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FREE RADICAL SCAVENGING POTENTIAL OF THE LEAVES OF *WITHANIA SOMNIFERA* L.: AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Withania somnifera L. is a well known plant in Ayurveda and Unani medicine. The present study was aimed to evaluate the antioxidant activity of the methanolic extracts of leaves of *W.somnifera*. The extract was subjected to assess its radical scavenging activities using the systems such as 1, 1–diphenyl, 2-picryl hydrazyl (DPPH), Superoxide scavenging, 2, 2'–azinobis, 3-ethyl-benzothiozline-6-sulphonic acid (ABTS), Nitric Oxide and Iron chelating activity. The extract showed best activity in scavenging of ABTS as it showed the lowest IC_{50} values(42.21 µg/ml) as well as potently scavenged the DPPH radical(44.25 µg/ml). The antioxidant property depends upon concentration and increased with increasing amount of plant fraction. The results of the study showed that leaves of *W.somnifera* possess significant antioxidant activity. Owning to these results, the plant has the potential to be used as a medicine against free radical associated oxidative damage.

Key words: *Withania somnifera*, Free radicals, DPPH, ABTS

INTRODUCTION

Withania somnifera L (family: Solanaceae*)* also known as ashwagandha, Indian ginseng, and winter cherry, it has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. It grows wildly in all drier parts of subtropical India, Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afganistan (Kokate *et al*., 1996; Singh *et al*., 2001; Gupta and Rana, 2007). It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy (Singh *et al*., 1982; Bone, 1996; Patwardhan, 1996). Also used in anxiety and depression (Archana and Namasivayam, 1999; Bhattacharya *et al*., 2001; Singh *et al*., 2001), chronic stress (Dhuley, 2000; Bhattacharya and Muruganandam, 2003).

Free radicals or ROS (reactive oxygen species) are highly reactive and potentially damaging transient chemical species and are continuously produced in the human body (Ali *et al.,* 2008). But there is a balance between generation of ROS and antioxidant system in organism. In pathological condition, ROS are over produced and result in lipid peroxidation and oxidative stress (Dharmishtha *et al.,* 2010). It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, skin ageing, diabetes mellitus, cancer, immuno suppression, neuro degenerative diseases and others (Branien, 1975; Ma *et al*., 2001; Gyamfi *et al.,* 2002; Devasagayam *et al*., 2004; Duang *et al.,* 2006). Phytoconstituents present in medicinal plant are important source of antioxidant and capable to terminate the free radical chain reaction (Oluwaseum and Ganiyu, 2008). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from aging related disease has intrigued scientist for a long time. It has been mentioned that antioxidant activity of plants might be due to their antioxidant constituents like polyphenoles (Duh *et al.,* 1999),

flavanoids, vitamin C, carotenoids and vitamin E (Sies and Stahl, 1995).

The present study was carried out to evaluate the antioxidant potential of methanolic extracts of leaves of *Withania somnifera L.* as till now not much attention has been paid towards this aspect of the plant.

MATERIAL AND METHODS

Leaves

The leaves of *W.somnifera* were collected in the month of June-September from the field of Jaipur region. It was washed with tap water, dried at room temperature and ground to fine powder. The species specimen was submitted in herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India and got the voucher specimen No. RUBL21148.

Chemicals

All the chemicals used were of analytical grade and purchased from Hi Media from Hi-media Laboratory Pvt. Ltd. Mumbai.

Plant extract

About 50g of the leaves were taken and extracted in a soxhlet extractor with methanol (0.2 L). At the end of extraction, it was passed through whatman filter paper No.1 (Whatman Ltd., England), and the extract was recovered. The crude extract was concentrated to dryness in a rotary flash evaporator under reduced pressure and controlled temperature (40–50^{o}C). The extract was preserved in vacuum desiccators for subsequent use for antioxidant activity.

DPPH radical scavenging assay

The radical scavenging activity was determined using DPPH as a free radical. To the methanolic solution of DPPH (1 mM) an equal volume of the test extract dissolved in alcohol was added at various concentrations from 2 to 1000 µg/mL in a final volume of 1.0 ml. An equal amount of alcohol was added to the control. The mixture was left at dark in room temperature and the absorption was monitored after 20 min by reading the absorbance at 517nm. L-ascorbic acid (Merck; M.W.176.13) was used as standard .Experiment was performed in triplicate (John, 1984; Sreejayan and Rao, 1996).

The antioxidant activity of the sample was expressed in terms of IC50 values (the concentration required to inhibit radical formation by 50% and compared with L-ascorbic acid, used as positive control. The results are expressed as antiradical efficiency (AE), which is 1000-fold inverse of the IC_{50} value ($AE = 1000/IC_{50}$).

Superoxide Scavenging Assay

Superoxide radical (O_2) was generated from the photoreduction of riboflavin and was deducted by nitroblue tetrazolium dye (NBT) reduction method. Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 mL of alkaline DMSO and 0.2 mL of NBT (20 mM) in phosphate buffer (pH 7.4) was added. The experiment was performed in triplicate (Govindarajan, 2003).The control tubes were also set up where DMSO was added instead of sample. The reaction mixture was illuminated for 30 mins and absorbance at 560 nm was measured against the control samples. A Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} value was calculated.

Iron chelating activity assay

The reaction mixture containing 1mL O-Phenanthroline, 2 ml Ferric chloride, and 2mL extract at various concentrations (2 to 1000 µg/ml) in a final volume of 5 mL was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of extract and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without extract. Experiment was performed in triplicate (John, 1984; Sreejayan and Rao, 1996; Benzie and Strain, 1996). A Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} value was calculated.

ABTS radical scavenging assay

The scavenging activity of test extract on ABTS (2,2´-azinobis(3-ethylbenzothiazoline- 6-sulfonic acid) radical cation was estimated according to the method of Sreejayan and Rao, 1996; John, 1984. To the reaction mixture containing 0.3 ml of ABTS radical, 1.7 ml phosphate buffer and 0.5 ml extract was added at various concentrations from 2 to 500µg/mL. ABTS solution without the sample served as a control. Absorbance of the solution was recorded at 734 nm. Experiment was performed in triplicate. A Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} value was calculated.

Nitric oxide radical scavenging assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. For the experiment, 0.3ml of sodium nitroprusside(5 mM), prepared in phosphate buffered saline(pH 7.4), was added to 1 ml of various concentrations(2 to 1000 µg/mL) of test compound. The test tubes were incubated at room temperature.

After the incubation of 5 hours, 0.5 ml of Griess reagent was added. The absorbance of the nitrite ions (chromophore) formed in the reaction was read at 546 nm. The reaction mixture without sample was used as control. The experiment was performed in triplicate (Sreejayan and Rao, 1996). Ascorbic acid was used as positive control. The IC_{50} value for each test extract as well as standard preparation was calculated

Statistical analysis: All the experiments were repeated at least three times. Results were reported as mean ± SE (not shown in graphs). IC_{50} values were also calculated by linear regression analysis. Percent Inhibition was calculated according to the formula:

% Inhibition = $[1 - (Abs_{SAMPLE} / Abs_{CONTROL})] \times 100$

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH is a free radical method used to evaluate antioxidant activity of compounds, plant extracts and foods (Soares et al, 1997). Antioxidants present in plant extracts reduce DPPH to 1, 1–diphenyl-2-Picryl hydrazine, a colorless compound and the degree of discolouration indicates the scavenging potentials of antioxidants at 517nm. The observed antioxidant activity of extract may be due to neutralization of free radical, DPPH, either by transferring of an electron or H^+ (Umamaheshwari and Chatterjee, 2008). Figure 1 shows the DPPH radical scavenging capacity of methanol extracts of leaves of *W.somnifera* with L-ascorbic acid as reference. The maximum scavenging activity was found to be 85.3% at 2ug/ml and 92.8% at 512 ug/ml respectively. The IC_{50} values of extract and standard were 44.25µg/ml and 72.0 µg/ml respectively (Table 1). The fraction showed higher % inhibition (stronger H-donating ability). This activity can also be contributed to high amount of Ascorbic acid, anthocynins, phenolics and flavanoidal contents (Alam et al, 2012).

Superoxide Scavenging Assay

The methanol extracts of leaves of *W.somnifera* quenched NBT free radical in a dose dependent manner because as the concentration of extracts increased, the NBT quenching activity was also increased. The superoxide scavenging ability of extract was found to be lower when compared with standard (Figure 2). This could be due to presence of relative concentration of other bioactives and mixtures of impurities or other nutrients in extract. The IC_{50} value of plant extract and standard was found to be 64.12 µg/ml and 72.32 µg/ml respectively(table 1) .The ability of extract to scavenge the NBT radical has also been related to inhibition of lipid peroxidation. The literature also supports that the glycowithanolides(Bhattacharya et al,1997) and sitoindosides VII-X isolted from the species posess antioxidant activity(Mishra and Singh, 2000).

Iron chelating activity assay

O-phenanthroline quantitatively forms complexes with Fe^{+2} which get disrupted in presence of chelating agents. The methanolic extract interfered with the formation of a ferrous- O-phenenthroline complex, thereby suggesting that the extract has metal chelating activity. Maximum chelating of metals ions was observed at 1000 µg/ml of extract is 81.4 % and standard was found to be 89% (Figure 3) respectively. Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. Iron is extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components(Smith et al,1992). This effect may be due to presence of polyphenols which has potent iron chelating capacity (Nabavi, 2008). The IC_{50} values of plant extract and standard was recorded as 46.15 µg/ml and 62.11 µg/ml respectively (Table 1).

ABTS radical scavenging assay

The ABTS method is widely employed for measuring the relative radical scavenging activity of H-donating and chain breaking antioxidants in plant extracts(Patel et al,2012). The capability of methanol extracts of *W.somnifera* to scavenge ABTS radical is shown in Figure 4 and compared to standard. As compared to other antioxidant assays done, it has shown best results as the IC_{50} value calculated for ABTS was 42.21ug/ml, the least(less the IC_{50} value, more the antioxidant potential),shown in table 1.

Nitric oxide radical scavenging assay

Nitric Oxide is a potent pleiotropic mediator of physiological process. Extract inhibit nitric oxide in a dose dependent manner (Figure 5) . The maximum nitric oxide radical scavenging activity of extract and standard was found to be 86.2% and 61.9% at 1000 µg/ml respectively (Table-30). The IC_{50} value of the methanol extract and standard was found to be 52.15 µg/ml and 31.14 µg/ml respectively (Table 1).

CONCLUSION

In the present study, the free radical scavenging activity of *Withania somnifera* was investigated. The results obtained in the study indicates that it possess remarkable antioxidant properties and could have great importance as therapeutic agents. By identification and isolation of antioxidant constituents from this plant will be promising application possibilities as natural antioxidant.

Table 1: IC_{50} value for different assays

Assays	IC_{50} value of extract (µg/ml)	IC_{50} value of standard (µg/ml)
DPPH	44.25±0.27	72
Superoxide Scavenging	64.12±0.23	72.32
Iron chelating	46.15±0.2	62.11
ABTS	42.21±0.3	38.46
Nitric oxide	52.15±0.25	31.14

Each value is expressed as mean ± S.E.(Standard Error) (n=3)

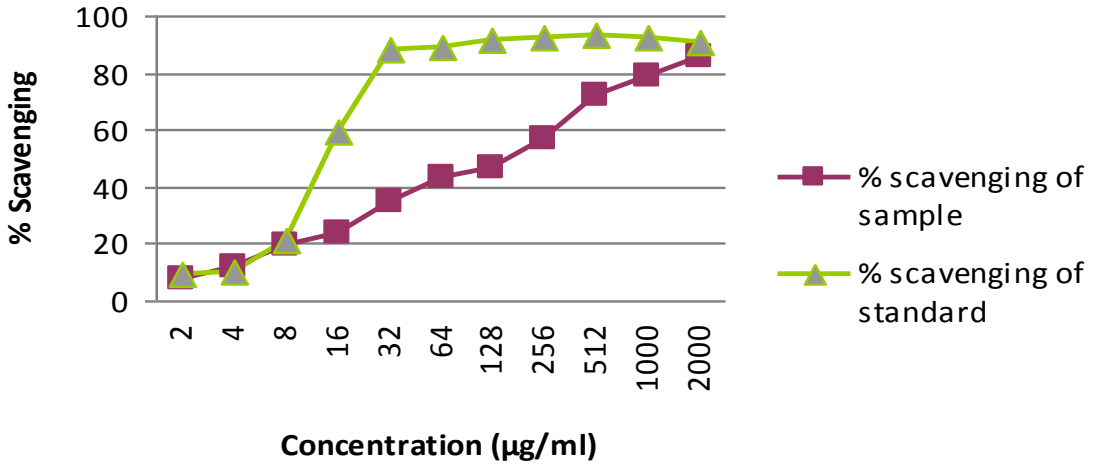

Figure 1: DPPH radical scavenging assay

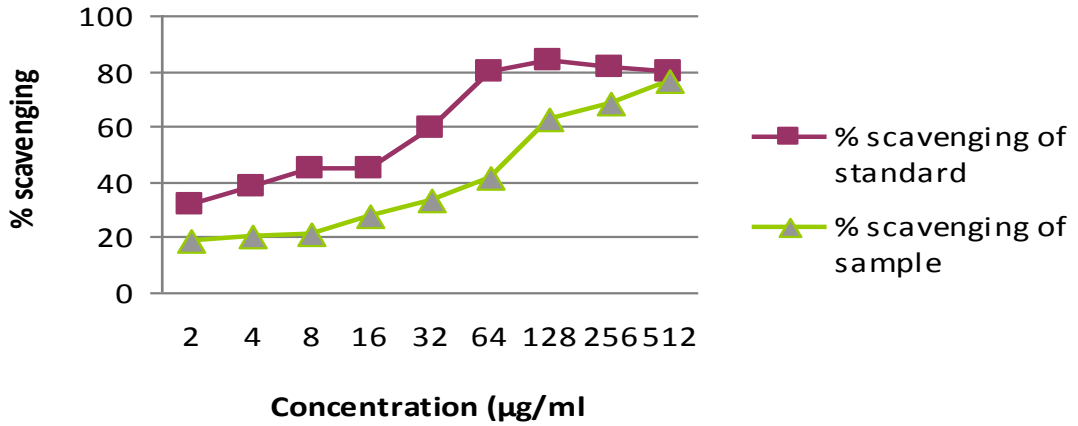

Figure 2: Superoxide scavenging assay

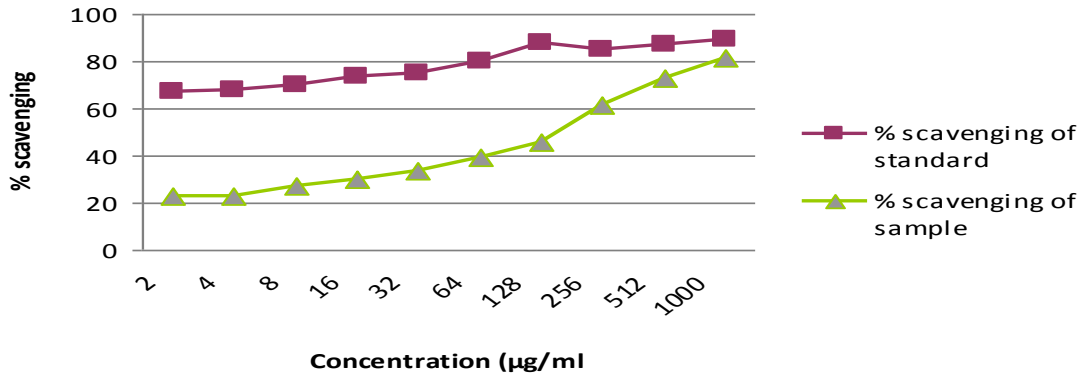

Figure 3: Ion chelating scavenging assay

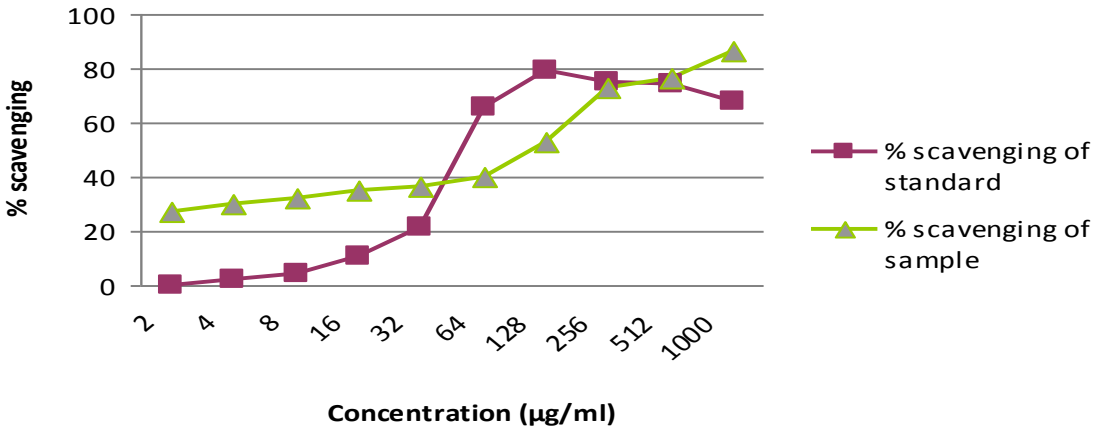

Figure 4: ABTS scavenging assay

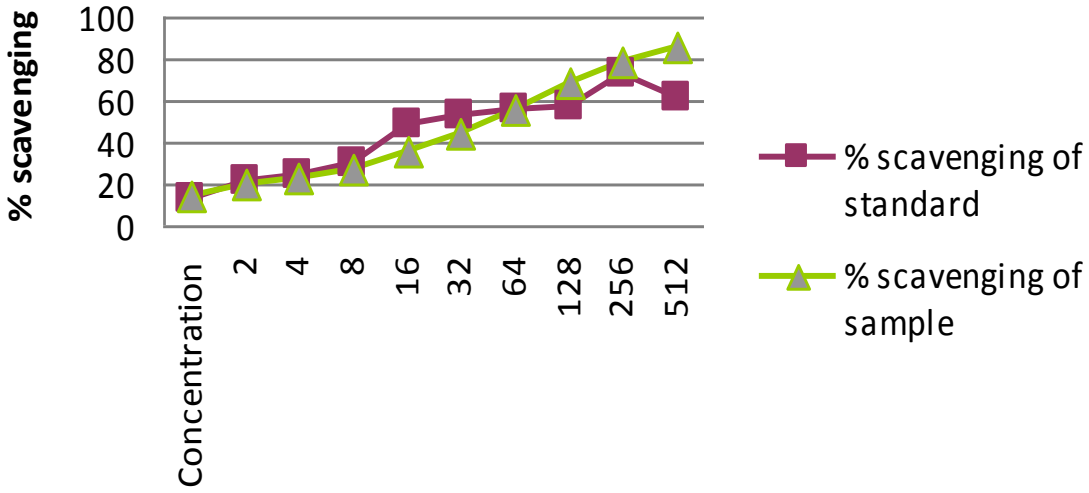

Figure 5: Nitric oxide scavenging assay

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