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Free Radical Scavenging and Lipid Peroxidation Inhibition Potential of *Hygrophila auriculata*

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Abstract – *Hygrophila auriculata* (K. Schum.) Heine is a wild herb commonly found in moist places on the banks of tanks, ditches and paddy fields throughout India and is one of the main sources of Ayurvedic 'Rasayana' drug. The free radical scavenging potential of 50% aqueous alcoholic extract of *H. auriculata* (HAEt) was studied for DPPH scavenging activity, nitric oxide, hydroxyl radical and ferryl bipyridyl complex scavenging activity along with lipid peroxidation and total antioxidant capacity inhibition using Thiobarbituric acid reactive substances (TBARS) in rat liver homogenate. Integral antioxidative capacity was determined by photochemiluminescence assay. HAEt showed good radical scavenging activity at various concentrations (200-1000 μg/ml) against 1,1 diphenyl, 2-picrylhydrazyl (DPPH) (32.32-77.02%) with moderate scavenging activity against Nitric oxide (12.46-52.84%), hydroxyl radical (11.69-55.26%), ferryl bipyridyl complex (17.66-58.67%) and lipid peroxidation (0.829-0.416 nmoles/mg protein). The above results indicate HAEt to be very effective antioxidant. It was suggested that the varied therapeutic activities claimed for the plant in the Indian indigenous systems of medicine may be in part due to the free radical scavenging and/ or potent antioxidant activity.
Keywords – *Hygrophila auriculata,* free radical scavenging, Lipid peroxidation, Photochemiluminescence assay

Introduction

There is increasing evidence that oxidative stress, defined as an imbalance between oxidants and antioxidant in favour of the former, leads to many biochemical changes and is an important causative factor in several human chronic diseases, such as atherosclerosis and cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders, and the aging process (Govindarajan *et al.,* 2003a). ROS such as $\cdot O_2^-$, H_2O_2 and OH are highly toxic to cells and cause severe tissue damage (Thomas and Kalyanaraman, 1999). Oxygen radicals catalyse the oxidative modifications of lipids and causes oxidative damage to DNA and proteins (Wiesman and Halliwell, 1996). Although medicinal plants are rarely used as antioxidants in traditional medicine, their claimed therapeutic properties could be due, in part to their capacity for scavenging oxygen free radicals, which may be involved in many diseases.

Hygrophila auriculata (K. Schum.) Heine Synonym: *Asteracantha longifolia* Nees (Family: Acanthaceae) is a wild herb commonly found in moist places on the banks of tanks, ditches and paddy fields throughout India and is one of the source of many Ayurvedic 'Rasayana' drug. Thus plant has been shown to possess hypoglycaemic activity in human subjects (Fernando *et al.,* 1989), hepatoprotective activity against paracetomol and thioacetamide intoxification in rats (Singh and Handa, 1995), diuretic, anabolic and adrogenic activity (Jayatilok, 1976). However to date no antioxidant investigations have been reported in this plant. Therefore, the present study was undertaken to investigate the free radical scavenging potential of the hydro alcoholic extract of *H. auriculata* (HAEt).

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Experimental

Plant material and extraction – Aerial parts of *H. auriculata* were collected from Rewa, Madhya Pradesh (India) during the month of September 2002. The plants were authenticated and the voucher specimen (LWG, 4609) was lodged in the departmental herbarium of National Botanical Research Institute. Aerial parts of *H. auriculata* (1 Kg) were air dried at room temperature and

coarsely powdered. The powder obtained (250 g) was macerated with 50% aqueous alcohol for a period of 24 h, filtered. The extract was then concentrated under reduced pressure and lyophilised (Labconco, USA) to get 14.9 g of the extract (HAEt).

Animals – Male Sprague Drawley rats (160-180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. These were kept in the departmental animal house at 26 ± 2°C and relative humidity 44-55% light and dark cycles of 10 and 14 hr respectively for one week before the experiment. Animals were provided with rodent diet (Amruth, India) and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals".

Free radical scavenging activity using DPPH – The ability of HAEt to scavenge the free radicals were estimated by *in vitro* method using a stable nitrogen centered radical viz. DPPH (Blois, 1958). Briefly 0.05 ml of extract dissolved in methanol was added to a methanolic solution of DPPH (100 µM, 2.95 ml) at different concentration (200-1000 µg/ml) and the absorbance was recorded at 517 nm.

Nitric oxide scavenging activity – Nitric oxide ($NO^{\bullet}$) radical scavenging activity was measured by using a spectrophotometer (Sreejayan and Rao, 1997). Sodium nitroprusside (5 mM, 1.5 ml) in phosphate buffered saline was mixed with different concentrations of HAEt (200-1000 µg/ml) dissolved in methanol and incubated at 25°C for 30 min. A control without test compound but with equivalent amount of methanol was taken. 30 min after incubation, 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethelene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethelene diamine was measured at 546 nm.

Hydroxyl radical scavenging activity – The ability of the test extract to scavenge the OH radical was determined using ascorbic acid iron-EDTA model · OH generating system. The standard reaction mixture consisted of 100 mM phosphate buffer, pH 7.4, 167 µM iron-EDTA complex, 0.1 mM EDTA, 2 mM ascorbic acid and 33 mM DMSO in a final volume of 3 ml. Iron catalyzed oxidation of ascorbic acid at 37°C was used to generate formaldehyde from DMSO. Appropriate controls, reaction mixtures without ascorbic acid, were maintained. HAEt was added to obtain final concentrations ranging from 50-400 µg/ml separately. D-Mannitol (50 mM) was used as a standard. The reaction was stopped by the addition of 1 ml of ice-cooled trichloroacetic acid (17.5% w/v). The decrease in formaldehyde formation due to scavenging or decreased formation of OH was assayed spectrophotometrically by the method of Nash (1953).

Chelation of Fe^{2+} ions – Concentration of free ferrous ions (Fe^{2+}) was estimated using chelating agent 2,2' bipyridyl (Govindarajan *et al.*, 2003). Briefly, the reaction mixture (1 mL) contained 50 µM $FeSO_4$, 50 µM NaCl (pH 7) and different concentrations of HAEt and were incubated for 30 min., at the end of which 2 ml of 2, 2' bipyridyl (1 mM) was added. Absorbance of ferrous-bipyridyl complex was measured at 525 nm against the blank devoid of ferrous sulphate.

Assay of lipid peroxidation – Randomly selected rats were fasted overnight and were sacrificed by cervical dislocation, dissected and abdominal cavity was perfused with 0.9% saline. Whole liver was taken out and visible clots were removed and weighed amount of liver was processed to get 10% homogenate in cold phosphate buffered saline, *p*H 7.4 using glass teflon homogeniser and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method (Okhawa *et al.*, 1979) with minor modifications (Govindarajan *et al.*, 2003b). Briefly, different concentrations of HAEt (200-1000 µg/ml) were added to the liver homogenate. Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm in a spectrophotometer (Pharmacia Biotech, India). The values of TBARS were calculated from a standard curve (absorption against concentration of tetraethoxypropane) and expressed as nmoles/mg of protein. Protein estimation was carried out by spectrophotometric method using Folin-ciocalteau reagent (Lowry *et al.*, 1951)

Photochemilumenescence assay – For the determination of the integral antioxidative capacity (AC) of the water soluble substances in HAEt the method of photochemil- uminscence (PCL) was used (Govindarajan *et al.*, 2004a). Apparatus used was Photochem® with Standard kit ACW (Analitik jena AG), where the luminol

plays a double role of photosensitizer as well as the radical detecting agent. Lyophilized extract was measured at 5 and 10 μg/ml concentration. A standard plot was plotted and the results were calculated in ascorbic acid equivalents (μmol/g).

Total antioxidant capacity – Total antioxidant capacity was measured according to spectrophotometric method of Preito *et al.* (1992). 0.1 ml of HAEt (10 mg/ml) dissolved in water was combined in a eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Statistical analysis – Linear regression analysis was used to calculate the IC_{50} values. Significance with respect to control was calculated with the help of SPSS 11.0 for Windows using paired *t*-test.

Results and Discussion

Oxygen radicals have been suggested to be involved in a number of clinical conditions including gastro intestinal tract lesions (Halliwell and Gutteridge, 1989). Furthermore, it has also been reported that the therapeutic properties of the traditional medicines could be due in part, to their capacity for scavenging oxygen free radicals which may be involved in many diseases as for example, in the case of plants used to treat inflammatory diseases, gastric ulcers and diabetes which could act by reducing the oxidative stress that takes place in cells undergoing these process (Desmarchelier, 1999; Govindarajan *et al.*, 2004b).

The scavenging capacity of HAEt was found to be 67.81% (Table 1) with the IC_{50} being 534 ± 2.69 μg/ml. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to it's odd electron, the methanolic solution of DPPH shows a strong absorption band at 517 nm. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour stoichometrically with the number of electrons taken up (Blois, 1958). Such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers. The inhibition was also found to be time dependent (Fig. 1).

Incubation of solutions of sodium nitroprusside in PBS at 25°C for 2 hr resulted in linear time dependent nitrite production, which was reduced by HAEt. The scavenging of nitric oxide by the extract was concentration dependent and the IC_{50} values were found to be 954 ± 3.66 μg/ml (Table 1). Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Miller *et al.*, 1992). Studies in animal models have suggested a role for

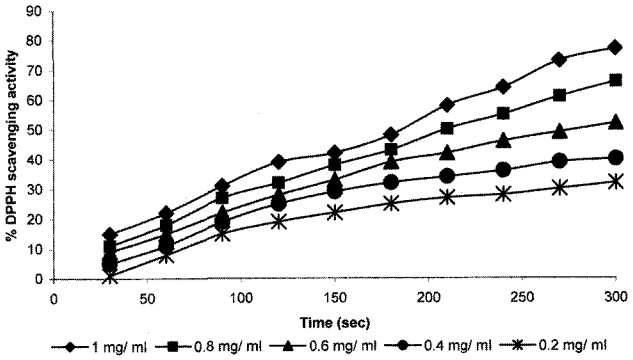

Fig. 1. Time dependent DPPH scavenging activity of HAEt at various concentrations.

Table 1. Free radical scavenging activity of the hydro alcoholic extract of *H. auriculata* (HAEt)

Conc. (μg/ml)	Inhibition (%)				TBARS (nmol/mg protein)
	DPPH	· OH radical	Nitric oxide	Ferryl bipyridyl complex	
1000	77.02 ± 1.25**	55.26 ± 1.33*	52.84 ± 0.36**	58.67 ± 1.77**	0.416 ± 0.62*
800	66.81 ± 1.36*	41.27 ± 1.87*	31.16 ± 0.42*	41.19 ± 1.13*	0.512 ± 1.62*
600	52.34 ± 0.62*	31.65 ± 1.94	24.28 ± 0.95	29.03 ± 2.17	0.621 ± 0.64*
400	40.65 ± 0.62*	11.69 ± 1.47	12.46 ± 1.62	17.66 ± 1.91	0.708 ± 0.42*
200	32.32 ± 0.16	-	-	-	0.829 ± 0.15
AA	88.60 ± 0.08*	-	72.6 ± 0.62	92.3 ± 0.91	-

**P<0.002, *P<0.01 as compare to control, Values are mean ± SED (n=6). AA: Ascorbic acid (100 μM)

NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease (Miller *et al.*, 1992). Nitric oxide is implicated in inflammation, cancer and other pathological conditions (Marlette, 1989). *H. auriculata* forms part of a number of Ayurvedic preparations prescribed as an anti-inflammatory agent. The potent nitric oxide radical scavenging activity justifies its use in the same.

HAEt also inhibited the hydroxyl radical in dose dependent manner (Table 1). The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides is produced (Miller *et al.*, 1992). Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases (Valentao *et al.*, 2002). Thus the decrease in the MDA level with the increase in the concentration of HAEt indicates the role of the extract as an antioxidant.

HAEt also had potent inhibition of the ferryl-bipyridyl complex. The results are summarized in Table 1. Inorder to test the possibility of the change in Fe^{2+}/ Fe^{3+} ratio, a separate experiment was performed. The extract inhibited the chromogen formation in a dose dependent fashion. Thus there is a possibility that the HAEt chelated the ferrous form and thereby removing the free iron out of the reaction system. The other possibility could be change of ratio of Fe^{2+}/ Fe^{3+}.

Table 1 shows that the HAEt inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. The values of TBARS were calculated from a standard curve (absorption against concentration of TEP) and expressed as nmoles/mg of protein (Table 1). IC_{50} values were found to be 668.74 ± 3.16 µg/ml. Plasma lipid peroxides appear higher than normal in Diabetes mellitus. The significance of oxidative stress in the disease pathology of diabetes is uncertain but is frequently proposed to be related to the hyperglycemia. Other possible sources include elevated plasma lipids leading to increased lipid oxidation by generation of H_2O_2 and decreased level of the antioxidant enzymes. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl perferryl complex (Gutteridge, 1985) or through OH radical by Fenton reaction (Halliwell, 1978). Ferryl-perferryl complex can also initiate lipid peroxidation in a similar manner as $OH^{\bullet}$, although it is less reactive than $OH^{\bullet}$, in iron induced lipid peroxidation, role of $OH^{\bullet}$ is not significant because little effect of tris and mannitol has been reported on this system (Tripathi and Sharma, 1998). HAEt shows good antioxidant activity suggesting the antidiabetic role of extract may be due to the antioxidant activity.

Photochemiluminescence method allowed precise as well as time and cost effective determination of the integral antioxidative capacity of the HAEt. Free radicals are generated in the instrument by means of photosensitizer. The free radicals thus generated were detected by their reaction with a chemiluminogenic substance. Luminol acts both as photosensitizer as well as the detecting reagent. In the presence of radical scavengers in the extract the intensity of the PCL was attenuated as a function of concentration. In this way the antioxidative capacity of the extract has been evaluated. The antioxidative capacity of HAEt was found to be 0.88 and 1.24 nmoles ascorbic acid/g equivalents for 5 and 10 µg/ml respectively.

The total antioxidant capacity of HAEt was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. The total antioxidant capacity of the extract was found to be 125.61 nmol/g ascorbic acid. Thus establishing the extract as an antioxidant.

Conclusion

The antioxidant and free radical scavenging activities of hydroalcoholic extract of *H. auriculata* used as a hepatoprotective and anti-diabetic drug in the Indian System of Medicine were determined *in vitro*. The results obtained suggest that the use of this plant extract widely in the treatment of many diseases, which include hepatic damage, diabetes etc. may be due in part to its antioxidant and free radical scavenging ability. Thus the varied therapeutic activity of the plant extract may be in part due to its antioxidant activity. The results obtained suggest that *H. auriculata* possesses potent antioxidant & free radical scavenging activity suggesting that ethnopharmacological approach in selecting the plant for study may be useful. The report of the efficacy of this plant as hepatoprotective and hypoglycaemic may be due to its antioxidant property.

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