Research Article

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FREE RADICAL-SCAVENGING POTENTIAL OF DIFFERENT EXTRACTS FROM *AZIMA TETRACANTHA* LAM.

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ABSTRACT

Oxidative stress resulted from free radicals and reactive oxygen species are associated with many diseases. Worldwide several studies are directed towards finding natural antioxidants of plant origin. Plants containing phenolic compounds have been reported to possess strong antioxidant activities. The aim of the present study was to investigate the phytochemical constituent of hexane, chloroform, ethyl acetate and methanolic root extracts and *in vitro* antioxidant activities of *Azima tetracantha* Lam. The different extracts of *A. tetracantha* root were studied for antioxidant potential by using different *in vitro* assays such as inhibition of DPPH, $ABTS^+$, Hydroxyl radical and superoxide anions. The total phenolic contents and ferric reducing antioxidant power of the extracts were also determined by using standard phytochemical reaction methods. Butylated hydroxyl toluene (BHT), Vitamin C and ascorbic acid were taken as standards. The different extracts of *A. tetracantha* root showed good dose dependant free radical scavenging activity in all the models. The total phenolic content of hexane, chloroform, ethyl acetate and methanolic root extracts was found to be 3.30±0.20, 4.20±0.20, 6.30±0.20 and 8.80±0.20 mg GAE/g respectively. Ferric reducing antioxidant power was also found to be increased with increase in extracts concentrations. All the results of the *in vitro* antioxidant assays revealed the free radical scavenging potential of *A. tetracantha* root and compared with standard antioxidants. These antioxidant activities may be endorsed to its high free radical scavenging and phenolic contents. Thus, our findings provide evidence that *A. tetracantha* is a potential source of natural antioxidants.

Keywords: Antioxidant activity, *Azima tetracantha*, DPPH, $ABTS^+$, FRAP.

INTRODUCTION

Many plants have nutritive value as well as they are the major source of medicine. The medicinal value of these plants lies in phytochemical constituents and the effectiveness of phytochemical in the treatment of various diseases may lie in their antioxidant effects that cause definite pharmacological action on the human body[1], which leads to drug discovery and it is referred to as "natural product screening"[2]. In recent years there is an increasing awareness among people in prevention of disease especially the role of free radicals in health and disease. Free radicals are continuously produced by the body's normal use of oxygen[3]. Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are produced by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process[4]. Free radicals can be either harmful or helpful to the body. When there is an imbalance between formation and removal of free radicals then a condition called as oxidative stress is developed in body. To counteract these free radicals body has protective antioxidant mechanisms which have abilities to lower incidence of various human morbidities and mortalities[5]. Antioxidant refers to a compound that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions and which can thus prevent or repair the damage done to the body's cells by oxygen. They act by one or more of the following mechanisms: reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. Epidemiological studies have shown that many phytonutrients of fruits and vegetables might protect the human body against damage by ROS. The consumption of natural antioxidant phytochemicals was reported to have potential health benefits. The antioxidant phytochemicals from plants, particularly flavonoids and other polyphenols have been reported to inhibit the propagation of free radical reactions, to protect the human body from disease and to retard lipid oxidative rancidity[6]. The medicinal value of the plants lies in phytochemical constituents that cause definite pharmacological action on the human body. *Azima tetracantha* Lam. locally known as "Mulsangu", is a rambling spinous shrub flowering throughout the year found in peninsular India, West Bengal, Orissa, India African countries and extending through Arabia to tropical Asia. In India and Sri Lanka the root, root bark and leaves were administered with food as a remedy for rheumatism[7]. This plant is considered as a powerful diuretic and is also used to treat rheumatism, dropsy, dyspepsia, chronic diarrhea and as a stimulant tonic for woman after confinement[8]. It is also used as food for various herbal medicines in Africa, India and Madagascar[9].

MATERIALS AND METHODS

Collection and preparation of crude extracts

The root of *Azima tetracantha* Lam (Salvodaraceae) was collected from Athamangalam (Lat. 10.46 ºN; Long, 79.49ºE), Nagapattinam District, Tamil Nadu, India during August to September 2012. Herbarium was deposited in Department of Botany, Annamalai University (AUBOT 262). The collected root was washed with tap water, then surface sterilized with 10 % sodium hypochloride solution to prevent contamination of any microbes. The samples were rinsed with sterile distilled water and allowed to shade dried under room temperature followed by oven drying at 50 ºC and then ground into powder using electric blender. One hundred grams of powder samples was packed in soxhlet apparatus and extracted with different organic solvents like non-polar to polar viz., hexane, chloroform, ethyl acetate and methanol for 72 hours[10]. The extracts were pooled and the solvent were evaporated under vacuum in a rotary evaporator (Heidolph, Germany) and the dried extracts were stored at 4 ºC for *In vitro* antioxidant activity.

Phytochemical screening of extracts

The hexane, chloroform, ethyl acetate and methanol extracts of *A. tetracantha* were used for qualitative phytochemical studies. Phytochemical like glycosides, terpenoids, proteins, amino acids, carbohydrates, flavonoids, tannins, saponins, phenolic compounds, alkaloids, fixed oil and fats were analyzed according to the standard method described by Kokate CK, 1994 [11].

Determination of total phenolic content

Total phenolic content was carried out following the Folin- Ciocalteu method by Singleton VL *et al*, 1965 [12]. 1 ml of crude extracts solution containing (125 to 1000 µg/ml) was added volumetric flask. 1 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) was added and allowed to stand at 22°C for 5 minutes; 7.5 % of 0.75 ml of sodium bicarbonate solution was added and mixed thoroughly. The samples were measured spectrophotometrically at 765 nm using spectrometer after 90 min at 22°C. The amount of total phenolics was determined as Gallic acid and equivalent and expressed as mg GAE/g dry weight.

DPPH radical scavenging activity

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to method of Blois MS, 1958 [13]. A methanol solution of the sample extracts at various concentrations (125, 250, 500 and 1000 µg/ml) was added to 0.5 ml of 0.1 mM methanolic solution of DPPH and allowed to stand for 30 min at 25ºC. The absorbance of the sample was measured at 517 nm. A 0.1 mM solution of DPPH in methanol was used as control, whereas L-ascorbic acid was used as reference standard. All tests were performed in triplicate. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

Percentage of inhibition = (A control – A sample/ A control) × 100

$ABTS^+$ scavenging assay

Radical scavenging ability was measured using 2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid ($ABTS^+$) as described by Winterbourne CC *et al*, 1975 [14]. $ABTS^+$ radical cations ($ABTS^+$) were produced by reacting $ABTS^+$ solution (7 mM) with 2.45 mM potassium persulphate. The mixture was incubated at room temperature in the dark for 12 to 16 h to yield a dark-colored solution containing $ABTS^{\bullet+}$ radicals and diluted for an initial absorbance of about 0.700 (± 0.02) at 734 nm. Aliquots (125, 250, 500 and 1000 µg/ml) of the different concentrations of extract were added to 1 ml of $ABTS^+$ solution. The absorbance was read at 734 nm after 6 minutes in a spectrophotometer. BHT was used as the standard. Appropriate solvents blanks were run in each assay. All determinations were carried out in triplicate and the percent of inhibition was calculated using the formula.

Percentage of inhibition = (A control – A sample/ A control) × 100

Superoxide scavenging activity

The superoxide scavenging ability of the extracts was assessed by the method of Elizabeth K *et al*, 1990 [15]. Superoxide anions were generated in samples that contained in 3.0 ml, 0.02 ml of the root extracts (125, 250, 500 and 1000 µg/ml), 0.2 ml of EDTA, 0.1 ml of NBT, 0.05 ml of riboflavin and 2.64 ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexes and the initial optical density was measured at 560 nm in a spectrophotometer (Hitachi U20). The absorbance was measured again at 560 nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

Percentage of inhibition = (A control – A sample/ A control) × 100

Hydroxyl radical scavenging assay

The extent of hydroxyl radical scavenging from Fenton reaction was quantified using 2'-deoxyribose oxidative degradation as described by Oyaizu M, 1986 [16]. The reaction mixture contained 0.1 ml of deoxyribose, 0.1 ml of $FeCl_3$, 0.1 ml of EDTA, 0.1 ml of H_2O_2, 0.1 ml of ascorbate, 0.1 ml of KH_2PO_4-KOH buffer and (125, 250, 500 and 1000 µg/ml) of plant extracts in a final volume of 1.0 ml. The mixture was incubated at 37ºC for 1 hour. At the end of the incubation period, 1.0 ml of TBA was added and heated at 95ºC for 20 minutes to develop the color. After cooling, the TBARS formation was measured spectrophotometrically (Hitachi U20) at 532 nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The percent TBARS production for positive control (Vitamin C) was fixed at

100 % and the relative per cent TBARS was calculated for the extract treated groups.

Percentage of inhibition = (A control – A sample/ A control) × 100

Ferric reducing antioxidant power

The reducing power of different extracts of *A. tetracantha* root was evaluated by Korycka Dahl M *et al*, 1978 [17]. The samples were mixed with 2.5 ml of 0.2 M Phosphate buffer (pH 6.6) and 2.5 ml of 1 percent potassium ferric cyanide. After that the mixture was incubated at 50ºC for 20 min, 2.5 ml of 10 percent TCA, 2.5 ml distilled water and 0.5 ml of 0.1 percent ferric chloride were added and then the absorbance was measured at 700 nm against a blank. The blank consist of all the reagents without the test sample. The reducing power of gallic acid was also determined for a comparison. High absorbance of the reaction mixture indicates strong reducing power.

RESULTS AND DISCUSSION

Preliminary phytochemical screening showed the presence of alkaloids, terpenoids, glycosides, saponins, protein, phenolic compounds like tannins and flavonoids (Table 1). The antioxidants from natural sources are the only alternative to synthetic antioxidants in counteracting the free radicals associated diseases. A great number of naturally occurring substances have been recognized to have antioxidant abilities and various *in vitro* methods have been used to assess their free radical scavenging and antioxidant activity. Therefore, in the present study hexane, chloroform, ethyl acetate and methanol in graded concentrations were assessed for their free radical scavenging and antioxidant activity in various *in vitro* models. It was observed that the test extracts scavenged free radicals in a dose dependent manner and antioxidant activity of hexane, chloroform, ethyl acetate and methanol was compared to standards such as BHT, Vitamin C, galllic acid and ascorbic acid.

Total phenolic content of extracts

Total phenolic content of hexane, chloroform, ethyl acetate and methanolic extracts obtained from *A. tetracantha* root, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents. The total phenolic content of hexane, chloroform, ethyl acetate and methanolic were found to be 3.30±0.20, 4.20±0.20, 6.30±0.20 and 8.80±0.20 mg gallic acid equivalent/g of extract, respectively.

DPPH radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical at room temperature which acts as an acceptor of electrons or hydrogen radicals to become a stable dia-magnetic molecule[16] and used as substrate to evaluate the free radical scavenging activities of the extracts. It involves the reaction of specific antioxidant with a stable free radical and results in decrease in absorbance which can be detected at 517 nm and it is due to scavenging of the radical by hydrogen donation which is measured by the change in color from purple to yellow. The scavenging effect of hexane, chloroform, ethyl acetate and methanol at the concentration of 1000 µg/ml was 63.6 %, 76.0 %, 88.6 % and 92.6 % respectively, when compared to the scavenging effect of L-ascorbic acid (95.0 %) at the same concentration with their IC_{50} values 800.02, 692.57, 563.91, 507.10 and 392.48 µg/ml respectively. The present investigation has shown that both the extracts exhibited DPPH radical-scavenging activity in a concentration dependant manner and results were compared with the standard compound as shown in Figure 1 indicating their abilities to act as radical scavengers.

$ABTS^+$ radical scavenging activity

The hexane, chloroform, ethyl acetate and methanol root extracts of *A. tetracantha* were fast and effective scavengers of the $ABTS^+$ radical. Figure 2 illustrates that hexane, chloroform and ethyl acetate extracts at 1000 µg/ml exhibited (63.3 %, 75.0 % and 85.6 %) $ABTS^+$ radical scavenging activity, which is lower than the methanol extracts (89.3 %) and standard compound BHT, which exhibited 97.0 % of radical scavenging activity. The IC_{50} values of hexane, chloroform, ethyl acetate, methanol and BHT was found to be 851.49, 755.26, 587.85, 524.58 µg/ml and 378.967 µg/ml. The results suggest that the methanol was more potent in antioxidant potential than ethyl acetate, chloroform and hexane.

Superoxide anion scavenging activity

Superoxide anion radical is one of the strongest reactive oxygen species and is also very harmful to cellular components[18]. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in absorbance at 560 nm with the plant extracts indicates their abilities to quench superoxide radicals in the reaction mixture. Figure 3 illustrates that hexane, chloroform and ethyl acetate extracts at 1000 µg/ml exhibited (32.20 %, 36.40 % and 55.50 %) superoxide radical scavenging activity, which is lower than methanol extract (68.50 %) and standard compound L-ascorbic acid, which exhibited 95.0 %. The IC_{50} values of hexane, chloroform, ethyl acetate, methanol and L-ascorbic acid was found to be 1549.29, 1422.89, 803.44, 481.11 and 392.48 µg/ml respectively.

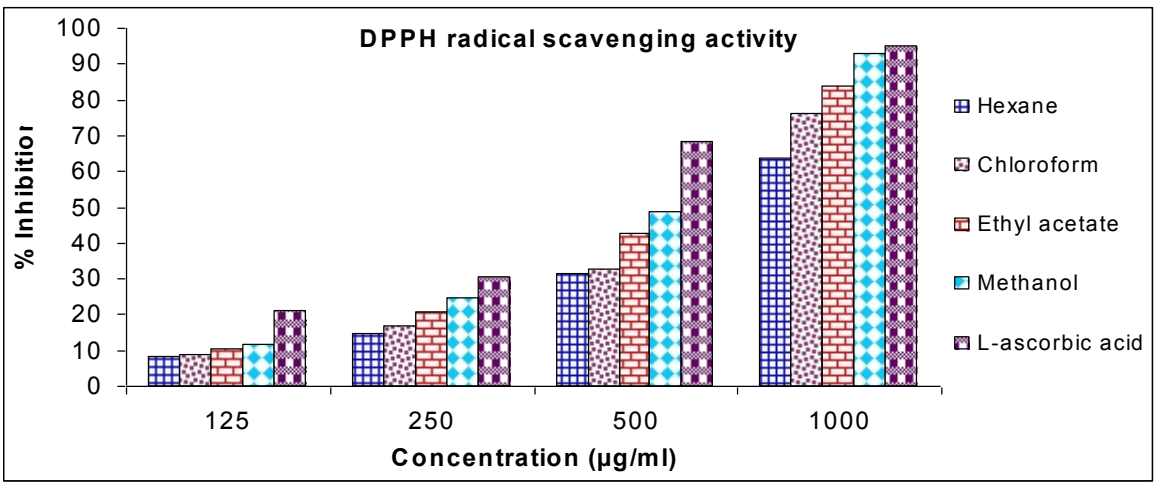
Hydroxyl radical scavenging activity

Hydroxyl radicals have a short half-life and are the most reactive and damaging oxygen species causing lipid per oxidation and cellular damage[19,20]. Hydroxyl radicals were produced in this study by incubating ferric-EDTA with ascorbic acid with 2- deoxy-2-ribose to generate malondialdehyde like product. This compound forms a pink chromogen upon heating with TBA at low pH[21].

Table 1: Preliminary phytochemical analysis of *Azima tetracantha* after successive extract with hexane, chloroform, ethyl acetate and methanol

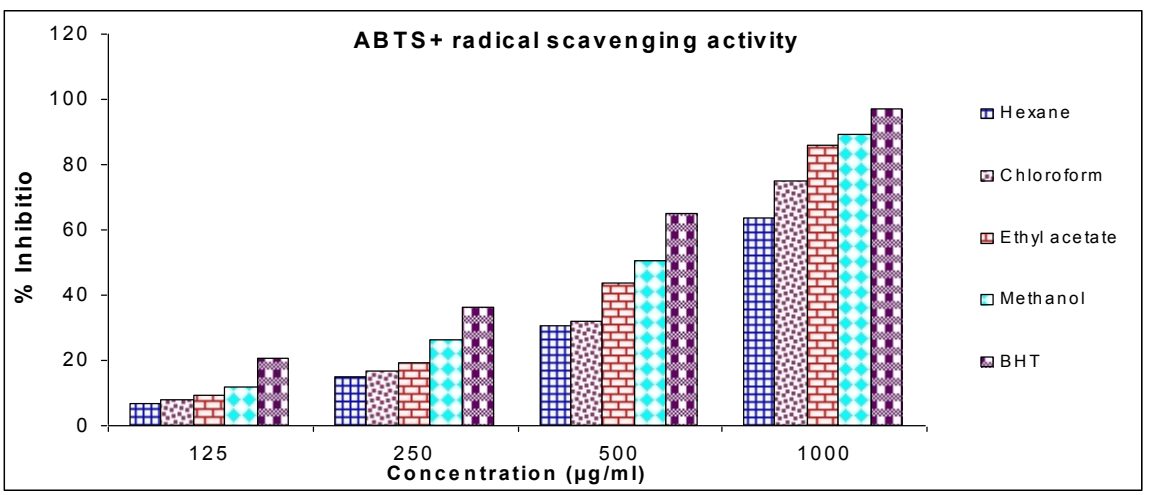
S. No.	Phytochemical compounds	Root			
		H	C	EA	M
1.	Alkaloids	-	+	+	+
2.	Carbohydrates	-	-	-	-
3.	Glycosides	-	-	-	+
4.	Saponins	-	-	+	+
5.	Protein	+	+	+	+
6.	Amino acids	+	-	-	-
7.	Fixed oil or Fats	-	-	-	-
8.	Phenolic compound	-	+	+	+
9.	Flavonoids	-	+	+	+
10.	Tannins	-	-	+	+
11.	Terpenoids	-	-	+	+

(+Present; - Absent), H – Hexane, C – Chloroform, EA– Ethyl acetate and M – Methanol)



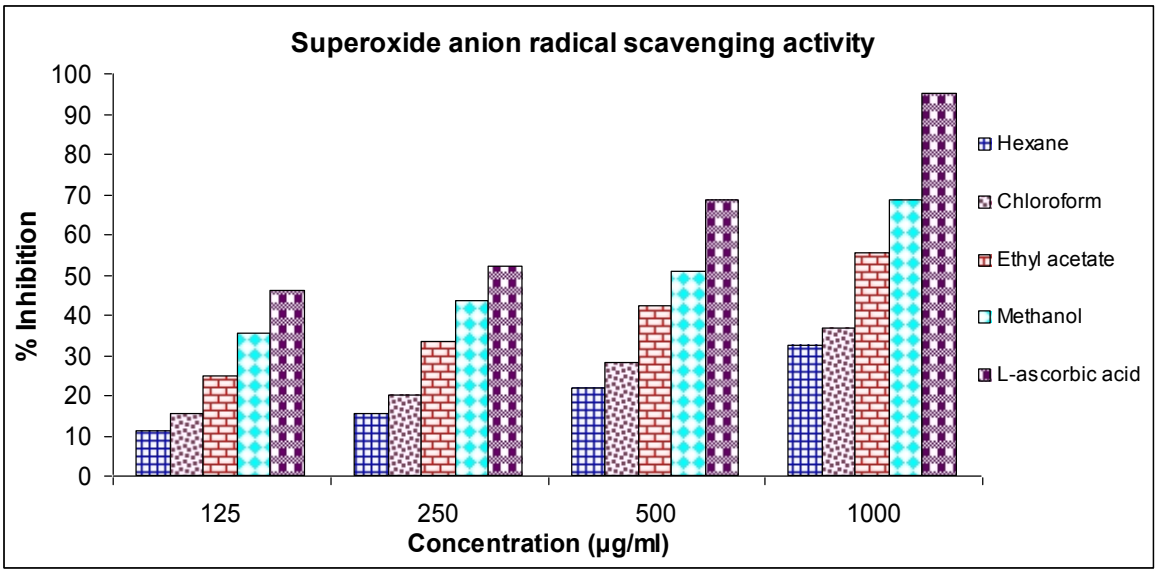
Values are means of triplicate determinations (n = 3)

Figure 1: DPPH free radical scavenging activity of different extracts of *A. tetracantha* and standards at various concentrations



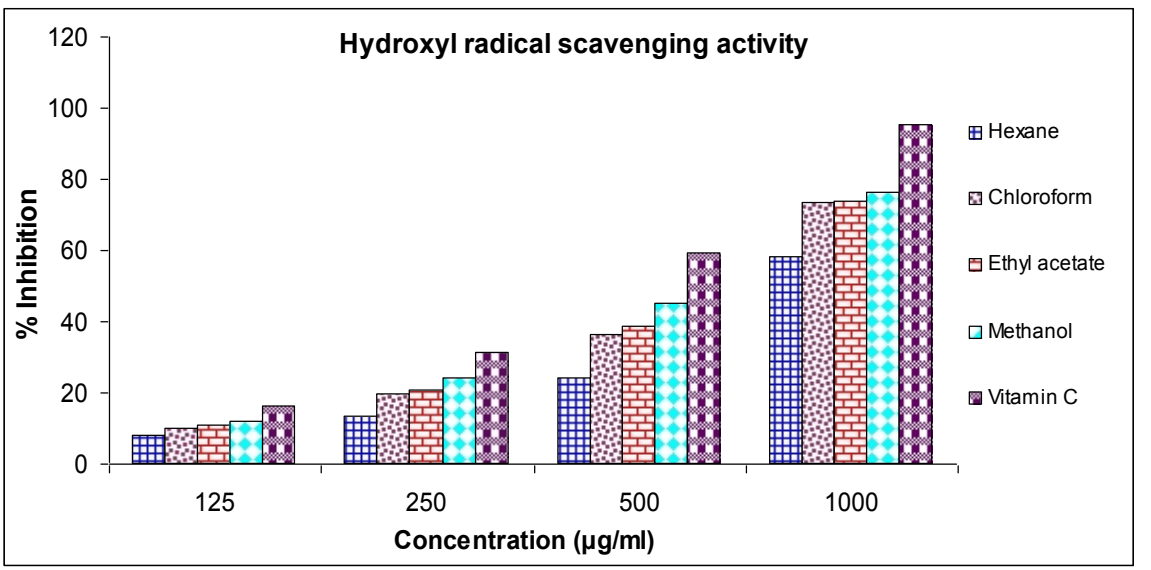
Values are means of triplicate determinations (n = 3)

Figure 2: ABTS^{+} free radical scavenging activity of different extracts of *A. tetracantha* and standards at various concentrations



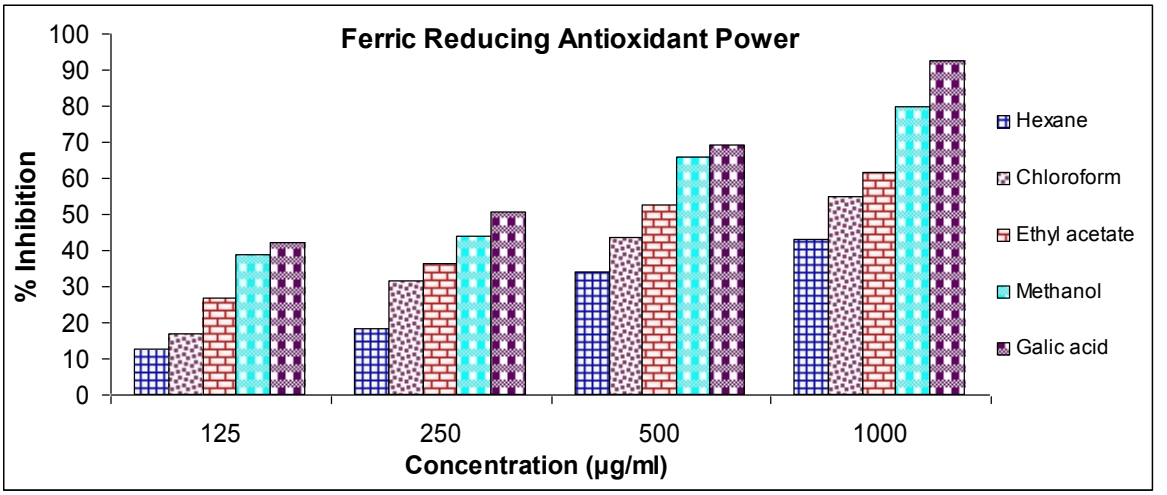
Values are means of triplicate determinations (n=3)

Figure 3: Superoxide anion radical scavenging activity different extracts of *A. tetracantha* and standards at various concentrations



Values are means of triplicate determinations (n = 3)

Figure 4: Hydroxyl radical scavenging activity of different extracts of *A. tetracantha* and standards at various concentrations



Values are means of triplicate determinations (n = 3)

Figure 5: Ferric reducing antioxidant power activity of different extracts of *A. tetracantha* and standards at various concentrations

Figure 4 illustrates the hydroxyl radical scavenging potential of hexane, chloroform, ethyl acetate and methanol extracts in comparison with the standard Vitamin C. When the hexane, chloroform, ethyl acetate, methanolic extracts and standard Vitamin C (1000 µg/ml) were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented the degradation of 2- deoxy-2-ribose. Hexane, chloroform and ethyl acetate at 1000 µg/ml exhibited (58.00 %, 73.23 % and 73.50 %) lower scavenging effect of OH^- than methanol (76.00 %) and standard Vitamin C (95.33 %). The IC_{50} value of hexane, chloroform, ethyl acetate and methanol extracts was found to be 887.68, 689.57, 679.63 and 620.70 µg/ml, in comparison with standard Vitamin C (430.72 µg/ml). The results suggest that the methanol is more potent than ethyl acetate, chloroform and hexane hydroxyl radicals when compared to Vitamin C. Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membranes and they are responsible for peroxidic reactions of lipids. It is thus anticipated that methanolic extract would show antioxidant effects against lipid per oxidation on bio membranes and would scavenge OH^- radicals at the stages of initiation and termination.

Determination of Ferric reducing antioxidant power

The reducing properties are generally depends on the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom[22]. Figure 5 shows the reducing capacity of hexane, chloroform, ethyl acetate and methanol extracts compared to standard gallic acid (125-1000 µg/ml) at 700 nm. Like the scavenging activity, the reducing power of both the extracts increased with increasing concentration. The result suggest that methanol extract has more reducing power than ethyl acetate, chloroform and hexane but not as efficient as standard Gallic acid.

CONCLUSION

In the present study results show that *A. tetracantha* root is good source of natural phenolic compounds. The methanolic extract of the *A. tetracantha* root showed better free radical scavenging capacity against different reactive oxygen species, among other extracts although with different efficiencies. The high content of antioxidants like phenolic compounds was found in theses extracts and it may impart health benefits by combating the free radicals in synergistic manner along with other compounds thus constitute part of the basis for the ethno pharmacological claim. Still extensive studies are needed to evaluate the phytochemical and pharmacological activities of specific lead compounds in order to use this plant as a probable source for the potential natural antioxidants.

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