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Radical scavenging activity of different extracts of *Withania somnifera* leaves

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SUMMARY

The free radical scavenging activity of the different leaf extracts namely, methanolic,chloroform and ethanolic of aswhagandha (*Withania somnifera*) were evaluated .The results revealed that among the three different leaf extracts of *W. somnifera*, methanolic extract exhibited more DPPH scavenging activity. The extent of ABTS radical scavenging was more effective in methanolic extract, the aqueous extract of the leaves decreased the effect induced by H_2O_2 marginally, albeit to a statistically significant extent. The methanolic extract of *W. somnifera* showed better scavenging of H_2O_2 than the other two extracts. It was observed that extracts were very effective in preventing the lipid peroxidation in the three membrane model sytems studied to a significant extent over the oxidant-challenged system. Here, again the results confirmed the findings with methanolic extract performing better compared to aqueous and chloroform extract. Though the components that elicit these responses are extracted into all solvents employed (water, methanol and chloroform), the most active principle seemed to concentrate in the methanolic extract.

Key words : Free radicals, Radical scavenging activity, Lipid peroxidation, *Withania somnifera*

Oxygen is essential for survival however, its univalent reduction generates several harmful reactive oxygen species (ROS) inevitable to living cells and highly associated with wide range of pathogenesis such as diabetes, liver damage, inflammation, aging, neurological disorder and cancer (Tripathi and Kamat, 2007).The high levels of reactive oxygen species and free radicals cause damage to nucleic acid, proteins and membrane lipids, the antioxidants in diet would terminate attacks by the free radicals and reduce the risk of these diseases (Terashima *et al.*, 2007).

Antioxidant compounds may function as free radical scavenger, complexers of pro-oxidant metal reducing agents and quenchers of singlet oxygen formation (Rajeswar *et al*., 2005). Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease (Baskar *et al*., 2006).

Medicinal plants, which form the back bone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies. This has been brought about by the acknowledgement of the value of medicinal plants as potential source of new compounds of therapeutic value and as sources of lead compound in the drug development (Kumar *et al.*, 2006). One such popularly used plant that is reported to have anti-tumour, radiosensitizer, anti-stressor, immunomodulatory, anti-inflammatory and antibacterial effects is *W.somnifera* Dunal, which is commonly known as Ashwagandha (Padmavathy *et al.*, 2005).

In all these medicinal preparation, it is the dry tubers that are exploited. Previous studies conducted in our laboratory showed the leaves and fresh tubers are also good sources of antioxidants and radical scavengers (Sumathi and Padma, 2008). The results were also confirmed using *in vitro* and *in vivo* models. In order to better understand the component responsible for all these activities, three different extracts of leaves in polar and non-polar solvents were analysed in the present study.

MATERIALS AND METHODS

W.somnifera noted for its medicinal property was maintained in medicinal plant garden of our University and leaves were taken as the sample for our study. The leaves were collected and washed in running tap water to remove the surface contaminants. The washed leaves were homogenized in three different solvents namely, water, methanol and chloroform using a micropestle. The homogenate was centrifuged at lower rpm to clarify the extract. The supernatant corresponding to the concentration of 1mg/µl was used for assay. The extracts were tested for their ability to scavenge the free radicals. The free radicals scavenging effects of the *W.somnifera* leaf extracts was assessed by analyzing its ability to scavenge DPPH, ABTS and hydroxyl radicals and antioxidant potential against the non-radical oxidant H_2O_2.

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DPPH Radical Scavenging Activity was assessed according to the method of Mensor *et al.* (2001). 3ml of 0.3mM DPPH- methanol solution was added to 2.5µl and 5.0µl of the plant sample. DPPH solution with methanol was used as a positive control and methanol alone acts as blank. When DPPH reacts with antioxidant, it is reduced and the color changes from deep violet to light yellow measured at 517mm. The per cent inhibition was calculated by the following formula:

$$\text{Scavenging capacity } \% = \frac{100 - A_0 - A_1}{A_0} \times 100$$

A_0 = Absorbance of control

A_1 = Absorbance in the presence of sample of plant extract and standard

ABTS Radical Scavenging Activity was assessed according to the method of Shirwaikar *et al.* (2006). ABTS radical cation ($ABTS^{+}$) was produced by treating ABTS solution (7mM) with 2.45mM ammonium persulphate. Different extracts of aqueous, methanol and chloroform extracts (0.5ml) were added to 0.3ml of ABTS solution and the final volume was made upto 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{test}}{\text{Control}} \times 100$$

Hydroxyl Radical Scavenging Activity was assessed according to the method of Elizabeth and Rao(1990). The reaction mixture contained deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA (0.1 Mm), H_2O_2 (1mM), Ascorbate (0.1mM) and KH_2PO_4 – KOH buffer (20mM, pH – 7.4). 20µl of plant extract was added such that the final volume was 1ml. Deoxyribose degradation was measured as TBARS and absorbance was measured at 532nm. H_2O_2 Scavenging Activity was assessed according to the method of Ruch *et al.* (1989). A 40mM solution of H_2O_2 was prepared in phosphate buffer (pH - 7.4). Different extracts at the concentration of 10mg / 10µl were added to H_2O_2 solution (0.6ml, 40mM). The total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm. A blank solution that contained phosphate buffer without H_2O_2 served as the blank. The percentage H_2O_2 scavenging activity of plant extracts was calculated using:

$$\% \text{ Scavenged } (H_2O_2) = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 = Absorbance of control

A_1 = Absorbance in the presence of sample of plant extract and standard.

Inhibition of H_2O_2 induced Lipid Peroxidation was assessed according to the method of Okhawa *et al.* (1979) and Dodge *et al.* (1963) in three membrane preparations RBC Ghost, liver homogenate and liver slices. Control tubes were prepared for each sample containing the respective plant extract (50µl corresponding to 20 mg.), membrane aliquot (RBC ghosts or liver homogenate) and TBS to make a final volume to 500µl. Pro-oxidant ($FeSO_4$ at 10µ moles final concentration) was not added to the control tubes. A blank containing no leaf extracts, no membrane aliquot, but only $FeSO_4$ and TBS was also prepared. An assay medium corresponding to 100% oxidation was prepared by adding all the other constituents except leaf extracts. The experimental medium corresponding to autooxidation contained only the membrane preparation. All the tubes were incubated at 37°C for one hour.

At the end of incubation, the samples, along with the homogenates prepared from the liver slices incubated with the oxidant H_2O_2 and extracts, were subjected to the TBARS quantification. The LPO reaction in all the tubes was arrested by the addition of 500µl of 70% ethanol. 1ml of 1% TBA was added to all tubes and heated in a boiling water bath for 20 minutes. After cooling to room temperature, added 500µl of acetone and measured the TBARS at 535nm in a spectrophotometer.

RESULTS AND DISCUSSION

Oxygen is a vital for all the living system, except for anaerobes. However, the paradox of aerobic life is that oxidative damage occurs at the key biological sites, threatening their structure and function. Oxygenic threat is met by an array of antioxidants that evolved in parallel with our oxygenic atmosphere (Benzie, 2003). Free radicals are generated as a by product or intermediates of aerobic metabolism and through reactions with drug and environmental toxins. The elevated cellular levels of free radicals cause damage to nucleic acid, protein and membrane lipids and are associated with many aging related problems including carcinogenesis and heart diseases (Jiao and Wang, 2000). The balance between the production and scavenging of ROS can therefore determine the susceptibility of the body to oxidative damage (Usoh *et al.*, 2005). The potential toxicity of free radicals is counteracted by a large number of cytoprotective enzymes and antioxidants that limit the damage (Dadheech *et al.*, 2006). Cell damage by free radicals has been

reported as the predominant mechanism of hepatotoxicity (Dahiru *et al*., 2005).

The free radical scavenging activity of different extracts of *W. somnifera* was studied under *in vitro* conditions. So, in the present study, attempt has been made to see the ability of different extracts to scavenge the free radical DPPH, ABTS and H_2O_2. Among the three different leaf extracts of *W. somnifera* methanolic extract exhibited higher dpph, abts and H_2O_2 scavenging activity followed by chloroform and aqueous extract which shows that the leaves of *W.somnifera* possess strong radical scavenging activity. The results are presented in Fig. 1. Different parts of *W.somnifera* namely, leaves, fresh and dry tubers tested for radical scavenging activity showed, leaves possessed strong radical scavenging effects compared to fresh and dry tubers (Sumathi and Padma,2009a).

Aqueous - methanolic extracts from *basil* and *laurel* (Dorman *et al*., 2005), hexane (Ramkumar *et al*., 2007), cold ethanolic extract of the leaves of *piper betle* showed promising antioxidant activity compared to that of synthetic antioxidant butylated hydroxyl toluene (Arambewela *et al*., 2006). The ethyl acetate fraction of leaves of *butea monosperma* Lam (Lavhale and Mishra,

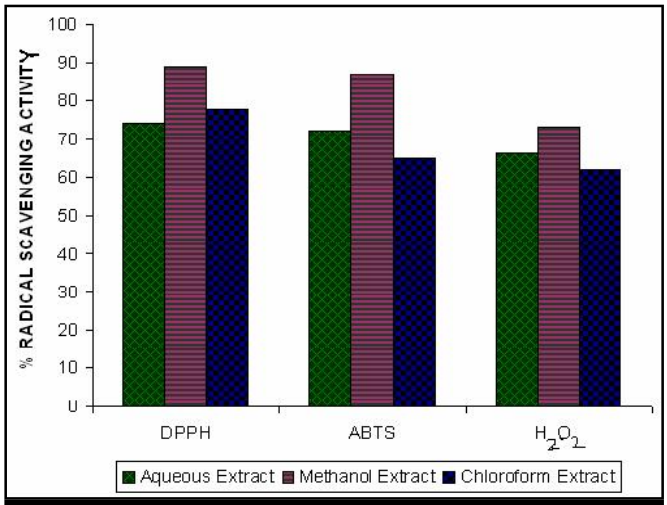

Fig. 1 : Radical scavenging activity of the different extracts of *W. somnifera*

2007), ethanolic extract of *Agaricus bisposus* (Fu *et al*., 2002) possessed free radical scavenging activity. Similar results were observed in our present study.

Similar results were observed in case of hydroxyl radical scavenging activity indicating methanolic extract found to have better scavenging activity compared to the other two extracts as depicted in Fig. 2. Similar results were observed in aqueous extract of *Cordyceps militaris* (Zhan *et al*., 2006) and *Poligomiun multiforum* (Shuang *et al.*, 2005), *Bhrama rasayana* (Rekha *et al.*, 2001) showed maximum inhibition of hydroxyl radical induced degradation. These studies supports the present findings.

The extent of lpo was tracked in three diverse membrane preparations, namely, goat rbc (Plasma membrane preparation), goat liver homogenate (Mixture of plasma membrane and internal membrane) and goat liver slices (Intact cells) and the ability of the extracts to inhibit LPO was observed. These models differed in their lipid composition and architecture. The values obtained are represented in Fig. 3.

In the present study, all the three extracts caused a substantial decline in the extent of lpO in the different membrane preparations. The methanolic extract caused better protection to all the three lipid preparations followed by aqueous and chloroform extracts. Among the membrane preparation, maximum protection was observed in the liver homogenate followed by goat rbc ghost and liver slices. Sumathi and Padma (2009b) have reported a similar study, where among the different parts of *W. somnifera* namely leaves, fresh and dry tubers tested, the leaves showed maximum protection in all the three membrane preparations and maximum protection in goat liver homogenate.

Ethanolic extract of *Teucrium polium* (Hasan *et al*., 2007), diethyl ether, ethyl acetate and n-butanol extracts of *Teucrium* species inhibited lipid peroxidation *in vitro* in rat liver homogenate (Panovska and Kulevanova, 2005). The antioxidant activity possessed by Turkish *Sideritus* species was reflected by the lipid peroxidation action (Guvenc *et al*., 2005). In single cell suspension too, the leaves rendered better protection compared to fresh and

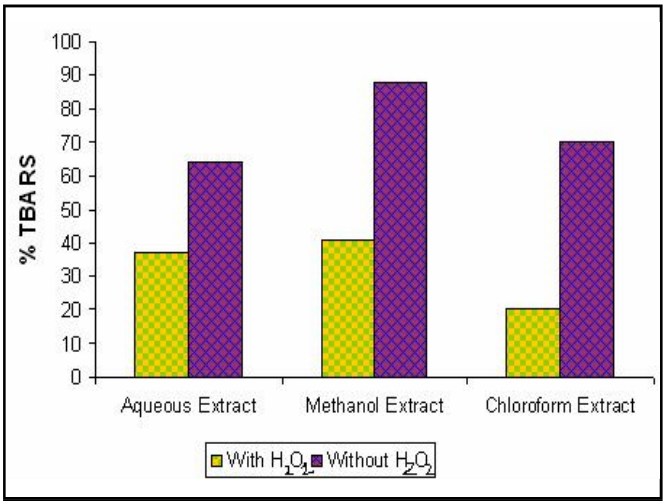

Fig. 2 : Hydroxyl radical scavenging activity of the different extracts of *W. somnifera*

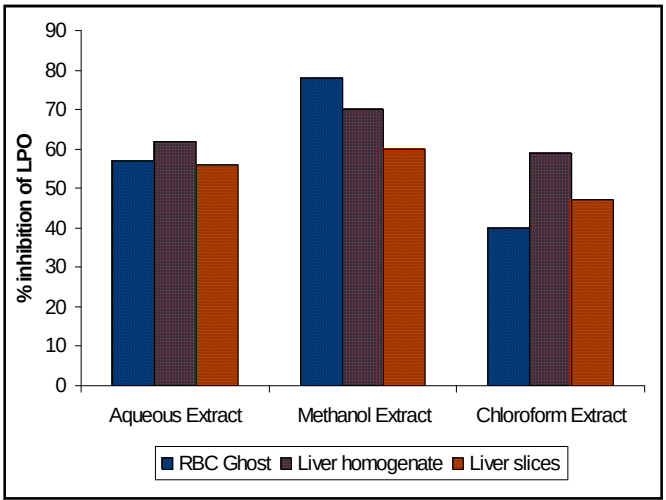

Fig. 3 : Effect of *W. somnifera* leaf extracts on lipid peroxidation in three different membrane models

dry tubers (Sumathi *et al*., 2007). Thus the present study showed the strong free radical scavenging and antioxidant activity of *W. somnifera* and this validates the use of leaves in medicinal preparation for disorders and disease caused by oxidative stress.

Conclusion:

Though the components that elicit these responses are extracted into all solvents employed (water, methanol and chloroform), the most active principle seemed to concentrate in the methanolic extract. The chloroform extract was comparatively less effective than the other two, implying that the presence of active component that render better free radical scavenging activity to tissues may include more polar substance than non-polar substances.

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