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Summary and Conclusion

Plant-based compounds have witnessed an exponential growth in validating their drug activity over the past decades. Ayurvedic and Unani systems of India have used plants under the genera *Withania* for several thousand years as medicinal herbs. There are a number of health benefits associated with *W. somnifera* which are considered to be economically and medicinally valuable. In Siddha, Ayurveda and Unani medicine, *W. somnifera* is used in more than 200 traditional formulations for treating a variety of disorders. Bioactive constituents and extracts from these plants have been shown to possess valuable properties such as anticonvulsant, antioxidative, anticancer and neurological effects with little to no or tiniest side effects. In order to overcome the co-morbidities associated with COVID-19, *W. somnifera* and its metabolites were highly recommended for consumption. There has been a great deal of demand for the raw materials of the plant on the global market due to the therapeutic potential of the plant.

Despite tremendous efforts on their conservation, overexploitations of these plants are leading to their extinction. It is expensive and time-consuming to propagate plants using traditional methods. As well as these factors, chemotype variation, disease susceptibility and seasonal constraints contribute to poor secondary metabolite production. It can therefore be concluded that *in vitro* propagation of these plants is an effective method for producing desirable bioactive compounds and has a tremendous potential for supplying these secondary metabolites to the pharmaceutical industry.

A number of reports indicate that the plant has therapeutic potential due to the presence of withanolides. Approximately 4000 naturally occurring steroidal lactones belonging to the solanaceae family are known as withanolides. The accumulation of withanolides is highly tissue specific in spite of the fact that all parts of the plant contain multiple withanolides. Therefore, it is important to select the right tissue for tissue culture propagation. Traditional medicinal practitioners

advocate the use of field grown roots rather than field grown shoots, which are reported to be toxic. According to our previous study, withaferin A is synthesized in the shoots and is transferred to the roots, while withanolide A is synthesized in the roots and is transferred to the shoots. The ratio between withaferin A and withanolide A plays a critical role in determining pharmaceutical activity. Whereas withaferin A is concedered as toxic in high concentration.

Thus, the present study on **A study on Neuroprotective potential of *in vitro* and field tissues of *Withania somnifera* using *Caenorhabditis elegans* model**. In this study, *in vitro* and field grown tissues of *W. somnifera* are used to develop a formulation, its standardization, comparative analysis of metabolites, and the validation of bioactivity using *C. elegance* as a model organism.

Four phases were involved in the study. The first phase will involve standardization of the powder prepared using *in vitro* (*in vitro* shoot and root) and field raised tissues (field shoot and root) of *W. somnifera* in accordance with the standard method described in Pharmacopias. Standardization is required in order to ensure the quality of herbal based formulation. As part of the standardization process, the four powders were evaluated for authenticity, biological parameters, chemical parameters, physical parameters and analytical profiling to produce an herbal product that meets quality assurance quality. Standardization of herbal based medicine also involves the analysis of phytochemicals, quality assessment, the fingerprinting of metal ions, and the profiling of the metabolome. Thus, the second and third phases are completed with a comparative chemical analysis. To this effect, HPTLC analysis was performed to determine the *withanolide* content and to investigate how it varies from tissue to tissue. ICP-MS was used to determine the metal ion fingerprint. A study was conducted to determine whether any metals accumulated in the powders above the permissible level established by the WHO. Moreover, comparative metabolome profiling has been conducted to examine the differences in metabolome content between field tissues and *in vitro* tissues. The final phase of the study involved the comparative efficacy of *in vitro* and deal grown *W. somnifera* extracts to prevent α-synuclein aggregation, its associated pathologies, potential for oxidative stress resistance and its capability for neuroprotection in Parkinson's disease-modelled *C. elegans*.

In organoleptic evaluation all sample were examined for the senses of appearance, taste, smell, and touch. In pharmacogenetic study the percentage of foreign substances/matter was analysed. All samples were detected with the foreign matter less than 0.5% which signifies their good quality and purity. The API permissible limits for the foreign matter of herbal powders are 0.5–2%. The physicochemical analysis the pH, total ash, water-soluble ash and acid insoluble ash, moisture content was determined. The pH of *W. somnifera* powders showed slight acid nature, which might because of the acidic salts that are present in dried raw materials. The result for moisture percentage of IR, IS, FR, and FS are found to be within the standard range as mentioned by API, but it is observed that the FR contains a comparatively higher moisture percentage. It might be due to the higher thickness of field grown roots and the soil moisture. Since the plant has been cultivated in the semi arid regions of India and is considered a rain fed crop. The ash value indicates the inorganic salts that are present in the powdered formulations. Notably higher ash content was observed in FR. It represents contamination, substitution, and adulteration of raw materials taken from the field. Moreover, the water soluble ash and acid insoluble ash value are also higher in FR. The water-soluble ash is that part of the total ash value which is soluble in water. Acid insoluble ash specifically indicates contamination with siliceous materials such as earth and sand.

The flowability of the FR and IR was observed to be poor with a tapped density of (0.548±0.01) (0.542±0.02) and bulk density (0.446 ± 0.01) (0.442±0.02) respectively. This was additionally confirmed by high values of Hausner's ratio (1.23 and 1.22) and Carr's index (18.61%, 18.75%). These comparative results designated that the angles of repose, Hausner’s ratio, and Carr’s Index, FR and IR are showing some cohesiveness and IS and FS showing good flowability. Further, the pathogenic microorganisms such as *Salmonella spp, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were also absent in the four variant powders. Significantly, a moderate level of the microbial load was observed in powder prepared using *in vitro* samples (IS and IR) and it is due to the sterilized environmental condition that maintained throughout the cultivation period.

The primary analyses of the determination of major phytochemicals showed the presence of carbohydrates, proteins, tannins, alkaloids, phenols, flavonoids, and steroids. It was also noted that the saponins were absent IR extract. Among the four *W. somnifera* powder extracts, it was observed that the level of TPC (112.36± 2.18 mg GAE/g) and TFC (68.50± 0.453mg CE/g) were found to be higher in IS. The phytosteroid content (TSC) was found to be higher in FS (78.75 ± 5.91mg CHOL/g) and TAC is higher in IR (51.08± 2.07mg CF/g). So, the total phenols and total flavonoid content were higher among the *in vitro* shoot of the plant. Free radical scavenging activity (FRSA) was compared between *W. somnifera* roots and shoots cultivated *in vitro* and field grown. Comparably the IS showed a significantly lower IC50 value (872.15±8.73 µg/ml) than the other *W. somnifera* extract. The field shoots FS and IR extracts showed the highest IC50 values indicating the least antioxidant potential compared to the IS extract. Thus, the phytochemical content of the extracts were highly correlated to its antioxidant potential. From the DPPH radical scavenging activity, it was evident that besides the other phytochemical content, the TPC and TFC mainly serves as the determining factor towards the antioxidant potential of *W. somnifera*.

A methanolic extract of *in vitro* and field grown tissues was used to quantify WA, WFA and WTN which are the major secondary metabolites of *W. somnifera*. The findings of WA, WFA content back up a prior study that found WFA build-up to be tightly related to leaf tissue rather than roots. 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 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). So, a tissue specific accumulation pattern of major pharmacologically active metabolites was observed in *W. somnifera*.

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. Except for Pb and Cr, all other heavy metals are under the WHO permissible limit in all samples. The Cr concentration is higher than the WHO permissible limit in FR (0.5082 mg/Kg) and the Pb concentration is higher in FS (0.5023 mg/Kg) and is higher than the WHO permissible limit and it is due to the environmental pollution. It was that the *in vitro* plants are entirely free from heavy metal contamination.

In this study, metabolic profiling was conducted on shoots and roots of *W. somnifera* grown *in vitro* and in the field. Based on the GC-MS profile, a total of 33 metabolites were detected, including 13 organic acids, 9 amino acids, 3 sugars, 3 alcohols, and 4 other compounds. Several metabolites were found to be significantly different between *W. somnifera* samples grown *in vitro* and those grown in the field. In particular, the valeric acid present in the FS and IS was not detected in the FR and IR. Linoleaidic acid, Linolenic acid, Fumaric acid, and Levulinic acid were not detected in the metanolic extracts of FR and IR. Aziridine was not detected in IS, but all other compounds, such as Squalene, Putresin, and 2-pyrollidinone, were detected in field grown samples of FR, IS, and IR. In root samples like FR and IR, four amino acids were not detected, including L-Valine, Aspartic acid, Glycine and Glutamine. All *in vitro* and field grown samples were positive for Mannose, Glucose, and Galactose. PCA was used to simplify the complexity of the GC-MS data by plotting the 33 metabolites against two principal components, PC1 and PC2, which explained about 85% of the variation in the data. Biplot construction revealed a clear distinction between the FR, IR and FS, IS along the PC1, which showed a positive correlation with most amino acids and organic acids, particularly squalene, the precursor of withanolide production.

Under standard and laboratory conditions, the study concludes by demonstrating the anti-aging and neuroprotective effects of *in vitro* and field grown *W. somnifera,* as well as the involvement of the IIS pathway in *W. somnifera* mediated lifespan extension in the *C. elegans* model system. A

number of biologically active metabolites are present in the *W. somnifera*, including withanolides. *W. somnifera* supplementation increases the survival rate of worms in both standard and stress conditions. The presence of biologically active constituents of *W. somnifera*, such as WA and WFA, may have an impact on the lifespan and health span of *C. elegans*. Through their potent antioxidant activity, *W. somnifera* extracts treatment reduces intracellular ROS levels and increases the lifespan of short-lived mev-1 mutant worms. These extracts have also been proven to contribute to endogenous detoxification. It should be noted that the up-regulation of the antioxidant genes sod-3, gst-4, and ctl-1,2,3 as well as a decline in oxidative stress levels support the findings and may be responsible for the longevity extension, stress tolerance, and neuroprotection mediated by *W. somnifera*. The *W. somnifera* developed *in vitro* is capable of protecting *C. elegans* from 6-OHDA-induced neurodegeneration and other PD-associated pathologies without evidently altering overall physiological functions. Furthermore, the *W. somnifera* treatment extends the lifespan of 6-OHDA-exposed worms as well. Therefore, the study demonstrated that extracts from *in vitro* tissues of *W. somnifera* are as effective as roots grown in the field. These findings may lead to the development of innovative pharmaceuticals for treating neurological and aging-related illnesses, extending the lives of patients and improving their quality of life.

It has been concluded that the Plant tissue culture technique is a viable alternative to overcome the problems associated with the limited availability of *W. somnifera* and the increasing demand for this species. The *in vitro* grown shoots of *W. somnifera* were found to exhibit higher antioxidant activity and require shorter cultivation periods than the field grown leaves in our systematic study. An analysis of the GC-MS and HPTLC metabolites of in vitro cultured shoots of *W. somnifera* revealed that they are capable of accumulating metabolites comparable to those of roots grown in the field. Based on the results of testing of *W. somnifera* based drugs in *C. elegans* as a model organism, it has been determined that the in vitro shoots are safe and do not affect the natural progeny production of *C. elegans*. Additionally, *in vitro* shoots provide excellent life and health span extension, oxidative stress resistance, and neuroprotection in comparison to field root extracts. Therefore, the null hypothesis is rejected.

Recommendations for further studies

1. In collaboration with industrialists, large scale production of the *in vitro* developed shoots can be attempted.
2. It is necessary to study the mechanisms involved in the *W. somnifera*-mediated neuroprotection using *C. elegans* and using higher animals.
3. The design of bioreactors, along with the scaling up of technologies for the growth of cultures in large scale industrial bioreactors, should be standardised.
4. For the findings of this study to be substantiated, clinical trials of the in vitro developed shoot extracts are required.