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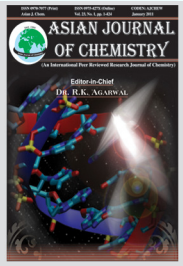

Antioxidant and Antimicrobial Activities of Extracts From Aerial Parts of *Alhagi pseudalhagi*

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In vitro total antioxidant capacity and antimicrobial activity of aqueous and organic extracts from aerial parts of *Alhagi pseudalhagi* extracts were investigated. Initially, the antioxidant components were extracted in methanol and later subjected to partitioning in solvents of different polarity. Antioxidant and radical scavenging activity of these extracts were evaluated using antioxidant assays such as 2,2'-azino*bis*(3-ethylbenzothiazoline-6-sulpohonic acid) (ABTS) radical cation scavenging, 2,2-diphenyl-1-picrylhydrazil (DPPH) scavenging, total phenolic contents and total antioxidant activity determination using ferric thiocyanate method. Using $ABTS^{\bullet+}$ decolorization assay *A. pseudalhagi* extracts showed a wide range of antioxidant activity. Trolox equivalent antioxidant capacity values ranged from 0.888-5.525 mM of trolox equivalents for different fractions of *A. pseudalhagi*. Using total phenolic contents and total flavonoid contents assays the amount of total phenolics and flavonoids for different fractions of *A. pseudalhagi* ranged from 10.657-21.759 mg/L of gallic acid equivalents (GAE) and 1.815-10.911 mg/L of quercetin equivalents, respectively. No significant co-relation was found between total phenolic contentss and trolox equivalent antioxidant capacity values. Employing potassium thiocyanate method, inhibition of lipid peroxidation comparable to trolox was exhibited by all the extracts. Antimicrobial activity of these extracts was also found against *Bacillus substilis*, *Escherichia coli*, *Salmonella typimorium*, *Pseudomonas aeruginosa* and *Aspergillus niger*. On the basis of the results obtained here *A. pseudalhagi* may be considered as a rich source of antioxidants and possessing potent antimicrobial activity.

Key Words: *Alhagi pseudalhagi*, ABTS radical cation, Trolox equivalent antioxidant capacity, Total phenolic contents.

INTRODUCTION

The enhanced production of reactive oxygen and nitrogen species (ROS/RNS) during oxidative stress conditions result in many disorders in living beings[1,2]. Apart from the defense mechanism of living bodies against ROS/RNS, many plant secondary metabolites like flavonoids, terpenoids, anthocyanidines *etc*., have been found effective against these radicals[3,4]. Many indigenous medicinal/herbal plants extracts or herbo-mineral formulations are considered effective against these diseases due to their active chemical constituents[5-7]. The increasing trend of using the plants/herbal extracts against certain diseases has attracted the food scientists to evaluate antioxidant activity of these plants[5,8,9].

Alhagi pseudalhagi (M. Bieb.) Desv. (Leguminosae) is commonly called camel's thorns. It is a spiny-branched shrub distributed throughout Indian Sub-continent, where it is used as a traditional herbal medicine[10-12].

Tanning agents, flavonoids, steroids, coumarins, anthocyanidines and catechins of organic acids, dyes and tanning agents have been reported previously in roots and the aerial part of plants of the genus *Alhagi*[13,14]. Vegetative parts of the plant contain about 0.33 % essential oil with a unique aroma. *Alhagi* is recommended as a source of vitamins, ascorbic acid, carotene, vitamin B, vitamin K and flavan glycosides with P-vitamin activity[15]. Alimova *et al*.[16] isolated 6 catechins and 4 proanthocyanidines from the aerial parts and roots of *A. pseudalhagi*.

Phenolic constituents previously reported from this plant include: rutin[17], (+)-catechin, (-)-epigallocatechin, (±)-gallocatechin and leucodelphinidin[18] and quercetin[19]. Singh *et al*.[20] isolated flavanone glycosides, naringenin 5-methyl ether 4'-glucoside and hesperitin 7-galactosyl(1→2)[rhamnosyl-(1→6)]glucoside, from the whole plant of *Alhagi pseudalhagi*. Different alkaloids such as -phenethylamine, N-methyl-phenethylamine, hordenine, 3,4-dihydroxy-phenethyltri-methylammonium hydroxide, N-methylmescaline and salsolidine were isolated[21]. The roots of this plant contain essentially the same alkaloids but in poorer yields. Preliminary pharmacological studies of the total alkaloids indicated that

some curative properties of the plant extract are due to these entities[21].

Fesenko *et al.*[22] confirmed the lipid peroxidation inhibition properties of Yantocine, a preparation derived from *Alhagi pseudalhagi* Desy, on a model of acute toxic hepatitis induced in albino rats. Khushbaktova *et al.*[10] demonstrated lipid peroxidation inhibition capacities of proanthocyanidins isolated from *A. pseudalhagi* Desy both in the myocardium and serum of rats and rabbits[10].

Survey of literature shows a few preliminary reports on antioxidant and lipid peroxidation inhibition potential of the plant extracts and its herbomineral formulations. There are almost no studies concerning the quantitative evaluation of antioxidant and antimicrobial activity of this plant extracts. The main objective of the this study is to quantitate the radical scavenging and antioxidant potential of different organic extracts of *A. pseudalhagi* by using a modified Trolox equivalent antioxidant capacity assay, ferric reducing antioxidant power (FRAP) assay, total phenolic contents assay, total flavonoid contents assay, DPPH free radical scavenging assay, total antioxidant activity using ferric thiocyanate method and metal chelating activity assay.

EXPERIMENTAL

Follin-Ciocalteau's reagent, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-azino*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, β-nicotianamide adenine dinucleotide reduced (β-NADH), nitrobluetetrazolium chloride (NBT), phenazine methosulphate (PMS), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, quercetin, iron(II) sulphate and potassium persulfate were purchased from Fluka (UK). All the solvents used were of analytical reagent grade and were purchased either from Merck or Panreac PA. (Germany). UV-1700 PharmaSpec UV-visible spectrophotometer, Shimadzu, Japan equipped with temperature control system was used for all the spectrophotometric measurements. The solutions were made in triplicate and each experiment was performed thrice.

Extraction of antioxidant components: The plant material was collected from Raiwind, Lahore (Pakistan). The finely ground shade-dried aerial parts of the plant (2.5 Kg) were exhaustively extracted with *n*-hexane at room temperature. The extract was concentrated by vacuum distillation and the residue obtained was re-soaked in methanol for 7 days at room temperature. The residue thus obtained from methanol extraction was suspended in water and partitioned successively in chloroform, ethyl acetate, 1-butanol and water fractions. Each fraction was concentrated using rotary evaporator. Sample standard solutions were obtained by dissolving 0.25 g each of the residue in 5 mL of the respective extraction solvent. The solutions were subjected to the antioxidant activity assays.

$ABTS^{\cdot+}$ scavenging potential: This assay is based upon the scavenging of $ABTS^{\cdot+}$ generated from a reaction between ABTS and potassium persulfate. The antioxidant components quench bluish green ABTS radical to colourless native ABTS form depending upon their nature and quantity on a time-dependant scale. In trolox equivalent antioxidant capacity assay, trolox is used as standard antioxidant for comparison purposes. For the determination of Trolox equivalent antioxidant capacity of the extracts, the procedure, as developed by Re *et al.*[23], was followed to study the antioxidant potential of standard antioxidant and plant extracts. 2,2'-Azino*bis*(3-ethylbenzothiazoline-6-sulpohonic acid) radical cation solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. The solution was equilibrated to a stable reading at 30 ºC. After addition of 10 µL of sample or standard antioxidant to 2.99 mL of diluted $ABTS^{\cdot+}$ solution (A = 0.700 ± 0.020), the absorbance was noted at 30 ºC, with exactly 1 min intervals for 6 min. Appropriate solvent blanks were run in each assay for accurate readings. The percentage inhibition (Inh %) of absorbance was calculated by the following formula:

$$\text{Inhibition } (\%)_{(\text{at } 734 \text{ nm})} = \left(1 - \frac{I_f}{I_o}\right) \times 100$$

where I_o and I_f are the absorbance of radical cation solution before and after addition of sample/standard antioxidants, respectively. The resultant data was plotted between concentration of antioxidants and that of trolox for the standard reference curve.

Total phenolic contents: For the determination of total phenolic contents, the method as developed by Slinkard and Singleton[24] was used. Stock solution of gallic acid was made by dissolving 0.5 g gallic acid in 10 mL of ethanol in a 100 mL volumetric flask and diluting to volume with double distilled water. To prepare standard solutions 0, 50, 100, 150, 250 and 500 mg/L gallic acid, 0, 1, 2, 3, 5 and 10 mL of phenol stock solution were added into 100 mL volumetric flask separately and then diluted to volume with double distilled water.

From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of double distilled water was added. Folin-Ciocalteu's reagent (200 µL) was added and mixed well. After 8 min, 600 µL of sodium carbonate solution (20 %) was mixed thoroughly in the solution. The solution was allowed to stand at 40 ºC for 0.5 h and absorbance of each solution was noted at 765 nm against the blank (without phenolic solution). A concentration *versus* absorbance linear plot was thus obtained. The concentration of total phenolic compounds of each plant extract in milligram of gallic acid equivalent (GAE)), was determined by using the following standard equation.

$$\text{Absorbance}_{(\text{at } 765 \text{ nm})} = 0.118x + 0.0824 \text{ (gallic acid (mg/L))}$$

Total flavonoid contents: The calorimetric method as described by Dewanto *et al.*[25], was followed to determine total flavonoid contents of plants samples. Briefly, 0.25 mL of plant extract or quercetin standard solutions was mixed with 1.25 mL of distilled water in a test tube followed by addition of 75 µL of 5 % $NaNO_2$ solution. After 5 min, 150 µL of 10 % $AlCl_3 \cdot 6H_2O$ solution was added and allowed to stand for 6 min. Added 0.5 mL of 1 M NaOH and the volume of the mixture was raised to 2.5 mL with distilled water and mixed well. Absorbance readings at 510 nm were taken immediately after mixing. Total flavonoid contents (mg of quercetin equivalent per gram of residue) of the plant extracts were calculated after comparison with the standard curve of quercetin.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity: Upon reduction, the purple colour of DPPH solution is changed to yellow diphenylpicrylhydrazine. DPPH radical scavenging activity was found following the method of Shimada *et al.*[26]. Briefly, DPPH solution (3 and 25 mg/L) in methanol was mixed with 100 µL of plant extract. The reaction progress of the mixture was monitored at 517 nm over a time period of 0.5 h. The percentage of the DPPH remaining ($DPPH_{rem}$ %) was calculated as:

$$DPPH_{rem}\ (\%) = \frac{[DPPH]_{t=t}}{[DPPH]_{t=0}} \times 100$$

where $[DPPH]_{t=0}$ = concentration of DPPH radical before addition of antioxidant sample and $[DPPH]_{t=t}$ = concentration of DPPH radical after reaction with antioxidant sample at time t. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min) was plotted for each fraction of the samples.

Total antioxidant activity in linoleic acid emulsion system by ferric thiocyanate method: Total antioxidant activity of the plant extracts was determined according to the method employed by Mitsuda *et al.*[27]. Briefly, the solution, which contained 100 µL the plant extract in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The solution was then incubated at 37 ºC in a sealed bottle, in dark. Solution without any plant extract was used as blank, while the solution containing 100 µL (50 µg/20 µL) of trolox was used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_2$ (20 mM in 3.5 % HCl) and thiocyanate solution (30 %) to ethanolic sample, the solutions were stirred for 1 min. The absorption values at 500 nm were noted after incubation for 5 min at 37 ºC, as lipid peroxidation values.

Metal chelating activity: Iron(II) chelating activity of plant extracts was estimated following the method of Dinis *et al.*[28]. Briefly, 100 µL of the extract was added to a solution of 2 mM Fe(II) chloride (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and total volume was adjusted to 4 mL with ethanol. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. The results were expressed as percentage of inhibition of ferrozine-Fe^{2+} complex formation. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Chelating activity}\ (\%) = \left[\frac{A_{Control} - A_{Sample}}{A_{Control}}\right] \times 100$$

where $A_{Control}$ and A_{Sample} are the absorbances of the control and the sample, respectively.

Superoxide anion radical scavenging activity: Superoxide anion radical scavenging activity was determined following the method previously used by Nikishimi *et al.*[29]. Superoxide anion radicals were generated by the PMS-NADH system. The reaction mixture contained 100 µL extract, 200 µM NBT, 624 µM NADH and 80 µM PMS in 0.1 M phosphate buffer (pH 7.4). After 2 min of incubation, absorbance was measured spectrophotometrically at 560 nm. The scavenging effect was calculated using the following formula:

$$\text{Scavenging}\ (\%) = \left[1 - \frac{A_s}{A_b} \times 100\right]$$

where A_s and A_b are the absorbances of sample and blank solutions at 560 nm, respectively.

Antibacterial and antifungal activity: Cup plate method was employed to study the preliminary antibacterial and antifungal activity of the organic extracts of *A. pseudalhagi* against *Bacillus substilis*, *Escherichia coli*, *Salmonella typimorium*, *Pseudomonas aeruginosa* and *Aspergillus niger*. Preparation of agar medium, nutrient broth, subculture and peptone water was done as per the standard procedure. The standard solutions of the sample were made by dissolving 10 mg each of *n*-hexane, chloroform, methanol and ethyl acetate residues in 1 mL of the respective solvent. The cups each of 9 mm diameter were made with a sterilized cork borer in a Petri dish which was previously streaked with the organisms. Benzyl penicillin and fluconazole were used as positive references for antibacterial and antimicrobial activity assays, respectively. The neat solvents were used as control. The solutions (2 µL) of each test compound and reference were added separately in the cups and the Petri dishes were subsequently incubated at 37 ºC for 24 h. Each experiment was performed in triplicate and the zones of inhibition and the zone of inhibition produced by each sample was measured in mm and averaged.

RESULTS AND DISCUSSION

$ABTS^{\cdot+}$ scavenging activity: The ABTS decolorization assay was applied to evaluate *in vitro* radical scavenging activity of organic and aqueous extracts of *A. pseudalhagi*. ABTS radical cation, produced *in vitro* conditions, has the reduction potential comparable to that of hydroxyl radical which is produced *in vivo* during metabolic reactions and stress conditions. All the antioxidative components lower in reduction potential than that of the ABTS radical cation, can reduce ABTS radical cation to its native form proportionate to their amount on a time scale. Trolox equivalent antioxidant capacity values were obtained by comparing the percentage inhibition values of extract samples with the standard trolox curve. Bar graphs are plotted for the Trolox equivalent antioxidant capacity values of each fraction of the sample (Fig. 1).

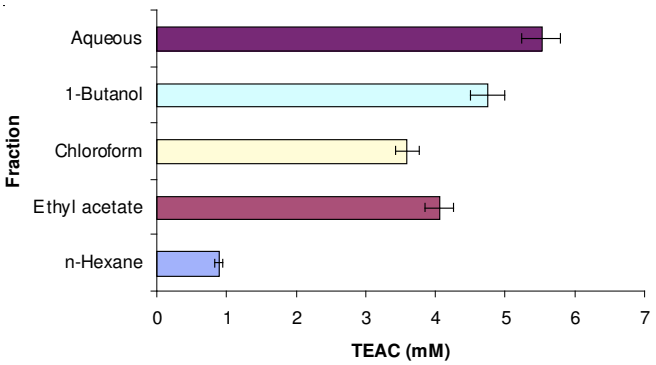

Fig. 1. ABTS radical cation scavenging capacity of different fractions of *A. pseudalhagi*

Trolox equivalent antioxidant capacity values ranged from 0.888-5.525 mM of trolox equivalents for *n*-hexane and aqueous fractions, respectively. With the exception of ethyl acetate a general trend of increase in the Trolox equivalent antioxidant capacity value with increasing polarity of extractive solvent is given Fig. 1. The somewhat greater trolox equivalent antioxidant capacity value obtained for ethyl acetate fraction may be attributed to greater solubility of antioxidant components in ethyl acetate. Likewise small Trolox equivalent antioxidant capacity values for relatively less polar solvent like *n*-hexane indicate low solubility of phenolic and other antioxidant components in non-polar medium.

Total phenolic and flavonoid contents: Total phenolic contents in the plant/herbal extracts and other samples are usually evaluated using Follin-Ciocalteu's reagent (FC reagent). On reduction by hydroxyl moieties of phenolic compounds yellow colour of FC reagent is reduced to blue colour proportionate to the amount of phenolic compounds present in the sample. This change in the colour is monitored spectrophotometrically at 765 nm. Total phenolic contents values of different fractions of *A. pseudalhagi* ranged from 10.657-21.759 mg/L of GAE for chloroform and 1-butanol fractions, respectively (Fig. 2a). Total flavonoid contents values of different fractions of *A. pseudalhagi* ranged from 1.815-10.911 mg/L of quercetin equivalents for ethyl acetate and chloroform fractions, respectively (Fig. 2b). The main active substances of this plant have been reported to be flavonods including flavanone glycosides, catechins and proanthocyanidins[16,20]. The presence of phenolic or polyhydroxyl moieties of these phenolic or flavonoids may considered as responsible for the high Trolox equivalent antioxidant capacity values of these extracts. However, no significant linear correlation between phenolic/flavonoid constituents and trolox equivalent antioxidant capacity values could be obtained. This may be justified on the grounds that phenolic moieties alone do not represent the total reducing charge of sample and at the same time some synergistic interactions amongst different antioxidative components in the sample may also be considered to contribute to the non-significant correlation.

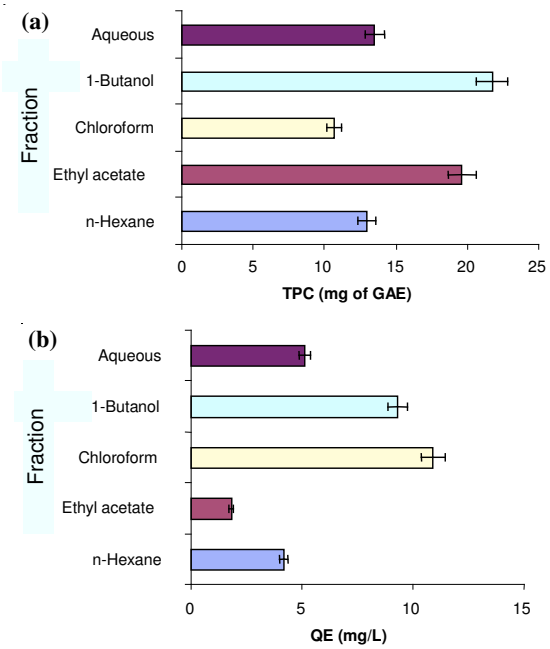

Fig. 2. Comparative profile of (a) total phenolic contents values (mg/L of GAE) and (b) total flavonoid contents values (mg/L of quercetin equivalents) of different fractions of *A. pseudalhagi*

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay: The DPPH free radical assay is based upon the measurement of the DPPH free radical scavenging capacity of plant extracts. The single electron on nitrogen in DPPH is reduced by a hydrogen atom donated by antioxidant compounds in the sample. The kinetics of the reaction of sample antioxidants with DPPH free radical was monitored spectrophotometrically by plotting the per cent DPPH remaining *versus* time. It is clear from the Fig. 3 that 1-butanol and ethyl acetate fractions of *A. pseudalhagi* contained high levels of DPPH radical scavenging agents. The kinetic curves obtained for all the samples are much steeper in the first 5 min, showing fast reaction of antioxidant components with DPPH radical. After 5 min, the curves become almost parallel to the time axis showing completion of reaction of most of the antioxidant agents. The major reduction in the absorbance in the first 5 min helps predicting that the majority of the antioxidant components present in the samples is fast-reacting. The slow and steady decrease in the absorbance even after 5 min indicates the presence of some slow-reacting antioxidant components in the samples. In case of 1-butanol the reduction in absorbance continued at somewhat faster pace even after 5 min. This behaviour shows that most of the antioxidant components in this fraction are relatively potent and fast reacting. On the basis of the data obtained, 1-butanol may be regarded as the most potent antioxidant fraction, which in turn also proves it to be the best extracting medium for *A. pseudalhagi* antioxidant components. The greater DPPH radical scavenging activity of organic extracts especially ethyl acetate and 1-butanol and less activity of aqueous fraction may be justified that the DPPH is only soluble in organic medium and thus more responsive to those antioxidant components which have greater solubility in organic medium.

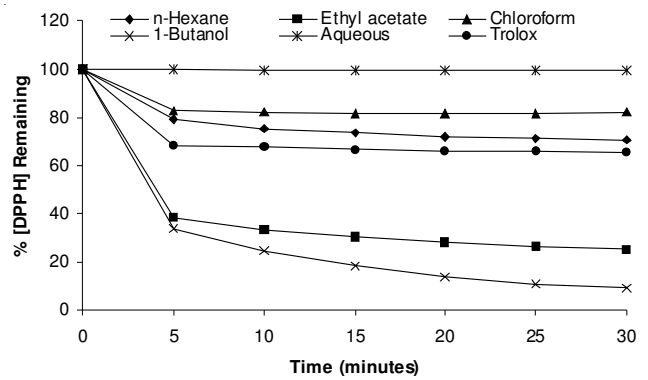

Fig. 3. Time course of DPPH radical scavenging activity of different fractions of *A. pseudalhagi*

Total antioxidant activity in linoleic acid emulsion systems by ferric thiocyanate method: The ferric thiocyanate method is based upon spectrophotometric measurement of Fe^{3+}-SCN complex in the reaction mixture. Peroxo radicals (ROO$^{\cdot}$), formed by air oxidation of linoleic acid, have the ability to oxidize Fe^{2+}-Fe^{3+}. The sample antioxidants scavenging cause delay in the oxidation of linoleic acid and thus inhibit formation

of ROO˙ and Fe^{3+}-SCN complex. The peroxidation value is noted spectrophotometrically as increase in the absorbance at 500 nm due to formation of this complex. Absence of antioxidant agents causes an unrestricted rise in the absorbance of blank. In case of standard or sample solutions, the antioxidants will try to inhibit or slow down oxidation of linoleic acid and will therefore, result into low peroxidation value. Thus a low peroxidation value indicates its capacity to inhibit peroxidation of linoleic acid and *vice versa*. Lipid peroxidation values of different fractions of *A. pseudalhagi* were determined as a function of time. Trolox was used as positive control. All the fractions of the plants demonstrated potent peroxyl radicals scavenging activity comparable to trolox (Fig. 4).

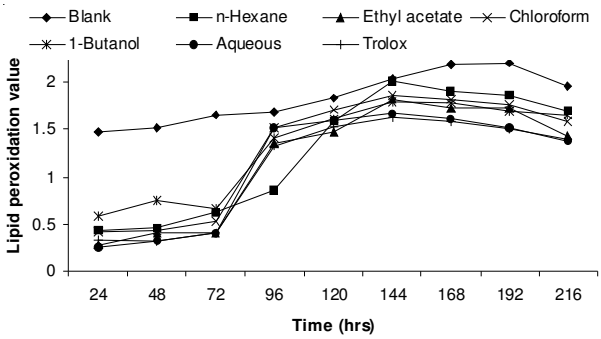

Fig. 4. Time-dependent lipid peroxidation values of different fractions of *A. pseudalhagi*

Metal chelating activity: The ability of plants extracts to make a complex with ferrozine was estimated following a procedure reported by Dinis *et al.*[28]. The complex of ferrozine with iron(II) can spectrophotometrically be measured at 562 nm. Polyphenolic compounds compete with ferrozine for the formation of this complex and thus cause a decrease in the absorbance. The results are expressed as percentage of inhibition of ferrozine-Fe^{2+} complex formation. Iron(II) found in the biological systems may act as pro-oxidants. A pro-oxidant does not act as harmful agent for biomolecules directly but facilitates production of such species which may cause damage to biomolecules. Polyphenolic compounds, thus by binding with Fe(II), do not allow them to act as pro-oxidant and thus add to an indirect defence mechanism against potentially harmful radical species. Per cent bound iron was calculated and graphed for all the plant extracts. Most of the extracts either did not respond to the test or the activity found was very small. The chloroform and 1-butanol fractions showed values of 10.149 and 21.072 in terms of percentage bound iron (Fig. 5). On the other hand *n*-hexane, ethyl acetate and aqueous fractions did not exhibit any detectable activity.

Superoxide anion radical scavenging activity: In living organisms superoxide anions produced by activated phagocytes plays an important role in killing microorganisms. On the other hand, superoxide anions are removed in the living bodies by superoxide dismutase[30]. In PMS-NADH system, superoxide anion radicals are produced from dissolved oxygen by PMS-NADH coupling reaction. The radicals reduce NBT to a blue colour compound. In the presence of antioxidant sample, a decrease in the absorbance at 560 nm indicates the consumption of superoxide anion radicals in the reaction mixture.

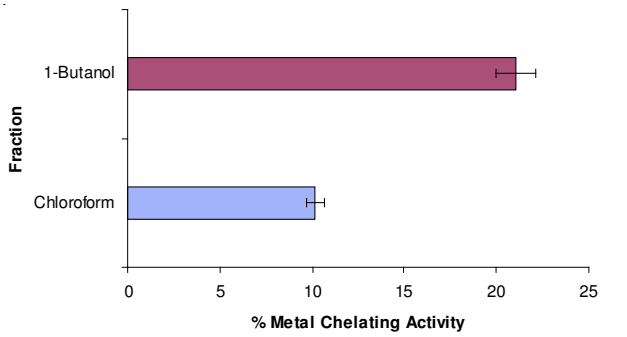

Fig. 5. Metal chelating activity of different fractions of *A. pseudalhagi*

Fig. 6 shows that aqueous fraction showed the highest superoxide anion radical scavenging activity followed by 1-butanol fraction. Ethyl acetate, chloroform and *n*-hexane fractions did not show any detectable scavenging activity.

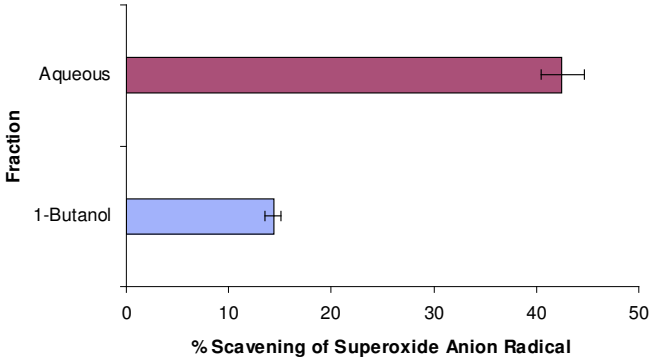

Fig. 6. Per cent scavenging activity of different fractions of *A. pseudalhagi*

Antimicrobial and antifungal activity: Cup plate method was used to evaluate antibacterial and antifungal activities of different fractions of *A. pseudalhagi*. Zones of inhibition were calculated in millimeter. The results obtained are shown in Table-1. It is evident that all the fractions of *A. pseudalhagi* have activity comparable to standards used against almost all the organisms tested.

TABLE-1
ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF DIFFERENT FRACTIONS OF *A. pseudalhagi*

Fraction/control/ standard	Zone of inhibition (mm)				
	B. subtilis	*E. coli*	*S. typimorium*	*P. aeruginosa*	*A. niger*
n-Hexane	20	11	n.d.	14	11
Chloroform	17	11	11	16	13
Methanol	13	11	n.d.	18	11
Ethyl acetate	11	13	n.d.	18	10
Control	n.d.	n.d.	n.d.	n.d.	n.d.
Benzyl penicillin	14	15	11	12	n.d.
Fluconazole	n.d.	n.d.	n.d.	n.d.	15

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