Antioxidant properties and stability of *aegle marmelos* leaves extracts

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Abstract *Aegle marmelos* (AM) leaves were extracted with methanol (ME), ethanol (EE), water (WE) and analyzed for antioxidant activities by DPPH radical scavenging method, reducing power and *in vitro* inhibition by Fenton's reagent—induced oxidation of lipid system. Stability of extracts to pH (4, 7 and 9) and temperature (100 °C, 15 min.) was studied. The three extracts showed varying degree of efficacy in each assay in a dose dependent manner. The inhibition of MDA formation in Linseed oil by EE (47%) was significantly ($P<0.05$) higher than WE (28%) and ME (23%) but less than α -Tocopherol (80%). WE showed maximum stability to high temperature. The antioxidant activity of EE at pH 4 was significantly higher ($P<0.05$) compared with WE and ME. At pH 7, the antioxidant activity of all the three extracts remained unchanged. Data indicates that potential exists for the utilization of *Aegle marmelos* as a natural antioxidant.

Keywords *Aegle marmelos* · Radical scavenging activity · Reducing power assay · Lipid peroxidation · Stability

Introduction

The importance of natural antioxidants for use as food additives or nutritional supplements has already been established (Bassiounny et al 1990, Mansour and Khalil 2000, Reddy et al 2005, Roy et al 2010). Antioxidants or ingredients having antioxidative properties are used extensively for improvement of food stability. With the focus is

being shifting towards finding alternatives for synthetic food ingredients, natural substances having antioxidative properties need to be further explored (Sachindra et al 2010). The search for safe and effective naturally occurring antioxidants is now focused on edible plants especially spices and herbs (Miliauskas et al 2004). Extracts from spices, herbs and hulls are reported to exhibit varying degrees of antioxidant activity (Koleva et al 2003, Miliauskas et al 2004, Dorman et al. 2003a, b, Takeoaka and Dao 2003). Some of the common spices have been evaluated and further screening of medicinal plants for their antiradical properties is of interest primarily to find out newer sources of natural antioxidants, functional foods and nutraceutical.

Aegle Marmelos (Bilwa, AM), a medicinal plant is found all over the sub Himalayan forests. Its history has been traced to the Vedic period, about 2000 BC. All parts of the tree have medicinal qualities (Chanda et al 2008). It contains volatile oils and pectin, the wood-ash is rich in minerals and is used in numerous products like candy, squash, coffee etc. Rana and Jain (1997) evaluated the anti fungal activity of essential oil isolated from leaves of the *Aegle marmelos* using spore germination assay and the oil established variable efficacy against different fungal isolations. (Shoba and Thomas 2001) reported the efficacy of methanol extract of AM in rodents against castor oil induced diarrhoea with reduction of both the induction time of diarrhoea and total weight of the feces. *Aegle Marmelos* is reported to have antidiarrheal (Shoba and Thomas 2001, Dhuley 2003), antiproliferative (Lampronti et al 2003), anti-inflammatory, antipyretic (Arulet al. 1997), and hypoglycemic (Upadhyay et al. 2004), (Kesari et al 2006, Narender et al. 2007) and antioxidant (Sabu and Kuttan 2004).

The fruit and bark of AM has been studied for antioxidant potency (Khanghan and Raul 1996, Riyanto and Mustafa 2000, Marzine and Gilbert. 2005), and the leaves are reported to be

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antidiabetic and antilipidemic in rats (Upadhyay et al. 2004, Sabu and Kuttan 2004). Limited information is available in the literature on the antioxidant aspects viz., components, activity and their stability. Hence, the present study was undertaken to analyze antioxidant components, activity and stability of various solvent extracts of AM leaves.

Materials and methods

Plant material

The selected plant material *Aegle marmelos* (AM) leaves were collected locally during September and October period, washed and dried in a hot air oven at 55 °C. Dry leaves were ground separately and passed through a 60 mesh sieve and kept in air tight containers at 4 °C until further use.

Determination of antioxidant components

In the dehydrated sample, different antioxidant components were estimated using standard methods. Ascorbic acid was determined according to the titrimetric method by the 2, 6-dichlorophenol-indophenol dye against KMnO_4 (Ranganna 1999). α -Tocopherol was extracted by direct saponification of dried sample and estimated based on formation of a red complex from reaction of α, α' -bipyridyl with ferrous ion due to reduction of ferric ion by tocopherol (Freed 1966). β -Carotene was quantified by open column chromatography, followed by measuring the absorbance of elute at 450 nm against standard β -carotene (Ranganna 1999). Reduced glutathione was determined based on the development of a yellow compound due to reaction of 5, 5-dithio (bis) nitro benzoic acid with compounds containing sulphhydryl groups (Beutler and Kelly 1963). Total phenols were extracted from a weighed portion (50–500 mg) of dried sample with 5 ml of 1.2 M HCl in 50% aqueous methanol for 2 h and with 70% acetone shaken for 2 h and analyzed by Folin-Ciocalteu micro method (Slinkard and Singleton 1967). Results were expressed as mg or g of Gallic acid equivalent g^{-1} dry weight. The content of flavonoids was determined by a pharmacopoeia method (Miliauskas et al 2004) using Rutin as a reference compound. The amount of Flavonoids in plant extracts in Rutin equivalents (RE) was calculated by the following formula,

$$X = \frac{(A.M_o.10)}{(A_o.M)}$$

Where, X- flavonoids content, mg/g plant extract in RE; A- the absorption of plant extract solution; A_o - the absorption pf standard Rutin solution; M- the weight of plant extract; g; M_o – the weight of Rutin in the solution, g.

Antioxidants extraction

A 15 g sample was extracted with 50 ml solvent, (methanol, ethanol and water separately) for 6 h in a mechanical shaker. The extracts were filtered and filtrates were evaporated at 40° under reduced pressure to dryness in a rotary evaporator (Superfit, India). The residue of each extract was stored in air-tight container at 4° until used. In case of water extract, the sample was added to boiling water and extracted for 15 min and filtered. The filtrate was freeze dried and stored at 4° C until further use.

Radical scavenging activity—(RSA)

The ability of extracts to scavenge DPPH radicals was determined according to the method of Blois (1958). Briefly, 1 ml of 0.1 mM DPPH solution was mixed with 3 ml of extract (containing 100–1000 μg) in methanol. The mixture was then vortex mixed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity expressed as % Radical Scavenging (RSA) relative to control using the following equation:

$$\text{RSA}\% = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing power assay

The ability of extracts to reduce iron (III) to iron (II) was determined in this method. The dried extract (200 μg) in 1 ml of the corresponding solvent was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of the potassium ferricyanide, ($\text{K}_3\text{Fe}(\text{CN})_6$; 10 g l^{-1}) then the mixture was incubated at 50° for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (100 g l^{-1}) was added to the mixture, which was then centrifuged at 1650 $\times$ g for 10 min. Finally supernatant solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl_3 (0.5 ml) (1 g l^{-1}) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power (Yildirim et al. 2003).

Inhibition of lipid peroxidation in vitro

Antioxidant activity

The protective action of the extracts against oxidation of lipid system was analyzed by the method of (Tamura and Yamagami 1994). An aqueous solution containing Linseed oil (1.5 mg/ml) was poured into a 30 ml test tube and diluted with 5 ml of Trizma–buffer solution (0.25 mM,

Table 1 Antioxidant components, total polyphenols and extract yield of different solvent extracts from *Aegle* leaves

Antioxidant component	Per 100 g
α - Tocopherol (mg) ^b	27
β - carotene (μ g) ^b	8600
Glutathione (m. moles) ^b	580
Ascorbic acid (mg) ^b	260
Total Flavonoids (mg) ^a	2.4
Total polyphenols (g) ^b	2.4
Polyphenol content ^c	
ME	9.9 (27.8)
EE	11.5 (12.3)
WE	8.9 (32.0)

^a mg/g extract, ^b per 100 g dry basis. ^c g of Gallic acid per 100 g of extract. Values in parenthesis indicate yield in g of extract per 100 g of dried leaves. ME methanol, EE ethanol, WE water extracts, $n=4$

pH 7.4) containing 0.2% sodium dodecyl sulphate (w/v) and 0.75 mM potassium chloride. Trizma buffer was prepared by diluting 6.075 g of Tris (Trihydroxymethyl)-amino methane and 11.184 g of potassium chloride with distilled water to 1 L after adjusting the pH of the solution to 7.4 Lipid peroxidation was initiated by adding Fenton's reagent (FeCl_3 -1 μ m and H_2O_2 0.5 μ m). Incubation was continued for 16 h at 37° in a dark place. The reaction was stopped by adding 50 μ l of 1% BHT alcoholic solution. The solution obtained was used for antioxidant activity assay.

Antioxidant activity assay

The dried plant extract (200 μ g) in 100 μ l of corresponding solvent was mixed with the solution (9.9 ml) mentioned above when necessary. BHT was used as a standard to evaluate the antioxidative activity of samples. The reacted solution obtained (1 ml) was used for TBA assay.

TBA assay

The degree of oxidation of oil was measured by the 2-thiobarbituric acid (TBA) assay (Ohkawa et al 1994). The

reacted solution (1 ml) mentioned above was mixed with 0.2% (w/v) TBA (3 ml) and 0.05 M sulfuric acid (2.5 ml) and the mixture was heated for 30 min in 95° water bath. After, the solution was then cooled in ice for 5 min; the colored substances were extracted by 4.0 ml of 1-butanol. The absorbance of 1-butanol layer was measured at 532 nm. Antioxidant activity (AOA) was expressed as percentage inhibition of lipid peroxidation relative to the control using the following equation:

$$\text{AOA}\% = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Heat and pH stability

The extracts were heated in a boiling water bath for 15 min and the residual antioxidant activity was determined by radical scavenging activity using DPPH as described previously. For pH stability, antioxidant extracts were incubated for 24 h at pH 4, 7 & 9 and the residual antioxidant activity was determined during the incubation period at different time intervals (0 min, 30 min and 24 h) as radical scavenging activity against DPPH (Arabshahi-Delouse et al. 2007).

Statistical analysis

Mean values of 4 determinations ($n=4$) ($p<0.05$) was subjected to one way ANOVA and Tukey's multiple comparison tests using SPSS software (version 11).

Results and discussion

Antioxidant component

The *Aegle* leaves were found to be a good source of several antioxidant components such as, β -carotene, glutathione, α -tocopherol, ascorbic acid and total polyphenols and flavonoids (Tables 1 and 2).

Table 2 Comparison of antioxidant properties and polyphenols of *Aegle* extracts and standards

Solvent	Radical scavenging activity DPPH, %	Percent Inhibition of lipid oxidation (%)	Reducing power (absorbance at 700 nm)
ME	81 $\pm$ 2.064 ^a	23 $\pm$ 2.00 ^a	0.232 $\pm$ 0.003 ^a
EE	87 $\pm$ 1.00 ^b	46 $\pm$ 1.15 ^b	0.246 $\pm$ 0.004 ^b
WE	93 $\pm$ 0.57 ^c	28 $\pm$ 2.51 ^a	0.349 $\pm$ 0.003 ^c
Standard	87 $\pm$ 0.52 ^{ba}	80 $\pm$ 5.03 ^{cb}	0.973 $\pm$ 0.002 ^{dc}

^a BHT, ^b α - Tocopherol, ^c Ascorbic acid, -standard conc. used 150 μ g and extracts 200 μ g;

ME-methanol, EE- ethanol, WE-water extracts, $n=4$

Fig. 1 DPPH radical scavenging activities (RSA) of methanol, ethanol and water extracts of *Aegle* leaves. BHT was used as positive control. % RSA relative to control. ME methanol, EE ethanol, WE water extracts

Radical scavenging activity

Free radicals which are involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathogens such as cancer and cardiovascular diseases among others (Dorman et al. 2003a, b). The DPPH radical scavenging has been widely used to evaluate the free radical scavenging ability of various natural products and has been accepted as a model compound for free radical originating in lipids (Porto et al 2000).

The radical scavenging activity (RSA) of the three solvent extracts (methanol, ethanol and water) as determined by DPPH assay at different concentrations is shown in Fig. 1. The scavenging ability of three extracts against DPPH was concentration dependent upto 300 µg., with increasing levels the RSA tended to decrease. This trend indicates that the scavenging potency of the extracts might reach a saturation point and at higher concentrations it may act as a pro-oxidant. Similarly, at higher concentrations phenolic com-

pounds are reported to enhance OH[•] formation and inhibit antioxidant enzymes (catalase and SOD) suggesting that the naturally occurring substances can have pro-oxidant effects under some reaction conditions (Sakihama et al 2002). At 200 µg concentration, WE showed maximum activity (92%) followed by EE (88%) and ME (78%) Table 2. These percentages can be considered as a full absorption inhibition of DPPH because after completing the reaction in the final solution always possesses some yellowish color and therefore its absorption inhibition compared to colorless methanol solution cannot reach 100% (Miliauskas et al., 2004).

Reducing power assay

The reducing power (electron donating capacity) of bioactive compounds is associated with antioxidant activity (Siddharaju et al 2002, Yen et al 1993). In the present study, the ability of extracts to reduce iron (III) to iron (II) was determined and was compared with that of a standard (ascorbic acid). All the three extracts showed some degree of electron donating capacity (Table 3) in which EE extract showed maximum reduction of iron (III) to iron (II) indicated by higher absorbance at 700 nm, but the capacities were inferior to that of ascorbic acid. It was interesting to note that despite containing the least amount of phenolics, WE was the most potent reducing agent.

Antioxidant activity (AOA)

The reducing power assay and radical scavenging activity assays are indicators of antioxidant activity. However, neither of these methods utilize a food or biologically relevant oxidizable substrate, hence no direct information on the protective properties of the extracts can be determined (Dorman et al 2003a, b). In present study, the effect of various extracts on the % inhibition of lipid peroxidation in Linseed oil (rich in PUFA) was determined by TBA method. All the three extracts, were capable in preventing the formation of TBARS generated by Fenton's reagent (Table 3), however EE was the most effective in inhibiting lipid oxidation. A similar effect was not observed at higher concentrations of extracts (>500 µg) indicating a

Table 3 Effect of pH and Temperature on the Antioxidant Activity of Methanol, Ethanol and Water Extracts of *Aegle* Leaves

Extracts conc. 200 µg; ME methanol, EE ethanol, WE water extracts, n=4

Solvent extract	0 min	30 min.	24 hrs	Before heat treatment	After heat treatment
4ME	73±1.52 ^{al}	71±1.73 ^{abl}	70±1.00 ^{al}	81±2.064	57±2.00
4EE	79±1.15 ^{bl}	72±0.57 ^{am}	75±1.52 ^{bn}	87±1.00	71±2.08
4WE	77±1.52 ^{al}	68±1.52 ^{bm}	72±2.00 ^{abm}	93±0.57	89±1.2
7ME	67±1.52 ^{al}	75±1.52 ^{bl}	72±2.51 ^{al}		
7EE	73±2.64 ^{al}	68±2.08 ^{al}	71±2.64 ^{al}		
7WE	80±1.00 ^{cl}	72±2.08 ^{am}	72±2.08 ^{am}		

pro oxidant role at higher concentrations. Sakihama (2002) reported that, the flavonoids and dihydroxycinnamic acids can nick DNA via the production of radicals in the presence of Cu and O₂. Phenoxyl radicals are reported to initiate lipid peroxidation while, Al, Zn, Ca, Mg and Cd have been found to stimulate phenoxyl radical-induced lipid peroxidation.

Heat stability

It is well known that many factors such as antioxidant concentration, temperature and pH of the media, processing treatment influence the antioxidant activity (Gazzani et al 1998). Table 3 shows the effect of temperature (100° C, 15 min.) on the stability of different solvent extracts. WE showed maximum stability as measured by RSA compared to ME and EE after heat treatment and similar results were found in case of *Raphanus sativus* where WE was stable and radical scavenging activity increased after heat treatment (Reddy et al 2010). In a study conducted by Gazzani et al (1998), the antioxidant activity of a number of vegetable juices was stabilized by boiling, suggesting that the initial pro oxidant activity was due to pro oxidases like peroxidases which are inactivated at high temperatures. The decline in antioxidant activity of extracts followed by heating at 100 °C might be related to either loss of naturally occurring antioxidants present in the extracts or formation of novel compounds having prooxidant activity (Arabshahi-Delouse et al 2007). Plant extracts are known to exhibit either a prooxidant or an antioxidant effect depending on the thermal processing and variety of the vegetable used (Castenmiller et al 2002, Arabshahi et al 2007, Gazzani et al 1998, Kaur and Kapoor 2000).

pH stability

The influence of pH on the stability of three solvent extracts (ME, EE, WE) is shown in Table 3. At pH 4, EE and WE exhibited significantly higher RSA initially which declined at 24 h in all the three extracts and the decline in RSA was minimum in ME (3%). At pH 7, WE exhibited significantly higher RSA initially whereas after 24 h, EE was more stable at pH 7. The activity of EE and WE after 24 h was comparable. In case of ME, the RSA after 24 h was similar to initial value indicating the stability at pH 7. In general, the extracts were found to be stable at both pH. At pH 9, none of the extracts exhibited ability to scavenge DPPH radicals as the absorbance was more than that of control, this may be due to the structural denaturation of natural antioxidant components and similar results were found by (Mansour and Khalil 2000) in fenugreek seeds and ginger rhizome extracts and *Rapahnus sativus* (Reddy et al 2010) extracts where the antioxidant activity decreased

by increasing the pH of media. The antioxidant activity of different extracts from cocoa by-products was found to be higher at alkaline pH (Azizah et al 1999). In an earlier study, it was observed that extracts from mint and carrot leaves showed higher antioxidant activity at pH 9 than pH 4 (Arabshahi-Delouse et al 2007). These differences might be due to different samples used, various compounds being extracted in each case and the method used to evaluate antioxidant potency.

Conclusion

Results of the present study reveal that the AM is a potential source of antioxidants which are responsible for the antioxidant activity. The stability to heat and pH of the different extracts of AM leaves extracts with strong antioxidant activity indicates its scope for utilization in food and biological systems. In addition to being consumed as healthy antioxidants, the compounds present in AM that are responsible for antioxidant activity could be isolated and then used as food additives to delay the oxidative deterioration of foods and as nutraceutical in food formulations against degenerative diseases.

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