

PHYTOCHEMICAL ANALYSIS AND EVALUATION OF ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *WITHANIA SOMNIFERA* ROOT

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ABSTRACT

Withania somnifera, also known as 'Ashwagandha', is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years. In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. The present study was performed to evaluate the phytochemicals and *in vitro* antioxidant activity of 70% methanolic extract of *W. somnifera* roots. The extract was used to determine the antioxidant potential using different tests including total antioxidant activity; efficiencies for scavenging of hydroxyl, superoxide, nitric oxide, singlet oxygen radicals, hypochlorous acid and inhibition of lipid peroxidation. The results obtained indicate that although *W. somnifera* has been widely used for centuries as immunostimulants in different ailments including neurodegenerative disorders, the 70% methanolic extract of the same also has possibility as an antioxidant and free radical scavenging agent.

Keywords: Phytochemical screening, reactive oxygen species, reducing power

INTRODUCTION

Since time immemorial, medicinal plants are of great importance to the health of individuals and communities. The medicinal plant products, which are derived from plant parts such as stem bark, leaves, fruits and seeds have been part of phytochemistry that produce a definite physiological action on the human body. The most important of these natural bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds¹. A number of reactive molecules are generated through various biological redox reactions such as superoxide radical (O_2^-), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) and nitric oxide (NO)² which can directly react with biological macromolecules such as proteins, lipids and DNA of healthy human cells and cause cell membrane disintegration, DNA mutation and protein damage. Deregulation of these reactive oxygen species (ROS) can further create cancer, atherosclerosis, cardiovascular disease, liver injury, ageing and inflammatory disease³. Antioxidants act as oxygen scavengers by interrupting the oxidation process by reacting with free radicals, chelating catalytic metals⁴. Some synthetic antioxidants were developed in the past few decades but they are suspected of having some adverse effects. Therefore, in search of suitable alternative natural antioxidants has received much attention to identify and develop more potent antioxidants of natural origin to replace synthetic ones. Different kinds of plant material have already been reported as natural antioxidant⁵.

Withania somnifera (L.) Dunal (Solanaceae) grows in many of the drier regions of Indian subcontinent and in tropical and subtropical zones of the Mediterranean region and northern Africa to Southwest Asia. In Ayurveda, *Withania* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life prolonging properties⁶. The plant was traditionally used to promote youthful vigor, endurance, strength, and health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells. The similarity between these restorative properties and those of ginseng roots has led to ashwagandha roots being called Indian ginseng⁷. The roots are used as a nutrient and health restorative in pregnant women and old people. The decoction of the root boiled with milk and ghee is recommended for curing sterility in women. The roots are also used in constipation, senile debility, rheumatism, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea⁸. It has been used owing to its antioxidant property to treat rheumatism and neurodegenerative disorders^{9,9}. Recently, it has been developed as an immunosuppressive agent for the inflammatory diseases^{10,11}. However, the scope for the current study lies in the fact that no earlier report has been made for the antioxidant capacity of the 70% methanolic root extract of *W. somnifera*.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), 2-deoxy-2-ribose, mannitol, lipoic acid, quercetin, sodium nitroprusside, 1,10-phenanthroline and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Folin-ciocalteu reagent, Mercuric chloride, Potassium Iodide, Anthrone, Vanillin, 2,4-Dinitrophenylhydrazine, Thiourea and *N,N*-dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. DPPH, gallic acid, (+) catechin and curcumin were obtained from MP Biomedicals, France. Catalase, reserpine and Sodium bi carbonate were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue, Lead acetate were purchased from BDH, England. D-glucose was procured from Qualigens Fine Chemicals, Mumbai. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

Plant Material and Extraction

The plant material was bought from local vendor of Kolkata, West Bengal, India. The root powder (100 g) of the dried root of *W. somnifera* was stirred using a magnetic stirrer with 1000 ml mixture of methanol: water (7:3) for overnight; then the mixture was centrifuged at 2850 x g and the supernatant decanted. The process was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator and lyophilized. The dried extract was stored at -20°C until use. Double distilled or MilliQ grade water was used to dissolve the extract and for further dilution during the experiments.

Animals

The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/ CPCSEA) approved the use of adult male Swiss Albino mice (*Mus musculus*), weighing 20-25 gm for experimentation. Each polypropylene cage contained 4 mice at a time; supplied with *ad libitum* laboratory diet and water. The mice were kept at 25 ± 2°C and 60 ± 5% humidity and normal photo cycle (12 h dark/12 h light).

Phytochemical Analysis

Qualitative tests

Phytochemical analysis of 70% methanol extract of *W. somnifera* root extract was carried out using standard qualitative methods as

described previously by Harborne and Baxter¹², Kokate et al¹³. The components analysed for phytochemicals were alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids anthraquinones, and triterpenoids.

Quantification of total phenolic content

The total phenolic content present in the 70% methanol extract of *W. somnifera* root was determined using Folin-Ciocalteu (FC) reagent by a formerly reported method¹⁴. Briefly, the plant extract (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.06% Na₂CO₃ (0.75 ml) solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. All tests were performed six times. The phenolic content was evaluated from a gallic acid standard curve.

Quantification of total flavonoid content

The amount of total flavonoid content was determined with aluminium chloride (AlCl₃) according to a known method¹⁴. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by NaNO₂ (0.03 ml, 5%). After 5 min at 25°C, AlCl₃ (0.03 ml, 10%) was added. After a further 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All tests were performed six times. The flavonoid content was calculated from a quercetin standard curve.

Quantification of carbohydrate content

Quantification of carbohydrate content was carried out using previously described method by Sadashivam et al¹⁵ with slide modification. 100 mg of the 70% methanolic extract of root was weighed into a test tube, hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. The volume was made to 100 ml and centrifuged. 0.25 ml supernatant was made up to 0.5 ml with distilled water and mixed with anthrone reagent (4 ml) and was incubated at 95°C for 8 min. After incubation, cooled rapidly and absorbance was measured of green to dark green colour at 630 nm. All tests were performed six times. The carbohydrate content was evaluated from a glucose standard curve.

Quantification of alkaloid content

Quantification of alkaloid content was carried out using previously reported by Dhruv et al¹⁶ with slide modification. To the 1 ml of extract (1 mg/ml) in water 0.1 ml of FeCl₃ (2.5 mM FeCl₃ in 0.5 M HCl) was added followed by addition of 0.1 ml 1,10 phenanthroline. After incubation for 30 min at 70°C the absorbance was taken at 500 nm. All tests were performed six times. The alkaloids content was evaluated from the reserpine standard graph.

Quantification of tannin content

This was assayed as described by Robert¹⁷ with a slight modification. 0.1 ml aliquots of 70% methanolic extract (1 mg/ml) in water were mixed with the 0.5 ml vanillin hydrochloride reagent (Mix equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solutions were mixed just before use, and avoid using even if it is slightly colour). After incubation for 20 min at room temperature the absorbance was measured of magenta-pink colour at 500 nm. All tests were performed six times. The tannin content was evaluated from the catechin standard graph.

In Vitro Antioxidant and Free Radical Scavenging Activity

Total antioxidant activity

The assay was performed on the ability of test sample to scavenge ABTS⁺ radical cation in comparison to trolox standard¹⁴. The ABTS⁺ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12–16 h in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the ABTS⁺ solution was equilibrated to 0.70 (± 0.02) by diluting with water at room temperature, then 1 ml was mixed with 10 µl of the test sample and the absorbance was measured at

734 nm after 6 min. All experiments were repeated six times. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC). To calculate the TEAC, the gradient of the plot for the sample was divided by the gradient of the plot for trolox.

DPPH radical scavenging assay

The complementary study for the antioxidant capacity of the plant extract was confirmed by the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay according to Mahakunakorn et al¹⁸, with slight modification. Different concentrations of the extract and the standard trolox were mixed with equal volume of ethanol. Then 50 µl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for a few minutes before the OD was measured at 517 nm. The measurement was repeated with six sets. The percentage of scavenging was calculated from the values of the control and the test samples.

Hydroxyl radical scavenging

Hydroxyl radical scavenging assay was performed by a standard method¹⁴. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe³⁺-ascorbate-EDTA-H₂O₂ system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH₂PO₄-KOH buffer (20 mM, pH 7.4); FeCl₃ (100 µM); EDTA (100 µM); H₂O₂ (1.0 mM); ascorbic acid (100 µM) and various concentrations of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Mannitol, a classical OH[•] scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

Superoxide radical scavenging

Measurement of superoxide anion scavenging activity was done based on the reduction of NBT according to a previously described method¹⁴. The nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (0–20 µg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed six times. Quercetin was used as positive control.

Nitric oxide radical scavenging

At physiological pH, nitric oxide generated from sodium nitroprusside (SNP) aqueous solution interacts with oxygen to produce nitrite ions measured by Griess Illosvoy reaction¹⁴. The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0–70 µg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Curcumin was used as a standard.

Peroxynitrite anion scavenging

Peroxynitrite (ONOO⁻) synthesis was carried out by the method as described by Beckman et al¹⁹. Acidic solution (0.6 M HCl) of 5 ml H₂O₂ (0.7 M) was mixed with 5 ml of 0.6 M KNO₂ on an ice bath for 1 s and 5 ml of ice-cold 1.2 M NaOH was added to the reaction mixture.

Excess H₂O₂ was removed by the treatment of granular MnO₂ prewashed with 1.2 M NaOH and the reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$).

Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity. The assay was performed according to a standard method¹⁴. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various doses of plant extract (0–200 $\mu\text{g}/\text{ml}$) and 1 mM peroxynitrite in a final volume of 1 ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The percentage scavenging of ONOO⁻ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as the reference compound.

Hydrogen Peroxide Scavenging Assay

As previously described, this activity was determined by FOX-reagent method using sodium pyruvate as a reference compound¹⁴. An aliquot of 50 mM H₂O₂ and various concentrations (0–2 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 μl of the reaction mixture was mixed with 10 μl HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 0.25 M H₂SO₄). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm.

Singlet oxygen scavenging

The production of singlet oxygen (¹O₂) was determined by monitoring *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a earlier reported method¹⁴. Singlet oxygen was generated by a reaction between NaOCl and H₂O₂ and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H₂O₂, 50 mM histidine, 10 μM RNO and various concentrations (0–200 $\mu\text{g}/\text{ml}$) of sample in a final volume of 2 ml. It was incubated at 30°C for 40 min and the decrease in RNO absorbance was measured at 440 nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times.

Hypochlorous acid scavenging

Hypochlorous acid (HOCl) was prepared just before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to pH 6.2 with 0.6 M H₂SO₄ and the concentration of HOCl was determined by taking the absorbance at 235 nm using the molar extinction coefficient of 100 M⁻¹ cm⁻¹. The assay was done according to a previously described method¹⁴. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM) and increasing concentrations (0–100 $\mu\text{g}/\text{ml}$) of plant extract. The assay mixture was incubated at 25°C for 20 min and the absorbance was measured against an appropriate blank. All tests were performed six times. Ascorbic acid, a potent HOCl scavenger, was used as a reference.

Reducing power

The Fe³⁺ reducing power of the extract was determined by an earlier method¹⁴. Different concentrations of the extract (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml FeCl₃ solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

Lipid peroxidation inhibition

The antioxidant capacity of the plant extract was alternatively measured by lipid peroxidation inhibition, following an earlier method²⁰. Brain homogenate was prepared by centrifuging Swiss Albino mice brain with 50 mM phosphate buffer and 120 mM KCl. An aliquot of the supernatant homogenate was mixed with plant extract of various concentrations, followed by addition of 0.1 mM FeSO₄ and 0.1 mM ascorbic acid, and incubated for 1 h at 37°C to generate the TBARS (Thiobarbituric acid reactive substance). After stopping the reaction with TCA, TBA was added and the absorbance of the supernatant was taken at 532 nm. All tests were repeated six times. Trolox was used as the standard.

Statistical Analysis

All data were reported as the mean \pm SD of six measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit). The IC₅₀ values were calculated by the formula, $Y = 100 \times A_1 / (X + A_1)$ where $A_1 = \text{IC}_{50}$, $Y = \text{response}$ ($Y = 100\%$ when $X = 0$), $X = \text{inhibitory concentration}$. The IC₅₀ values were compared by paired *t*-test, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Phytochemical Analysis

The phytochemical screenings of chemical constituents related to biological activity of the plant root extract are alkaloids, flavonoids, phenolics, carbohydrates, tannins and terpenoids (Table 1). The quantitative phytochemical estimation indicate that *W. somnifera* root extract contains significant amount of phenolic, flavonoids, carbohydrate, tannin and alkaloid content (Table 2) which confirms its antioxidant property. Phenolics content are very important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator²¹. They also act as radical scavenger due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process²². The phytochemical analysis showed that *W. somnifera* extract has 180.80 \pm 0.01 mg/100 mg extract gallic acid equivalent phenolic content and 136.97 \pm 0.01 mg/100 mg extract quercetin equivalent flavonoid content, carbohydrate content was found to be 19.01 \pm 0.03 mg/100 mg extract glucose equivalent, alkaloid was 119.7 \pm 0.58 mg/100 mg extract reserpine equivalent and tannin was 0.6 \pm 0.01 mg/100 mg extract catechin equivalent (Table 2). Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity²³. Also, the carbohydrates in food are of major interest in relation to chronic diseases. Different types of carbohydrates give rise to different glycaemic responses, and also able to stimulate lipogenesis^{24,25}. Moreover, in the medicinal effects described in the ayurvedic, siddha, folk and chinese traditional recipe tannins, phenolic acids, flavonoids and alkaloids are the important ingredients to prevent against oxidative stress and decrease the activity of cholinesterase and xanthine oxidase and also alleviating the mucus secretion in the airway glands²⁶. The results acquired in this study thus suggest the identified phytochemical compounds may be the bioactive constituents and this plant is proving to be a valuable reservoir of bioactive compounds of substantial medicinal merit.

Table 1: Preliminary (Qualitative) phytochemical analysis of 70% methanolic extract of *W. somnifera* root.

PHYTOCHEMICALS	ROOT EXTRACT
Phenolics	+
Flavonoids	+
Carbohydrate	+
Alkaloids	+
Tannins	+
Terpenoids	+
Triterpenoids	-
Anthraquinones	-
Saponins	-
Glycosides	-

'+' Represents presence of the phytoconstituent; '-' represents absence of the phytoconstituent

Table 2: Quantitative estimation of some phytochemicals

PHYTOCHEMICAL	AMOUNT PRESENT
Total phenolics (mg/100 mg extract gallic acid equivalent)	180.80 ± 0.01
Total flavonoids (mg/100 mg extract quercetin equivalent)	136.97 ± 0.01
Carbohydrate (mg/100 mg extract glucose equivalent)	19.01 ± 0.03
Alkaloid (mg/100 mg extract reserpine equivalent)	119.7 ± 0.58
Tannin (mg/100 mg extract catechin equivalent)	0.6 ± 0.01

Data are expressed as mean ± S.D, (n=6).

Total antioxidant capacity

The reaction between ABTS and potassium persulfate results in the formation of a blue coloured chromophore, ABTS⁺. The total antioxidant activity of extract was calculated based on the decolourization of the ABTS⁺, which was measured spectrophotometrically at 734 nm. After addition of the plant extract and trolox, this pre-formed radical cation was converted to ABTS on a concentration dependent manner (Fig. 1). The results are compared with trolox and the TEAC value was found to be 0.033 ± 0.01 (Table 3). The obtained TEAC value demonstrates the extract as a poor antioxidant.

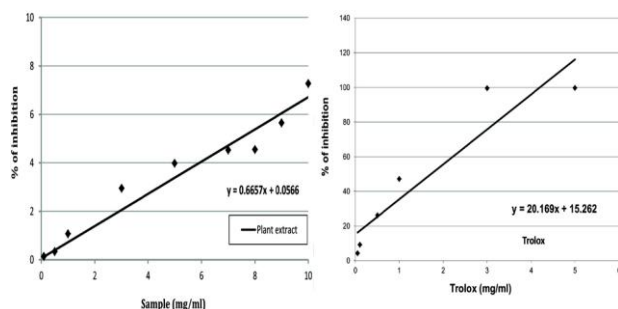


Fig. 1: Total antioxidant activity: Total antioxidant activity of *W. somnifera* extract and reference compound trolox on ABTS radical cation decolourization assay. The percentage of inhibition was plotted against concentration of sample. All data are expressed as mean ± S.D. (n=6).

DPPH radical scavenging assay

The inefficiency of the plant extract in the scavenging assay of DPPH radical (Fig. 2) furthermore assured the fact that the extract is not a good antioxidant, since the study on TEAC and DPPH scavenging can be observed as complementary to each other²⁷. The extracts showed unconvincing DPPH radical scavenging activity starting from very high concentration of extract compared to the standard. The IC₅₀ values (Table 3) of the root extract and standard ascorbic acid were 650.37 ± 107.18 µg/ml and 5.29 ± 0.28 µg/ml respectively.

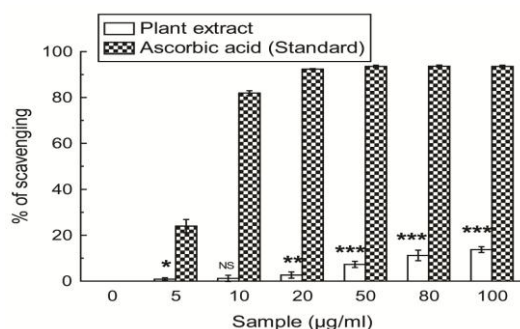


Fig. 2: DPPH radical scavenging activity of *W. somnifera* and standard ascorbic acid. Each value represents mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and *p < 0.001 vs 0 µg/ml. NS = Non-significant**

Hydroxyl radicals scavenging activity

The hydroxyl radical is one of the reactive free radicals formed in biological systems. It causes enormous damage on biomolecules of the living cells²⁸. In course of the Fenton reaction, hydroxyl radicals are formed by incubating Ferric-EDTA with ascorbic acid and H₂O₂ at pH 7.4 that cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH. As the *W. somnifera* extract or standard mannitol is added to the reaction mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked (Fig. 3). The IC₅₀ values of extract and standard were found as 1808.69 ± 391.16 µg/ml and 580.33 ± 20.23 µg/ml, respectively (Table 3) which indicates that the plant extract is not a better hydroxyl radical scavenger than standard mannitol.

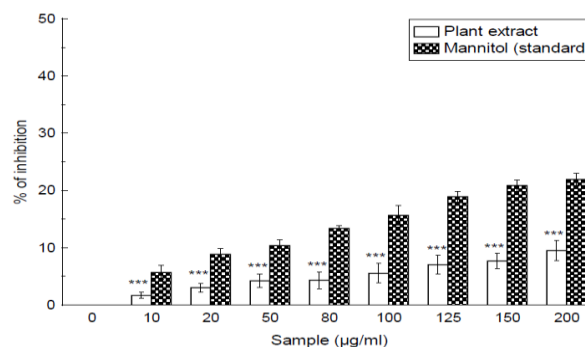


Fig. 3: Hydroxyl radical scavenging: Hydroxyl radical scavenging activity of the *W. somnifera* extract and the reference compound mannitol. The data represent the percentage of inhibition of deoxyribose

Table 3: Comparison of the antioxidant and free radical scavenging capacities of 70% methanolic crudes of *W. somnifera* and standard reference compounds

NAME OF ASSAY	<i>W. SOMNIFERA</i> ROOT EXTRACT	STANDARD	VALUES OF STANDARD COMPOUNDS
TEAC Values	0.033 ± 0.01	-	-
∅ IC ₅₀ values of the extracts for free radical scavenging capacity for:			
DPPH	650.37 ± 107.18***	Ascorbic acid	5.29 ± 0.28
Hydroxyl radical (OH [•]) scavenging	1808.69 ± 391.16***	Mannitol	571.45 ± 20.12
Nitric oxide radical (NO) scavenging	405.91 ± 145.84**	Curcumin	90.82 ± 4.75
Peroxynitrite (ONOO ⁻) scavenging	3427.18 ± 1212.65***	Gallic acid	876.24 ± 56.96
Singlet oxygen (¹O ₂) scavenging	234.49 ± 37.69***	Lipoic acid	46.15 ± 1.16
Hypochlorous acid (HOCl) scavenging	328.99 ± 35.92***	Ascorbic acid	235.96 ± 5.75
Lipid peroxidation	284.13 ± 146.66**	Trolox	6.76 ± 0.17

∅ Unit of IC₅₀ for all activities is µg/ml. Data are expressed as mean ± S.D, (n=6), where- **p < 0.01, ***p < 0.001.

Superoxide anion scavenging capacity

Superoxide anion is also implicated as harmful reactive oxygen species. In biological system cellular components get damaged in contact with superoxide anion. PMS-NADH coupling reaction yields dissolved oxygen which in turn changed into superoxide radicals. These superoxide radicals can be measured by its ability to reduce NBT. The ability of the plant extract and the reference compound quercetin to quench superoxide radicals from reaction mixture is

reflected in the decrease of the absorbance at 560 nm. However, the results obtained for the plant extract were not in accordance to the expected trend and hence not shown.

Nitric oxide scavenging assay

Nitric oxide radical is well known as it has an important role in various types of inflammatory process. The production of nitric oxide radical at a sustained levels result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis²⁹. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. *W. somnifera* extract also moderately inhibits nitrite formation by directly competes with oxygen to react with nitric oxide. Curcumin was used as a reference compound. The scavenging activity of extract and curcumin was shown in fig. 4 and 405.91 ± 145.84 $\mu\text{g/ml}$ and 90.82 ± 4.75 $\mu\text{g/ml}$ were determined as IC_{50} , respectively (Table 3). The present study proved that the nitric oxide scavenging activity of the studied extract is not as good as the standard curcumin.

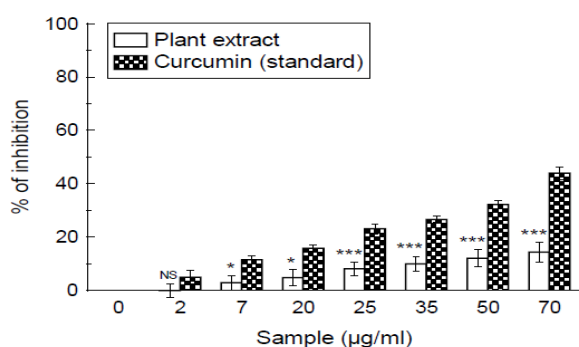


Fig. 4: Nitric oxide radical scavenging: The nitric oxide radical scavenging activity of *W. somnifera* extract and standard curcumin. The data represents the % of nitric oxide inhibition. Each value represents mean \pm S.D. (n=6). * $p < 0.05$ and * $p < 0.001$ vs 0 $\mu\text{g/ml}$. NS = Non-significant**

Peroxynitrite anion Scavenging assay

Moreover, the toxic effect of NO increases greatly upon reaction with superoxide radical and resulting formation highly reactive peroxynitrite anion (ONOO^-), especially its protonated form peroxynitrous acid (ONOOH)³⁰. Figure 5 shows the peroxynitrite scavenging activity of the plant extract in a concentration dependent manner. The calculated IC_{50} was 3427.18 ± 1212.65 $\mu\text{g/ml}$ which was lower than that of the reference compound gallic acid ($\text{IC}_{50} = 876.24 \pm 56.96$ $\mu\text{g/ml}$) (Table 3) indicating that the sample is more potent scavenger of peroxynitrite than gallic acid.

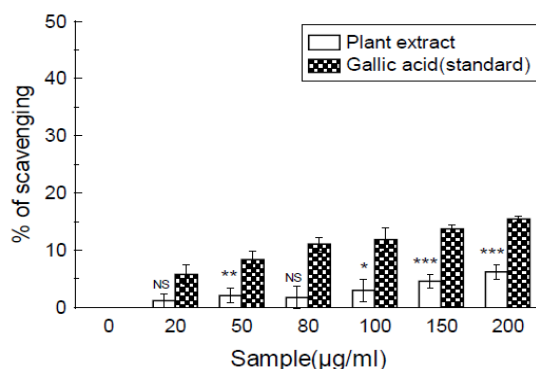


Fig. 5: Peroxynitrite anion Scavenging: The peroxynitrite anion scavenging activity of *W. somnifera* extract and standard gallic acid. Each value represents mean \pm S.D. (n=6). * $p < 0.05$, ** $p < 0.01$ and * $p < 0.001$ vs 0 $\mu\text{g/ml}$. NS = Non-significant.**

Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide, a weak oxidizing agent and cross cell membrane rapidly, reacts with Fe^{2+} and possibly Cu^{2+} ions to form the damaging toxic hydroxyl radical. The scavenging activity of the methanolic plant extract was found to be so poor to the standard sodium pyruvate; the figure and the IC_{50} value were hence not given.

Singlet oxygen scavenging activity

Singlet oxygen, a high energy form of oxygen, was generated in the skin upon UV-radiation. It induces the hyperoxidation, oxygen cytotoxicity and decreases the antioxidant activity³¹. The IC_{50} value of test sample was found to be 234.49 ± 37.69 $\mu\text{g/ml}$ whereas that of lipoic acid was found to be 46.15 ± 1.16 $\mu\text{g/ml}$ (Table 3). The IC_{50} value of the extract was higher than the reference compound. The present study indicates that the *W. somnifera* extract has singlet oxygen scavenging activity but not as much compared to the standard lipoic acid (Fig. 6).

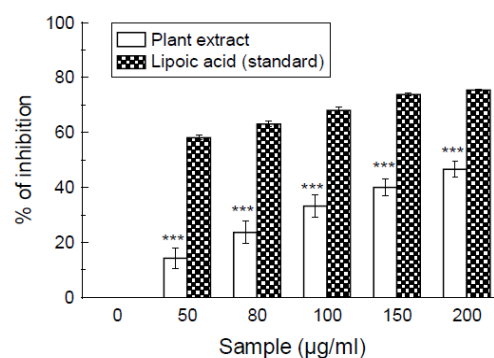


Fig. 6: Singlet oxygen scavenging: Effect of *W. somnifera* plant extract and standard lipoic acid on the scavenging of singlet oxygen. The results are mean \pm S.D. of six parallel measurements. * $p < 0.001$ vs 0 $\mu\text{g/ml}$.**

Hypochlorous radical scavenging Activity

Hypochlorous acid is another harmful ROS. At the sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of this ROS^2 . HOCl has the ability to inactivate the antioxidant enzyme, catalase through break down of heme prosthetic group. The inhibition of catalase inactivation in the presence of the extract signifies its HOCl scavenging activity. The obtained results (Fig. 7) indicate that the standard ascorbic acid ($\text{IC}_{50} = 235.95 \pm 5.75$ $\mu\text{g/ml}$) is the better scavenger than the plant extract ($\text{IC}_{50} = 328.99 \pm 35.92$ $\mu\text{g/ml}$) (Table 3). So, it is anticipated that *W. somnifera* is nearly as efficient scavenger of HOCl as the standard.

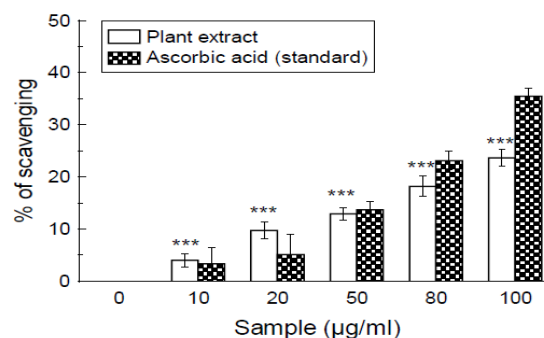


Fig. 7: HOCl scavenging: Hypochlorous acid scavenging activity of *W. somnifera* plant extract and standard ascorbic acid. All data are expressed as mean \pm S.D. (n=6). * $p < 0.001$ vs 0 $\mu\text{g/ml}$.**

Reducing Power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. As illustrated in fig. 8, at 0.2 mg/ml and at 1.0 mg/ml, the absorbance of the plant extract

were 0.012 and 0.052, respectively, while the absorbance of the standard ascorbic acid remained thoroughly constant at around 0.47. This result indicates that the activity of the extract, although present, is very low in comparison to that of the standard ascorbic acid.

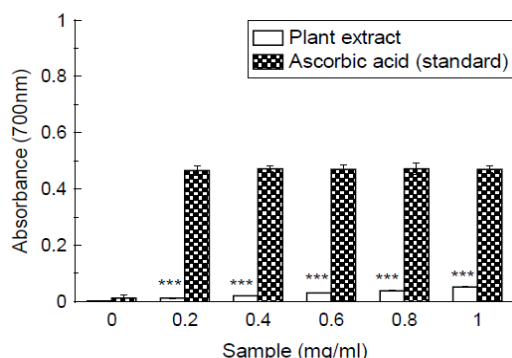


Fig. 8: Reducing Power: The reductive ability of extract and standard ascorbic acid. The absorbance (700nm) was plotted against concentration of sample. Each value represents mean \pm S.D. (n=6). * $p < 0.001$ vs 0 mg/ml.**

Inhibition of lipid peroxidation

Lipid peroxidation is initiated through iron catalysed generation of ferryl-perferryl complex or hydroxyl radicals that accelerates peroxidation by decomposing lipid hydro-peroxides into peroxy and alkoxy radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids that eventually yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. As can be seen in fig. 9 and from the IC_{50} values of the plant extract and standard trolox being $284.13 \pm 146.66 \mu\text{g/ml}$ and $13.52 \pm 0.33 \mu\text{g/ml}$ (Table 3), respectively, the gradual decrease of the MDA measured as inhibition of lipid peroxidation with increasing concentration of the sample establishes the role of the sample as an antioxidant.

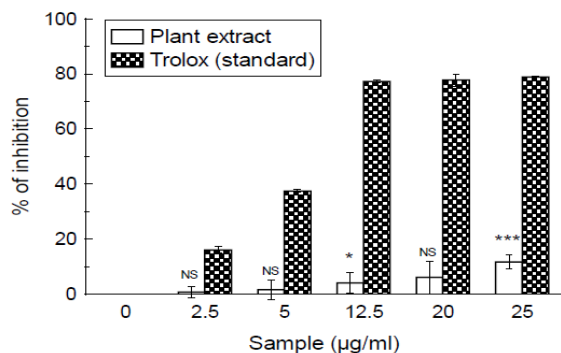


Fig. 9. Lipid peroxidation inhibition: The inhibitory effect of *W. somnifera* plant extract and standard trolox on lipid peroxidation phenomenon shown as % inhibition of peroxidation of lipids from mice brain homogenate. The results are mean \pm S.D. of six parallel measurements. * $p < 0.05$ and * $p < 0.001$ vs 0 $\mu\text{g/ml}$. NS = Non-significant.**

CONCLUSIONS

Based on the results of this study, it may be concluded that 70% methanolic extract of *W. somnifera* root possess mild free radical scavenging and antioxidant as well as iron chelating properties *in vitro*. These activities might be due to the presence of phenolics, flavonoids, tannins, alkaloids and carbohydrates contents. Further studies of the plant material by extracting with different solvents in evaluating the antioxidant and immunomodulatory potential, both *in vitro* and *in vivo* may be interesting. A correlation study of the active principles to the bioactivity of this plant may also be substantially important.

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