplements are frequently contaminated with bacteria and fungi, with levels of CFU/g exceeding both national (Agência Nacional de Vigilância Sanitária, 2010) and international standards (Umair et al., 2017). The most contaminated herbal medicines are those in liquid or semi-solid pharmaceutical forms for oral use, and they are also used by the elderly population. A lack of control over moisture levels during the transportation and storage of herbal medicines. In addition, an inability to control the temperature of liquid forms and finished botanical products may have led to the growth of microorganisms. A further factor that leads to contaminating the herbal based medicines are enteric pathogens of public health importance is that most of them are prepared in an open environment under unhygienic conditions (Zank *et al.,* 2017). The present study compares the presence of viable microorganisms in *in vitro* and field grown plant tissues of *W. somnifera*.

In the process of drug production, there is the possibility of biological contamination occurring at any point in the process. The contamination of medicinal plants can be caused by the environment in which they are grown, how they are dried and processed, how they are stored and transported, or the manufacturing processes for ready-made pharmaceutical products (Chan, 2003). It has been reported that various medicinal plants from India are contaminated with fungi or contain mycotoxin levels that exceed the WHO tolerance levels (Sahoo *et al.,* 2010). As Roy (2003) points out, the chemical composition of raw materials can be affected by fungal contamination, resulting in a decrease in herbal drug potency. Moreover, when contaminated materials are ingested, these fungi produce a variety of toxic effects (Dubey *et al.* 2008). Although limited research has been conducted on bacterial contamination of Ayurveda herbal medicines (Kalaiselvan *et al.,* 2010), this contamination may pose a health risk when it occurs due to the bacteria itself, the toxins it produces, and a reduction in the therapeutic potency of the drugs. All *Withania* preparations in the

present study had bacterial counts that were below WHO standards (WHO, 2007), indicating no health risk associated with their consumption.

There have been many studies that measured the number of bacteria in herbal materials and herbal medicines (Kosalec *et al.,* 2009, Danladi *et al.,* 2009, Khattak, 2009, Famewo *et al.,* 2016, Govender *et al.,* 2006, Kneifel *et al.,* 2002, Yesuf *et al.*, 2016), indicating that these products pose risks when consumed. The presence of *E. coli*, *Salmonella spp (*species*)*., *P. aeruginosa*, and *S. aureus* was not detected by microbiological analyses in both *in vitro* and field cultivated samples. This indicates that there was no fecal contamination and that proper hygiene conditions were maintained throughout the process of preparing and storing these herbal preparations. It can be concluded that the samples are safe to consume as *E. coli* was not detected in any of the four samples, indicating no fecal contamination (Esimone *et al.,* 2007). Further, this study did not detect the presence of *S. aureus*, which is known for its potential to cause staphylococcal gastroenteritis, scalded skin syndrome, and folliculitis, among other diseases. According to a study which also assessed the microbial quality of herbal formulations developed from field grown raw materials, 47.6% of samples were contaminated with *E. coli*, 33.3% with *Salmonella spp*., and 71.4% with *S. aureus* in a study that also found 37.4% contaminated with *E. coli*, 33.3% with *Salmonella spp*. (Esimone, 2007). The presence of pathogenic bacteria such as *E. coli*, *Shigella*, *S. aureus*, and *P. aeruginosa* was also reported in other studies (Esimone *et al.,* 2007, de Sousa Lima *et al.,* 2020). Contamination of herbal medicines may have resulted from unsafe collection, transportation, drying, preparation, storage, and dispensing processes

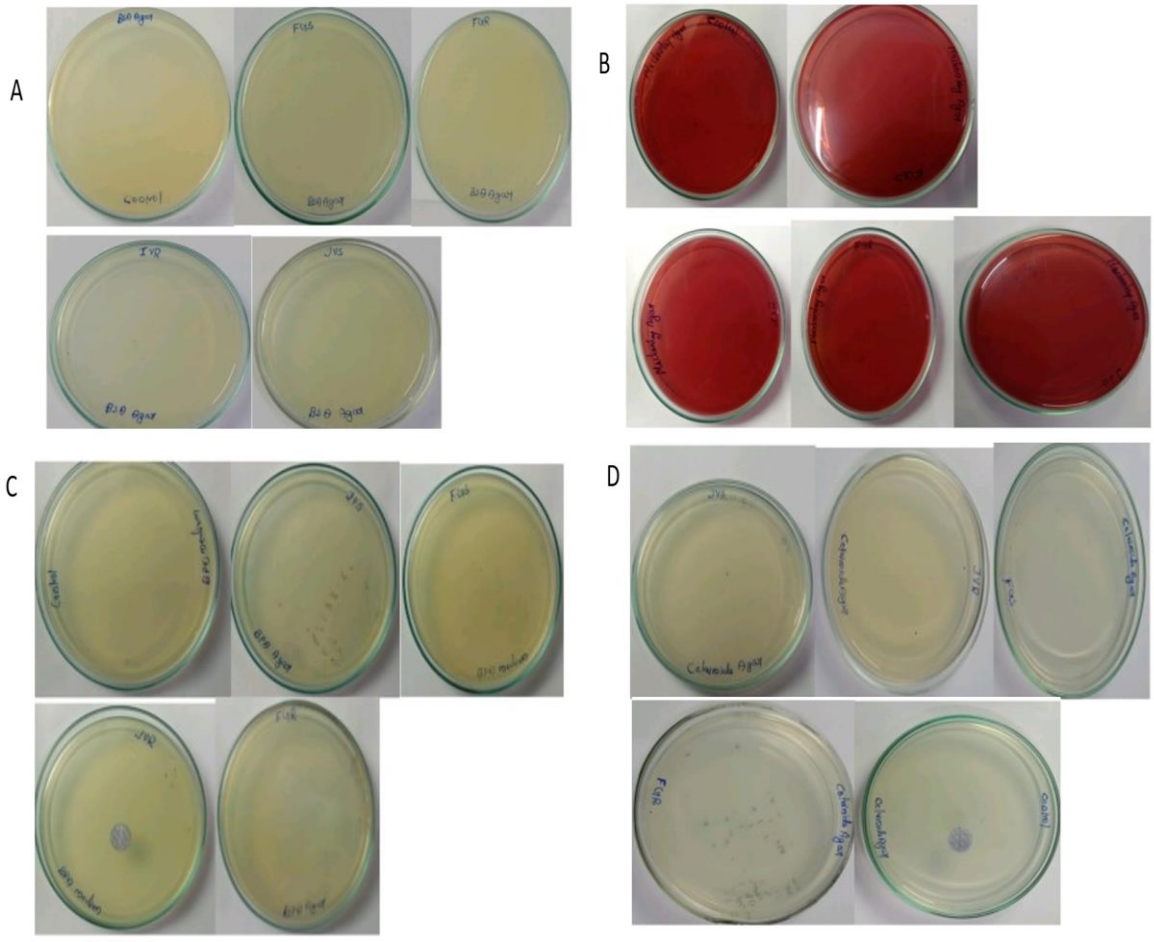
Herbal products that contain microbial contaminants can be reduced or inactivated, thereby adversely affecting patients who take these medicines (Rajput *et al., 2012).* Microbial load is completely absent from *in vitro* plants that are grown in controlled and sterile conditions. As well, four *W. somnifera* powders were found to have a microbial load within API limits for specified pathogens. According to this study, *in vitro* developed plants are microbiologically safe for human consumption. Data on microorganism quality control are presented in (**Tables 4.5 and 4.6**).

Table 4.5: Microbial screening for potentially Pathogenic Bacteria

Pathogenic Bacteria	FR	IR	FS	IS	Limit as per API
Escherichia coli	ND	ND	ND	ND	A
Salmonella ebony	ND	ND	ND	ND	A
Pseudomonas aeruginosa	ND	ND	ND	ND	A
Staphylococcus aureus	ND	ND	ND	ND	A

All experiments were performed in triplicates. Samples- IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root. ND-not detected, A- Absent. FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots, API (Ayurvedic pharmacopeia of India).

Plate 4.1: Determination of pathogenic Bacteria



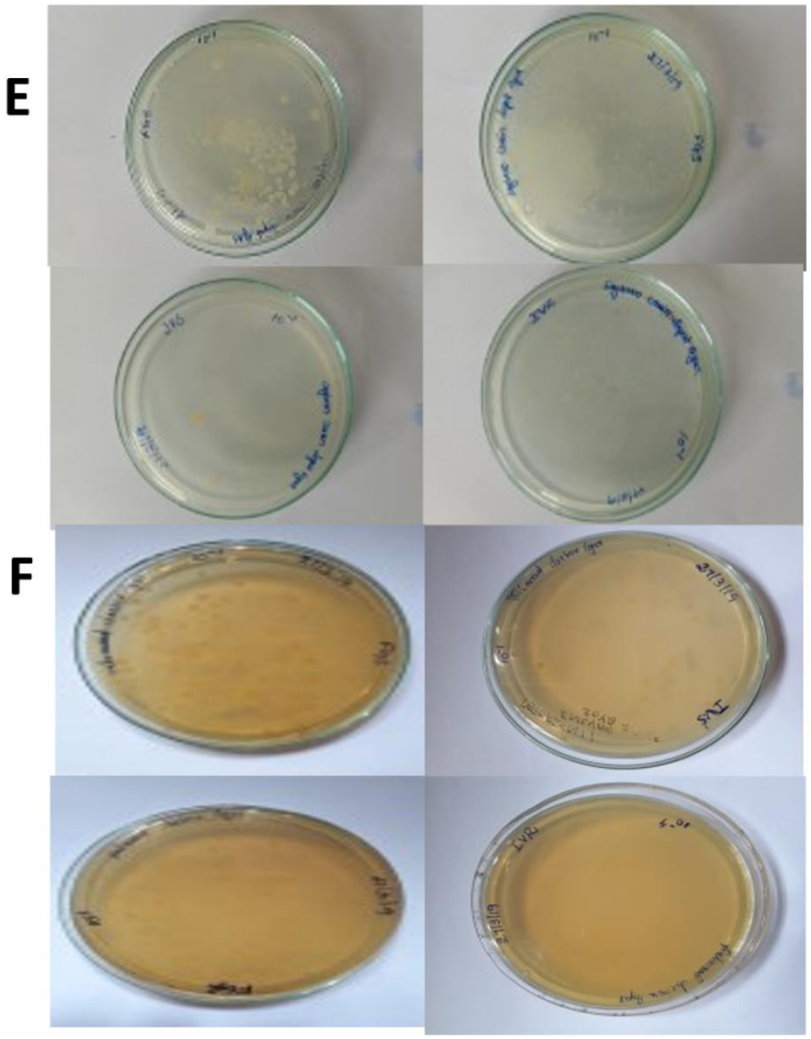
The plates of Microbiological limit test of four samples FR, IR, FS, and IS. (a) isolation of *Escherichia coli* in MacConkey agar, (b) *Staphylococcus aureus* in Mannitol salt agar (MSA) (c) Salmonella *spp*. in Cetrimide agar (CA) (d) Pseudomonas aeruginosa in Nutrient agar (NA).

Table 4.6: Microbial screening to identify total Fungal count and total Bacterial Count

Parameters	FR	IR	FS	IS	Limit as per API
Total microbial plate count	32 CFU/g	12 CFU/g	40 CFU/g	10 CFU/g	10^5/g
Total Fungal Count	20 CFU/g	8CFU/g	17 CFU/g	5 CFU/g	10^3/g

*All experiments were performed in triplicates. IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root. CFU - Colony forming unit/gram.FR- Field grown roots, IR-*In vitro* Root, FS- Field shoot, IS-*In vitro* shoots.

Plate 4.2: Determination of total Bacteria and total Fungus count



The plates of microbiological screening test of four samples FR, IR, FS, and IS. (e) isolation of bacterial species in NA (Nutrient agar) (f) Isolation of fungal species in PDA (Potato Dextrose agar).

4.2. Comparative analysis of chemical constituents and metal ion profiling of *in vitro and field* grown of *W. somnifera*

Plants are excellent sources of phytochemicals. There are a number of them that exhibit interesting biological and pharmacological properties. Environmental stress factors result in inconsistencies in the phytochemical composition of plants. Several factors can influence the chemical composition of crude drugs, including their geographical location, the period and time of harvest, the part of the plant used for drug preparation, and the method of separation. Analyzing pharmacologically active metabolites is a crucial step in the standardization of drugs or herbal raw materials. Accordingly, the present study attempted to compare the accumulation patterns of phytoconstituents and total metabolites of *in vitro* and field developed tissues of *W. somnifera*. In accordance with standard protocols, alkaloids, flavonoids, phytosterols, and alkaloids were screened and quantified.

A great deal of attention have been focuse on trace metals in herbal mediciens in recent years because of their adverse side effects on health of the human being and the environment. Humans are exposed to heavy metals through contaminated soils and plants. As well as accumulating in the food chain, metals also tend to accumulate in the body. Because heavy metals have a low absorption rate, even at very low concentrations, they can harm humans (Korfali *et al.,* 2013a, Korfali *et al.,* 2013b). A variety of minerals play a vital role in the physiological and biological functions of the human body, including zinc, copper, iron, manganese, and chromium. But the absorption beyond certain permissible limits can, however, result in toxic effects. Several health problems have been associated with excessive consumption of metals through diets. Immune dysfunction, heart problems, fetal malformations, impaired psychological and neurological function, and gastrointestinal cancer are just a few of the issues that may result (Mahan *et al.,* 2012, Singh *et al.,* 2011). Many Asian, South American, and African herbal remedies have been reported to contain heavy metal contamination (Rahimi *et al.,* 2012, Saper *et al.,* 2004, Harris *et al.,* Saeed *et al.,* 2011*,* Alwakeel *et al.,* 2008). As a result of environmental pollution, medicinal plants grown in the field are more likely to accumulate heavy metals. The present study aims to examine how metal ions

accumulate in plant tissue and how they differ in *in vitro* and field grown *W. somnifera* tissues.

4.2.1. Qualitative & quantitative evaluation of phytoconstituents

In the development of quality control profiles for herbal medicines, the quantitative and qualitative estimation of phytoconstituents is one among the most significant. The majority of medicinal botanicals contain high concentrations of phenols and flavonoids. As a result of these flavonoids and phenols, medicinal herbs are thought to be capable of exhibiting antioxidant properties (Alagar Yadav *et al.,* 2011). A comparison was made between the methanolic extracts of root and shoot of *in vitro* raised plants (IS, IR) and those obtained from field grown plants (FS, FR). A phenol, flavonoid, alkaloid, phytosterol, and flavonoid screening was conducted on the methanolic extracts. *Withania* extracts were analyzed using the Folin-Ciocalteu method to determine total phenolic content (TPC) which is expressed as gallic acid equivalents (GAE) per gram. A calibration curve was plotted to the total flavonoid content (TFC), total phytosterol content (TSC), and total alkaloid content (TAC) of the extracted samples, and the results are expressed as catechin equivalents (CE), cholesterol equivalents (CHOL) and caffeine equivalents (CF) per gram of extract respectively. As a result of the primary analyses of phytochemicals, carbohydrates, proteins, tannins, alkaloids, phenols, flavonoids, steroids, and saponins were found to be positive for all four *Withania* root and shoot samples. Saponins are found to be absent in *vitro* root samples (IR). The preliminary phytochemical screening was shown in (**Table 4.7**).

Table 4.7: Preliminary Phytochemical screening of *W. somnifera* tissues

phytoconstituent	FR	IR	IS	FS
Alkaloids	Y	Y	Y	Y
Flavonoids	Y	Y	Y	Y
Tannins	Y	Y	Y	Y
Steroids	Y	Y	Y	Y
Phenols	Y	Y	Y	Y
Carbohydrates	Y	Y	Y	Y
Saponins	Y	N	Y	Y

*All experiments were performed in triplicates Phytochemical screening of *in vitro* shoots and field tissues of *W. somnifera*. (Y) – Dectected,(N) - Not Dectected. IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

Table 4.8: Quantitative phytochemical analysis of *in vitro* shoots and field tissues of *W. somnifera*

	TPC (mg GAE/g)	TFC (mg CE/g)	TSC (mg CHOL/g)	TAC (mg CF/g)
FR	56.86 ± 7.27	21.97 ± 2.36	77.50 ± 3.54	47.58 ± 2.87
FS	92.23 ± 5.67	38.82 ± 4.31	78.750± 5.91	40.58± 2.87
IR	84.37± 6.61	28.71± 0.453	65.00±14.14	51.08± 2.07
IS	112.36±2.18	68.50± 0.453	73.75±15.90	47.58± 0.42

*Data is represented as Mean ± SD of triplicate values (n=3). GAE: Gallic acid equivalent, CE: Catechin equivalent, CHOL: Cholesterol equivalent, CF: Caffeine equivalent. Samples- IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

Phytosteroids, alkaloids, flavonoids, and phenols were present in all *in vitro* and field grown tissues. It was observed that the level of TPC and TFC were found to be higher in IS. In contrast, a higher accumulation of TAC was observed in the IR. In terms of TPC, the increased content of phenol in the FS is comparable to the comparative quantification of total phenolics in various parts of the *W. somnifera* by Fernado and colleagues. According to their findings, phenol accumulation occurs in the order of leaf> pods> flower> stem> root respectively (Fernando *et al.,* 2013). Plant-derived phenols serve as an antioxidant source and provide protection against disorders associated with oxidative stress (Soobrattee *et al.,* 2005). The TPC of the methanolic extracts of IS ranged 112.36± 2.18 GAE mg/g and was lowest in FR 56.86 ± 7.27 GAE mg/g. It is noteworthy that the range of phenols found in IS comparable to that is found in FS (92.23 ± 5.67 GAE mg/g). Similarly, the highest level of flavonoid was also exhibited by IS (68.50±0.453 mg CE/g) and found to be lowest in FR (21.97 ± 2.36 mg CE/g). The phytosterol and alkaloid contents of the *W. somnifera* tissues were not highly variable, despite the wide variation in phenol and flavonoid content. Interestingly, the phytosteroid content is almost the same in field grown samples FS (78.75 ± 5.91 mg CHOL/g) and FR (77.50 ± 3.54 mg CHOL/g). Moreover, the phytosteroid content of IS comparable to those of field samples and it is observed in the shoot 78.75±5.91 CHOL/g and in roots 77.50

± 3.54 CHOL/g. IR contain comparably less phytosteroid content and it ranges 65.00±14.14 mg CHOL/g. Interestingly, the accumulation pattern of the alkaloid is found similar in FR and IS and measured as 47.58 mg CF/g in IS and 47.58±0.42mg CF/g in FR. While IR contains higher alkaloid (51.08 mg CF/g) and FS has lower alkaloid content (40.58 mg CF/g). The pattern of accumulation of dominant phytochemicals in IS substantially higher than observed in field tissue extracts.

There is a greater importance to phenolic content than other phytoconstituents since it is related to primary antioxidant activity and free radical termination from body. A phenolic compound's redox properties make it capable of donating hydrogen to form hydrogen bonds, chelating metal ions, reducing oxidizing agents, and quenching singlet oxygen (Rice-Evans *et al.,* 1995). The TFC of *in vitro* and field samples were conducted using the procedure by Hithamani and Srinivasan's (2014). A comparative analysis of root and leaf extracts of *H. radicata* was carried out by Senguttuvan *et al.,* (2014). In methanolic extracts of roots and leaves, phenolic content was 3.75 mg GAE/100g and 5.04 mg GAE/100g, respectively. Another study found that *C. asiatica* shoot culture (372.5 mg/g GAE) exhibited higher TPC for GAE than callus culture (182.5 mg/g GAE) and cell suspension culture (70 mg/g GAE) (Roy *et al.,* 2018). *W. somnifera* root extract also contained 49.39 mg GAE/mg phenolic content, as reported by Pal *et al.,* (2015). The phenolic content of plants may enhance their antibacterial, anticancer, anti-inflammatory, antiviral, antiallergenic, and immunostimulant properties (Liu *et al.,* 2008). Vaggasiya *et al.* (2011) evaluated the TPC of selected Indian medicinal plant species. Acetone and methanol were used to extract the plant samples. Methanolic extracts showed the highest TPC (88.63%). Similarly, Chandra *et al.* (2014) found that W. *somnifera* and *C. intybus* have antioxidant properties. The methanolic extracts of both plants contained high concentrations of phenolic compounds. According to Siddique *et al.,* (2014), alcoholic and water extracts of *Nepeta bracteata* have TPCs of 326.28 mg GAE / g and 319.14 mg GAE/g, respectively. Additionally, Paul (2017) reported that indigenous root and imported root of *W. somnifera* contained 0.52 mg/ml and 0.39 mg/ml of TPC, respectively.

A type of polyphenolic phytochemical called flavonoids contributes to the flavour and color of different fruits. Six major subclasses of flavonoids can be distinguished: flavonoids (apigenin, luteolin), flavonoids (quercetin, myricetin), flavanones (naringenin, hesperidin), and catechins or flavanols (fruits and vegetables). There have been approximately 5000 and odd reports of various flavonoids (such as epicatechin, gallocatechin), anthocyanidins (such as cyanidin, pelargonidin), and iso flavonoids (such as genistein, daidzein). The majority of plant flavonoids are attached to sugars (glycosides), although they are rarely found as glycones (Ross and Kasum, 2002). Flavonoids exhibit a range of activities including antiallergic, anticancer, antithrombotic, anti-inflammatory, vasoprotective, tumour inhibitory, antiviral, and antioxidant properties (Pourmorad *et al.,* 2006). *In vitro* and *in vivo* extracts of *W. somnifera* roots, shoots, and leaves contain flavonoids (Pietta, 2000). A study by Chandra *et al.,* (2014) estimated the TFC in methanolic extracts of *W. somnifera*. In addition to their chemical and biological activities, flavonoids have been shown to have effective free radical scavenging properties. It was reported by Chaudhuri *et al.,* (2014) that *W. somnifera* methanolic extract contains high levels of flavonoids (136.97 mg/g CE). In another study higher TFC was reported in methanolic extract of *Thymus vulgaris* that grown locally (Hossain *et al.,* 2013). According to Roy *et al.,* (2018), different extracts of *C. asiatica* have different concentration of TFC. Approximately 275 mg CE/g of TFC was found in the shoot culture, followed by 145 mg QE/g in the callus culture, and 100.07 mg CE/g in the suspension culture. Based on different solvent systems, Senguttuvan *et al.,* (2014) estimated the TFC in the leaf and root. In contrast to chloroform root extract (14.31 mg RE/100 mg) and ethyl acetate root extract (14.28 mg RE/100g), ethyl acetate leaf extract (17.79 mg RE/100g) contained the most flavonoids.

Steroid compounds are a large class of natural products that have been extensively studied. The use of steroidal hormones is found in the treatment of a variety of diseases. They are particularly effective at combating inflammation and curing certain skin conditions like eczema and dermatitis. Plant-derived steroidal compounds have a wide range of therapeutic properties, including antitumor, immunosuppressive, hepatoprotective, and antimicrobial activity. As

plant growth stimulators, some steroidal compounds are used in agrochemicals. In the field of agriculture, some steroidal compounds stimulate plant growth. In addition to alkaloids the steroids also contain nitrogen atoms with a molecular weight from 100 to 900 Daltons. About one-fifth of all plants contain alkaloids and used to protect themselves against herbivores and pathogens with alkaloids. The cytotoxicity of alkaloids is one of their common biological properties. As a result, they have been used in different systems of medicine for centuries (Nobori *et al.,* 1994).

According to Gulati *et al.,* (2017), different genotypes of *W. somnifera* root contain different quantity of alkaloid. Debnath *et al.,* (2015) quantitatively analyzed the TPC of HWS-08-18 genotype of *W. somnifera* root. It was found that the range of TPC varied from 1.15 to 1.34 grams (%). Quantitative phytochemical analysis of *Naravalia zylanica* aerial parts revealed a higher TAC (0.86%) TPC (0.72%) or TFC (0.56%) (Sutharsingh *et al.,* 2011). An alkaloid content of leaf and root powders of *H. radicata* was reported by Senguttuvan *et al.,* (2014). The TAC was higher in leaf powder (4560.21 mg/100g) than in root powder (3800.83 /100g).

4.2.2. DPPH radical scavenging activity of methanolic extracts of *W. somnifera*

Phytoconstituents of natural drugs are being quantified to evaluate their antioxidant activity. A number of plant species contain antioxidant compounds (Pourmorad *et al.,* 2006), which have been linked to the presence of phenolic compounds (Pietta, 2000). These plants are used primarily as feedstock for the production of plant-based antioxidants (Exarchou *et al.,* 2002). As unstable compounds, free radicals steal electrons from other compounds to become stable. Randomly, they can damage other compounds or cells. Free radical reactions can cause cancer, hypertension, cardiac infarctions, atherosclerosis, rheumatism, and cataracts (Ostrowska and Rzemy, 1998). To combat various diseases and health issues, free radical scavenging may be the most effective solution. By preventing oxidative reactions, plant phenolics, and flavonoids act as powerful antioxidants. Most medicinal plants contain high levels of phenols and flavonoids. The antioxidant properties of medicinal herbs are attributed to flavonoids and phenols (Alagar Yadav *et al.,* 2011). Extracts prepared using

methanol IR, IS, FR and FS were subjected to quantification of Free Radical Scavenging Activity (FRSA) using 2, 2-diphenyl-1-picryl- hydrazyl-hydrate (DPPH), a stable free radical.

A dose-dependent escalation in DPPH activity was observed in methanolic extracts. IC50 values were used to evaluate the scavenging potential of the methanolic extracts (**Table 4.9**). An IC50 value indicates how much plant extract is need to scavenge 50% of free radicals. As a result, a higher IC50 value indicates that more plant extract is needed to bring the activity, indicating a lower antioxidant capacity.

Table 4.9: Free Radical Scavenging Activity (FRSA) of *W. somnifera* tissues

Treatment groups	DPPH IC_{50} (µg/ml)
IR	3571.4±5.56
FR	506.77±2.97
IS	872.15±8.73
FS	3002.50±6.13

Each value is indicated as the mean ± SD of triplicate values(n=3). Samples- IS-*In vitro* shoot, FR-Field root and FS- Field shoot, IR-*In vitro* root.

The FRSA of the FS was found to be 3002.50±6.13 µg/ml and FR showed 506.77±2.97 IC50 values. Similarly, the IS showed a significantly higher IC50 value 872.15 ± 8.73 than the FR. The FR observed the least IC50 value compared to other *Withania* tissues. Thus, a decrease in IC50 value was observed on FR indicating an increase in the antioxidant potential of the FR. Also, an increase in the FRSA of FS indicates a decrease in antioxidant potential. In comparison to IS, the antioxidant potential of FR was comparable and showed only a slight difference. In contrast, the field shoots FS and IR extracts showed the highest IC50 values, indicating that they have the lowest antioxidant potential as compared to the FR and IS extracts.

An extract of the leaf of *W. somnifera* was reported to have potent antioxidant properties by Sumathi *et al.,* (2007). *W. somnifera* imported root and indigenous root methanolic extracts were found to have IC50 values of 18 µL/mL and 12 µL/mL, respectively, by Paul *et al.* (2017). Based on the results of Alam *et al.* (2017), we conclude that the phenolic and flavonoid content of plant

extracts determines their antioxidant activity. In *in vitro* developed *C. intybus* showed higher radical scavenging activity (70.4%), whereas *in vitro* grown calluses of the same plant exhibited the lowest radical scavenging activity (30.5%). A study conducted by Roy *et al*., (2018) examined the antioxidant activity of callus culture, shoot culture, and cell suspension culture of *C. asiatica*. The IC50 value of the callus culture was found to be higher than that of the shoot (19 g/mL) and cell suspension (21.25 g/mL) cultures. Additionally, the present study supported to the finding of Sharma *et al.,* (2012), who evaluated the antioxidant activity of leaf extracts of *W. somnifera*. The antioxidant potential of ethanol, methanol, and hexane extracts from six green leafy vegetables was examined by Sahu *et al.* (2013). All six plants showed high DPPH scavenging activity in methanolic extracts. Using aqueous, alcoholic, and methanolic extracts of *W. somnifera* and *C. intybus*, Chandra *et al.,* (2014) demonstrated that methanolic extracts were the most effective at scavenging free radicals.

In vitro grown *W somnifera* leaves showed higher TPC and TFC than field plants (Dewir *et al.,* 2007). A methanolic extract IS exhibited the highest radical scavenging activity, and these same extracts also contained the highest amounts of flavonoids and phenolics. Clearly, there is a strong correlation between phenolic content and FRSA. The TPC is also strongly correlated with antioxidant capacity (Olajuyigbe and Afolayan, 2011). A positive correlation has been found between the total phenolic and flavonoid content and antioxidant activity of 21 selected medicinal plants by Mustafa *et al.,* (2010). Aside from their chelating properties, flavonoids also possess antioxidant properties. According to Sharififar *et al.,* (2009), this activity depends on the hydroxyl group structure and substitution pattern. Phenylpropanoid metabolic pathways are known to be responsible for flavonoid biosynthesis. These findings indicate that the phenylpropanoid-flavonoid metabolic pathway is activated in *W. somnifera* samples, which suggests that flavonoids contribute significantly to antioxidant activity.

In this regard, it is important to note, despite the high correlation between phytochemical content and antioxidant activity of methanolic extracts, FS extracts with relatively lower phytochemical contents were shown to have the least radical scavenging activity as compared to IS. Also, the IS and IR extracts

with the highest phytochemical content exhibited effective radical scavenging activity. Based on Pearson's correlation test in MS Excel 2007 (**Table 4.10**), the quantity of phytochemical and antioxidant activity were correlated. In the correlation analysis, the antioxidant potential of the plant extracts was highly correlated with the total phenol content and the total flavonoid content, with R = 0.80 (a negative correlation with the IC50 indicates a positive correlation with antioxidant potential). In *W. somnifera* extract, a similar correlation was observed between phenol content and antioxidant activity, suggesting a role for total phenol content in determining antioxidant activity (Kumar *et al.,* 2018). Among the four phytochemicals, phenol and flavonoid showed correlation coefficients of 0.166 and -0.257 with DPPH activity (The least IC50 values indicate that lower concentrations of the extract are required to exhibit 50% activity).

Table 4.10: Pearson's correlation coefficients of antioxidant activity (DPPH) with the phytochemical content of *W. somnifera* extract

	Phenol	***flavanoid***	***phytosterol***	***alkaloid***	***DPPH***
Phenol	1				
Flavonoid	0.910114	1			
Phytosterol	-0.14935	0.066061	1		
Alkaloid	-0.15732	-0.12207	-0.8001	1	
DPPH	0.166516	-0.25702	-0.50484	-0.07722	1

The table shows the correlation of phytochemical content to that of the antioxidant potential of the plant.

Accordingly, the antioxidant potential of the plant extract is highly correlated with its phenol and flavonoid content. According to a similar study, phenol content is correlated with antioxidant potential, supporting the hypothesis that phenols contribute significantly to the antioxidant properties of plants (Piluzza *et al.,* 2011). Additionally, the TPC and TFC were highly correlated with a correlation coefficient of 0.910. The total alkaloid and phytosterol, which showed a high correlation (r=0.540), had the least correlation with antioxidant activity, with correlation coefficients of -0.077 and -0.504, respectively. The antioxidant potential of the methanolic extracts was significantly correlated with their phytochemical content, however, it is noteworthy that the FS extracts with lower phenols and flavonoids, than the IS extracts showed less radical

scavenging activity. Furthermore, the IR extracts with high flavonoid and phenol content displayed good radical scavenging properties.

4.2.3. Quantitative analysis of withanolides in *in vitro* and field tissues

The secondary metabolism of plants plays a crucial role in enhancing self-protection abilities and capturing environmental data. As far as roots are concerned, the production of specialized metabolites can be achieved by either induced growth of transformed roots (hairy roots) by *Agrobacterium rhizogenes* or by promoting the growth of adventitious roots by interplaying with auxins in culture conditions (Martin *et al.,* 2008).

Extractions are the first step in the analysis of medicinal plants, since it is necessary to separate and characterize the desired chemical components. Since methanol has been reported to provide a high yield of extracts when used as an extracting solvent (Mohammed and Atik, 2011), so that, in the present study we have chosen methanol as extraction solvent. Further, methanol was found to be the most commonly used solvent for extracting therapeutically desired active constituents, followed by ethanol, chloroform, and ethyl acetate (Gupta *et al.,* 2012). It is important to consider the nature of the bioactive compound when selecting a solvent system. To extract the bioactive compound from natural products, different solvent systems are available.

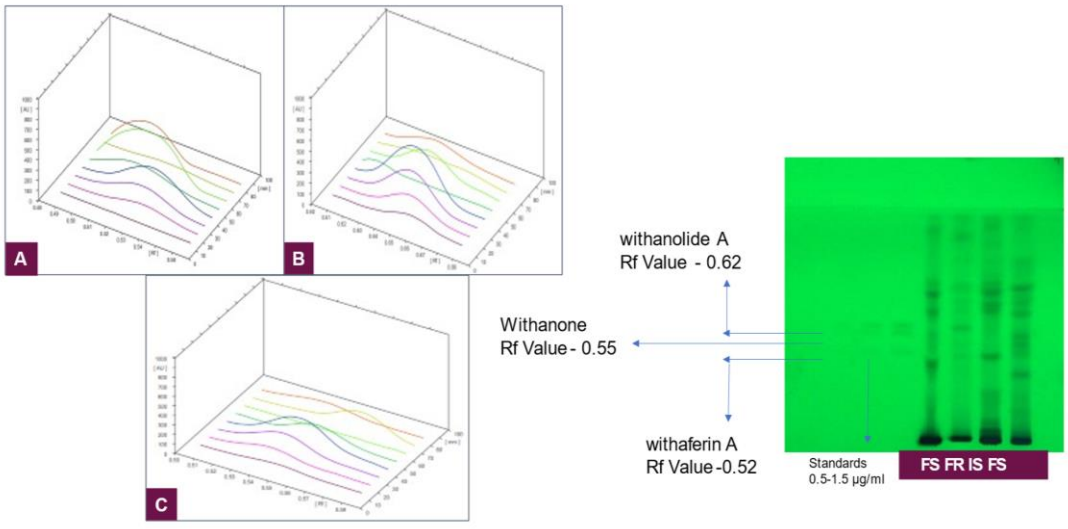
In this method, the mobile phase provided good separation and resolution between the peaks of two withanolides standards, Withanolide a (WA) and Withaferin A (WFA). Accordingly, Preethi *et al.,* (2014) concluded that toluene: ethyl acetate: formic acid was the most appropriate solvent system out of the four. A similar mobile phase had been used by Sharma *et al.,* (2007) for estimating the amounts of WA and WFA in various parts of *W. somnifera*. Most studies on *Withania* plant have been conducted using the same mobile phase.

A methanolic extract of *in vitro* and field grown tissues was used to quantify WA, WFA and Withanone (WTN) which are the major specialised metabolites of *W. somnifera*. To quantify these major withanolides the standard solutions of these compounds were prepared (0.1 mg/mL). Using multi-wavelength scanning, the absorption spectra of the three standards were determined (λmax). A standard wavelength of 234 nm, 223 nm, and 231 nm

(maximum of standards) was used to detect withanolide WA, WFA, and WTN. The detection was performed using, a unique fingerprint profile developed for withanolide standards and crude samples. A qualitative assessment of the plates was provided by analyzing the Rf values of the separated compounds. According to Reich and Schibli (2008), the strength of the solvent used directly influences the position of the sample after separation (Rf value).

In analyzing the HPTLC profiles, it was possible to identify withanolides with characteristic bands, their positions, and their intensities. Following derivatization with 10% sulphuric acid, a characteristic fluorescence emitted Yellow for WA and WFA, and orange WTN was observed under UV light at 366 nm (Nano meter) , which could be used to identify these withanolides among other separated compounds in a mixture. By standardizing the mobile phase, it was possible to successfully separate and quantify the withanolides from crude extracts with accurate Rf values. Multi-sample HPTLC analysis using this standardized method can be used for high throughput analysis and quantification of marker compounds.

PLATE 4.3. comparison of Withanolides accumulation pattern using HPTLC



a) b)

a) Scanning of the sample tracks showing standard peaks A. Withanolide A, B. Withaferin A and C. Withanone. b) HPTLC plate viewed at 254nm showing standards 1. Withanolide A (Rf 0.62), 2. Withaferin A (Rf: 0.52) and 3. Withanone (Rf: 0.55) . along with samples IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

Table:4.11- Comparative analysis of major chemical constituents – Withaferin A, Withanolide A, and Withanone

Samples	WFA (mg/g DW)	W A (mg/g DW)	WTN (mg/g DW)
FR	1.99 ±0.4	2.83±0.4	1.29±0.7
IR	0.48± 0.4	1.67±0.4	1.26±0.2
IS	2.76±0.8	1.36±0.4	2.58±0.4
FS	5.74±0.9	0.19±0.4	3.02±0.8

*Data is represented as Mean ± SD of triplicate values (n=3). WFA-Withaferine A, WA-Withanolide A, WTN- Withanone, IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

HPTLC was carried out to measure the WA, WFA and WTN. The 45-day-old *in vitro* plant had the highest accumulation of main components including WA and WFA, as previously reported (Senthil *et al.,* 2015). So, the comparative study, was carried out using 45 days in IS & IR with FR and FS. IR preferentially accumulate WA, and WFA accretion was observed predominantly in shoot tissues. In accordance with the type of tissue and the cultivation period, the assembly pattern of these two medically relevant phytochemicals varies (Senthil *et al.,* 2015). The findings back up a prior study that found WFA buildup to be tightly related to leaf tissue rather than roots (Praveen *et al.,* 2010). FS tissues contain higher concentration of WFA (5.74±0.9 mg/g DW) than IS (2.76±0.8 mg/g DW). The WA concentrations of FR (2.83±0.4 mg/g DW) and IR (1.069.72 mg/g DW). WFA was found in comparatively less concentrations in FR (1.26±0.2 mg/g DW) but negligible in IR (0.48± 0.4). In FS, the WA level was 0.19±0.4 mg/g DW, while in IS, it was found 1.36±0.4 mg/g DW. The tissue specific accumulation pattern of WFA (b) and WA obtained from the HPTLC experiment were shown in. The WTN content is higher in FS (5.74±0.9 mg/g DW) and lowest in IR (1.26±0.2) and comparable to those of FR (1.29±0.7 mg/g DW). As per this study the accumulation pattern of WTN of FS and IS were comparable. The IS contain (2.58±0.4 mg/g DW) (**Table:4.11**). So, this result emphasized that the WTN concentration is also linked in shoot tissues more than root of *W. somnifera* in order to determine the slope, intercept, and correlation coefficient for the calibration over the concentration range, we used least square linear regression analysis **(Plate.4.1).**

4.2.3. Comparison of metal ion profiling of *Withania* tissues using ICPMS

The medicinal and nutritional properties of plants are partly attributed to their chemical constituents, including metal ions. A variety of therapeutic actives are formed in medicinal botanical by the presence of metals. Trace elements play a crucial role in the biosynthesis and metabolism of plants as cofactors for enzymes. The plant cells are also metabolically dependent on these trace elements (Rajurkar and Damame, 1997). The accumulation of metals in organisms through plants or food chain is the most serious environmental concerns today, not just because many of these metals are phytotoxic to the crops themselves, but also because they may have harmful effects on animals. It is therefore imperative that heavy metals be monitored in crops, medical botanicals and other foods to protect the public from harmful impacts.

Some metals are also essential nutrients (B, Mg, P, K, Ca, Fe, Mn, Zn, Cu, and Mo) but toxic at high concentrations, while others (Hg, Co, Cr, Ni, Ar, Cd, and Pb) have no biological significance for plants or humans (Radojevic, 1999). Nevertheless, all elements may be harmful to organisms in high concentrations. There is a great deal of knowledge regarding the nutritional roles of elements, the essentiality of trace elements, as well as the biochemical and pathological effects of these elements. Generally, metals may interact directly with Deoxyribonucleic acid (DNA). There is a high affinity for metals with Nucleic acids, due to their large number of nucleophilic centers. Crosslinks between DNA strands may be the most serious interaction between metals. The DNA was observed after exposure to Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} by Eichhorn, 1979. As well as reacting with DNA bases, ions such as Ni2+ and Cd2+ can also react with phosphate groups. A variety of DNA lesions have been reported as a result of nickel exposure. Damage to DNA strands and cross-links between DNA proteins are both included in these types of damage. As a result of either *in vitro* or *in vivo* exposure to nickel, DNA synthesis has been inhibited (Frisk *et al.,*1997).

Herbal medicines can be contaminated with metal, which is one of the biggest threats to human health. Numerous reports have suggested that metal accumulation patterns in medicinal plants depend on soil properties and climatic

conditions where they are grown (Anjorin *et al.,*2010). In contrast, metal contamination in medicinal plants causes toxicity in the human body. To this effect, the present study analyzed the difference in essential and potentially toxic metal ions of *in vitro* grown tissues and tissues of field germinated *W. somnifera*. The essential metal ions like B, Mg, P, K, Ca, Fe, Mn, Zn, Cu, Mo and the potentaily toxic metals like Hg, Co, Cr, Ni, Ar, Cd, and Pb were analysed using ICP-MS.

Table:4.12: Comparison of essential metal ion profiling of *Withania* tissues using ICP-MS

Elements	FR (mg/Kg)	IR (mg/Kg)	IS (mg/Kg)	FS (mg/Kg)	Permissible limit as per WHO (mg/Kg)
11B	5.97± 0.055	10.90±0.084	21.97±0.051	33.86±0.476	-
24Mg	1424.08± 0.577	506.35±1.154	1348.32±0.577	8085.97±3.214	2000
31P	489.9210±1.527	2180.57±0.577	3879.80±0.595	2002.90±1.154	-
39K	1515.97±1.154	13009.2903±1.754	22109.40±0.577	28276.92±0.593	-
44Ca	3381.47±1.154	1617.10±0.594	1411.68±1.732	8689.19±0.577	-
55Mn	9.86±1.002	48.98±0.666	57.95±0.583	46.57±1.163	200
57Fe	13.55±0.519	5.99±0.140	12.00±0.092	9.900±0.173	20
63Cu	3.89±0.465	1.54±0.031	2.04±1.729	8.290±0.587	1 to 12
66Zn	6.60±0.230	16.95±0.057	32.00±0.645	15. 75±0.937	12 to 60
95Mo	0.308±0.584	4.92±0.539	2.81±0.484	0.810±0.031	200

*Data is represented as Mean ± SD of triplicate values (n=3)., 11B - Boron,24Mg – Magnesium 31P- Phosphorus, 39K -Potassium, 44Ca-Calcium, 55Mn -Manganese, 57Fe-Iron, 63Cu- Copper, 66Zn -Zinc, 95Mo- Molybdenum. Samples - IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

Table:4.13: Comparison of potentially toxic metal ion profiling of *Withania* tissues using ICP-MS

Parameters	FR mg/Kg	IR mg/Kg	IS mg/Kg	FS mg/Kg	Permissible limit as per WHO
52Cr	0.5082±0.052	0.0094±0.006	0.0076±0.005	0.2079±0.001	0.002 to 0.2
59Co	0.2009±0.209	0.1376±0.068	0.1502±0.074	0.0065±0.108	-
60Ni	0.8972±0.004	0.0653±0.016	0.0032±0.001	0.9866±0.001	0.1 to 5
75As	0.0418±0.001	0.0016±0.020	0.0045±0.002	0.0962±0.101	1.0 to 10.0
111Cd	0.3203±0.211	0.0076±0.006	0.0055±0.012	0.31±0.102	0.002 to 0.5
202Hg	0.3203±0.721	0.0036±0.065	0.0003±0.017	0.5023±0.021	0.001- 0.04
208Pb	0.1115±0.231	0.0049±0.021	0.0079±0.042	0.1252±0.068	0.3 to 10

*Data is represented as Mean ± SD of triplicate values (n=3). 52Cr- Chromium 59Co- Cobalt, 60Ni-Nickel, 75As- Arsenic, 111Cd- Cadmium, 202Hg- Mercury, 208Pb – Lead. Samples - IS-*In vitro* shoot, FR- Field root and FS- Field shoot, IR-*In vitro* root.

Boron (B) plays an important role in the growth and development of plants and animals. Numerous studies have demonstrated that this element is involved in several biological processes. Additionally, it is used for the treatment of diseases conditions such as cancer and some neurological disorders. The excess concentration of boron, however, results in toxicity in cells (Haseeb *et al.,* 2018). One of the unique characteristics of B is that it does not uptake as ions. Boric acid is added to plant tissue culture media as a source of B. Through their roots, plants acquire boron in the form of boric acid, which accumulates predominantly in their leaf tips (Raven, 1980, Nable, 1988). That may explain the relatively higher concentration of B in both leaf powder FS and IS than FR and IR. Among the leaf tissue the FS accumulated with highest concentration of B (33.87 mg/Kg) and IS (21.97mg/Kg). The FR contain the least concentration of B(5.97 mg/Kg) and IR contain (10.90 mg/Kg).

The Magnesium (Mg) and Potassium (K) elements are specifically important to the plant and are involved in intracellular physiological functions (Aparna and Rajitha, 2021), and about 75% of Mg from leaf tissue is involved in

protein synthesis, while the remaining 15–20% is associated with chlorophyll pigments (White and Broadley, 2009). Another major peculiarity of leaf Mg is that it is bound to a central atom in the tetrapyrrole ring of both chlorophyll molecules (Wilkinson,1990). Both K and Mg have prominent roles tightly related to photosynthesis and photosynthetic translocation. This might be the reason for leaf powder being accumulated with a high concentration of Mg. The FS and IS contain 8085.97 mg/Kg and 1348.32 mg/Kg respectively. While the permissible limit of Mg as per WHO is 2000 mg/kg. However, excessive intake of Mg from food supplements or medicines often results in diarrhea that can be accompanied by nausea and abdominal cramping (Ranade and Somberg, 2001). The Mg accumulation was observed least in IR (506.35 mg/kg) and FR contains (1424.08 mg/kg). Moreover, in humans, K is an essential metal that is diuretic in nature. This is a critical factor that controls blood pressure and the better function of the brain and nervous system and thereby helping to prevent stroke. The K is associated with appropriate bone health and the prevention of osteoporosis (He and MacGregor, 2008, Susan and Lanham, 2008). But hyperkalaemia disease is a condition that is affected by excessive potassium levels in the blood and ultimately leads to kidney disease. The concentration of K is highest in FS (28276.92 mg/kg) and comparable IS (22109.40 mg/kg). Notably, the K concentration of IR is comparable to FR. But IR contains least concentration (13009.29 mg/kg) and FR contains (1515.97 mg/kg).

The highest Calcium (Ca) concentration was observed in the powder prepared using field grown leaves of *W. somnifera* (FS-8689.1884 mg/Kg). The Ca uptake and distribution are always influenced by several factors. Normally the roots uptake the calcium and transfer it to shoots. Interestingly, the Ca concertation is least in IS (1411.68 mg/Kg). Among the root tissues FR contain (3381.47 mg/Kg) and IR was shown 1617.10 mg/Kg concentration of Ca. It is reported that the leaves generally increase in Ca with their age (Wallace and Mueller, 2008) and this might be a reason for elevated Ca concentration in FS. Ca functions as a major constituent of bones and teeth, regulation nerve impulses, and muscle function, and respiratory disorders and cardiac failure (Robin and Peter, 2010).

Manganese (Mn) is an obligatory trace element for plant as well as animals and act as an enzyme cofactor for several biological reactions (Valeria *et al.,* 2005). Mn is found in metalloproteins, such as pyruvate carboxylase, and in cytoplasmic glial enzymes, such as glutamine synthetase. Mammals with manganese deficiencies suffer from severe skeletal and reproductive abnormalities. As set by FAO/WHO, the permissible limit for medicinal plants is 200 mg/kg and a daily intake of 11 mg/day. As a result of adding MnSO4 to the tissue culture medium, a higher Mn concentration was observed in *in vitro* tissues IS (57.95 mg/Kg) IR (48.98 mg/Kg as compared with field grown shoot tissues FS (46.57 mg/Kg). It was also noticed that the Mn concentration in IR is comparatively very low (9.86 mg/Kg). Exposure to excessive doses of Mn can have affects the lungs and the brain adversely (Barceloux, 1999). There is evidence that manganese stimulates several signaling pathways responsible for apoptosis, and it also induces caspase-dependent apoptosis in PC12 cells (Hirata, 2002). A lifelong neurological disorder known as Manganism can also be caused by Mn toxicity. An insufficient supply of Mn is associated with a variety of health repercussions, including generalized growth impairment, defects in birth, reduced fertility, impaired bone formation, as well as altered lipid, protein, and carbohydrate metabolisms (Keen and Zidenberg-Cherr, 1997, Finley and Davis, 1999).

Iron is involved in a wide variety of metabolic processes in almost all living organisms, including transport of oxygen, synthesis of DNA, and electron transport. It performs a variety of biological functions, including the production of energy and the supply of oxygen. As a result of the formation of free radicals, high concentrations of iron may cause tissue damage. Iron metabolism disorders are among the most prevalent diseases of humans, encompassing a wide spectrum of conditions with diverse clinical manifestations ranging from anemia to iron overload and possibly to neurodegenerative diseases (Gurzau *et al.,* 2003). According to the WHO, the permissible limit for the range of Fe in medical herbs is 20 mg/kg, while its daily intake is between 10 and 28 mg. Consequently, all *in vitro* and field grown tissue extracts that were studied had Fe levels that were below WHO permissible limits. Normally, the roots of the plant absorb Fe and transport it to the shoot, since Fe is essential for

mitochondrial and chloroplast metabolism. It has been reported that Fe deficiency results in anemia, and that overdose is associated with nausea, diarrhea, dizziness, and sometimes liver damage (Obi *et al.,* 2006). The present analysis the Fe concentration was observed highest in FR (13.55 mg/kg) and it is comparable with IS (12.00 mg/kg). The Fe is found to be the least in IR (5.99 mg/kg) and FS contains (9.90 mg/kg).

It is essential for many enzymes to contain Copper (Cu), such as, cytochrome c-oxidase, superoxide dismutase (SOD), lysyloxidase, as well as ceruloplasmin. However, Cu is potentially toxic because it is a powerful free radical generator. There is evidence that copper plays a role in many neurological conditions, including Alzheimer's, Prion's disease, Wilson's disease, and Amylotrophic lateral sclerosis (Merce, 2001). In addition, the concentration of Cu in the FS is the highest (8.2903 mg/Kg) and the lowest (1.541 mg/Kg) in IR. The concentration of Cu in FR and IS are found 3.891 mg/Kg and 2.039 mg/Kg respectively. Each *in vitro* and field grown sample contains less than the FAO/WHO permissible level of Cu. Copper is an indispensable element for humans because it is a key component of enzyme systems such as cytochrome oxidase, ceruloplasmin, and enzymes that oxidize iron in the blood. Wilson's disease (Shoaib *et al.,* 2017) can also be caused by a deficiency of Cu. Despite this, excessive consumption of Cu can lead to severe and life-threatening organ dysfunctions. As a result of Fenton type reactions, causes toxicity DNA breaks, membrane damage, and mitochondrial damage (Chukhlovin *et al.,* 2001).

As an integral component of a wide range of enzymes, zinc (Zn) plays catalytic, structural, and/or regulatory functions. The role of zinc in the structure and function of biomolecules has also been demonstrated. A decline in growth velocity is usually the first symptom of Zn deficiency in infants. It is possible to have an impaired appetite. When the depletion is severe, mood changes are accompanied by irritability and lethargy. An impaired immune system, especially T-cell function, increases infection susceptibility (Onyamborko *et al.,* 1990). It is possible to experience symptoms of Zn intoxication either in the lungs or in the intestinal tract. Zn plays a vital role in synthesis of DNA, growth and development of organs, development of brain, behavioral response, reproduction, fetal development, bone formation, and wound healing. (Piao *et al.,*

2003). The presence of high concentrations of Zn in *in* v*itro* results in neurotoxicity. After exposure to 300-600 μM Zn for 15 min, cultured mouse cortical neurons became swollen and granular, and they were destroyed after 24 hrs (Zatta *et al.,* 2003). Zn induces swelling of mitochondria and cell bodies in cell cultures. As a result of Zn induced cytotoxicity, DNA and RNA (Ribonucleic acid) synthesis are inhibited, cellular protein metabolism is compromised, and Ca2+ is antagonized (Walter *et al.,* 2003). According to Wilhelm *et al.,* 2001, Zn exposure induces inhibition of Glutathione disulfide (GSSH) reductase early in the process. During the first 1-2 hrs following exposure, inhibition occurs, which precedes the depletion of glutathione (GSH) or an increase in GSSH. The Zn was found to be highest in IS (32.002 mg/Kg). Comparatively similar concentrations were observed in IR (16.95 mg/Kg) and FS (15. 75 mg/Kg). Notably, a low concentration of Zn was observed in FR (6.601 mg/Kg). But all Zn concentration was found below the WHO permissible limit.

Molybdenum (Mo) is a transition element and a major constituent of diverse metalloenzymes such as xanthine oxidase, aldehyde oxidase, nitrate reductase, and hydrogenase (Soetan *et al.,* 2010). The present study, it is observed that the Mo concentration is highest in IR (4.922 mg/Kg) and lowest in both FS (0.810 mg/Kg) and FR (0.308 mg/Kg). The excess supplementation of Mo in the biological system may manifest some toxic effects called 'Molybdenosis' further results in diarrhea, anorexia, pigmentation of hair, neurological disturbances, and premature death.

In vitro and field grown *W. somnifera* were analyzed for concentrations of heavy metals including Chromium (Cr), Cobalt (Co), Nickel (Ni), Arsenic (As), Cadmium (Cd), Mercury (Hg), and Lead (Pb). A strict recommendation has been made by WHO regarding the presence of these metals in medicinal plants. The presence of even the tiniest concentration of these metals can be extremely toxic to an organism. Based on the results of these studies, it was found that there was a considerable deviation in the range of heavy metals between the plants grown in the field and those grown in *in vitro* condition. Comparatively, the heavy metal content of field cultivated varieties was higher than that of *in vitro* samples. The higher heavy metal content in FS and FR is possible due to environmental

pollution. However, all other heavy metals, except for Pb and Cr, are within the WHO permissible limit.

Pb, a trace element that is unimportant, affects the nervous system, cardiac system, and reproductive system, as well as the skeleton, muscles, and blood (Johnson, 1998). Comparatively high concentrations of Pb were observed in field grown tissues FS (0.1252 mg/Kg) and FR (0.1115 mg/Kg) and *in vitro* tissues observed minute concentrations of Pb IR (0.0049 mg/Kg) and IS (0.0079 mg/Kg). Cd is a powerful cell poison that can cause a variety of different types of damage, including cell death and increased proliferation of cells. Several tissues may be adversely affected by oral exposure to cadmium (Lopez *et al.,* 2003). Cd also affects the nervous system, leading to neurological disorders such as learning disabilities and hyperactivity in children. Cd induces oxidative stress in neuronal cells, which results in protein damage and subsequent neurodegeneration. It is also known to increase the production of free radicals in the brain and to interfere with the cellular defense mechanism against oxidation. As for the other toxicological effects of Cd on human health, they specifically involve the liver, kidney, and renal tract (Li *et al.,* 2012) damage. The present study emphasized the Cd concentration is higher in FR (0.320 mg/Kg) and FS (0.310 mg/Kg).

Normally, Chromium (Cr) does not bioaccumulate in animals and plants. So elevated amounts of the metal present in food chain as well as the environment are exceedingly toxic to the animals as well as plants. In humans, toxicity is manifested as liver and kidney disfunction, as well as skin disease such as lesions or rashes on the skin (Mandina *et al.,* 2013). Similarly, the Cr was also detected higher in FS (0.2079 mg/Kg) But not more than the WHO permissible value. In this study, it was found that the Cr concentration of FR exceeded (0.5082 mg/Kg) the WHO permissible limit (0.002 to 0.2 mg/Kg). But the IS and IR are found to be the lowest. *i.e.*, IS (0.0076±0.005) and IR (0.0094±0.006).

Furthermore, Cobalt (Co) is a moderately infrequent element and a requisite nutrient to mammals in the form of vitamin B12 (cobalamin) (Leyssens et al., 2017). The permissible level of Cobalt (Co) in medicinal herbs set by the

FAO/ WHO is 0.48 ppm (Kumar *et al.,*2018, Soylak *et al.,* 2012). The human body contains around 1 mg of Co and 85% is in the form of vitamin B12 metal is mandatory for the management of anemia associated with malabsorption of vitamin B12 in ulcers in stomach especially gastric ulcer. Among the four different *Withania* tissue powders the FR was given the higher value (0.201 mg/Kg) FS showed lest value (0.0065 mg/Kg). The IS and IR contain lest values, 0.1376 mg/Kg and 0.1502 mg/Kg respectively. Even though a high level of Co also leads to toxicity mainly affects hematological, cardiovascular, neurological, and endocrine defects (Catalani *et al.,* 2012, Apostoli *et al.,* 2013).

Calcium channels allow Ni 2+ to cross cell membranes and compete with Ca2+ for specific intracellular receptors (Barceloux, 1999). One of the common side effects is allergic dermatitis known as nickel itch (Gatebe *et al.,* 2012). In the present study, the Ni concentration was found to be lest in *in vitro* tissue IR (0.0653 mg/Kg) and IS (0.0032 mg/Kg), while in traditionally cultivated *Withana* tissues showed a higher accumulation pattern of Ni (FR 0.8972 mg/Kg) FS (0.9866±0.001 mg/Kg) but it is lower than the Permissible limit by WHO.

Mercury (Hg) could bioaccumulate causing deleterious health impacts on human health. Excessive exposure to Hg may cause tremors, irritability, abnormal excitement, memory loss, insomnia, lethargy, delirium, hallucinations, and gingivitis (Harada *et al.,* 1999). The Pb content was also found higher in field tissues than *in vitro* tissues of *W. somnifera*. The accumulation is in the order of FS>FR>IS>IR. But all values are within the WHO recommended values (0.3 to 10 mg/Kg). The FS contain (0.1252±0.068 mg/Kg) and FR (0.1115±0.231 mg/Kg). The *in vitro* plant tissues IS (0.0079±0.042 mg/Kg) and IR (0.0049±0.021 mg/Kg).

While minor exposure to Arsenic (As) leads to vomiting nausea, reduced number of white blood cells (WBC) and red blood (RBC), disturbance in cardiac rhythm, injury to blood vessels, and tingling effect in the hands and feet. Since the elevated exposure creates skin blackening, hypertension, severe consequences in the cardiovascular system, and hepatic impairment were also observed at higher doses (Martin *et al.,* 2009). All the *in vitro* and field grown samples were found to be below the WHO permissible limit for Ar (1.0 to 10.0

mg/Kg). The FR was showed (0.0418±0.001 mg/Kg) and FS (0.0962±0.101 mg/Kg). The IS and IR contain (0.0045±0.002 mg/Kg) and (0.0016±0.020 mg/Kg) respectively.

The ICPMS analysis clearly indicates the essential metal accumulation pattern and heavy metal contamination in *in vitro* and field cultivated *W. somnifera*. The plants from field cultivation always have a risk of heavy metal accumulation. Continuous consumption of heavy metal contaminated herbs arises a plethora of health risks in mammals as well as plants. These findings highlighted that the *in vitro* cultivated plants are free from heavy metal contaminations and accumulated with a higher range of potentially useful elements.

4.3 Comparative metabolic profiling of the *in vitro* developed shoots of *W. somnifera* with field grown tissues

Organisms produce metabolites throughout their life cycle under all possible conditions, which constitute the metabolome of the organism. Cells, tissues, and organs of plants are affected by global changes in gene transcription and protein activity. Variations in these properties often result in inconsistent formation of metabolites in plants. The deviation in these metabolome leads batch to batch variation in pharmaceutical product and finally the entire therapeutic properties of a herbal medicine. Because of this commercializing a plant based medicine is difficult since different constituents may have varying health effects as a result of complex multi-target interactions of plants with environmental factors**.** Therefore, health promoting properties of commercial *W. somnifera* and its and its formulations are challenching.

It is becoming increasingly desirable to analyze the metabolome fingerprints of extracts of medicinal herb or raw materials in order to standardize drugs and establish their scientific basis for pharmacological action. In the present study, GC-MS techniques were used to analyze the metabolomes of leaf and root extracts from *W. somnifera* developed *in vitro* (IS, IR) with those of traditionally cultivated tissues (FR, FS). Performing a comprehensive chemical analysis is essential for the establishment of a correlation between complex chemical mixtures and molecular pharmacology, as well as for the understanding

of complex cellular processes and biochemical pathways through the analysis of metabolites to genes. (Nakabayashi et al., 2009).

4.3.1 Gas chromatography- Mass spectroscopy analysis (GC-MS)

As a result of recent advances in analytical techniques such as chromatography, mass spectrometry, and nuclear magnetic resonance (NMR), metabolomics has been revolutionized. From the identification of metabolites to the extrapolation of metabolomic data towards exploring metabolic changes, metabolomics has become an important tool. Additionally, the development of technical tools is combined with the development of software tools for the analysis and processing of data into innovative workflows for metabolomics. In addition, it facilitates the study of many unknown regulatory networks, genes and pathways that regulate plant growth and metabolism (Chang *et al.,* 2021). A metabolic fingerprinting of plant extracts is also an important part of the standardisation of drugs. For sensitive identification and quantification of metabolites, most metabolomic studies employ MS-based techniques, such as GC-MS.

A mass spectrometer detects ionic fragments of each compound that elutes from a gas chromatography column after it is ionized and fragmented. Each ionic fragment can be identified by its m/z ration, which serves as a tag for a particular metabolite. Using AMDIS (Automated Mass Spectral Deconvolution and Identification System) software (D'Arcy and Mallard, 2004), the ionic fragments, peak areas, and accumulation patterns of the compounds were retrieved. By screening metabolites using the Golm metabolome database (GMD) and the NIST (National Institute of Sciene and Technology) commercial library, the higher probability of match was determined. Appendix 6 displays the GC-MS peaks obtained for each sample. Among 33 compounds identified, 13 were organic acids, 10 were amino acids, 4 were alcohols, 3 were sugars, and 4 were other compounds **(Table 4.12)**. It was found that all sugars, alcohols, and metabolites such as squalene, 2-pyrollidinone, and putrescine could be detected in plant tissue extracts, both *in vitro* and in the field. While 2- pyrollidinone's role in plants has not yet been explored, it is believed to contribute to *Brassica*

oleracea's anticancer activity (Thangam *et al.,* 2013). Korean red ginseng has also been found to contain this alkaloid compound (Hyun *et al.,* 2020).

All *in vitro* and field-grown samples contained butanoic acid, which was higher in shoot samples. However, the FS showed an elevated concentration of butanoic acid. It is interesting to note that neither FS nor IS extracts contained valeric acid. In FS, valeric acid concentrations are higher than IS, possibly due to environmental stress factors. As a result of humic acid stress, sugarcane leaf levels of valeric acid also increased (Aguiar *et al.,* 2018). Likewise, three valeric acid derivatives were detected in mulberry yu-711 under drought stress (Ackah *et al.,* 2021). The GC-MS analysis of *Solanum khasianum* methanolic extracts revealed the presence of phytol, myo-inositol, and valeric acid in the leaf and alanine, pthalic acid, and 2-pyrollidinone in the root tissues (Chirumamilla *et al.,* 2022), suggesting similar metabolic profiles among the families of Solanaceae.

The metanolic extracts of FR and IR did not show the presence of organic acids namely Linoleaidic acid, Linolenic acid, Fumaric acid and Levulinic acid. With the exception of Aziridine, which was not detected in IS, all other compounds, such as Squalene, Putresin, and 2-pyrollidinone, were detected in field grown samples of FR, IS, and IR. A total of four amino acids were not detected in root samples such as FR and IR, including L-Valine, Aspartic acid, Glycine and Glutamine. All *in vitro* and field grown samples tested positive for the sugars Mannose, Glucose, and Galactose. *In vitro* cultured samples and field grown samples had significantly different levels of soluble sugar like glucose. It is noteworthy that glucose levels are comparably higher in root samples than those in leaf samples, while mannose and galactose levels are much higher in leaf samples than in field grown root samples.

Myo-inositol is a commonly found biologically active sugar alcohol that plays an important role in signal transduction in plants (Chhetri, 2019). Solanaceae species (*S. pimpinellifolium and S. lycopersicum*) responded to salt stress by increasing myo-inositol levels (Razali *et al.,* 2018). Both FS and IS showed higher quantity of myo-inositol levels. The GC-MS data indicated a clear difference between the tissues of field cultivated tissues and the *in vitro* grown shoot cultures in terms of metabolic content.

Table 4.14: GC-MS identification of metabolites in methanol extracts of *W. somnifera*

Class	Metabolites	KEGG ID	RT (min)	Fragmentation ion (m/z)	FR	IR	FS	IS
Organic acids	Propanoic acid	C00163	10.4895	73, 147, 117	Y	Y	Y	Y
	Pthalic acid	C01606	23.0005	149, 177, 127	Y	Y	Y	Y
	Valeric acid	C00803	22.2948	60, 73	ND	ND	Y	Y
	Palmitic acid	C00249	27.9812	77, 57, 60	Y	Y	Y	Y
	Linoleaidic acid	5283446	31.0012	67, 81, 95	ND	ND	Y	Y
	Linolenic acid	C06427	29.2997	79, 67, 217	ND	ND	Y	Y
	Stearic acid	C01530	32.003	73, 57, 60	Y	Y	Y	Y
	Fumaric acid	C00122	17.1305	245, 73, 147	ND	ND	Y	Y
	Butanoic acid	C00246	20.8145	174, 73, 147	Y	Y	Y	Y
	Levulinic acid	11579	10.8764	73, 143, 147	Y	Y	Y	Y
	Oxalic acid	C00209	11.0072	71, 72, 90	Y	Y	Y	Y
	Succinic acid	C00042	11.4987	55, 45, 101	Y	Y	Y	Y
	Myristic acid	C06424	23.9865	60, 127, 405	Y	Y	Y	Y
Alcohols	Diethylene glycol	C14687	14.884	52, 73, 117	Y	Y	Y	Y
	Glycerol	C00116	15.9116	73, 147, 205	Y	Y	Y	Y
	Myo-inositol	C00137	30.8423	73, 217, 305	Y	Y	Y	Y
	Phytol	C01389	30.8221	73, 71, 305	Y	Y	Y	Y

*The table represented the presence of organic acid and alcohols in the samples Y- detected, N- not detected. Samples: FR- Field grown root, IR - *I n vitro* root , IS- *In vitro* shoot and FS field grown shoot. RT- Retention time, KEGG- Kyoto Encyclopedia of genes and Genomes.

Table 4.14: GC-MS identification of metabolites in methanol extracts of *W. somnifera*

Class	Metabolites	KEGG ID	RT (min)	Fragmentation ion (m/z)	FR	IR	FS	IS
Amino acids	L- valine	C00183	14.543	72, 73, 55	N	N	Y	Y
	Aspartic acid	C00049	16.9834	73, 147,115	N	N	Y	Y
	Glycine	C00037	16.0342	174,73,86	N	Y	Y	Y
	Proline	C00148	18.0023	84, 56, 85	Y	Y	Y	Y
	Asparagine	C00152	21.0931	73, 116, 333	Y	Y	Y	Y
	Glutamic acid	C00025	19.0346	73, 246, 350	Y	Y	Y	Y
	Glutamine	C00064	23.4563	73, 158, 350	N	N	Y	Y
	Leucine	C00123	26.9846	117, 110, 274	Y	Y	Y	Y
	Tryptophan	C00078	37.0214	73, 147, 259	Y	Y	Y	Y
Sugars	Mannose	C00936	29.0012	73, 204, 147	Y	Y	Y	Y
	Glucose	C00221	26.9542	73, 147, 205, 160	Y	Y	Y	Y
	Galactose	C00984	28.4639	73, 205, 319	Y	Y	Y	Y
Others	2-Pyrrolidinone	C11118	10.5321	84, 85, 56	Y	Y	Y	Y
	Putrescine	C00134	24.9865	73, 215, 378, 175	Y	Y	Y	Y
	Squalene	C00751	39.6842	69, 81, 95	Y	Y	Y	Y
	Aziridine	-	10.8426	98, 99, 100	Y	Y	Y	N

*The table represented the presence of organic acid and alcohols in the samples Y- detected , N- not detected. Samples: FR- Field grown root, IR - I n vitro root , IS- In vitro shoot and FS field grown shoot. RT- Retention time, KEGG- Kyoto Encyclopedia of genes and Genomes.

Table 4.15: Relative percentage of the compounds detected in *in vitro* and field samples using GC-MS

Metabolites	FR	IR	IS	FS
Propanoic acid	0.0097	0.000599	0.0102	0.1460
Pthalic acid	0.0010	0.00306	0.00476	0.0058
Valeric acid	-	-	0.00429	0.0631
Palmitic acid	0.2410	0.00306	0.0326	0.1040
Linoleaidic acid	-	-	0.0182	0.0267
Linolenic acid	-	-	0.0182	0.0353
Stearic acid	0.2410	0.0334	0.0733	0.1210
Fumaric acid	-	-	0.00429	0.0084
Butanoic acid	0.0163	0.0043	0.0512	0.0047
Levulinic acid	-	-	0.000226	0.0023
Oxalic acid	0.000037	0.0024	0.0189	0.0259
Succinic acid	0.0316	0.0244	0.124	0.3550
Myristic acid	0.2410	0.00758	0.131	0.1040
Diethylene glycol	0.0007	0.00518	0.00812	0.0002
Glycerol	0.0098	0.00499	0.0287	0.0101
Myo-inositol	0.1760	0.0449	1.98	0.0141
Phytol	0.0830	0.000128	0.000254	0.0620
L- valine	-	-	0.009473	0.0190
Aspartic acid	-	-	0.000352	0.0062
Glycine	0.0031	-	0.0102	0.0097
Proline	0.1009	0.0596	0.0549	0.1542
Asparagine	0.0057	0.00427	0.00513	0.0139
Glutamic acid	0.0893	0.0596	0.0363	0.0662
Glutamine	0.0728	0.0573	0.0363	0.0648
Leucine	0.0017	0.00024	0.0014	0.0038
Tryptophan	0.00008	0.000039	0.0002	0.0003
Mannose	0.7050	0.508	0.837	0.9910
Glucose	0.1310	0.0283	0.0732	0.0455
Galactose	0.7250	0.558	0.00302	0.1780
2-Pyrrolidinone	0.0091	0.0133	0.066	0.0316
Putrescine	0.0003	0.062	0.043	0.0021
Squalene	0.0428	0.0449	1.98	0.0588
Aziridine	0.0017	0.000124	-	0.0004

*Table represents the peak area of the samples for each metabolite. Samples FR- Field grown root, IR – *I n vitro* root, IS- *In vitro* shoot and FS field grown shoot.

4.2.2 Principal component analysis (PCA)

Statistical analysis of metabolomic data requires advanced multivariate techniques that account for more than one experimental factor to analyse the biological data with a greater number of variables than samples. One approach to characterizing metabolomic data variation is principal component analysis (Chang *et al.,* 2021). Due to the difficulty of understanding the bulk GC-MS data, a simple principal component analysis was conducted using XLSTAT. It is one of the most efficient statistical techniques for analysing large numbers of variables and explaining them in terms of smaller numbers of variables without incurring loss of information. In this manner, PCA can assist in understanding the nature of the obtained multivariate data. Using PCA, the present study has identified differences in metabolite accumulation patterns between *in vitro* and field grown *W. somnifera* tissues. In this study, identified and scored the major sources of variation in metabolic data among the sample extracts in order to provide a clear picture of metabolic variation among plant extracts. So, the GC-MS data was subjected to PCA . *W. somnifera* tissues were analysed *in vitro* and the field tissues to distinguish the total metabolites that vary between them. Using PCA, the 33 metabolites were categorized into three principal components (PC1, PC2, and PC3). An analysis of principal components reveals the contribution of each metabolite to each component in terms of loading values, which represent correlations between variables and factors. The principal component (PC) with the larger loading value is presumed to have the greatest impact on the metabolite on the respective quadrant. As a result, the PCA showed that all metabolites contributed most towards PC1 and PC2 when compared to the other principal components.

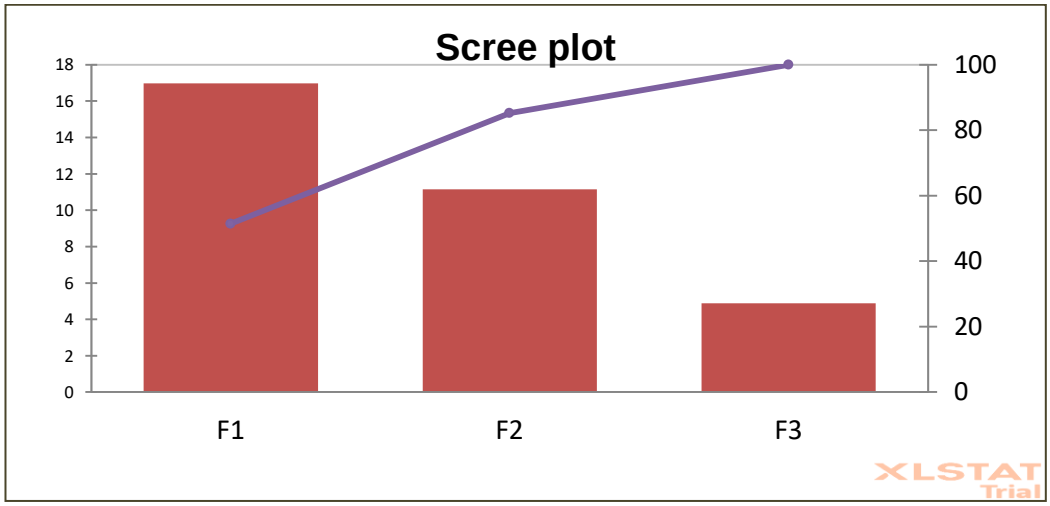
The PCA further reduces n-dimensional data to a smaller number of dimensions. The eigenvalue reflects the quality of this projection. Based on the principal component analysis, PC1 and PC2 showed the most variation (**Table 4.15**) and (**Figure 4.3)** The principal components PC1 (51.43%) and PC2 (33.79%) together accounted for (85.21%) of the variance.

Table 4.16: Eigen values and percentage variability of the principal components

Principal components	PCA		
	Eigen value	Variability (%)	Cumulative (%)
PC1	16.970	51.43	51.43
PC2	11.150	33.79	85.21
PC3	4.880	14.79	100.00

The PCA involving *in vitro* and field grown samples and showed 3 principal components PC1-Principle component 1, PC2-Princilple component 2 and PC3- Principle component 3 .

Figure 4.3: Scree plot representation of principal components.

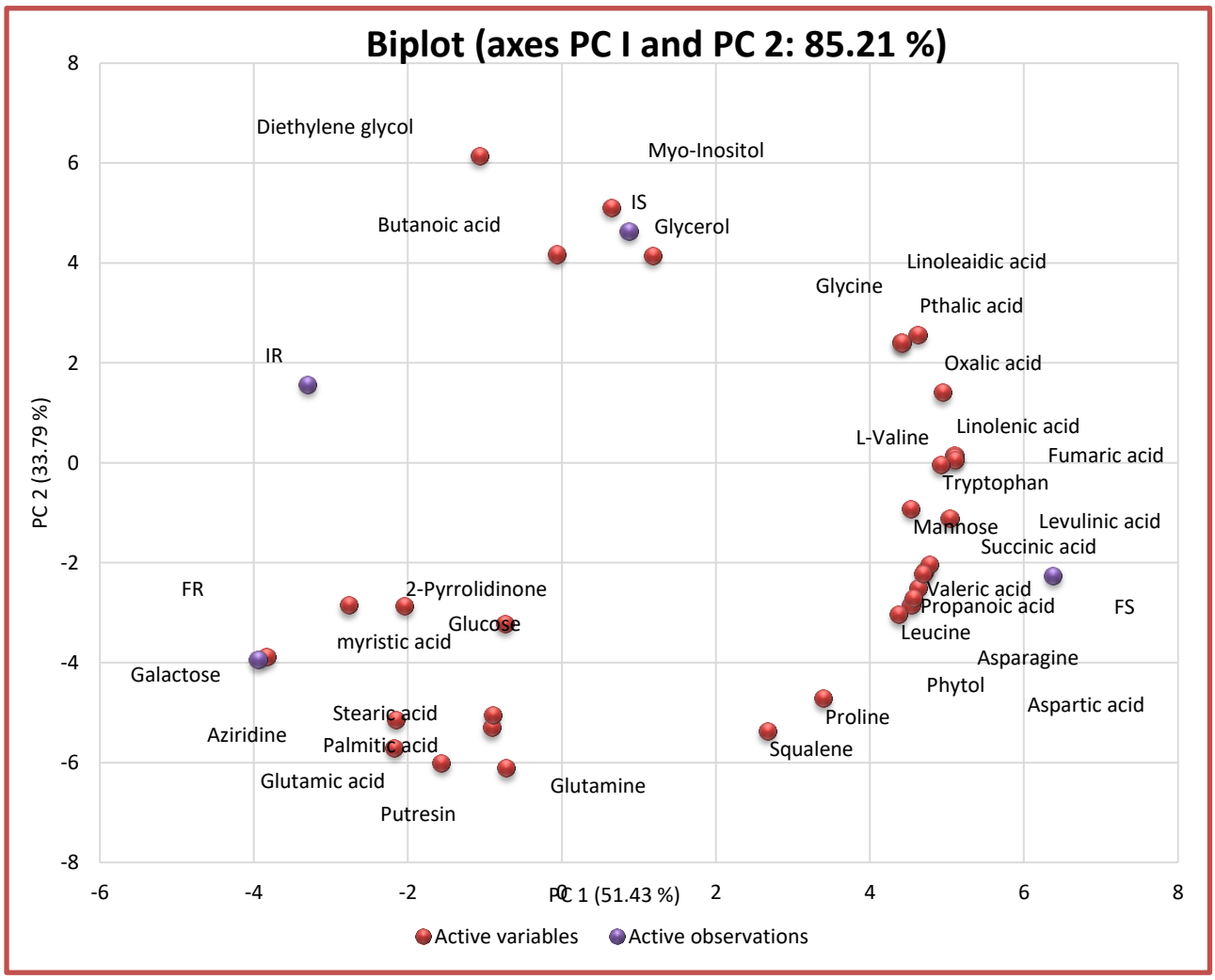


The scree plot representation principle components and showed 3 principal components F1-Principle component 1, F2-Princilple component 2 and F3- Principle component 3

The samples and their metabolites were initially plotted against PC1 and PC2. In (**Figure 4.5**), the PCA biplot is shown (PCA. PC1 is represented by the horizontal axis (X axis) and PC2 is represented by the vertical axis (Y axis). Metabolites obtained by GC-MS are represented by red vectors. The length of a vector shows the quality of representation of a metabolite in a particular dimension. Vectors with longer lengths are better represented on PC dimensions. From the biplot, it can be seen that all the metabolites under study have good representation in both dimensions. Furthermore, the biplot demonstrates a clear differentiation between samples grown *in vitro* (IS and IR) and those grown in the field (FS and FR) along PC1.

The shoot cultures FS and IS were highly positive for PC1 which is also positively correlated to squalene, the key precursor of withanolide biosynthetic pathway (Yousefiana *et al*., 2018). The other compounds positive for PC1 include organic acids (Propanoic acid, Pthalic acid, Succinic acid, Levulinic acid, Lenoleaidic acid, Valeric acid, Oxalic acid, Linolenic, Glutamine acid, Fumaric acid), amino acids (Leucine, Proline, Glutamine and Glycine, Asparagine, Tryptophan, Aspartic acid and L. Valine and a few alcohols like Myoinsitol, Glycerol and Phytol and suger Mannose. The PC2 contain the organic acids like Butanoic acid, Myristic acid, Steric acid, Palmic acid, Glutamic acid sugers such as Glucose and Galactose and other compounds like Diethyl glycol, 2 pyrolidinone, Putresin and Aziridine.

Figure 4.4: PCA biplot of IR, IS, FR and FS



PCA biplot with PC1 along X axis and PC2 along Y axis. The red vectors represent the metabolites obtained in GC-MS. Samples FR- Field grown root, IR – *In vitro* root, IS- *In vitro* shoot and FS field grown shoot.

4.4 Comparison of Neuroprotective activity and among *in vitro* and field grown tissues in *C. elegans*.

Each living organism faces the challenge of aging, and humans are no exception. Many neurological disorders have been associated with this risk factor (Niccoli and Partridge, 2012). As a matter of fact, many genetic pathways affect aging as well as provide neuroprotection, and some of these pathways are evolutionarily conserved (Kenyon, 2005, 2010, de la Monte and Wands, 2005, Bartke, 2008). The progress of an organism is directly related to the environment were it is exposed to. The biology of an organism can be analysed based on this fact. Whenever any ecological parameter changes, it is likely that the organism's biology will be affected. Non-parasitic nematode *C. elegans* undergoes behaviour and physiological changes similar to higher mammals including humans on aging (Kirkwood, 2011), making it an excellent model for biological research. Animals show sarcopenia, become infertile and suffer severe muscle damage as they age, and their locomotion becomes hindered. It is caused by the accretion of oxidized proteins and lipofuscin (Klass, 1977, Johnson, 2003).

C. elegans is a transparent organism that allows the observation of morphological and developmental changes within the individual worms. Due to its ability to reproduce within two to three days, as well as the fact that wild type animals generally produce 10-15 eggs in their uterus (Fielenbach and Antebi, 2008), this is an attractive option. Genetically, it shares 80% of the characteristics of humans (Braeckman and Vanfleteren, 2007, Bell *et al.,* 2009). Due to their short lifespan and ease of culture, these nematodes are easy to monitor during experimental treatments. There has been much use of this model organism in the study of pharmacological effects and gene interaction (Rand *et al.,* 1995). *C. elegans* can be prolonged or postponed from physiological aging by pharmacological interventions with antioxidant supplements and phytochemicals (Collins *et al.,* 2006, Lucanic *et al.,* 2013). This method is therefore both cost-effective and efficient, as it reduces the use of animals and allows high-quality microscopic imaging to be captured (Flecknell, 2002).

Due to this, the present study attempted to understand the multidimensional animal model system *C. elegans* in order to investigate and compare the efficacy and safety profiles of *in vitro* (*in vitro* root (IR) *in vitro* shoot (IS) and field root (FR) and field shoot (FS) of *W. somnifera*. Furthermore, this study also compared the longevity and neuroprotective efficacy of the *in vitro* and field cultured tissues of *W. somnifera*. It also provided some insights into the underlying mechanisms involved.

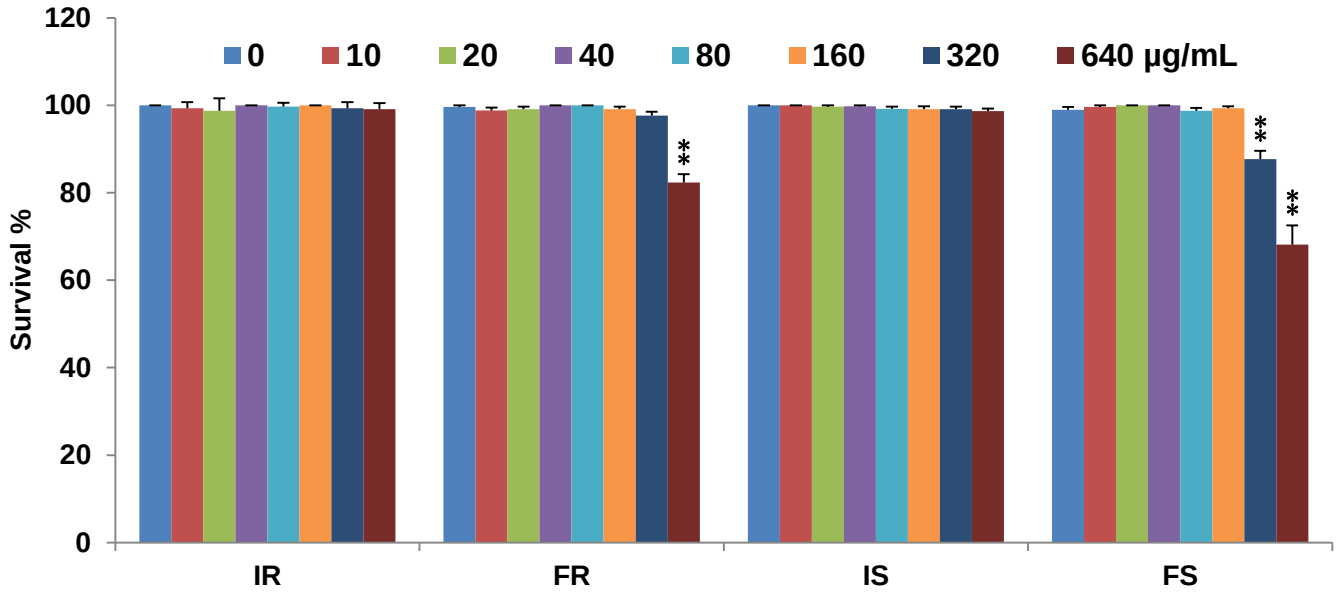
4.4.1. *Safety evaluation of Withania extracts on C. elegans*

Tartrazine and sulphanilic acid were tested for toxicity on worm *C. elegans* at various concentrations (0.5 mM to 3.0 mM) and growth and development were assessed. AFB1 and T2 mycotoxin toxicity was studied using *C. elegans* as a model organism by Yang *et al.,* (2015). Therefore, toxicity and progeny production of wild-type worms were used to assess the safety properties of *W. somnifera* extracts. A study was conducted to determine the viability of worms after treatment with various pharmacological doses of *W. somnifera* shoot extracts grown *in vitro* and in the field. The survival and reproduction of *C. elegans* were affected differently by acute exposure to different pharmacological dosages of *W. somnifera* extracts (IR, FR, IS, FS; 0, 10, 20, 40, 80, 160, 320, and 640 g/mL). The results showed that IR and IS extracts did not adversely affect normal survival, reproduction rate, and progeny production. Among the tested doses, FR extract at 640 µg/mL and FS extract at 320 and 640 µg/mL were evaluated as toxic and significantly ($p<0.01$) reduced the percentage survival of wild-type worms when compared to that of unexposed worms (**Figure 4.5**). In addition, 160-640 µg/mL FS extract significantly affects the overall reproduction rate and progeny development (**Figure 4.6**). Hence, the toxic concentrations of *W. somnifera* extracts were excluded from the further lifespan extension experiments, and non-toxic concentrations were used to study their life-promoting efficacy in *C. elegans*.

It was found that WFA concentrations in FS were (5.74±0.9 mg/g DW), IS was (2.76±0.8 mg/g DW), and FR was (1.99±0.4 mg/g DW). WFA concentrations in FS are double those in FS compared to other tissue samples. It was comparable to the report by Kirti Vaishnavi *et al.,* 2012. At higher concentrations,

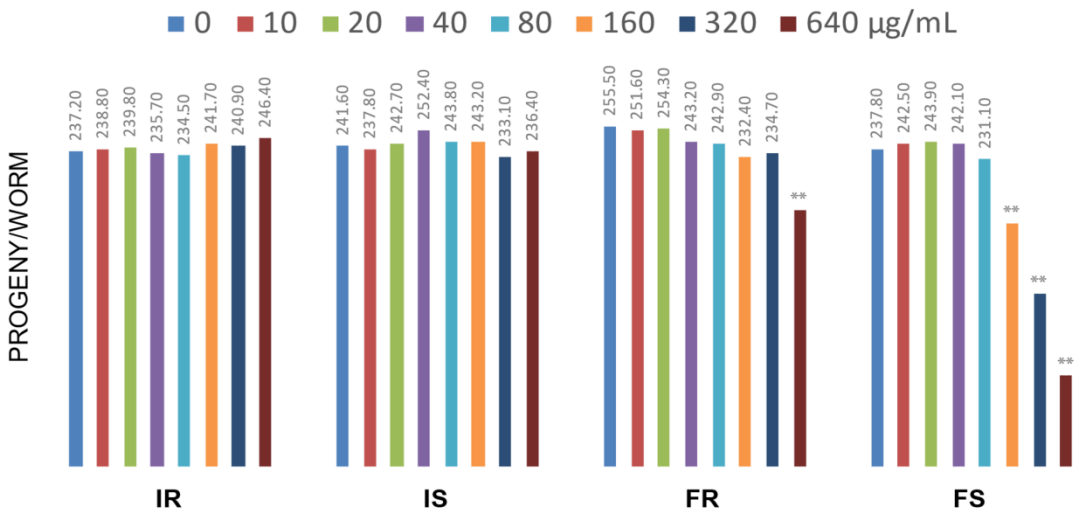
WFA is reported to be toxic. WFA has been classified as a toxic compound by the European Food Safety Authority (EFSA). The reason is its enormous ability to kill tumor cells in cancer therapy (Rinku Dutta *et al.,* 2019). It has been reported that WFA has cytotoxic effects on RPE cells (Taimur *et al.,* 2012) in in vitro experiments. It was shown that intraperitoneal administration of aqueous-methanol root extract caused 50% lethality in mice at 1076 mg/kg, and that equimolar combinations of sitoindosides VII and VIII and WFA (SG-2) caused a LD50 of 1564±92 mg/kg (Grandhi *et al.,*1994). Its toxicity led to the formation of different withaferin A analogs; among them, the 3-azido derivative and oxidized forms of the epoxide are more cytotoxic than the parent (Yousuf *et al.,* 2011). Different analogs of withaferin A can modulate its apoptotic activity as well (Llanos *et al.,* 2017). As a whole, the current study demonstrated that WFA is an essential phytoconstituent for the plant's pharmacological activity. But the concentration of WFA, however, is extremely relevant.

Figure 4.5: Toxicity measurement of *W. somnifera in C.elegans*



*The data presented in the graph is the toxicity effect of *W. somnifera* extract (IR, IS, FR,FS) of Wild type *C. elegans* at different concentrations such as 10, 20, 40, 80, 160, 320 and 640 µg/ml on NGM plates at 20°C. The percentage survival of wild-type worms when compared to that of unexposed worms

Figure 4.6: Effect of *W. somnifera* extract on Natural reproduction in *C. elegans*



Natural reproduction or overall reproduction rate and progeny development of *C. elegance* in different concentrations of *W. somnifera* extract such as 10, 20, 40, 80, 160, 320 and 640 µg/ml on NGM plates. The results are calculated from three independent biological trials vs. control. IR -*in vitro* root, IS *in vitro*- shoot, FR- field grown root, FS- field grown shoot.

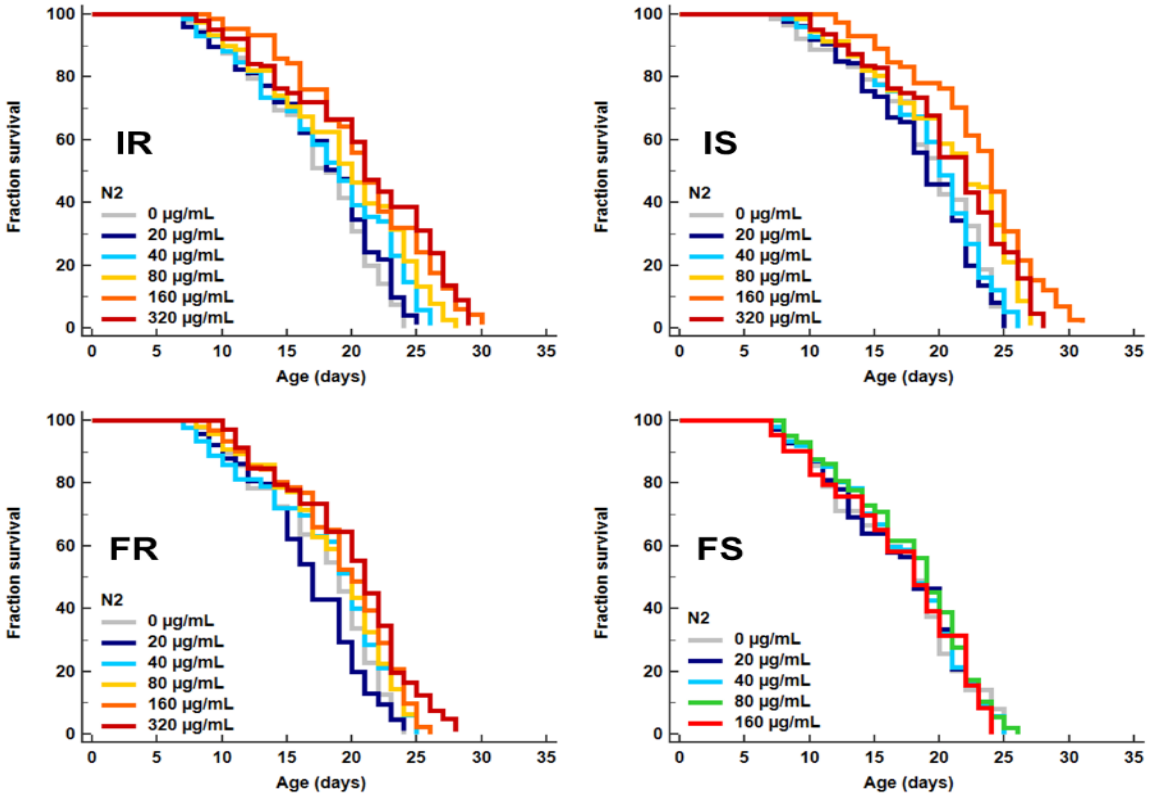
4.4.2. Determination of lifespan extension potential of *W. somnifera* in wild type *C. elegans*

Genetics and/or dietary interventions have been found to increase both healthspan and/or lifespan (Chen *et al.,* 2013, Lucanic *et al.,* 2013; Argyropoulou *et al.,* 2013), and phytochemicals are promising in this regard. It is necessary to emphasize that phytochemicals are non-nutritive components that occur naturally in plants and carry substantial biological effects. It is estimated that vegetables and edible fruits were accumulated with plenty of biologically active phytochemicals, which are likely to interact in a number of ways in preventing disease and promoting health (Surh, 2003, Argyropoulou *et al.,* 2013). The principal component of the Indian herbal drug Ashwagandha (root of *W. somnifera*) is Withanolide A (WA), a steroidal lactone (Baitharu *et al.,* 2014). Many neurological disorders can be treated with WA, including AD-associated amyloid pathology, neuritis regeneration, synapses that have been damaged, and axonal outgrowth, among others. (Zhao *et al.,* 2002, Kuboyama *et al.,* 2002, 2005, Baitharu *et al.*, 2014, Kurapati *et al.,* 2013). Additionally, it can be used as an adaptogen to reduce stress on the body, *W. somnifera* is also renowned for

its use in Ayurvedic medicine for a variety of age-related ailments (Winston, 2019). The Ayurvedic term Avarada refers to *W. somnifera*, which means rejuvenation or youthfulness.

In a previous study, *W. somnifera* root extract was shown to slow down and regulate the aging process and extend healthy longevity in *C. elegans* (Kumar *et al.,* 2013). Even so, the specific biological mechanism underlying *W. somnifera* extract for life-prolonging effects is still unclear. Therefore, the current study set out to scrutinize the effect of *W. somnifera* extract on the lifespan of *C. elegans* and explore its genetic mechanism. To investigate whether *W. somnifera* extracts have an effect on lifespan, age-synchronized L4 stage wild-type worms were treated with different concentrations until they died. Epigallocatechin-3-gallate (EGCG; 100 µM), a green tree polyphenol, was used as a positive control. Under standard conditions (at 20°C on NGM plates), wild-type worms lived an average of 17.32±0.40 days and a maximum of 24 days. We found that different pharmacological concentrations of *W. somnifera* extracts have different effects on the lifespan of wild-type worms **(Figure 4.7)**. It was observed that 160 µg/mL of IR and IS, and 320 µg/mL of FR were the highly effective concentration and could extend the mean lifespan of *C. elegans* by 21.07% (20.97±0.46 days; $p<0.0001$), 21.41% (22.94±0.39 days; $p<0.0001$), and 12.14% (19.86±0.43 days; $p<0.0001$), respectively. Other tested concentrations of IR (20, 40, 80, and 320 µg/mL) and IS (80, and 320 µg/mL) significantly extend the mean lifespan of *C. elegans* ($p<0.05$, $p<0.01$), and they also exhibited hormesis-like, dose-dependent biphasic effect. FR extracts displayed a concentration-dependent effect, while FS extracts did not show any effects on the lifespan of *C. elegans* at any of the concentrations tested ($p>0.05$). It is crucial to note that the lifespan extension induced by IR and IS extracts is significantly higher than the effects that are produced by FR extract at higher concentrations. These results suggest that 160 µg/mL of IR and IS, and 320 µg/mL of FR are more effective in promoting the mean lifespan of *C. elegans* and that these optimal concentrations were used in most of the subsequent experiments **(Table 4.15** (Statistical data)**)**

Figure 4.7: Effect of *W. somnifera* on Life span extension in *C.elegans*



Effect of different pharmacological concentrations of *W. somnifera* extracts on the lifespan of *C. elegans*. Survival curves were graphed using the Kaplan-Meier survival method and analysed by a log-rank test.

Table 4.17: Effect of *W. somnifera* extracts on the lifespan wild-type *C. elegans*

Genotype	Treatment (µg/mL)	Mean survival (Mean±SEM)	Maximum lifespan (days)	Sample size (N)	Censored	% Change	P Value
In vitro* grown roots (IR) of *W. somnifera							
N2 (Wild)	0	17.32±0.404	24	137	7		
	20	17.67±0.410	25	154	11	(+) 2.02	0.2004
	40	18.29±0.466	26	143	8	(+) 5.61	0.0014
	80	19.24±0.465	28	150	8	(+) 11.11	0.0001
	160	**20.97±0.456**	**30**	**134**	**5**	**(+) 21.07**	**0.0001**
	320	20.78±0.525	29	140	5	(+) 20.00	0.0001
In vitro* grown shoots (IS) of *W. somnifera							
N2 (Wild)	0	18.90±0.416	25	144	13		
	20	18.51±0.404	25	135	10	(-) 2.03	0.2885
	40	19.34±0.375	26	152	13	(+) 2.34	0.5411
	80	20.74±0.468	27	129	12	(+) 9.75	0.0001
	160	**22.94±0.391**	**31**	**157**	**16**	**(+) 21.41**	**0.0001**
	320	20.86±0.439	28	141	13	(+) 10.38	0.0001

Genotype	Treatment (µg/mL)	Mean survival (Mean±SEM)	Maximum lifespan (days)	Sample size (N)	Censored	% Change	P Value
Filed grown roots (FR) of *W. somnifera*							
N2 (Wild)	0	17.71±0.408	24	125	9		
	20	16.73±0.408	24	115	12	(-) 5.56	0.0572
	40	18.07±0.455	25	134	12	(+) 2.02	0.0723
	80	18.67±0.392	25	141	10	(+) 5.37	0.0172
	160	19.20±0.428	26	122	8	(+) 8.37	0.0006
	320	**19.86±0.431**	**28**	**137**	**11**	**(+) 12.14**	**0.0001**
Filed grown shoots (FS) of *W. somnifera*							
N2 (Wild)	0	17.06±0.424	25	153	15		
	20	17.12±0.436	24	138	11	(+) 0.36	0.7452
	40	17.45±0.405	25	149	9	(+) 2.28	0.8070
	80	17.91±0.413	26	144	0	(+) 4.98	0.2499
	160	17.14±0.456	24	132	4	(+) 0.46	0.7310
Positive control							
N2 (Wild)	EGCG (100 µM)	25.08±0.611	37	173	16	(+) 44.80	0.0001

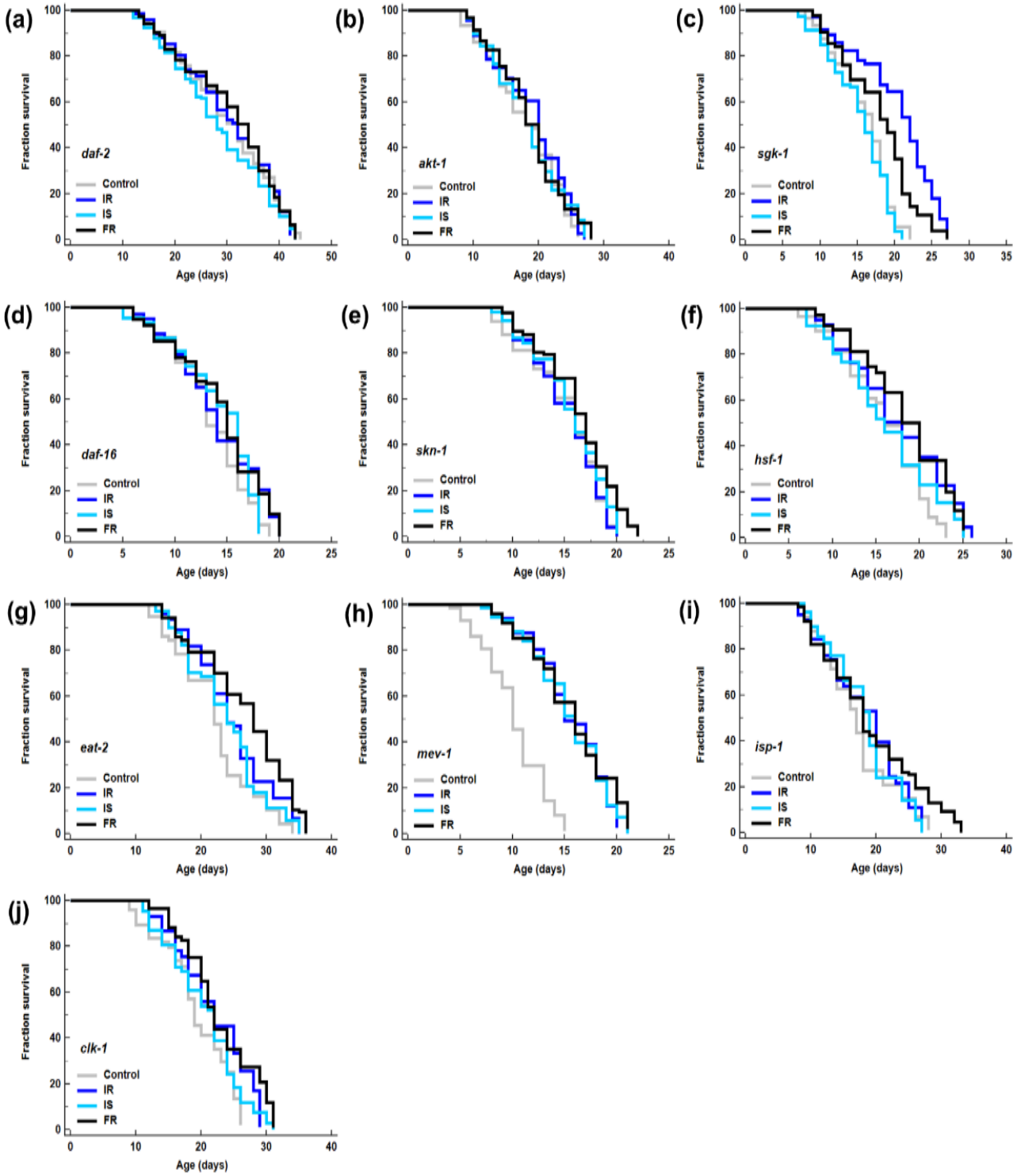
The statistical data of lifespan extension ability of *W. somnifera* extract (IR, IS, FR, FS) of wild-type *C. elegans* at different concentrations such as 20, 40, 80, 160, 320 and 640 µg/ml on NGM plates at 20°C. The results are calculated from three independent biological trials and presented as the mean ± SEM. #p<0.01 vs. N2; *p<0.05 and **p<0.01 vs. control. (IR (*in vitro* root), IS (*in vitro* shoot), (FR- field grown root, FS- field grown shoot.

4.4.3. Study the rudimentary genetic mechanism of *W. somnifera* mediated lifespan extension in mutated *C. elegans*

Several genes and cellular signaling pathways that control the process of aging in mammals are conserved and well-established in *C. elegans* (Kenyon, 2010). Among others, insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS), mitochondrial electron transport chain complexes (mETC), and dietary restriction (DR) are playing an important role in regulating aging and longevity (Kenyon, 2010). Reduced IIS signaling or mutation in daf-2, the *C. elegans* homolog of the IGF-1 receptor, increases lifespan depending on DAF-16, the *C. elegans* homolog of the forkhead box O (FOXO) transcription factor (Uno *et al.,* 2016). Moreover, the daf-2 mutation affects the activity of downstream targets akt-1 and sgk-1, while AKT-1 functions in parallel to SGK-1 to mediate the signaling. The IIS pathway has also been shown to regulate lifespan by modulating the activity of SKN-1 and HSF-1 transcription factors (Blackwell *et al.,* 2015). From invertebrates to mammals, DR is the most

constant environmental intervention known to enhance lifespan (Seo *et al.,*2013). The DR model consisted of worms with a loss of function mutation in eat-2. The eat-2 is a component of the nicotinic acetylcholine receptor that controls pharyngeal pumping, and its mutation causes a reduction in food intake. In addition, mitochondrial respiration has been shown to enhance the lifespan of many organisms, including budding yeast, Drosophila, and mice (Bargmann *et al.,* 1993, Kapahi *et al.,* 2017). With this understanding, we performed genetic screening to identify the potential mechanism underlying *W. somnifera* extracts-mediated lifespan extension using specific *C. elegans* strains deficient in the IIS pathway (*daf-2*(e1370), *akt-1*(ok525), *sgk-1*(ok538), *daf-16*(mgDf50), *skn-1*(zu67), *hsf-1*(sy441)),mitochondrial electron transport chain complex (mev-1(kn1), *isp-1* (qm150), *clk-1* (e2519)), and DR (*eat-2*[ad1116]). The results showed that IR extract significantly extended the lifespan of *sgk-1*, clk-1, *sgk-1*, *hsf*-1,eat-*2*, and mev-1 mutant worms, indicating that IR extract does not require these genes for lifespan extension. Simultaneously, IR extract fails to extend the lifespan of worms carrying a loss of function mutation in *daf-2*, *akt-1*, *skn-1*, and *isp-1*, showing that *daf-2*, *akt-1*, *skn-1*, and *isp-1* are required for the lifespan extension observed in IR extract treated worms. In addition, the lifespan of daf-16 mutant worms was marginally increased by the IR extract, indicating the partial requirement. As shown in **(Figure 4.8, Table 4.17)** (Statistical data), IS extract treatment significantly extended the lifespan of *eat-2* and *mev-1* worms. However, no corresponding lifespan extension was observed in *daf-2*, *akt-1*, *sgk-1*, *daf-16*, *skn-1*, *hsf-1*, *isp-1,* and *clk-1* worms. These results clearly indicated that IS extract acted on the IIS pathway and mitochondrial respiration but independently of the DR-like mechanism. In addition, FR extracts significantly prolong the lifespan *sgk-1*, *hsf-1*, *eat-2*, *mev-1*, *isp-1*, and *clk-1* as seen wild-type worms, while no significant changes in the lifespan were observed in *daf-2*, *akt-1*, *daf-16*, and *skn-1*. These data strongly indicated that the FR extract required the IIS pathway, but not mitochondrial function and DR for lifespan extension in *C. elegans*. In lifespan experiments, we found that all *W. somnifera* extracts notably ($p<0.0001$) extended the lifespan of mev-1 mutant worms under standard conditions.

Figure 4.8: Effect of *W. somnifera* extracts on the lifespan of mutant *C. elegans*



*Lifespan of *C. elegans* strains deficient in components of (**a-f**) IIS pathway (daf-2, akt-1, sgk-1, daf-16, skn-1, and hsf-1), (**g**) DR (eat-2), and (**h-j**) mitochondrial respiration (mev-1, isp-1, and clk-1). Survival curves were graphed using the Kaplan-Meier survival method and analyzed by a log-rank test.

The succinate dehydrogenase cytochrome b560 subunit, an integral membrane protein that is a subunit of mitochondrial respiratory chain complex II, is encoded by mev-1 in *C. elegans* (succinate-CoQ oxidoreductase). MEV-1 is essential for oxidative phosphorylation, and its absence resulted in aberrant energy metabolism and increased intracellular ROS, resulting in a shorter lifespan (Ishii *et al.,* 1998). These observations suggest that *W. somnifera* extracts could prolong the lifespan of short-lived *mev-1* mutant worms through their potent antioxidant activity and their involvement in the endogenous detoxification pathway. These results are mostly in accordance with previous findings (Bargmann *et al.,* 1993, Mohankumar *et al.,* 2019).

Table 4.18: Genetic requirements for lifespan extension mediated by *W. somnifera* extracs

Genotype	Treatment (µg/mL)	Mean survival (Mean±SEM)	Maximum lifespan (days)	Sample size (N)	Censored	% Change	P Value
daf-2	Control	30.07±0.773	44	137	8		
	IR	30.35±0.751	42	144	12	(+) 0.95	0.9988
	IS	28.38±0.798	43	132	8	(-) 5.60	0.1228
	FR	30.73±0.741	43	154	15	(+) 2.20	0.7453
akt-1	Control	17.92±0.505	26	122	12		
	IR	18.94±0.481	27	136	11	(+) 5.64	0.1074
	IS	18.29±0.501	27	116	4	(+) 2.03	0.4573
	FR	18.63±0.468	28	127	7	(+) 3.94	0.4063
sgk-1	Control	15.90±0.343	22	120	5		
	IR	19.38±0.450	27	142	10	(+) 21.90	0.0001
	IS	16.29±0.366	21	114	7	(+) 2.47	0.2229
	FR	18±0.429	27	126	7	(+) 13.20	0.0001
daf-16	Control	13.45±0.336	19	109	4		
	IR	14.12±0.397	20	103	4	(+) 5.00	0.0560
	IS	14.12±0.368	20	106	8	(+) 4.97	0.1216
	FR	14.33±0.384	20	115	6	(+) 6.57	0.0240
skn-1	Control	15.08±0.310	20	134	9		
	IR	15.23±0.295	20	120	5	(+) 0.97	0.9580
	IS	15.62±0.287	20	142	11	(+) 3.59	0.1113
	FR	16.38±0.314	22	127	5	(+) 6.59	0.1052
hsf-1	Control	15.89±0.444	23	112	4		
	IR	17.56±0.508	26	123	6	(+) 10.52	0.0001
	IS	16.22±0.529	25	107	4	(+) 2.05	0.1267
	FR	18.41±0.491	25	108	8	(+) 15.85	0.0001

Genotype	Treatment (µg/mL)	Mean survival (Mean±SEM)	Maximum lifespan (days)	Sample size (N)	Censored	% Change	P Value
eat-2	Control	22.20±0.550	34	115	3		
	IR	24.84±0.535	35	127	12	(+) 11.93	0.0001
	IS	24.78±0.568	35	109	4	(+) 11.63	0.0001
	FR	26.71±0.626	36	121	7	(+) 20.34	0.0001
mev-1	Control	10.20±0.258	15	130	11		
	IR	15.53±0.281	20	152	15	(+) 52.26	0.0001
	IS	15.40±0.307	21	145	11	(+) 51.02	0.0001
	FR	15.61±0.350	21	122	5	(+) 53.07	0.0001
isp-1	Control	17.26±0.517	28	116	6		
	IR	18.33±0.537	27	123	10	(+) 6.19	0.2052
	IS	18.30±0.497	27	110	4	(+) 5.61	0.3965
	FR	20.23±0.654	33	129	6	(+) 17.18	0.0001
clk-1	Control	19.37±0.475	26	122	10		
	IR	21.98±0.519	29	114	7	(+) 13.46	0.0001
	IS	20.60±0.489	31	130	5	(+) 6.36	0.0811
	FR	22.87±0.462	31	145	10	(+) 18.04	0.0001
NL5901	Control	10.24±0.305	14	103	5		
	IR	12.68±0.393	18	100	9	(+) 23.89	0.0001
	IS	14.71±0.356	21	122	11	(+) 43.68	0.0001
	FR	12.79±0.417	19	108	11	(+) 24.93	0.0001
	EGCG	14.65±0.419	22	94	8	(+) 43.15	0.0001

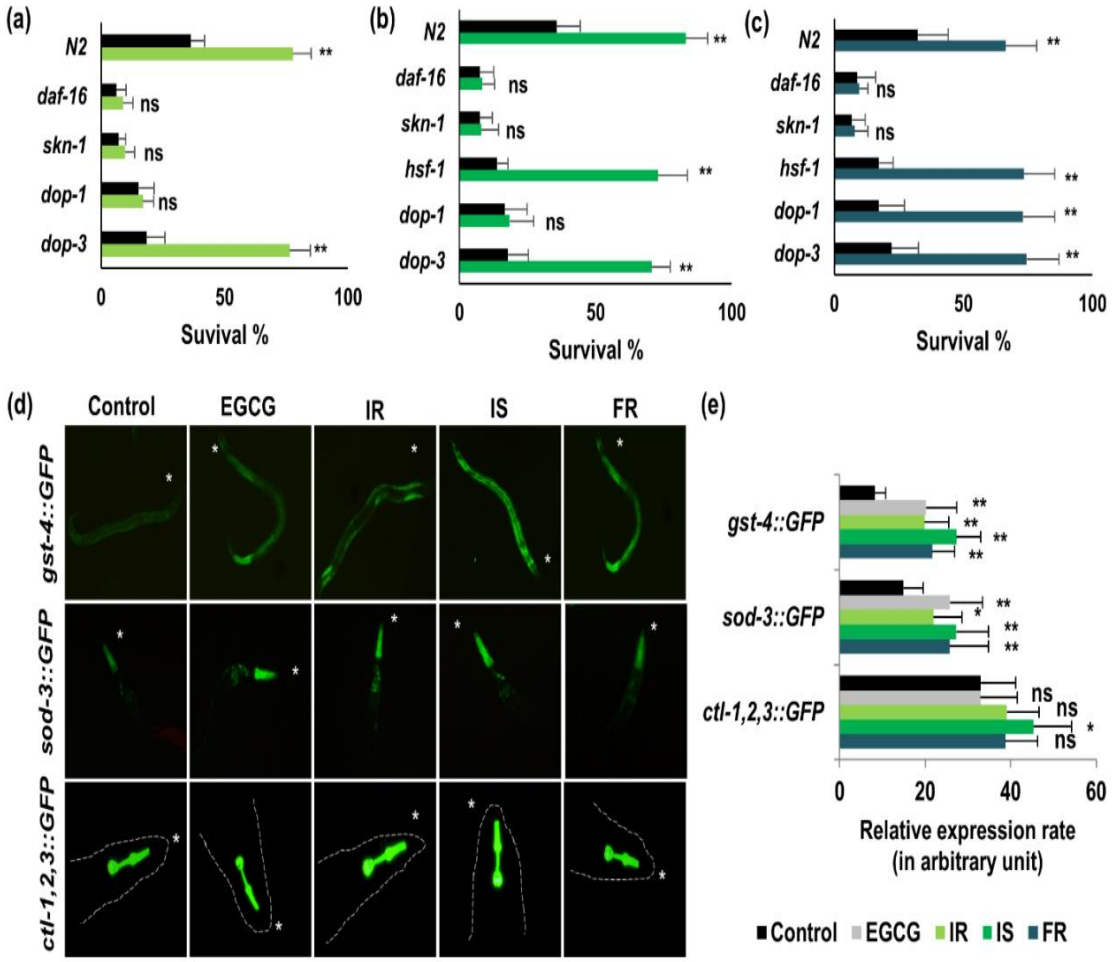
The statistical data presented in the table are Genetic requirements for lifespan extension mediated by *W. somnifera* extracts. Lifespan of *C. elegans* strains deficient in components of the IIS pathway (daf-2, akt-1, sgk-1, daf-16, skn-1, and hsf-1), DR (eat-2), and mitochondrial respiration (mev-1, isp-1, and clk-1). The results are calculated from three independent biological trials and presented as the mean ± SEM. #p<0.01 vs. N2; *p<0.05 and **p<0.01 vs. control. (IR- *in vitro* root), IS – *in vitro* shoot), (FR- field grown root).

4.3.4. Evaluation of stress resistance capability of *Withania* extracts in oxidative stress condition.

Various reports have found that a longer lifespan is linked to better survival under oxidative stress situations. (Mohankumar *et al.,* 2020, Bargmann *et al.,* 1993, Mohankumar *et al.,* 2018, Lithgow *et al.,* 1995, Devagi *et al.,* 2018). Therefore, to study whether *W. somnifera* extracts affect stress resistance in *C. elegans*, wild-type worms previously treated with all three *W. somnifera* extracts were exposed to oxidative stress. The percent survival of wild-type worms pretreated with IR. IS and FR extracts were significantly increased by about 77.69%, 83.07%, and 66.44%, respectively, compared to the untreated control groups (34.74%) (**Figure. 4.9 a-c**). In daf-16 and skn-1 mutant worms, the improved tolerance to oxidative stress is lost, but not in dop-3 mutants.

These findings revealed that functional DAF-16 and SKN-1 were mandatory for stress protection in all *W. somnifera* extracts, but not DOP-3. dop-3 is a homolog of mammalian D2 dopamine receptors that regulates the basal slowing response and stress resistance in well-fed animals. IR and IS extract treatment fail to protect the worms deficient in dop-1, suggesting the involvement of DOP-1 in IR and IS extract-mediated stress protection. dop-1 encodes a D1-like dopamine receptor and regulates mechanosensory behaviors. Conversely, FR extract significantly enhanced the survival rate of dop-1 mutants under oxidative stress conditions, as seen in wild-type worms. This result showed that dop-1 is not required for oxidative stress resistance following FR extract treatment. Overall, the stress-protecting properties of IR and IS extract were dependent on daf-16, skn-1, and dop-1; however, FR extracts were only dependent on daf-16 and skn-1. These results are consistent with the lifespan experiments. Increased expression of stress-protective genes is linked to higher survival under oxidative stress conditions. (Mohankumar *et al.,* 2018, 2020, Shanmugam *et al.,* 2017, Lithgow *et al.,* 1995, Govindan *et al.,* 2018). Therefore we investigated the effects of *W. somnifera* extracts on the expression of stress-responsive genes viz., gst-4, sod-3, and ctl-1,2,3 using transgenic reporter strain stably expressing gst-4::GFP, sod-3::GFP, and ctl-1,2,3::GFP transgene. These genes are direct transcription targets of DAF-16 (sod-3 and ctl-1,2,3) and SKN-1 (gst-4) transaction factors and offer conserved protection against various stresses (Kumar *et al.,* 2013, Uno and Nishida, 2016, Soo *et al.,* 2020). As a result, the expression levels of gst-4::GFP and sod-3::GFP, were significantly upregulated by all three *W. somnifera* extracts ($p<0.05$ or $p<0.01$), but ctl-1,2,3::GFP expression was upregulated by IS extract alone ($p<0.05$) (**Figure. 4.9 d** and **e**). These results suggest that enhanced activation of DAF-16 and SKN-1 downstream target genes (gst-4, sod-3, and ctl-1,2,3) by *W. somnifera* extracts suppresses the oxidative stress and prolongs the lifespan of *C. elegans*.

Figure. 4.9: Effect of *W. somnifera* extracts in oxidative stress and expression of antioxidant defense genes in *C. elegans*



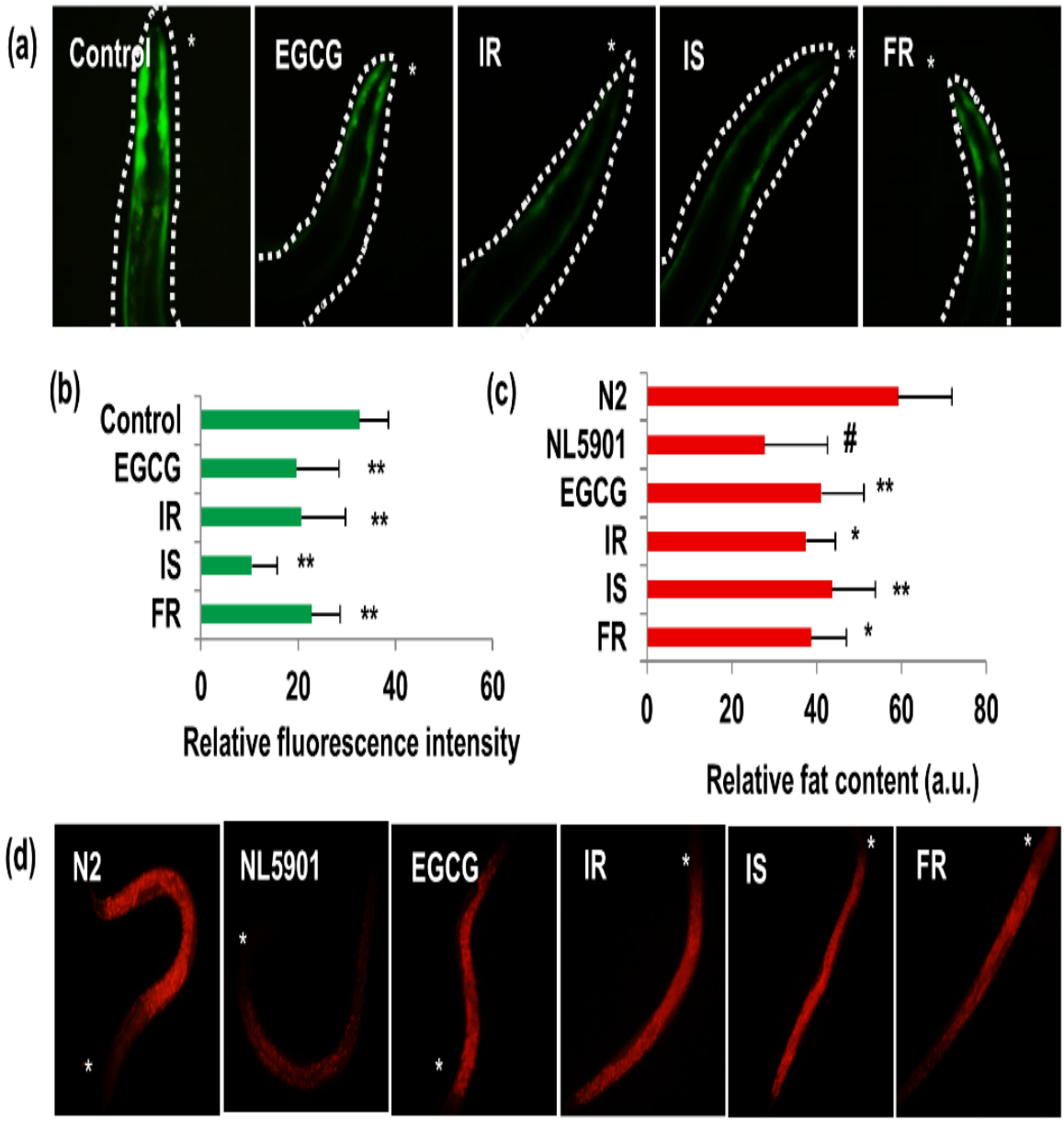
Effect of *W. somnifera* extracts on (**a-c**) oxidative stress tolerance and (**d, e**) expression of antioxidant defense genes in *C. elegans*. The results are calculated from three independent biological trials and presented as the mean ± SEM. *p<0.05, **p<0.01, and ns-not significant. In the fluorescence micrograph, the anterior region of *C. elegans* was marked with an asterisk (*).

4.3.5. Neuroprotective efficacy of *W. somnifera* extracts

The *C. elegans* has been used extensively to study several neurological disorders and aging (Kenyon, 2005, 2010, Markaki and Tavernarakis, 2010, Li and Le, 2013). A short life-cycle, fully sequenced genome, 60–80% human gene counterparts, and amenability to classical and reverse genetics make it an attractive model for aging neuroscience. *C. elegans* has such characteristics making it an ideal platform for the discovery of anti-aging and neuroprotective compounds. A number of investigators have successfully used the *C. elegans* model system to identify compounds that have an impact on the neurobiology of aging (Marvanova and Nichols, 2007; Petrascheck *et al.,* 2007; Cho *et al.,* 2010; Argyropoulou *et al.,* 2013, Lucanic *et al.,* 2013, Fu *et al.,* 2014).

Most neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease have a strong link to aging as a risk factor for start and progression and amyotrophic lateral sclerosis. Mounting experimental evidence indicates that delaying the aging process through pharmacological interventions can inhibit the pathologies of neurodegenerative diseases (Seo *et al.,* 2013). Given the life-promoting properties of *W. somnifera* extracts and since *W. somnifera* has been reported to have profound neuropharmacological effects (Dar and Muzamil, 2010), we were curious to check whether the *W. somnifera* extracts show neuroprotective effects in *C. elegans*. In this study, Parkinson's disease (PD) models of *C. elegans* were used to assess the neuroprotective properties of *W. somnifera* extracts. PD is the second most common degenerative condition that can be seen in elderly populations across the world. The two most important pathological hallmarks of PD are neuronal loss in the midbrain and typical aggregation of misfolded protein α-synuclein in brain tissue (Antony *et al.,* 2013). The human ortholog gene for α-synuclein is absent in *C. elegans*; however, the transgenic expression of α-synuclein gene, fused with a yellow fluorescent protein (α-synuclein::YFP) in body wall muscle cells, allows us to quantitatively examine the aggregation pattern *in vivo*. Control and *W. somnifera* extract treated NL5901 worms (animals expressing α-synuclein in body wall muscle cells) were examined for α-synuclein aggregation levels at day 6 adulthood. As shown in (**Figure 4.10 a**), all three *W. somnifera* extracts significantly reduced the fluorescence intensity of α-synuclein::YFP in NL5901 worms. It was observed that the aggregation of α-synuclein was decreased to 36.68%, 68.01%, and 30.25% in NL5901 worms treted with IR, IS, and FR extracts, respectively. Interestingly, the efficacy of IS on α-synuclein aggregation was more potent than that of the positive control EGCG and reduced the aggregation by 46.84% ($p<0.01$ vs. EGCG). No significant changes in α-synuclein protein aggregation were observed between EGCG, IR, and FR treatments (**Figure 4.10 b**).

Figure 4.10: Effect of *W. somnifera* extract on α-synuclein aggregation and lipid content in worms expressing α-synuclein.



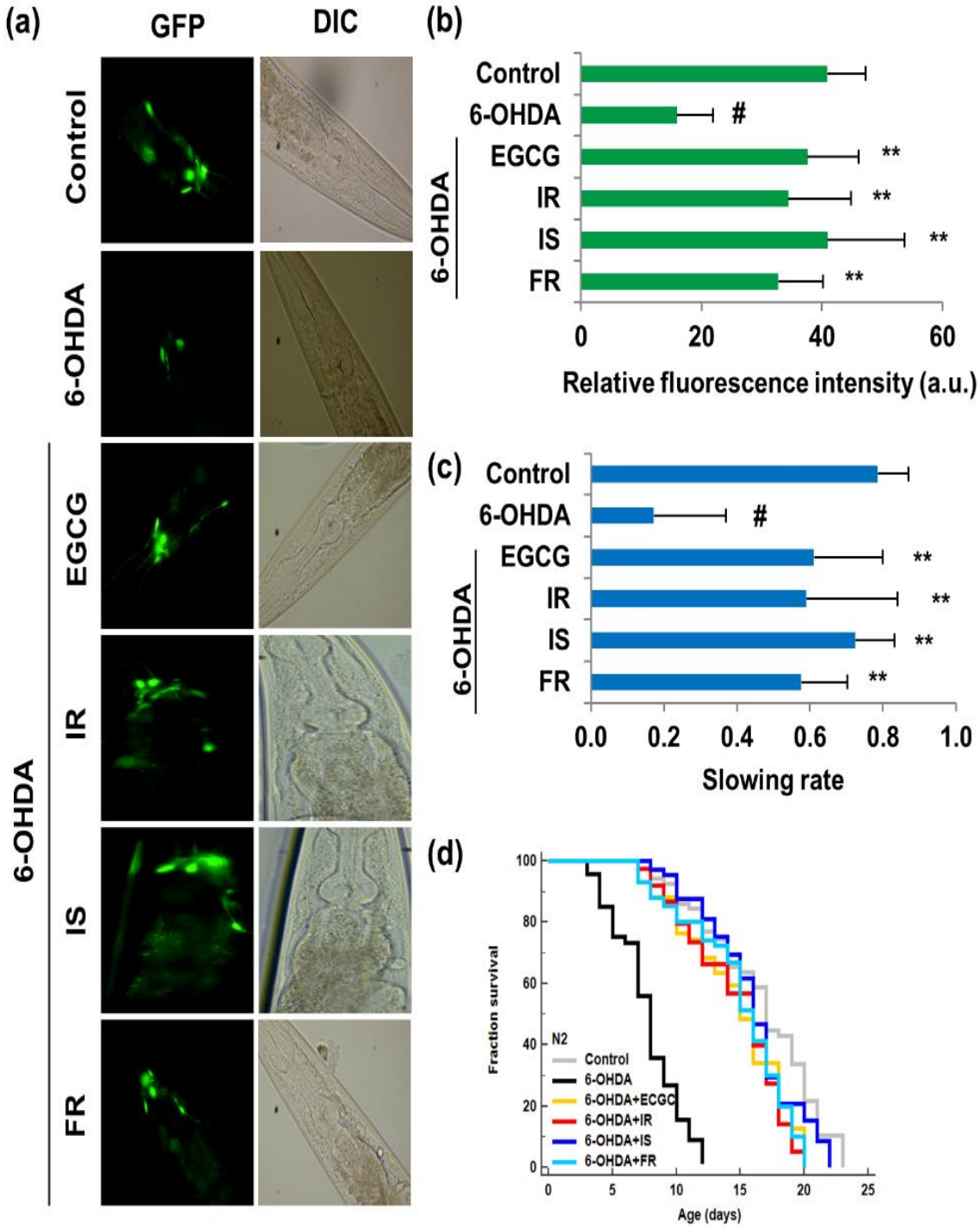
Effect of *W. somnifera* extract on (**a**, **b**) α-synuclein aggregation and (**c, d**) lipid content in worms expressing α-synuclein. The results are calculated from three independent biological trials and presented as the mean ± SEM. #p<0.01 vs. N2; *p<0.05 and **p<0.01 vs. control or NL5901. In the fluorescence micrograph, the anterior region of *C. elegans* was marked with an asterisk (*).

In addition, *W. somnifera* extracts (IR, IS, and FR) significantly expanded the lifespan of NL5901 worms by 23.89%, 43.68%, and 24.93%, receptively **(Table 4.16:)**. Since the pathogenesis of PD is associated with altered lipid and fatty acid levels due to the excessive accumulation of misfolded α-synuclein (Ruipérez *et al.,* 2017), therefore, we measured the lipid content in NL5901 worms using Nile Red staining. The results showed that NL5901 worms

exhibited dramatically lower lipid content (53.27%) compared to wild-type worms, however, treatment with *W. somnifera* extracts restored lipid levels throughout the body of worms that expressed the α-synuclein. The effect of IS extract on lipid levels in NL5901 worms was comparable to that of EGCG **(Figure 4.10 c and d**). These data strongly confirmed that *W. somnifera* extracts have the potential to reduce Parkinson's and associated pathologies.

To further explore the neuroprotective effects of *W. somnifera* extracts, the viability of dopaminergic (DAergic) neurons in worms exposed to 6-OHDA was measured. Neuronal viability was analyzed by quantifying the expression of GFP (Green Fluorescent Protein) transgene labeled in all eight DAergic neurons of BZ555 *C. elegans*. 6-OHDA, a catecholaminergic neurotoxin, was used as an inducer of experimental PD models. 6-OHDA enters DAergic neurons via dopamine-active transporter and disrupts neuronal functions (Simola *et al.,*2007). As shown in (**Figure 4.11 a**), 6-OHDA exposure reduced the GFP intensity by up to 60.98% in BZ555 worms compared to unexposed control groups, indicating their neurotoxic properties. Furthermore, when the worms were treated with IR, IS, and FR extracts, the neurons displayed enhanced GFP expression in all DAergic neurons (**Figure 4.11 b**). Moreover, treatment with IR, IS, and FR extracts increased the basal slowing response to food to 0.59±0.25, 0.72±0.11, and 0.58±0.13 in worms exposed to 6-OHDA (0.17±0.20). Untreated control group worms exhibited 0.79±0.08 as the slowing rate to food. It has been well established that dopamine-mediated neural networks control the slowing response to food in *C. elegans* (**Figure 4.11 c**). We next assessed the life-promoting efficacy of *W. somnifera* extracts in worms exposed to 6-OHDA. The results showed that 6-OHDA exposure reduced the mean lifespan by 53.39% (7.72±0.27 days); in contrast, treatment with IR, IS, and FR extracts extended the lifespan of worms intoxicated with 6-OHDA to 14.62±0.36, 15.97±0.36, and 14.93±0.37 days, respectively (**Figure 4.11 d, Table 4.19)** (Statistical data)**)**. These results indicated that *W. somnifera* extracts not only protect neurons from degeneration but also extend the lifespan of worms.

Figure 4.11: Neuroprotective efficacy of *W. somnifera* extracts against 6-OHDA-induced, degeneration of DAergic neurons, food sensing behavior, and lifespan.



Neuroprotective efficacy of *W. somnifera* extracts against 6-OHDA-induced (a, b) degeneration of DAergic neurons, (**c**) food sensing behavior, and (**d**) lifespan. The results are calculated from three independent biological trials and presented as the mean ± SEM. #p<0.01 vs. control; **p<0.01, vs. 6-OHDA. Survival curves were graphed using the Kaplan-Meier survival method and analyzed by a log-rank test.

Table 4.19: Life promoting ability of *W. somnifera* extracts in presence of neurotoxins

Genotype	Treatment (µg/mL)	Mean survival (Mean±SEM)	Maximum lifespan (days)	Sample size (N)	Censored	% Change	P Value
N2 (Wild)	Control	16.57±0.409	23	121	5		
	6-OHDA	7.72±0.265	12	93	4	(-) 53.39	0.0001#
	6-OHDA+ EGCG	14.61±0.406	20	102	6	(+) 89.15	0.0001*
	6-OHDA+ IR	14.62±0.356	20	113	0	(+) 89.32	0.0001*
	6-OHDA+ IS	15.97±0.364	22	106	8	(+) 106.77	0.0001*
	6-OHDA+ FR	14.93±0.369	20	116	11	(+) 93.29	0.0001*

The statistical data presented in the table are the life-promoting efficacy of *W. somnifera* extracts against 6-OHDA-induced worms. The results are calculated from three independent biological trials and presented as the mean ± SEM. #p<0.01 vs. N2; *p<0.05 and **p<0.01 vs. control or NL5901. samples - IR (*in vitro* root), IS (*in vitro* shoot), FR (field grown root), FS (field grown shoot).