Evaluation of in-vitro Cultured Cells of *Withania somnifera* for Antioxidant Activity

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Abstract

*Withania somnifera* (Solanaceae), commonly known as ashwagandha, is known to have anti-inflammatory, antitumor, anticonvulsive and immunosuppressive properties. In the present study, antioxidant potential of *in vitro* cultured cells and roots of *W. somnifera* was evaluated within the concentration range of 5-100 µg/ml using *in vitro* studies viz. free radical scavenging capacity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical cation decolourization assay, scavenging of nitric oxide radical and total antioxidant capacity. Ascorbic acid was used as standard compound. Among antioxidant screening models tested, ethanolic extracts of *W. somnifera* cells from transformed callus cultures had shown better antioxidant potential in comparison to that of roots and ascorbic acid. Therefore the results justify the therapeutic application of *W. somnifera* as an antioxidant in the indigenous system of medicine.

Key words: Antioxidant, Free radical scavenging capacity, *Withania somnifera*, Withanolides.

Introduction

Generation of free radicals is known to be involved in the development of various ailments like cancer, diabetes, liver cirrhosis, nephrotoxicity, Alzheimer’s disease, parkinsonism and inflammatory responses (1-4). These free radicals are produced in body as by products of biological redox reactions (5) and their concentrations exist in a dynamic equilibrium with antioxidants to quench and/or scavenge and then to protect the body against harmful effects of free radicals. Reactive oxygen species (ROS) mainly causes cumulative damage of DNA, proteins, lipids and membranes and thus oxidative stress. This may result in chromosomal aberrations and/or genetic alterations leading to carcinogenesis (6). Furthermore, imbalance between pro-oxidant and antioxidant homoeostasis results in various degenerative diseases. Presently, the use of complimentary and alternative therapy and especially the utilization of phytoconstituents have been significantly increasing worldwide. This is due to better acceptance of herbal products than synthetic drugs because of lesser side effects and better compatibility thus improving patient tolerance even on long-term use (7). Crude extracts from plants and a number of phytochemicals are known to posses excellent antioxidant potential and may serve as successful lead molecules in therapeutic armamentarium against these diseases.

*Withania somnifera*, also known as ashwagandha or Indian ginseng has been an important herb in the Ayurvedic and indigenous medical systems for more than 3,000 years (8). It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides.

In vitro culture of *Withania somnifera*
At present, 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. The principle withanolide in the Indian variety of the plant is withaferin A. This drug is known to have anti-inflammatory (9), antitumor (10), anticonvulsive (11) and immuno-suppressive properties (12). Preparation of crude extract from natural sources by solvent extraction method generally resulted in low content of therapeutically active phytoconstituents due to presence of impurity. Therefore crude extract prepared from in vitro cultured cells may provide a suitable alternative. Keeping this in view, callus cultures of W. somnifera was established in the present study. Crude extract from in vitro cultured cells of W. somnifera was evaluated for its antioxidant potential and compared to that of roots.

Materials and methods
Collection of plant material and germination of seeds

Plant materials (seeds and roots) of W. somnifera were collected from Neemuch district of Madhya Pradesh. The dried roots of the plant were used for extraction. Seeds were used to develop in vitro plants for initiation of cell cultures. Seeds of W. somnifera were washed in 1% savlon and then treated with 0.1% bavestin and rinsed five to six times with sterile double-distilled water (SDDW). Surface sterilization was performed using 70% v/v ethanol treatment for 30 s and rinsed thrice with SDDW. This was followed by treatment with 0.01% w/v mercuric chloride for 5 min and rinsing with SDDW for four to five times. For aseptic germination, sterilized seeds were then placed on Murashige and Skoog (MS) medium (13) with 30 g/l sucrose and 7 g/l agar at 25±2°C in a 16/8-h light/dark cycle with a light intensity of 1,200 lux.

Initiation of transformed callus cultures

Hypocotyls were used as explants for transformation by Agrobacterium tumefaciens strain (MTCC 2250) procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Explants from 25-day-old in vitro germinated plants were used for culture initiation studies. For this, bacterial colonies were cultured for 2 days on solid yeast mannitol broth medium (YMB) at 25±2°C. The culture (2% v/v) was reinoculated in liquid YMB medium and grown till they achieved an optical density of ~ 1.0 at 600 nm. The suspension was then centrifuged at 6,000 g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 5 ml of fresh liquid YMB media. This concentrated culture was used further for the infection of plant materials.

Forty explants were kept in a sterile plate, pricked manually with a 24-gauge metal needle (~5 wound per cm²), dipped in Agrobacterium culture, and incubated for 5 min. The liquid YMB medium without bacteria was applied to the explants as a control. The infected explants were preincubated for cocultivation at 25±2°C for 48 h on sterile MS medium, solidified with 10 g/l agar. The infected explants were then transferred to an antibiotic, cefotaxime (1 g/l) containing MS medium to check the overgrowth of bacteria and were incubated at 25±2°C in 16/8-h light/dark regime. The transformed cultures were then transferred to fresh MS medium containing 1 g/l cefotaxime. Axenic cultures were obtained by subsequent subculture to fresh MS medium for every 7 days containing the antibiotic. Axenic cultures of W. somnifera were maintained on 100 ml of MS medium solidified with 8 g/l agar in 500 ml Erlenmeyer flasks by transferring 2 g
of fresh water to each flask. Cells were harvested after 20 days for extraction.

**Preparation of extracts and phytochemical tests**

The collected roots and in vitro cultured cells of *W. somnifera* were air dried at 27±2°C, powdered and stored in an air tight container at 27±2°C till further use. These dried and powdered roots and cells (200 g each) were accurately weighed and defatted with 1 litre petroleum ether (40-60). It was then extracted separately with 1 litre ethanol in a soxhlet for 36 h. The extracts were filtered, evaporated to dryness under vacuum and stored in the desiccators for use in subsequent experiments. The qualitative chemical investigations of ethanolic extracts obtained from in vitro cultured cells and air dried roots of *W. somnifera* were carried out to check the presence of various phytoconstituents (15).

**Assessment of anti-oxidant activity**

The assessment of anti-oxidant activity was carried out using following methods:

1. **Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity**

For the present study, the samples were prepared in different concentrations i.e. 5-100 µg/ml in methanol. The samples of above concentrations were mixed with 3 ml of 100 µM DPPH prepared in methanol and final volume was made up to 4 ml with methanol. The absorbance of the resulting solutions, in triplicate, and the blank (with same chemicals except sample, if required) were recorded after 20 min at 25±2°C against ascorbic acid. The disappearance of color was read spectrophotometrically at 517 nm using a Shimadzu visible spectrophotometer. Radical Scavenging Capacity (RSC) in percent was calculated by following equation:

\[
\text{RSC (\%)} = 100 \times \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}
\]

Where;

**RSC** = Radical Scavenging Capacity,  
**A**<sub>blank</sub> = Absorbance of blank,  
**A**<sub>sample</sub> = Absorbance of sample,  

From the obtained RSC values, the IC<sub>50</sub> were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization (16).

2. **ABTS radical cation decolorization assay**

ABTS radical cation (ABTS<sup>•+<sup>) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulfate and the mixture was allowed to stand in dark at 25±2°C for 12-16 h before use. For this study, different concentrations (5-100 µg/ml) of the ethanolic extracts (2 ml) were added to 1.2 ml of ABTS solution and the final volume was made up with ethanol to 4 ml. The absorbance was read at 745 nm and the experiments were performed in triplicate (17).

3. **Scavenging of nitric oxide radical**

Nitric oxide is generated from sodium nitroprusside and measured by Griess’ reaction (18,19). Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations (5 -100 µg/ml) of the ethanolic extracts dissolved in phosphate buffer saline and the tubes were incubated at 25±2°C for 5 h. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylenediamine was recorded at 546 nm. The experiment was repeated in triplicate (20).
Total antioxidant capacity

Ethanolic extracts of *W. somnifera* (100 µg) were added, in triplicate, to a mixture of ammonium molybdate (4 mM) and sodium phosphate (28 mM) in 0.6 M H₂SO₄ in total volume of 2 ml in eppendorff tubes and kept at 95 ±2°C for 90 min and the absorbance was measured at 695 nm after cooling at 25±2°C (21).

IC₅₀ was calculated by using formula:

\[
b = \frac{\Sigma x \cdot y}{\Sigma x^2}
\]

\[
a = y - bx
\]

\[
\text{IC}_50 = a + b (50)
\]

where, \(b\) = Regression coefficient of \(x\) on \(y\); \(a\) = Intercept of the line; \(x\) = Concentration in µg/ml; \(y\) = % Scavenging; \(x\) = Mean of concentration; and \(y\) = Mean of % scavenging.

Statistical analysis

The results were expressed as mean values. The significance of statistical analysis was performed by ANOVA followed by Dunnett’s test and P values (< 0.05 and < 0.01) implied significance.

Results and Discussion

In present study, transformed callus cultures of *W. somnifera*, capable to produce withaferin A, were developed and evaluated for antioxidant potential. DPPH radical scavenging capacity, nitric oxide scavenging effect and ABTS assay were used for establishment of antioxidant potential of *W. somnifera* in comparison to ascorbic acid as standard compound within the concentration range of 5-100 µg/ml. Extraction of roots and cells of *W. somnifera* using soxhlet apparatus resulted in crude extracts with yields of 13.4 % w/w and 12.3 % w/w respectively. Preliminary phytochemical screening of ethanolic extracts revealed the presence of steroids and alkaloids. Their presence was further confirmed qualitatively by thin layer chromatographic studies. DPPH is a relatively stable free radical and this method determines the ability of ethanolic extract of *W. somnifera* roots (TER) and in vitro cultured cells (TEC) to reduce the DPPH radical to the corresponding hydrazine by reacting with the hydrogen donors in the antioxidant principles (22). DPPH radicals convert the unpaired electrons to the paired one and the solution loses colour stoichiometrically depending on the number of electrons taken up (23). The dose dependant inhibition of DPPH by extracts (TEC and TER) and ascorbic acid is given as fig. 1. IC₅₀ values of 32.55 µg/ml, 40.4 µg/ml and 52.5 µg/ml were obtained from ethanolic extracts from cells from callus cultures (TEC), roots (TER) and ascorbic acid respectively. Ethanolic extracts of *W. somnifera* exhibited better antioxidant potential in comparison to ascorbic acid as evidenced by lower IC₅₀ values respectively in DPPH assay.

![Fig. 1: DPPH scavenging capacity of *W. somnifera* extracts and ascorbic acid](image)

Fig. 1: DPPH scavenging capacity of *W. somnifera* extracts and ascorbic acid [TEC - Total extract from in-vitro culture cells, TER - Total extract from roots, Asc. acid - Ascorbic acid]

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The ABTS decolourization assay results in direct generation of ABTS radical mono cation prior to addition of antioxidant components instead of in presence of it. The concentration dependent inhibition/scavenging properties of TER, TEC and ascorbic acid towards ABTS are given as fig. 2. TEC had exhibited comparatively higher antioxidant potential with IC\textsubscript{50} value of 41.8 µg/ml in comparison to TER and ascorbic acid with respective IC\textsubscript{50} values of 48.9 µg/ml and 51.56 µg/ml.

Chemically NO is very unstable under aerobic condition and it produces nitrite and nitrate upon reacting with O\textsubscript{2} through intermediates like NO\textsubscript{2}, N\textsubscript{2}O\textsubscript{4} and N\textsubscript{3}O\textsubscript{4}. In the present study, incubation of sodium nitroprusside in phosphate saline buffer had resulted in generation of nitrite, which was reduced by ethanolic extracts of \textit{W. somnifera}. This effect may be due to competition of antioxidant compounds present in extract with oxygen to react with nitric oxide (24), which ultimately leads to inhibition of generation of nitrite. Effect of different concentrations of ethanolic extracts (TER and TEC) on nitric oxide scavenging capacity was determined and results are presented as fig. 3. The extracts, TEC and TER, exhibited scavenging potential with IC\textsubscript{50} values of 17.55 µg/ml and 25.69 µg/ml respectively. These values were significantly lower than ascorbic acid (46.67 µg/ml) used as standard in the assay indicating higher antioxidant activity of extracts of \textit{W. somnifera}. Total antioxidant potential of extracts was also determined by formation of phosphomolybdenum complex under acidic conditions. Ethanolic extract of \textit{in vitro} cultured cells exhibited slightly higher total antioxidant activity (326) in comparison to that of roots of \textit{W. somnifera} (264).

\textbf{Conclusion}

The present study proved promising antioxidant potentials of ethanolic extracts of \textit{W. somnifera}.
*W. somnifera*. It is reported that secondary products have good antioxidant activities. Therefore the antioxidant activity of *W. somnifera* may be attributed to the presence of these compounds. Furthermore ethanolic extract of *in vitro* cultured cells had shown better free radical scavenging capacity in comparison to roots. This might be due to presence of other compounds/impurities in ethanolic extracts from roots. Hence these results support the view that some traditionally used Indian medicinal plants are a promising source of potential antioxidants.

**References**


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