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Free Radical Scavenging Activity of *Azima tetracantha* Leaves–An *In Vitro* Study

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Abstract: ***Free radical scavenging activity of ethanolic extract of Azima tetracantha leaves were carried out for proving its utility in free radical mediated diseases including diabetic, cardiovascular, cancer etc. The ethanolic extract was screened for in vitro antioxidant activity by oxygen and nitrogen radical scavenging such as DPPH scavenging, superoxide anion radical scavenging, metal chelation, reducing power activity and total antioxidant assay at different concentrations. Throughout the studies leaves extract showed marked antioxidant activity. The antioxidant activity of the leaves extract may be due to the phytochemicals present in it. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioactive compounds in the leaves of Azima tetracantha. The results of the present study concluded that the plant extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including aging.***

Keywords: Antioxidant activity, *Azima tetracantha*, Radical scavenging, Reactive oxygen species

1. Introduction

In living organisms, various reactive oxygen and nitrogen species (ROS/RNS) e.g., superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), nitric oxide radicals ($NO^{\bullet}$) and non-radical compounds, can be formed by different mechanisms. It is unavoidable one because of they are continuously produced by the body's normal use of oxygen. Such species are considered to be important causative factors in the development of diseases such as diabetes, stroke, arteriosclerosis, cancer, and cardiovascular diseases and the aging process (Velavan, 2011; Alma et al, 2003). This effect was significantly reversed by prior administration of antioxidant providing a close relationship between free radical scavenging activity (FRSA) and the involvement of endocrinological responses (Wiseman and Halliwell, 1996).

The recent abundant evidence suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of natural antioxidants in the maintenance of human health and prevention and treatment of diseases (Niki, 2010). Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan et al, 2007). The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthrocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene, and tocopherol are known to possess antioxidant potential (Prior, 2003). With this background and abundant source of unique active components harbored in plants. The chosen medicinal plant namely as *Azima tetracantha* leaves L belongs to the *Salvadoraceae* family. Therefore, the present study were to investigate the free radical scavenging activity of *Azima tetracantha* leaves through the free radical scavenging such as DPPH scavenging, superoxide anion radical scavenging, metal chelation, reducing power activity and total antioxidant assay.

2. Materials and Methods

In Vitro Antioxidant Activity

DPPH Assay

The scavenging ability of the natural antioxidants of the plant extract towards the stable free radical DPPH was measured by the method of Shimada et al., (1992). Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

$$\text{Radical scavenging activity}(\%) = 100 - \frac{A_C - A_S}{A_C} X\ 100$$

Where $A_{C\ =}$ control is the absorbance of the control and $A_{S\ =}$ sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.* (1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Superoxide anion scavenging activity assay

The scavenging activity of the *Azima tetracantha* towards superoxide anion radicals was measured by the method of

Liu et al. (1997). Superoxide anions were generated in a non-enzymatidc phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$,

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis et al. (1994). To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

Chelating rate (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu (1986) with slight modifications. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate $[K_3Fe(CN)_6]$ (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis: Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

3. Results and Discussion

DPPH Assay

DPPH radical scavenging activity of plant extract of BABE and standard ascorbic acid are presented in Fig.1 and Table 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.*, 2003). Recently, the use of the DPPH$^{\bullet}$ reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH$^{\bullet}$ free radical by a scavenger (A-H) causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region. The effect of antioxidants on DPPH$^{\bullet}$ is thought to be due to their hydrogen donating ability (Sindhu and Abraham, 2006). The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 54.73µg ml^{-1} and 34.91 µg ml^{-1} respectively**.** The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

Total antioxidant activity

The yield of the ethanol extract of the plant and its total antioxidant capacity are given in Fig. 2 and Table 1. Total antioxidant capacity of BABE is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extract (Prieto *et al.*, 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract. The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 54.55µg ml^{-1} and 42.41 µg ml^{-1} respectively**.**

Table 1: *In vitro* antioxidant study of *Azima tetracantha*

Concentrations (µg/ml)	*DPPH*	*DPPH (Standard)*	*Total Antioxidant*	*Antioxidant (Standard)*
20	18.20±1.27	25.6±2.04	12.50±0.88	22.35± 1.80
40	36.34±2.55	61.26±4.90	28.12±1.97	51.23± 4.09
60	55.45±3.88	88.98±7.11	53.12±3.72	72.54± 5.80
80	72.73±5.09	99.34±7.94	84.37±5.91	86.35± 6.91
IC-50	54.73	34.91	54.55	42.41

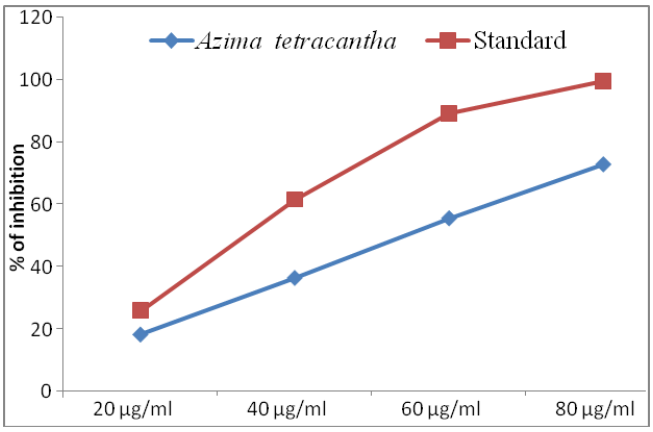

Figure 1: DPPH radical scavenging activity of *Azima tetracantha*

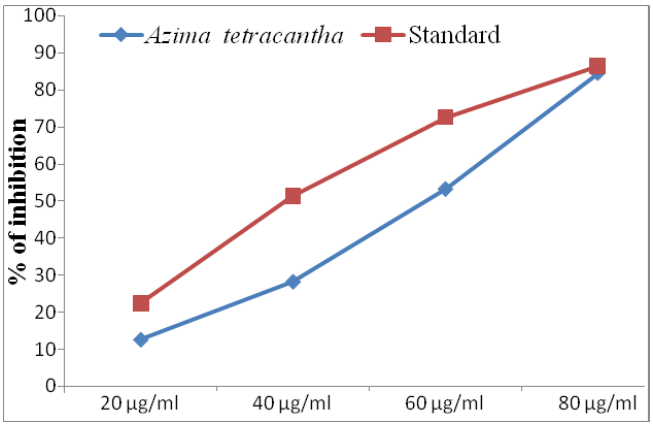

Figure 2: Total Antioxidant Assay of *Azima tetracantha*

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which are very harmful to the cellular components in a biological system (Korycka-Dahl & Richardson, 1978). The superoxide anion radical scavenging activities of the extract from *Azima tetracantha* assayed by the PMS-NADH system were shown in Fig 3 and Table 2. The superoxide scavenging activity of *Azima tetracantha* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *Azima tetracantha* was 55.93µg ml^{-1} and ascorbic acid were 31.62µg ml^{-1}**.** These results suggested that *Azima tetracantha* had notably superior superoxide radical scavenging effects.

The ferrous ion chelating activity

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the presence of aqueous extract of *Azima tetracantha* , indicating that have chelating activity with an IC_{50} of 50.83µg ml^{-1} and ascorbic acid was 30.96µg ml^{-1} (Fig. 4 and Table 2). Ferrous ion can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliwell, 1991). Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that form bonds with a metal are effective as secondary antioxidants because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion (Gordon, 1990). Thus, *Azima tetracantha* demonstrate a marked capacity for ion binding, suggesting their ability as a peroxidation protector that relates to the ion binding capacity.

Table 2: *In vitro* antioxidant study of *Azima tetracantha*

Concentrations (µg/ml)	Superoxide Anion	Superoxide Anion (Standard)	Fe^{2+} Chelating Agent	Fe^{2+} Chelating Agent (Standard)
20	14.28±1.00	31.25 ± 2.50	15.48±1.08	35.23 ± 2.81
40	28.57±2.00	64.23 ± 5.13	30.86±2.14	65.21 ± 5.28
60	57.14±4.00	89.54 ± 7.16	65.46±4.58	78.51± 6.28
80	75.00±5.25	98.51 ± 7.88	80.76±5.92	98.65 ± 7.89
IC-50	55.93	31.62	50.83	30.96

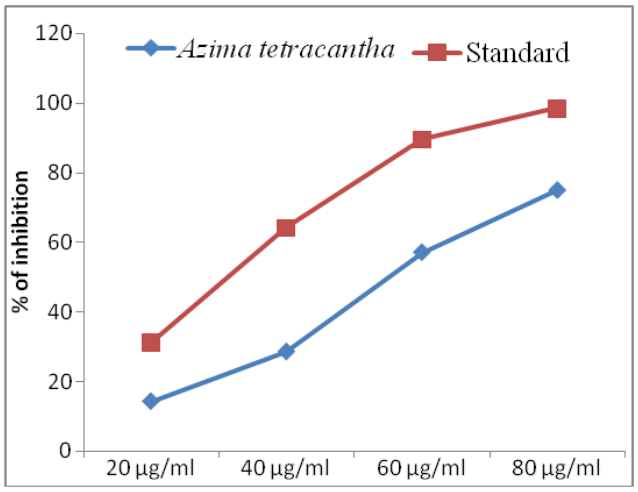

Figure 3: Superoxide radical scavenging activity of *Azima tetracantha*

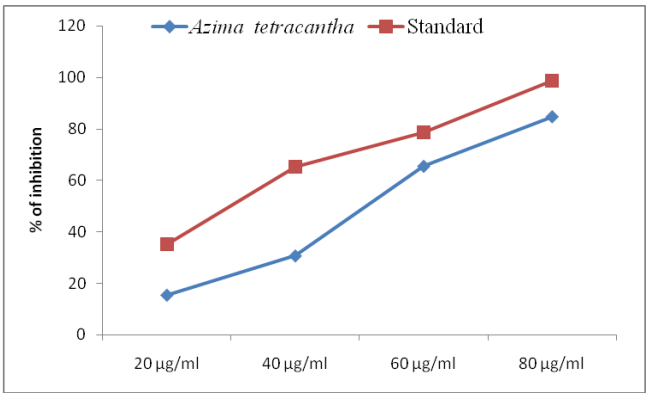

Figure 4: Iron chelating activity of *Azima tetracantha*

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Azima tetracantha* . The reducing capacity of a compound may serve as a significant indicator for its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Yildirim *et al.*, 2000). Fig. 5 and

table 3 depicts the reductive effect of *Azima tetracantha* . Similar to the antioxidant activity, the reducing power of *Azima tetracantha* increased with increasing dosage. All the doses showed significantly higher activities than the control indicating that *Azima tetracantha* consist of hydrophilic polyphenolic compounds that cause the greater reducing power.

Table 3: Reducing power activity of *Azima tetracantha*

Concentrations (µg/ml)	*Reducing Power Assay*	*Reducing Power Assay (Standard)*
20	0.24 ±0.017	0.41± 0.03
40	0.44 ±0.031	0.7 ± 0.05
60	0.68 ±0.048	0.89± 0.07
80	0.82 ±0.057	0.98 ± 0.08

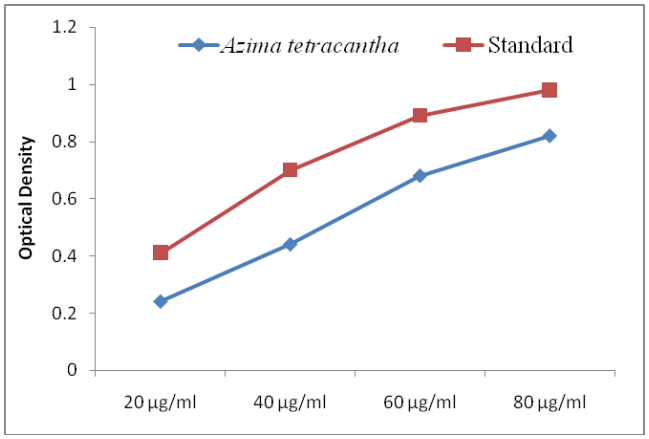

Figure 5: Reducing power activity of *Azima tetracantha*

4. Conclusion

On the basis of the results of this study, it clearly indicates that *Azima tetracantha* leaves had powerful *in vitro* antioxidant capacity against various antioxidant systems as DPPH, superoxide anion scavenging and metal chelator. From our results, the antioxidant activity of *Azima tetracantha* leaves was concentration dependent. The extracts could exhibit antioxidant properties approximately comparable to commercial synthetic antioxidants as ascorbic acid. From the above assays, the possible mechanism of antioxidant activity of *Azima tetracantha* leaves includes reductive ability, metal chelator, hydrogen donating ability and scavengers of superoxide and free radicals.

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