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Original Article

SCAVENGING OF FREE RADICALS AND TOTAL PHENOLS OF METHANOL EXTRACT OF *AZIMA TETRACANTHA* LAM

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ABSTRACT

Objective: To report the antioxidant activity, phenolic acid profile of the *Azima tetracantha* methanolic extract and find the correlation between the total phenolic content and their relative antioxidant activity

Methods: The total antioxidant activity, hydroxyl radical and hydrogen peroxide scavenging of *A. tetracantha* methanol extract was evaluated by adopting various standard methods. Total phenolic content was estimated from dried methanolic extract and the phenolic acids in the extract were identified by HPLC.

Results: The methanolic extract of *A. tetracantha* showed free radical scavenging in a concentration dependant manner. The studies showed that the extract possess high free radical scavenging property, especially against hydroxyl radicals (67.61±0.42%). The correlation analysis results showed linear relation between phenolics content of methanolic extract and antioxidant assays (R^2=0.97, 0.94, 0.99 and 0.98, significance at P<.0.001). The phenolic acid profile showed the presence of both benzoic acid and cinnamic acid derivatives, well known for their antioxidant potential.

Conclusion: The current observations suggested that the methanol extract of *A. tetracantha* exhibited multiple antioxidant activities, by scavenging free radicals. So, it can be concluded that the extract is a source of natural antioxidant, suggesting its traditional use as a rejuvenating agent.

Keywords: *Azima tetracantha*, Total antioxidant activity, Hydroxyl radical, Hydrogen peroxide, Total phenols**,** HPLC analysis.

INTRODUCTION

Almost all the degenerative diseases are initiated by free radical oxidation. Free radicals have the potential to oxidize proteins, lipids and DNA in cells and inhibit the oxidative mechanisms that lead to degenerative diseases. Antioxidants play an important role as health protecting factors since they have the ability to trap such free radicals. Most of the antioxidant compounds are derived from plant sources. Recently, there is an increased interest in the therapeutic value of medicinal plants as antioxidants.

Phenolic acids, polyphenols and flavonoids from plants are well known scavengers of free radicals. The present study investigates the quenching effect of methanolic extract of one such medicinal plant, *Azima tetracantha* (Salvadoraceae), on different free radicals, *in vitro,* and also the relationship of total phenolic content with its antioxidant activity.

MATERIALS AND METHODS

Plant material and extraction

Leaves of *Azima tetracantha* were collected from Vembayam, Trivandrum. A voucher specimen has been submitted in the Herbarium, Dept. of Botany (KUBH 5813). The leaves were cleaned and freed from foreign materials. They were then minced, air dried and powdered. [1].

The powdered samples were extracted with methanol (ME) using Soxhlet apparatus. The extract obtained was subsequently concentrated under reduced pressure and the residue collected. The extractive value was calculated and the residue was used for further antioxidant studies.

Antioxidant Assays

In the present study, the total antioxidant capacity was measured using ferric reducing antioxidant power (FRAP) assay and trolox equivalent antioxidant capacity **(**TEAC) assay. Hydroxyl radical (FRBR assay) and hydrogen peroxide scavenging were also determined to identify the antioxidant potential of *Azima tetracantha*. All the assays were correlated with the total phenolic content of the extract.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was conducted using the method described by Wong *et al.* [2]. The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer at pH3.6, 10 mM 2,4,6tri-(2-pyridyl)-s-triazine (TPTZ) solution and 20 mM $FeCl.6H_2O$ at the ratio of 10:1:1. The extract (200µl) at varying concentrations (100-600µg ml^{-1}) was added with 3 ml of the FRAP reagent and the reaction mixture was incubated in a water bath at 37°C for 30 minutes. The increase in absorbance was measured using a spectrophotometer (Shimadzu, Japan) at 593 nm. Ascorbic acid was used as the reference compound. The antioxidant capacity based on the ability to reduce ferric ions of the extracts was calculated using the formula,

$$\text{Percent of inhibition } (\%) = \frac{\text{A593 of sample} - \text{A593 of control}}{\text{A593 of sample}} \text{x } 100$$

Where, A593 is the absorbance at 593nm.

Trolox Equivalent Antioxidant Capacity (TEAC) /ABTS assay

ABTS is 2,2-azinobis- (3-ethylbenzothiozoline-6-sulphonate). ABTS substrate working solution was prepared by adding 25µl of 3% H_2O_2 solution to 10 ml of ABTS substrate solution. Assays were performed in 96 well plates with Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin- E analogue, as standard. Trolox standard (10µl) and test samples (10µl) were taken in the well plate to which 20µl of myoglobin and 150µl of ABTS substrate working solution were added. The plate was incubated for five minutes at room temperature. The reaction was terminated by adding 100µl of stop solution. The end point absorbance was read at 405 nm using a Multiskan microplate reader [3]. A standard curve was prepared for the Trolox standard and percent inhibition values were obtained by multiplying millimolar Trolox equivalent (mMTE) values of methanol extract. By 100.

Hydroxyl radical scavenging activity (Fenton Reaction Based Radical (FRBR) assay)

Hydroxyl radical scavenging activity was measured by the ability of the different fractions of methanol extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) [4][5]. The reaction mixture in a final volume of 1.0 ml contained 100 µl of 2-deoxy2- ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 µl of the fractions at various concentrations (100-600 µg/ml) in buffer, 200 µl of 1.04 mM EDTA and 200µM $FeCl_3$ (1:1, v/v), 100 µl of 1.0 mM hydrogen peroxide (H_2O_2) and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37°C for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. One ml of 1% thiobarbituric acid (TBA) and 1.0 ml 2.8% trichloroacetic acid (TCA) were added to the test tubes and was incubated at 100ºC for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Quercetin, in varying concentrations, (100-600µg ml^{-1}), was used as a positive control.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the methanol extract of *A. tetracantha* was determined by the method of Ruch *et al* [6]. Plant extract (4 ml) prepared in distilled water at various concentrations were mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against the blank solution containing the plant extract without H_2O_2.

$$\text{Inhibition (\%)} = \frac{(\text{Abs.(C)} - \text{Abs.(S)})}{\text{Abs.(C)}} \text{x100}$$

Where Abs. C= Absorbance of control

Abs. S= Absorbance of sample

Estimation of Phenols

Quantification of phenols was done by Folin-Ciocalteau method [7]. Folin-Ciocalteau reagent (0.5 ml) and 2 ml 20% Na_2CO_3 was added to varying concentrations (0.1 to 0.6mg/ml) of methanol extract. It was kept in a boiling water bath for 5 min till a white precipitate was formed and was then centrifuged at 5000 rpm for 5 min. Gallic acid was used as the reference phenolic group. The absorbance of the clear supernatant was read at 650 nm against the blank and the results are expressed as gallic acid equivalents (GAE).

Phenolic compound identification

HPLC Analysis: The extract was analysed using a liquid chromatography (Shimadzu, Japan). Separation was achieved on a reverse phase C18 column, temperature at 24°C. The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B (0.5% (v/v) acetic acid in water). The elution profile was as follows: 0 min 10% A in B, 28.6 min 60% A in B, 30 min 10% A in B. The flow rate was 1 mL/min, and injection volume was 20µL. Absorption was measured at 290 nm. Detection was carried out in UV-visible detector. The eluted components were identified based on the retention time by comparison with retention time of reference standard. The phenolic compounds present in the samples were characterized according to their UV-vis spectra and identified by their retention times in comparison with those of commercial standards (Himedia and Sigma-Aldrich).

Statistical analysis

The scavenging activity and the total phenol content are the results of three independent analyses and is expressed as mean ± standard deviation (SD). Data was analyzed by regression analysis using SPSS v.17.0 program.

RESULTS

FRAP

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay. The inhibition of ferric radicals by methanol extract of *A. tetracantha* was in a concentration dependant manner (Fig.1). Regression studies have revealed a significant inhibition (r^2= 0.9738, p= 0.001) of ferric ions by the extract. The concentration of extract as well as ascorbic acid, to inhibit 50% of the radicals was found to be a concentration less than 100 µg ml^{-1}.

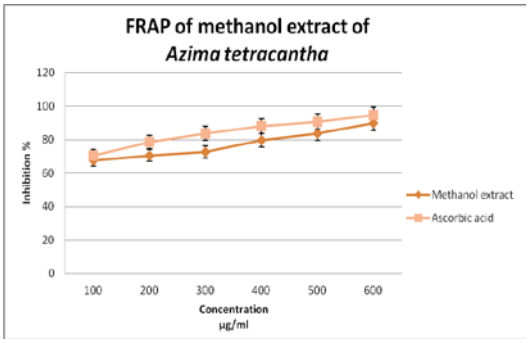

Fig. 1: Ferrous reducing antioxidant property of methanol extract of *A. tetracantha*

ABTS

This assay helps to assess the scavenging activity of the extract against the radical ABTS, which is expressed as Trolox equivalent antioxidant capacity (TEAC). The results indicate an increase in TEAC value as the concentration increases and are the average of three independent analyses. The mean TEAC value for *A. tetracantha* methanol extract was 0.1794±0.32 mMTE. The standard curve for Trolox and the calculated percentage inhibition of ABTS radical is expressed in Figure 2 and 3 respectively. The standard curve of Trolox showed the equation as y= 1468x + 0.7905 with an r^2 value of 0.989. Significant regression relating the percentage inhibition and concentration of methanol extract was observed at p<0.01 (r^2= 0.9474).

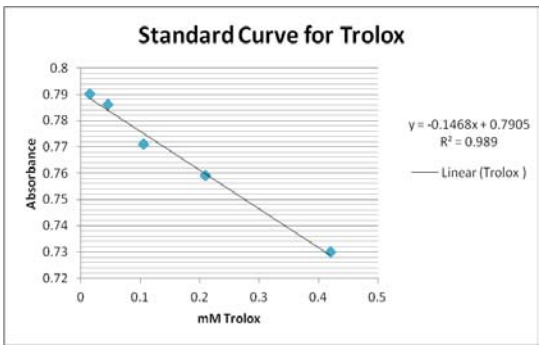

Fig. 2: Standard curve for Trolox

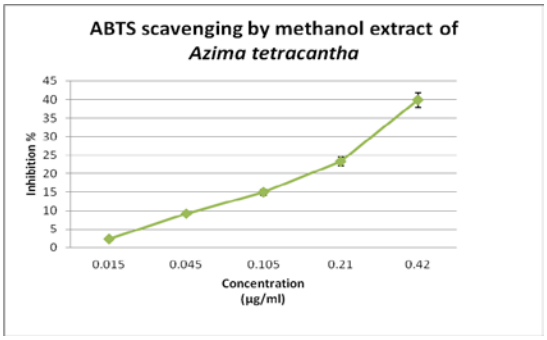

Fig. 3: ABTS scavenging by methanol extract of *A. tetracantha*

Hydroxyl Radical

Hydroxyl radical was generated by Fenton reaction, which degrades deoxy ribose. The results show a dose response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Fig. 4). The average scavenging of hydroxyl radical by methanol extract was 67.61±0.42% and that of quercetin was 77.31±0.15%. Inhibition of hydroxyl radicals by the methanol extract was significant at $p<0.05$ ($r^2= 0.9995$, $p=0.01$), on regression analysis. IC_{50} was calculated to be by linear regression of plots and was observed as 106 µg ml^{-1} for methanol extract.

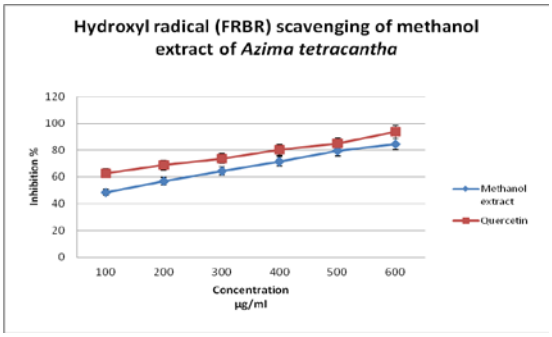

Fig. 4: Hydroxyl radical scavenging of methanol extract of *A. tetracantha*

Hydrogen peroxide scavenging assay

The ability of *Azima tetracantha* leaf extract to scavenge H_2O_2 was measured spectrophotometrically. The scavenging was in a concentration dependant manner and was comparable to that of the standard, ascorbic acid (Fig. 5). The mean total scavenging of hydrogen peroxide was observed as 65.70±0.80% and that of ascorbic acid was 72.44±0.28%.

There was a significant regression relating scavenging ($r^2= 0.9890$, $p<0.01$) of hydrogen peroxide by the methanol extract. Moreover, the IC_{50} was found to be 0.156 µg ml^{-1} for methanol extract.

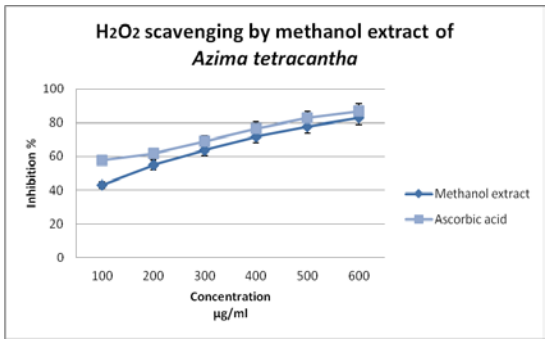

Fig. 5: H_2O_2 scavenging by methanol extract of *A. tetracantha*

Total Phenols

The total phenols present in varying concentrations of methanol extract was measured and shown in Table 1. The total phenolic content varied from 64.62± 0.25 to 90.21±0.20mg GAE, with an average amount of 79.05±0.37mg GAE phenols in methanol extract of *A. tetracantha* leaves.

Phenolic compound identification

HPLC Analysis: The phenolic acid composition in methanol extract was identified by HPLC analysis. In the present observation both benzoic acid as well as cinnamic acid derivatives was identified. The benzoic acid derivatives of gentisic acid (2.6 min), benzoic acid (3.6 min), gallic acid (3.7), salicylic acid (32.7) and vanillin (33.5) and cinnamic acid derivatives of cinnamate (29.2 min), rosmarinic acid (30.4), ferulic acid (31.7), were identified from methanol extract of *A. tetracantha*, by comparison with the corresponding standard compounds (Fig. 6).

Table 1: Total phenolic content in leaves of *Azima tetracantha*

Concentration (mg/ml)	Total phenols content (mg GAE)
0.1	64.62±0.25
0.2	72.41±0.40
0.3	78.10±0.59
0.4	82.01±0.57
0.5	86.96±0.22
0.6	90.21±0.20

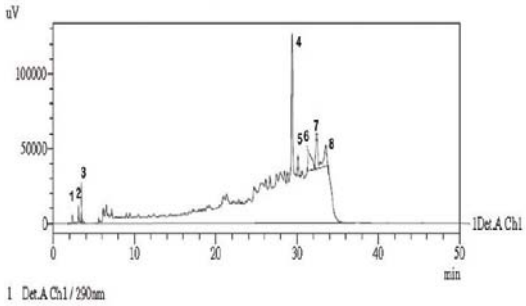

Fig. 6: HPLC chromatogram of *A. tetracantha* extract recorded at 290 nm. Peaks related to phenolic acids are indicated, 1. Gentisic acid, 2. Benzoic acid, 3. Gallic acid, 4. Cinnamic acid, 5. Rosmarinic acid, 6. Ferulic acid, 7. Salicylic acid, 8. Vanillin

Correlation between the phenols and the antioxidant assays

Correlation between the amount of phenols and the different antioxidant assays were determined (Fig. 7). The phenolic content and the percentage inhibition of different antioxidant assays show high correlation coefficient values (r^2), indicating the contribution of phenolics to the antioxidant activity (Table 2). Among the different assays performed, a strong correlation could be noticed for the total phenol content and the scavenging of hydroxyl radicals ($r^2= 0.9963$), by the methanol extract. The results of this study thus provide evidence that the contribution of phenols in scavenging hydroxyl radicals is the highest.

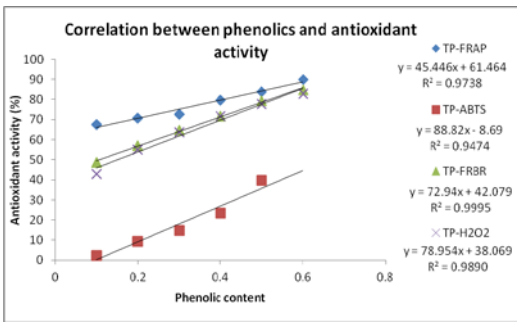

Fig. 7: Correlation between total phenolic content of *A. tetracantha* and antioxidant activity.

DISCUSSION

The total antioxidant activity of *Azima tetracantha* methanol extract was measured using FRAP and ABTS radical scavenging assays. Both the assays showed significant quenching of ferric radicals and ABTS radicals to their corresponding ferrous and $ABTS^{*+}$ radicals.

FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex (Fe^{3+}-TPTZ complex) to its ferrous coloured form in the presence of antioxidants. This suggests that a lower concentration was enough to reduce Fe^{3+}- TPTZ complex to Fe^{2+}- TPTZ complex. Only a marginal difference in ferric radical scavenging was observed between the methanol extract of *A. tetracantha* and ascorbic acid in the present study. Wong *et al* [8] reported a strong correlation between the mean values in DPPH and FRAP assays. Accordingly, this correlation suggests that the compounds present in the methanol extract of *A. tetracantha,* capable of reducing ferric to ferrous ions will also be able to reduce DPPH radicals, as well.

Table 2: Correlation of total phenol and antioxidant assays

Antioxiant assays	Regression equation	Pearson coefficient value (R^2)
FRAP	y= 45.446x+61.464	0.9738
TEAC/ ABTS	y=88. 82x +8.69	0.9474
FRBR	y=72.94x+42.079	0.9995
H_2O_2	y= 78.954x+38.069	0.9890

ABTS assay is based on the inhibition of the absorbance of the radical $ABTS^+$ which has a characteristic long wavelength absorption spectrum [9]. The principle of the antioxidant assay is the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS to produce a radical cation, $ABTS^{*+}$. It was observed that an extract concentration equivalent to 0.3986 mM Trolox was required to scavenge about 40% of ABTS radical. At the highest concentration studied. Hence, the concentration at which 50% of the radicals were scavenged was found to be a concentration greater than 0.420µg ml^{-1}. Higher TEAC values have been reported for *Spondias pinnata,* stem bark (0.78±0.02) [10] than *A. tetracantha* (0.18±0.02). In another study however, it was observed that metabolic leaf extracts of *Alpinia nigra* exhibited potent scavenging effects against ABTS radical with an IC_{50} value 28.32 µg ml^{-1} [11]. Greater efficiency of *A. tetracantha* leaf extracts in scavenging ABTS radical, over *A. nigra* is evident from this observation.

Hydroxyl radicals are important active oxygen species causing lipid peroxidation and enormous biological damage. These radicals are formed in free solution by their ability to degrade deoxyribose into fragments [12]. Methanol extract of *A. tetracantha* inhibited about 84.63% of the radicals at a concentration of 0.6mg ml^{-1} in comparison to the known scavenger, quercetin, which scavenged only about 77.31% of hydroxyl radical at the same concentration. A similar, dose dependent hydroxyl radical scavenging activity was observed for all the extracts of *A. tetracantha*, studied by Thendral *et al* [13]. However, the studies done by Muthuswamy *et al* [14] showed that the methanolic extract of *A. tetracantha* exhibited an inhibition percent of 82.2%, while the ethyl acetate extract exhibited 75.1% inhibition against hydroxyl radical and only at its highest concentration, 1000µg/ml. From this information it appears that methanol extract of *A. tetracantha*, could efficiently scavenge hydroxyl radicals and that the presently collected sample of *A. tetracantha* exhibited the best value when compared to the earlier reports.

Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be cytotoxic by giving rise to hydroxyl radicals in the cells. Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolics, which donate electron to H_2O_2, thus reducing it to water [15]. Nadaroglu *et al* [16] has reported a similar, significant scavenging of hydrogen peroxide by the aqueous and ethanol extracts of Iris (*Iris germanica*) in comparison to that of BHA, BHT and α- tocopherol, in a concentration dependant manner.

On the whole, it is evident from the different scavenging assay results that the methanol extract of *A. tetracantha* has a high free radical scavenging property, especially against hydroxyl radicals. Free radical scavenging property serves as an indicator of the possible antioxidant property of *A. tetracantha*.

Several authors had reported correlation between antioxidant activities of plant materials with the content of phenolic compounds. [17][18]. However, plants vary in content and structure of phenolic compounds such as number of phenolic rings, aromatic substitution, glycosylation and conjugation with other phenolic compounds or organic acid and therefore are likely to vary in their antioxidant properties. In addition, the *in vitro* antioxidant studies as described here for the methanol extract of *A. tetracantha* provide only relevant clues for further investigation.

CONCLUSION

Methanol extract of *A. tetracantha* leaves exhibited a concentration dependant free radical scavenging property as observed from the different antioxidant assays attempted. Highest activity was exhibited against the hydroxyl radical. A significant correlation could also be observed between the exhibited antioxidant activity and the total phenolic content. Antioxidant property of *A. tetracantha* may be one of the reasons for its use as a rejuvenating drug in the traditional medicinal practices.

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