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Research Article

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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Maintenance of *In Vitro* Tissues

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

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

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

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

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

Maintenance of *Caenorhabditis elegans*

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

Preparation of Plant Extract

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

Assay using *C. elegans* as a Model Organism

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

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

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

Statistical Analysis

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

RESULTS AND DISCUSSION

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

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

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

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

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

The full assessment of nematode growth and development of the wild population at various doses of plant extract is shown in Figures 2 and 3. In the laboratory, *C. elgans* are easily grown on agar plates, with *E. coli* (OP50) as a food source. Visual inspection of the bacterial turbidity on top of the agar demonstrated that the growth of *E. coli* (OP50) was unaffected by the various doses of plant extract employed in the toxicity assay. The analysis's findings showed that worms can be cultivated successfully on 96-well plates using a solid medium. First, we attempted to determine the maximum number of worms that can develop in a single well without depleting the available nutrients before the end of the reproduction cycle (six days).

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

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

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

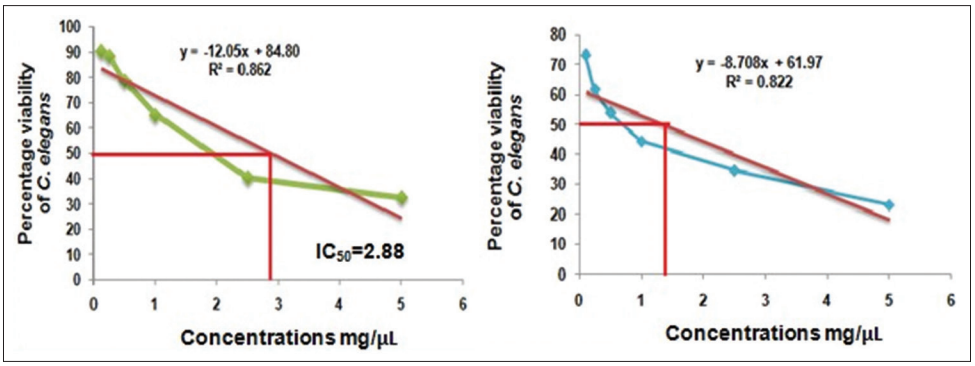

Figure 1: Influence of *W. somnifera* shoot/leaf samples on the percentage viability of *C. elegans*

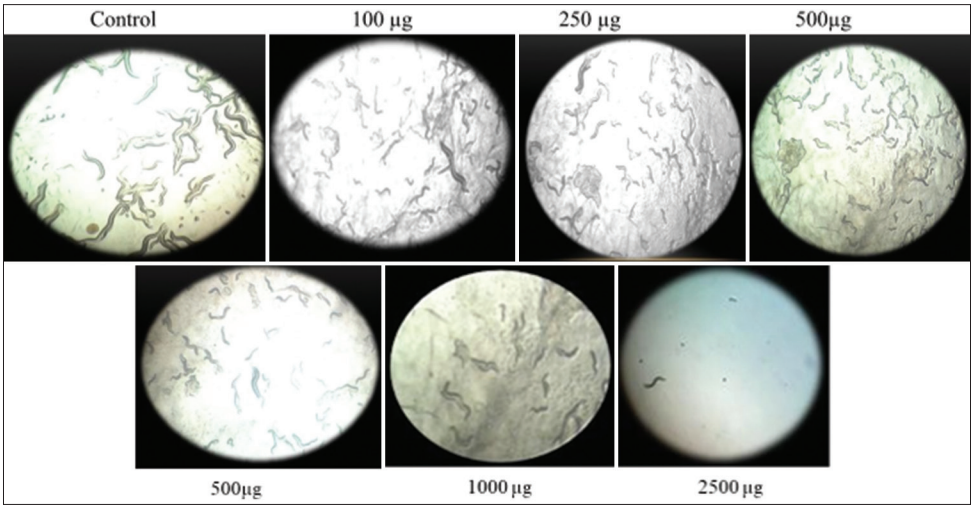

Figure 2: The plates indicate the toxicity effects of *in vitro* cultivated shoot samples of *W. somnifera* at different concentrations that affect the growth of *C. elegans*

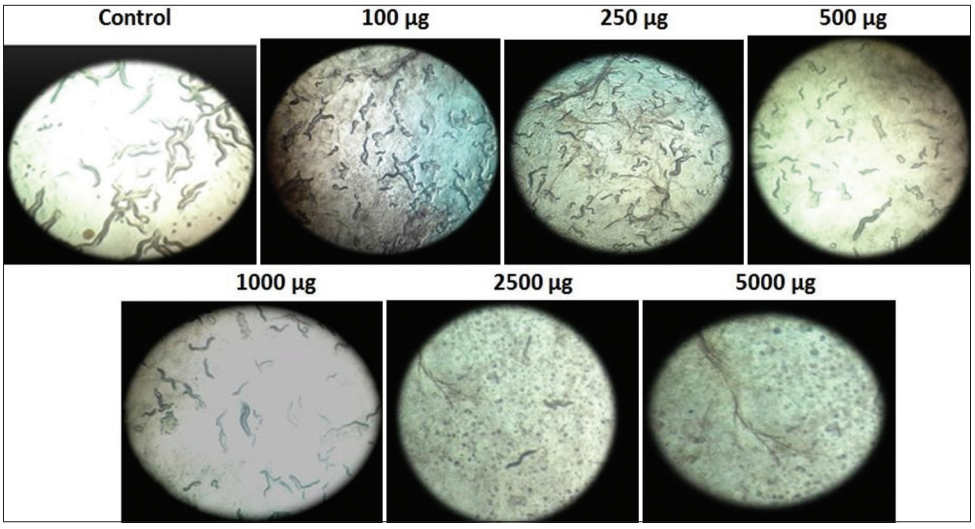

Figure 3: The plates indicate the toxicity effects of field grown shoot samples of *W. somnifera* at different concentrations that affect the growth of *C. elegans*

In order to determine cytotoxicity by plate-based assay, the worms were exposed to different concentrations (0-5.0 mg/µL) of *in vitro* and *in vivo* shoot extract of *W. somnifera*. Among the different concentrations, a higher concentration of plant extract above 1.0 mg/µL caused premature mortality of the worms. Exposure to concentrations between 0.1 mg/µL to 0.5 mg/µL showed a beneficial effect on an increasing developmental delay with associated delay in the reduction of nutrients.

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

CONCLUSION

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

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