

In vitro studies on antioxidant and free radical scavenging activities of *Azima tetraacantha*. Lam leaf extracts

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Abstract

The leaves of *Azima tetraacantha* (Salvadoraceae) commonly known as "mulluchangu" is best known for its medicinal properties. In the present study, the antioxidant potency of successive extracts (petroleum ether, hexane, ethylacetate & methanol) was investigated, by determining the secondary metabolites such as flavonoids, tannin and phenolic compounds. The free radical scavenging activity of various solvent extracts of the leaves was assessed by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide radical (NO), superoxide anion, hydroxyl radical and lipid peroxide generating models. Reducing power and Vit E level in the leaves were also measured. The results show that the methanol extract of the plant *A. tetraacantha* contains significant quantities of the antioxidant principles compared to the other solvent extracts. Similar observations were seen in scavenging the free radicals by the methanolic extract of the leaves of *A. tetraacantha*. Thus the therapeutic property of the leaves of the plant *A. tetraacantha* can be attributed to the antioxidant principles which scavenge the free radicals responsible for pathological severity.

Keywords: *Azima tetraacantha*, phytochemicals, antioxidants, free radical scavenger.

Introduction

Oxidative stress is a crucial etiological factor to the pathophysiology of a variety of degenerative or pathological conditions such as aging, cancer, coronary heart disease, Alzheimer's disease, atherosclerosis and inflammation (Pong, 2003; Sandhya *et al.*, 2010). Human body has multiple mechanisms especially enzymatic and non enzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage (Anderson, 1999). However due to the overproduction of reactive species and/or inadequate antioxidant defense, it culminates in severe or continued oxidative stress. The harmful action of the free radicals can, however, be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (Balakumar *et al.*, 2010). Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and have been added to food stuffs during food processing but they may possess toxic side effects and also acts as carcinogens (Anagnostopoulou *et al.*, 2006). The safety and toxicity of synthetic antioxidants have been important concerns; therefore, attention has been focussed on the use of natural antioxidants for inhibition or protection from oxidative damage (Howell, 1986). In recent years attention has been directed in utilizing natural antioxidants substantially (Shahidi *et al.*, 2006; Vetrivel Rajan & Shanmugavalli, 2009). Secondary metabolites of plants such as flavonoids, anthocyanins, carotenoids and

vitamins have been reported as promising antioxidants (Kivits *et al.*, 1997). Plant-derived antioxidants function as singlet and triplet oxygen quenchers, peroxide decomposers and/or chelators of the catalytic metal ions (Robak & Marcinkiewicz, 1995).

Traditionally herbal medicines are currently used in protecting and restoring health. People of all continents and civilizations used plants in one form or the other. *Azima tetraacantha* Lam., (Salvadoraceae) commonly known as "mulluchangu" is a glabrous, rigid, rambling, thorny shrub commonly called Bee sting bush found in Africa, India, and Madagascar. Several medicinal properties are attributed to this plant in the Indian system of medicine and included in the check list of traded medicinal plants. The ethno botanical survey reveals the usage of this plant as an unique folk medicine by the adivasis (tribal) (Hebbar *et al.*, 2004; Mohamed Al-Fatimi *et al.*, 2007; Murthy *et al.*, 2008; Vikneshwaran *et al.*, 2008; Raghupathy *et al.*, 2008; Ignacimuthu *et al.*, 2008). The root, root bark and leaves are administered with food as a remedy for rheumatism (Chopra *et al.*, 1956; Kirtikar *et al.*, 1984). It is a powerful diuretic given in rheumatism, dropsy, dyspepsia and chronic diarrhea and as a stimulant tonic after confinement (Nadkarni, 1976). The leaves are found to contain azimine, azcarpine, carpine and isorhamnitine-3-O-rutinoside etc., (Rall *et al.*, 1967; Williams & Nagarajan, 1988, Bennet *et al.*, 2004). Friedelin, lupeol, glutinol and β -sitosterol were isolated from the petroleum ether extract of the leaves of *A.*

tetracantha (Rao & Prasada Rao, 1978). The seeds of this plant have been found to possess novel fatty acids along with other fatty acids (Daulatabad *et al.*, 1991). Antimicrobial activity was also reported for this plant (Siva *et al.*, 2008). *A. tetracantha* leaf powder was assessed for its anti-inflammatory activity (Ismail *et al.*, 1997). The benzene, chloroform and aqueous extract of leaves of *A. tetracantha* were screened for analgesic activity in mice using hot plate method (Nandgude *et al.*, 2007). The ethanolic leaf extract of *Azima tetracantha* Lam. was investigated for hypoglycemic and hypolipidemic activity in alloxan-induced diabetic albino rats (Begum *et al.*, 2009). However, information pertaining to the systematic studies on the antioxidant properties of *A. tetracantha* is lacking. In view of the above fact, in the present study the possible antioxidant activity of the various solvent extracts petroleum ether, hexane, ethylacetate and methanol were investigated by employing different *in vitro* free radical scavenging models.

Materials and methods

Plant collection and extraction

The fresh leaves of *A. tetracantha* were collected during the month of September from the foothill of Sirumalai Hills, Dindugal, Tamil Nadu, India. Botanical identification was performed by Dr. T. Sekar, Dept. of Botany, Pachaiyappas's College where the voucher specimen has been deposited. The leaves were washed in fresh water to remove adhering dust and then dried under shade. The air dried and powdered leaves were extracted in soxhlet extractor successively with petroleum ether, hexane, ethyl acetate and methanol. The successive extracts were evaporated to dryness and then stored residue was used for analysis.

Methods

Preliminary phytochemical screening of the four successive extracts (Petroleum ether, hexane, ethylacetate & methanol) were performed (Kokate, 2001). The flavonoid content of the extracts was measured using colorimetric assay as described by Chang *et al.*, 2002. The amount of tannins in the extracts was determined using Folin-Ciocalteu reagent according to the Schanderl (1970) procedure. The level of total phenolic compounds in the extracts were determined by Folin-Ciocalteu reagent using gallic acid as the standard (McDonald *et al.*, 2001). Total Vitamin E content was measured according to the method of Prieto *et al.* (1999). The reductive potential of the extracts were determined according to the method of Oyaizu (1986).

Lipid peroxidation inhibitory capacity was assayed in rat liver homogenate according to the method adopted by Ohkawa *et al.*, 1979. The free radical scavenging activity of the four extracts were measured *in vitro* by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay by the method of Koleva *et al.* (2002). The influence of extracts on the generation of nitric oxide was measured according to the method described by Green *et al.* (1982). The ability of extracts to scavenge superoxide radicals was measured

by the method of Kakkar *et al.* (1984). Hydroxy radical scavenging activity was performed as described by Halliwell *et al.* (1987).

Statistical analysis

Experimental results were expressed as mean \pm SEM of three parallel measurements.

Results and discussion

Phytochemical analysis

The phytochemical results confirms the presence of alkaloids, phenolic compounds, reducing sugars, flavonoids, tannins and glycosides in all the four successive extracts but in variable quantities (Table 1). These are the phytochemicals which are essential in many medicinal plants responsible for the antioxidant property either by scavenging free radicals or by preventing their formation (Patricia *et al.*, 2005). The reported medicinal property of the plant might be due to the presence of these bioactive components in *A. tetracantha*. Phytochemical such as steroids, saponins, quinones and anthroquinones were found to be absent in all the four test extracts.

Effect on lipid peroxidation

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids and also damages the biomolecules. Transition metal ions, such as iron and copper, are known to stimulate LPO through various mechanisms (Halliwell & Gutteridge, 1984). These metal ions may generate hydroxyl radicals to initiate the lipid peroxidation process and/or propagate the chain process via decomposition of lipid hydro peroxides (Braugher *et al.*, 1987). In this investigation the lipid peroxidation of rat liver homogenate was triggered by Fe (II)-ascorbate, and the end products of the process were measured in terms of thiobarbituric acid-reactive substances (TBARS) formed. All the extracts significantly inhibited the lipid peroxidation. Maximum inhibition was observed with methanol extract (IC_{50} 99.53 \pm 6.17 μ g/ml) followed by the ethyl acetate (IC_{50} 119.05 \pm 2.05 μ g/ml), petroleum ether (IC_{50} 1430.5 \pm 44.5 μ g/ml) and hexane extracts (IC_{50} 1538.5 \pm 19.5 μ g/ml) (Fig 1). The scavenging effect was comparable to that of the standard ascorbic acid with IC_{50} value of 76.75 \pm 1.36 μ g/ml. From the study it was evident that ability of the extract to inhibit lipid peroxide level may be due to the chelation of Fe^{2+} or trapping of free radical or chain breaking nature of the extract.

Effect on scavenging DPPH radical

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, e.g., a free radical-scavenging antioxidant, the absorption strength is decreased and results in decolorization (yellow colour) with respect to the number of electrons captured (Blois, 1958). More the decolorization more is the reducing ability. This test has been the most accepted

Fig. 1. Inhibition of lipid peroxidation

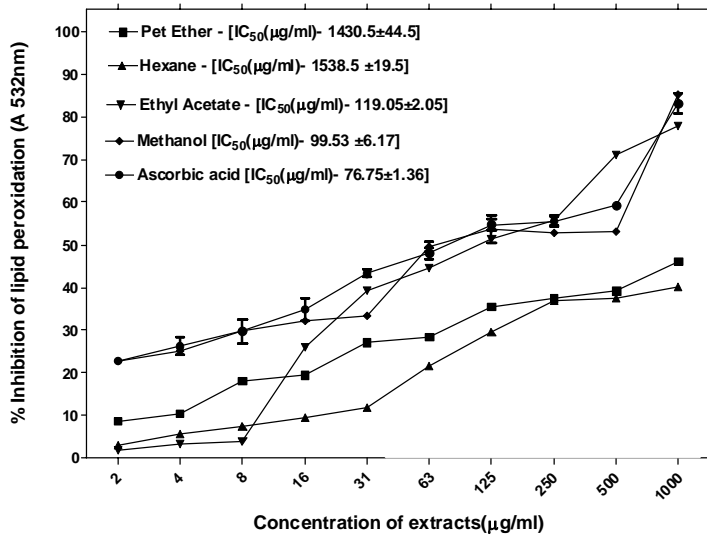


Fig. 2. Scavenging of DPPH radical

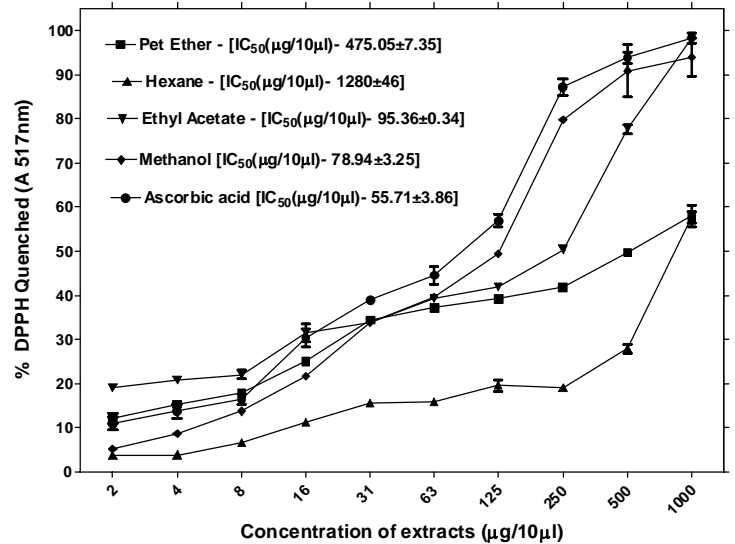


Fig. 3. Nitric oxide scavenging activity

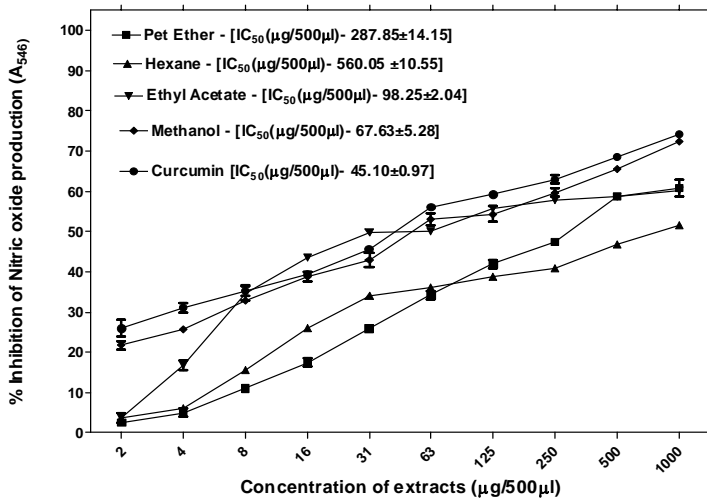


Fig. 4. Inhibition of superoxide anion

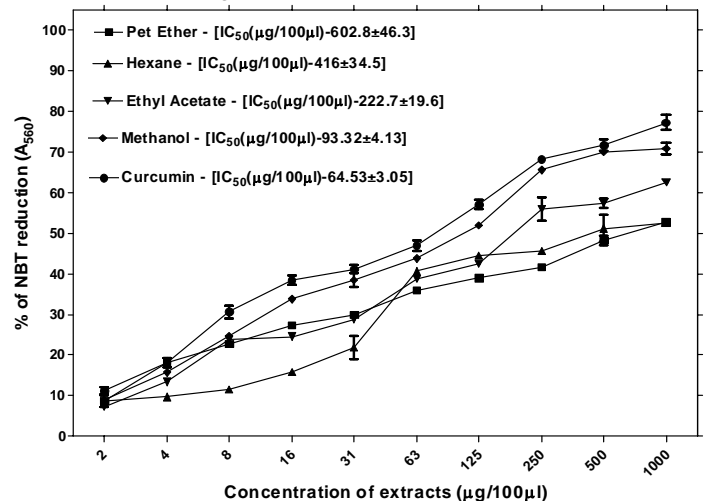
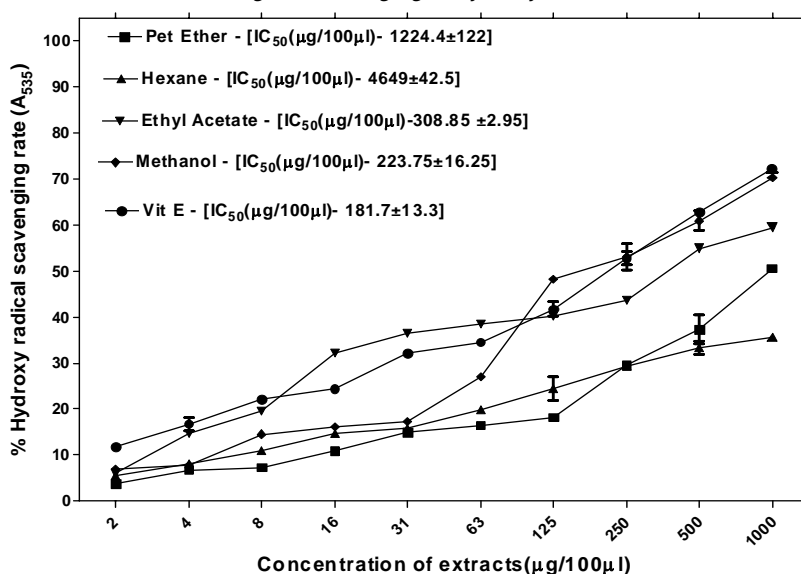


Fig. 5. Scavenging of hydroxyl radical



model for evaluating the free radical scavenging activity of any new drug. The percent DPPH scavenging activities of *A. tetraacantha* extracts were summarised in (Fig. 2). At all concentrations tested, *A. tetraacantha* exhibited a dose-dependent DPPH radical-scavenging activity. The order of the scavenging activity was found to be methanol (IC_{50} $78.94 \pm 3.25 \mu\text{g}/10 \mu\text{l}$) > ethyl acetate (IC_{50} $95.36 \pm 0.34 \mu\text{g}/10 \mu\text{l}$) > petroleum ether (IC_{50} $475.05 \pm 7.35 \mu\text{g}/10 \mu\text{l}$) > Hexane (IC_{50} $1280 \pm 46 \mu\text{g}/10 \mu\text{l}$). The scavenging effect was comparable to that of the standard ascorbic acid with IC_{50} value of $55.71 \pm 3.86 \mu\text{g}/10 \mu\text{l}$. The results suggested that the medicinal property exhibited by the plant might be due to the radical scavenging activity. Our results are in tune with earlier investigations that the methanol extracts of *Pterocarpus santalinus* exhibited significant DPPH radical inhibition (Arokiyaraj *et al.*, 2008).

Inhibition of NO radical production

Nitric oxide (NO^{\cdot}) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities (Nathan & Hibbs, 1991). Despite the possible beneficial effects of NO^{\cdot} , its contribution to oxidative damage is also reported. This is due to the fact that NO^{\cdot} can react with superoxide to form the peroxy nitrite anion, which is a potential oxidant that can decompose to produce OH^{\cdot} and NO^{\cdot} (Pacher *et al.*, 2007). Large amounts of NO^{\cdot} may lead to tissue damage. It would be interesting to develop potent and selective inhibitors of NO^{\cdot} for potential therapeutic use (Nowakowska, 2007). The ability to scavenge 50% of NO^{\cdot} radical was found to be IC_{50} $67.63 \pm 5.28 \mu\text{g}/500 \mu\text{l}$ for methanol > ethyl acetate (IC_{50} $98.25 \pm 2.04 \mu\text{g}/500 \mu\text{l}$) > petroleum ether (IC_{50} $287.85 \pm 14.15 \mu\text{g}/500 \mu\text{l}$) > hexane (IC_{50} $560.05 \pm 10.55 \mu\text{g}/500 \mu\text{l}$) (Fig. 3). The scavenging effect was comparable to that of the standard curcumin with IC_{50} value of $45.1 \pm 0.97 \mu\text{g}/500 \mu\text{l}$. So it could be presumed that the methanol extract could act as a potential therapeutic agent for the control of oxidative damage.

Inhibition of superoxide anion radical production

Superoxide is biologically important as it can form singlet oxygen and hydroxyl radical (Korycka-Dahl & Richardson, 1978). Overproduction of superoxide anion radical, contributes to redox imbalance and associated with harmful physiological consequences (Pervaiz & Clement, 2007). Superoxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan. From the present study it was found that the inhibition was seen in a dose dependent manner (Fig. 4). Extracts found to possess the activity in the order of their scavenging effect methanol (IC_{50} $93.32 \pm 4.13 \mu\text{g}/100 \mu\text{l}$) > ethyl acetate (IC_{50} $222.7 \pm 19.6 \mu\text{g}/100 \mu\text{l}$) > hexane (IC_{50} $416 \pm 34.5 \mu\text{g}/100 \mu\text{l}$) > petroleum ether (IC_{50} $602.8 \pm 46.3 \mu\text{g}/100 \mu\text{l}$). The scavenging effect was comparable to that of the standard curcumin with IC_{50} value of $64.53 \pm 3.05 \mu\text{g}/100 \mu\text{l}$. The antioxidant activity is afforded, not only by

phenolic compounds, but also has important contributions from other superoxide anion radical scavengers, such as essential oils, carotenoids and vitamins (Moure *et al.*, 2001).

Scavenging effect on hydroxy radical

Hydroxy radical is a highly reactive radical formed in biological systems and capable of damaging almost every molecule found in living cells (Halliwell, 1991). This radical has the capacity to induce carcinogenesis, mutagenesis and rapidly initiates lipid peroxidation (Rajesh Manian *et al.*, 2008). In vitro, (OH^{\cdot}) were generated by a mixture of Fe^{3+} -EDTA, H_2O_2 and ascorbic acid and were assessed by monitoring the degraded fragments of deoxyribose, through malonaldehyde (MDA) formation. If any drug scavenges the hydroxyl radical, they may either scavenge the radical or may chelate the Fe^{2+} ion making them unavailable for the Fentons reaction. Here the successive extracts were tested, where they found to exhibit a dose dependent hydroxyl radical scavenging activity (Fig. 5). The hexane extract was found to exhibit the scavenging activity with IC_{50} $4649 \pm 42.5 \mu\text{g}/100 \mu\text{l}$, followed by petroleum ether extract (IC_{50} $1224 \pm 122 \mu\text{g}/100 \mu\text{l}$), ethyl acetate (IC_{50} $308.85 \pm 2.95 \mu\text{g}/100 \mu\text{l}$) and the methanol extract with the highest scavenging activity (IC_{50} $223.75 \pm 16.25 \mu\text{g}/100 \mu\text{l}$). The scavenging effect was comparable to that of the standard Vitamin E with IC_{50} value of $181.7 \pm 13.3 \mu\text{g}/100 \mu\text{l}$. The radical scavenging power of the extracts can be accounted by the presence of flavonoid, tannin, phenols and vitamins. Plant extracts containing polyphenols are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical (Mi-Yae Shon *et al.*, 2003) or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the aromatic ring (Becana & Klucas, 1992).

Bioactive antioxidant levels:

Flavonoids are a group of effective antioxidants which are present abundantly throughout the plant kingdom. Flavonoid and related compound are effective in scavenging DPPH radical (Apati *et al.*, 2003), hydroxyl radical and in metal-chelating capacity (Lean *et al.*, 1999). Flavonoids are found to exhibit numerous biological activities like vasodilatory, anticarcinogenic, antiinflammatory, antibacterial, immune-stimulating, anti-allergic, and antiviral effects (Middleton & Kandaswami, 1992). In the present study the methanol extract was found to possess significant amount, 40mg/gm when compared to other extracts (Table 1).

Tannins, the high molecular weight phenols, act as a good scavenger of free radical either by donating hydrogen atom or by reducing them. This property is attributed by the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution and specific functional groups present in the tannins (Hagerman *et al.*, 1998). The results show that the methanol extract contains more tannin content when compared to the other extracts (Table 2). Thus polar

fraction may have more polyhydroxy phenolics such as tannins, which may be acting synergistically, with other phytoconstituents to exhibit its antioxidant property.

Table 1. preliminary phytochemical analysis of *Azima tetraacantha* leaf extracts

Phytoconstituents	Pet. ether	n-Hexane	Ethylacetate	Methanol
Steroids	-	-	-	-
Alkaloids	+++	+++	+++	+++
Phenolic Compounds	++	++	++	+++
Reducing sugars	+	+	+	+
Flavonoids	+	+	+	++
Tannins	++	++	++	++
Glycosides	-	+	-	+++
Saponins	-	-	-	-
Antraquinones	-	-	-	-
Quinones	-	-	-	-
Proteins	+	+	++	++

+ - mild ; ++ - moderate ; +++ - more

Table 2. Comparison of extraction yield, flavonoid content, tannin content and total phenolic content of different successive extracts obtained from leaves of *Azima tetraacantha*

Extracts	Yield ^a (%w/w)	Flavonoid ^a (mg/gm extract)	Tannin ^a (mg/gm extract)	Total phenolic compounds ^a (mg/gm extract)
Petroleum Ether	1.50	15.47±0.40	2.70±0.10	33.08±0.09
n- Hexane	0.280	5.48±0.28	0.84±0.06	46.53±1.34
Ethyl Acetate	1.97	33.54±0.12	35.18±0.69	66.19±3.41
Methanol	27.17	40.01±0.06	41.24±0.10	84.25±2.41

^a Each value is expressed as mean± SEM (n=3).

Phenols are very important plant constituents because of their radical scavenging ability due to the hydroxyl groups (Hatano *et al.*, 1989). The total phenolic content of the four successive extracts were quantified with Folin Ciocalteu reagent. The phenolic content of the successive extracts are shown in Table 2. The phenolic content of methanolic extract was found to be high followed by ethylacetate, hexane and petroleum ether extracts. This observation suggests that the phytochemicals necessary for the antioxidant and radical scavenging activity are present abundantly in the polar fraction than the non polar fractions. This might be the reason for the methanolic extract to possess more phenol content when compared to other extracts. The pronounced antioxidant activity of the extract of *A. tetraacantha*, manifested as inhibition of lipid peroxidation (LPO), scavenging of hydroxyl, nitric oxide and superoxide radicals, was possibly due to its

high phenolic content. Polyphenols are known to exhibit a variety of biological actions such as free radicals scavenging, metal chelation, modulation of enzyme activity and more recently to effect signal transduction, activation of transcription factors and gene expression (Bito *et al.*, 2000).

Ascorbic acid is a very good reducing agent and free radical scavenger. It is also reported to prevent the damage to RBC membranes by interacting with superoxide and hydroxy radical in plasma, and also by influencing α -tocopherol in inhibition of LPO by recycling the tocopherol radical (Beyer, 1994). Interestingly the ascorbic acid content of *A. tetraacantha* extracts was found to be in the order methanol extract > ethyl acetate > hexane > petroleum ether. α -tocopherol could also directly react with a variety of oxy radicals, including the peroxy radical, OH[•], O²⁻ and singlet oxygen (Littarru *et al.*, 1984). Our study reveals the order of α -tocopherol content of the extracts as follows petroleum ether > methanol > ethyl acetate > hexane. So the vitamins E and C present in *A. tetraacantha* leaves are quenchers of free radicals and good reducing agents (Table 3). Thus the results confirm that *A. tetraacantha* could be an important source of natural antioxidants.

Conclusion

Results of this study show that *A. tetraacantha* leaves are good source of natural phenolic compounds. The methanolic extract of the *A. tetraacantha* leaves showed better free radical capacity against different reactive oxygen /nitrogen species, among other extracts although with different efficiencies. The high content of antioxidants like phenolic compounds, flavonoids and vitamins found in these extracts, may impart health benefits by combating the free radicals in synergistic manner along with other compounds and thus constitute part of the basis for the ethno pharmacological claim.

Thus *A. tetraacantha* shall further be subjected to systematic *in vivo* studies for the preventive action against cancer, cardiovascular and neurological disorders.

Acknowledgement

Thendral Hepsibha, acknowledges Mother Teresa Women's Univ. for opportunity given to do the work and thanks Dr. A.

Geetha, Associate Professor in Biochemistry, Bharathi Women's College, for her constant encouragement and help throughout the research work.

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Table 3. Reducing capacity and vitamin E content of *Azima tetraacantha* extracts

Extracts	Reducing capacity ^a (gm extract which contains 1 gm equivalent of Vit C)	Vit E Content ^b (gm extract which contains 1 gm equivalent of Vit E)
Petroleum Ether	51.41±0.84	10.29±0.14
n- Hexane	41.19±2.3	19.48±0.38
Ethyl Acetate	27.57±0.28	14.96±0.09
Methanol	17.19±0.39	11.11±0.17

^a Mean of triplicate determinations ± SEM (n=3). ^b Mean of triplicate determinations ± SEM (n=3)

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