



## **Evaluation of Antioxidant Potential and Phytochemical investigations on *Aegle marmelos* (L.) Corr**

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### **ABSTRACT**

*Herbal medicine constitutes major component in earlier and traditional medical systems evolved in India especially in Ayurveda. Aegle marmelos (L.) Corr. commonly known as Bael is an important medicinal plant of India and it is considered as "Mahaphala" (Fruit with all qualities) in Ayurvedic System of medicine. A. marmelos is utilized as an effective ethnomedicine as astringent, antidiarrheal, antidysentric, demulcent, antipyretic and anti-inflammatory treatment. Plant also contains compounds useful for treatment of several diseases including cancer, diabetes, and cardiovascular diseases. Current investigation was aimed to evaluate potential of this plant as an effective antioxidant source. A. marmelos fruit and leaf extracts prepared using various solvents exhibited a significant variability regarding their antioxidant activity. The different extracts from leaf and fruit tissues were determined for their antioxidant potential through DPPH assay, FRAP analysis, reducing power, total antioxidant capacity method and metal chelation ability. The methanol extract of leaf and hexane extract of fruits exhibited relatively higher antioxidant activity as compared to other solvents. Flavonoids and total phenolic content was also determined in different extracts. Presence of bound flavanoid like chalcones (Butein-4'-glucoside) and aurones was also recorded in the extracts by UV Spectrum. Higher flavanoid content was not paralleled with high antioxidants activity in various extracts. The hexane extract of fruit exhibited most effective antioxidant properties whereas extracts prepared using water and phosphate buffer exhibited only a marginal antioxidant activity.*

*Keywords: Flavonoid; Phenolic compounds; DPPH; FRAP*

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### **INTRODUCTION**

*Aegle marmelos* (L.) Corr. (Family: Rutaceae) is reported to be important plant in several ethnobotanical and ethnomedicinal studies for its anti-diabetic, anti-ulcer, anti-inflammatory, anti-pyretic, anti-oxidant and anti-microbial activity [44]. Several plant parts of *A. marmelos* are reported to be medicinally important [13, 21, 23]. The Garasia tribal in Rajasthan (India) take powder made from roots, stem bark and leaves to cure diabetes, orally with water [4]. Fruit is considered as most natural medicinal fruit [43, 49] and the pulp of ripe fruit is used for treating chronic stomach ailments [50]. Fruit pulp is reported to be stimulant, antipyretic and antiscorbutic [3]. Fruit juice is used as a very good coolant and consequently it is very popular in tropical and arid parts of India [4].

The consumption of fruits and vegetables has been inversely associated with morbidity and mortality from degenerative diseases [36, 37]. In last few decades extensive research has been carried out to explore the dietary constituents, responsible for this association. The human health benefits of phytochemicals have been a major area of research, due to their anti oxidant potential. However, despite the strong evidence that many groups of phytochemicals are potentially active as antioxidants both *in vitro* and *in vivo*, our knowledge about the biological function of phytochemicals as antioxidants is sketchy. Antioxidants appear to play a major role in the protective effect of plant foods [37, 38, 59].

*A. marmelos* is a rich source of variety of phytochemicals including aegeline, aegelenine, marmelosine, marmelin, *o*-methyl hayordinol, alloimperratorin methyl ester, *o*-isopentanyl hayordinol, linoleic acid, cineole, *p*-cymene, citronella, citral, cuminaldehyde, D-limonene, eugenol, tannins, phlobatannins, flavon-3-ols, leucoanthocyanins, anthocyanins and flavonoid glycoside [29]. This wide array of phytochemicals makes *A. marmelos*, an appropriate plant with nutraceutical importance. Most of these compounds have been reported to possess antioxidation and free radical scavenging activities [11, 14, 22, 25, 42, 57]. Various compounds present in *A. marmelos* leaf extracts are considered good antioxidants and are

capable of inhibiting lipid peroxidation [35, 58]. The overall antioxidant capacity is a reflection of the combined activity of a wide range of compounds which may include phenolics, peptides, organic acids, enzymes, Maillard reaction products and possibly other minor components [18].

In previous studies, investigations on *A. marmelos* antioxidant potential of various plant parts have been carried out [46, 51]. However no detailed investigation on antioxidant potential of fruit pulp and leaf extracts of *A. marmelos*, has been put forth. Keeping this in view the current investigation was primarily aimed at evaluation of antioxidant properties and identification of phytochemicals in leaf and fruit extracts from *A. marmelos*. In addition, present study also evaluates several methodologies employed in studying anti oxidant properties of fruit and leaf extracts of *A. marmelos*. Methods evaluated were ET-based assays, Folin-Ciocalteu reagent (FCR), ferric ion reducing antioxidant power (FRAP), and DPPH, Phospho-molybdenum assay and also procedure involving chelation of metal ions [56].

## MATERIALS AND METHODS

### *Plant species*

*A. marmelos* (L.) Corr. (Family: Rutaceae) is medium to small size tree. Ripe fruit is sweet, aromatic, cooling and nutritive. While raw, unripe fruit is astringent, digestive, stomachic and demulcent, ripe fruit is used in treating constipation, chronic dysentery and dyspepsia (29). Flowering and fruiting takes place between March and April, in general. *A. marmelos* is distributed in sub mountainous regions and plains almost throughout India, from sub Himalayan forests, Bengal, Central and in Burma [12, 34].

### *Collection of Plant Material*

Leaves and fruits were collected from wild trees at Jaipur and were shade dried without any external application. Deveined Leaves and pulp (separated from seeds) were kept in shade till there was no measurable change in biomass was recorded. Dried material was powdered manually using mortar and pestle and sieved through 1 mm sieve. Powdered plant material was stored in refrigerator in airtight containers.

### *Extraction*

100 g leaf material was extracted with several solvents separately and solvents used were ethanol, methanol, ethyl acetate, phosphate buffer (pH 6.6) and water. For fruit extracts, ethanol, hexane, methanol, phosphate buffer and water were used as solvents. All extraction work was carried out using Soxhelt apparatus. Extracts were evaporated to dryness and powdery extracts were dissolved in methanol to a final concentration as per the requirement of analysis.

### *Antioxidant assays*

#### DPPH photometric assay

Crude extracts were subjected to 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay as reported by Yamaguchi et al. (60). Each sample stock solution ( $1.0 \text{ mg.ml}^{-1}$ ) was diluted to concentrations of  $500 \mu\text{g ml}^{-1}$ ,  $250 \mu\text{g ml}^{-1}$ ,  $100 \mu\text{g ml}^{-1}$ ,  $50 \mu\text{g ml}^{-1}$  and  $10 \mu\text{g ml}^{-1}$  in methanol. Reaction sample contained 1 ml of a 0.3 mM DPPH methanolic solution and 2.5 ml of sample. Reaction mixture was incubated at  $32^\circ\text{C}$  i.e. room temperature for 30 min. The absorbance was measured at 518nm against blank (1 ml of methanol + plant extract). The percentage of DPPH decolorization in reaction sample was calculated according to the equation:

$$(\%) \text{ Decolorization} = [1 - \text{Absorbance in sample} / \text{Absorbance in control}] \times 100$$

DPPH solution plus methanol was used as a negative control whereas DPPH solution plus 1 mM quercetine was utilized as positive controls.

#### Rapid screening of antioxidant by dot-blot DPPH staining and TLC analysis

An aliquot (3 ml) of each dilution of plant extract was carefully loaded on a TLC layer (Silica gel 60 F<sub>254</sub>; Merck) and allowed to dry. Each sample was loaded in order of decreasing concentration on the TLC plate. The staining of the silica plate was done following the procedure of Soler-Rivas et al. (54). TLC plate with the dry spots was further developed by placing upside down for 10 seconds in a 0.4 mM DPPH solution. Stained silica layer revealed a purple back-ground with yellow spots at the location of the sample exhibiting free radical scavenger capacity. The intensity of the yellow color reflects the amount and nature of radical scavenger present in the sample. For TLC analysis plant extracts samples were loaded individually onto the baseline of the layer, which was then developed with toluene: ethyl acetate (93:7, V/V) and chloroform:ethyl acetate (60:40, v/v). The layer was dried and stained with DPPH solution, as described earlier.

#### Ferric Reducing Ability of Plasma (FRAP) Assay

FRAP assay for assessing antioxidant property was done following Soler-Varga et al. [53]. FRAP reagent was prepared using acetate buffer (25 ml, pH 3.6), 2.5 ml of 10 mM 1,2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM FeCl<sub>3</sub>.6H<sub>2</sub>O solution in dH<sub>2</sub>O. The working solution was always prepared fresh for analysis. Standard solution of FeSO<sub>4</sub> was used for calibration (in a range of 100-1000  $\mu\text{mol.L}^{-1}$ ). Samples

were observed for 5 min at 593 nm at 25°C. Values were determined using the calibration curve of FeSO<sub>4</sub>. The relative activities of samples were assessed by comparing their activities with that of L-ascorbic acid.

#### **Determination of Total Flavanoid Content**

The AlCl<sub>3</sub> method [26] was used for determination of the total flavanoid content of the sample extracts. 1.5 ml of extract was added to equal volume of 2% AlCl<sub>3</sub>.6H<sub>2</sub>O (w/v in methanol). The mixture was vigorously shaken and incubated for 10 min and absorbance was read at 367 nm. Flavanoid contents were expressed as mg quercetine equivalent.g<sup>-1</sup> dry weight.

#### **Metal chelating effect**

Chelation of transition metal ions was evaluated as per modified procedure adapted from Miranda et al. [32] by Shimadzu UV-visible spectrophotometer. The control reaction mixture for chelation contained 0.9 ml 10 mM phosphate buffer (pH 7.4), 50 µl 1.0 mM FeSO<sub>4</sub> and 50 µl 1.0mM EDTA. Whereas to the test sample (devoid of EDTA) 50 µl of plant extract (20 mg.ml<sup>-1</sup> concentration) was added to determine chelation effect. Transition ion spectra were scanned from 200 to 900 nm.

#### **Determination of Total Phenolic Compounds**

Total phenolic compounds were determined following the Folin-Ciocalteu method [41]. The reaction mixture containing 1 ml extract, 10.0 ml dH<sub>2</sub>O, 2.0 ml of Folin-Ciocalteu phenol reagent was incubated at room temperature for 5 min, and then 2.0 ml sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. The contents of phenolic compounds were expressed as mg quercetine equivalent.g<sup>-1</sup> dry weight.

#### **Evaluation of antioxidant capacity by phosphomolybdenum method**

The total antioxidant capacity of extracts was evaluated by employing the method reported by Prieto et al. (39). An aliquot of 0.1 ml of sample solution (0-100 µg.ml<sup>-1</sup>) was combined with 1ml of reagent solution (600 mM H<sub>2</sub>SO<sub>4</sub>, 28 mM Na<sub>2</sub>PO<sub>4</sub> and 4mM (NH<sub>4</sub>)<sub>2</sub>MoO<sub>4</sub>). The tubes were placed on water bath for 90 min at 95°C. The samples were cooled to room temperature and the absorbance of the aqueous solution of each was measured at 695 nm against blank. Water soluble antioxidant capacity was expressed as equivalents of ascorbic acid (µM.g<sup>-1</sup> of extract).

#### **Measurement of Reducing Power ability**

The reducing power of the crude extracts was determined according to Yen and Chen [62]. The crude extract (0 µg.ml<sup>-1</sup>, 5 µg.ml<sup>-1</sup>, 10 µg.ml<sup>-1</sup>, 25 µg.ml<sup>-1</sup>, 50 µg.ml<sup>-1</sup>, 75 µg.ml<sup>-1</sup> and 100 µg.ml<sup>-1</sup>) was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Thereafter an equal volume of 1% (v/v) trichloro acetic acid was added to the mixture and centrifuged at 6,000 rpm for 10 min. The upper layer of the solution was mixed with dH<sub>2</sub>O and 0.1% FeCl<sub>3</sub> in a ratio of 1:1:2, and the absorbance were measured at 700 nm. Butylated hydroxyl toluene (BHT) was used as standard.

#### **Thiobarbituric acid- reactive-substances assay**

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction, as described by Dhindsa et al., (15). Plant extracts (0.2 g) were mixed in 2 mL of 20% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 3500 g for 20 min. 1.5 ml of 20% TCA containing 0.5% (w/v) TBA and 100 µl 4% butylated hydroxytoluene (BHT) in ethanol were added to 0.5 ml of the aliquot of the supernatant. The mixture was heated at 95°C for 30 min and was then quickly cooled in ice. The contents were centrifuged at 10,000 g for 15 min and the absorbance was measured at 532 nm. The value of non-specific absorption was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mmol/L.cm<sup>-1</sup>.

#### **UV-visible spectrum for the identification of flavanoids**

UV-visible spectrums of leave and fruit extracts for flavanoid identification were analyzed in accordance to the table described by Mabry et al., [28]. Extracts were prepared in ethanol, methanol, diethyl ether and ethyl acetate using 100g powder of dried leaves and fruit. These mixtures were continuously stirred for 8 hours on shaker and then incubated overnight at room temperature. Filtered extracts were stored in glass vials. Spectra were recorded on Shimadzu UV-visible spectrophotometer from 190 to 700 nm.

#### **Data analysis**

The data for antioxidant enzyme activities were subjected to one-way analysis of variance (ANOVA) at 5% significance level to evaluate the significance of the observed differences in different treatments. Mean and standard error were determined for the replicate values. The differences in the mean values of a given parameter for different groups were tested for significance (P<0.05) using Tukey's multiple comparison procedure.

## **RESULTS**

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (24). 50% scavenging capacity (SC<sub>50</sub>) of DPPH free radical by fruits

and leaf extracts of *A. marmelos* has been presented in figure 1 and 2. Highest active radical scavenging was recorded in methanol extract of leaves of *A. marmelos* ( $143.82 \pm 0.62 \mu\text{g ml}^{-1}$ ). For fruit, hexane extract exhibited maximum free radical scavenging capacity as compared to other extracts ( $SC_{50} = 154.95 \pm 0.92 \mu\text{g.ml}^{-1}$ ). The free radical scavenging activity in the leaf extracts of *A. marmelos* was in the following order: Methanol > Water > Ethanol > Phosphate buffer > Ethyl acetate (Fig.1). However the order of DPPH assay of fruit extracts in *A. marmelos* was observed as: Hexane > Methanol > Phosphate buffer > Ethanol > Water (Fig. 2). For qualitative detection of reactive scavenging capacity (RSC) in various plant extracts, the extracts were applied on TLC layer and developed in two solvent systems. All extracts of *A. marmelos* showed numerous migrating spots having strong intensities on TLC plate developed on toluene/ethyl acetate solvent and chloroform-ethyl acetate. The number of white spots and their migration was variable depending on TLC solvent system. The chromatogram of leaf extracts of *A. marmelos* developed on chloroform/ethyl acetate solvent system revealed more migrating spots as compared to toluene/ethyl acetate system. Maximum number of spots was recorded for methanol extract of *A. marmelos* leaf. Whereas hexane extract of *A. marmelos* fruit showed more migrating spots when developed in toluene-ethyl acetate solvent as compared to chloroform-ethyl acetate. Dot-Blot DPPH staining procedure for leaf extract of *A. marmelos* was also carried out. For ethanol and phosphate buffer extracts, a pronounced activity was observed at all the dilutions tested. Water and ethyl acetate extracts shown this activity only at higher concentration i.e.  $400 \text{ mg.ml}^{-1}$  whereas intermediate concentration of methanol extract revealed this activity.

Significant variability was recorded in FRAP value of leaves of *A. marmelos* extracted with different solvents ( $F_{4,16} = 8626.22$ ,  $P < 0.0001$ ) (Table 1). The order of FRAP assay values in *A. marmelos* fruit extracts prepared using different solvents was as: Hexane > Methanol > Phosphate buffer > Ethanol > Water. In case of leaf extracts, the maximum antioxidant activity was recorded in phosphate buffer and ethanol extracts. The order of FRAP assay for leaves extracts of *A. marmelos* in different solvents was as: Phosphate buffer > Ethanol > Methanol > Ethyl acetate > Water.

The present investigation, the flavanoid content of leaves extracts correlate with these assays (Fig. 1). Flavanoid content in different leaf extracts of *A. marmelos* was significantly variable ( $F_{4,16} = 88378.76$ ,  $P < 0.0001$ ) in current investigation. Flavanoid content (Table 1) in different leaf extracts was in following order: Methanol > Ethyl acetate > Phosphate buffer > Ethanol > Water. (Fig. 1). Similarly in *A. marmelos* fruit extracts, the flavanoid content varied significantly ( $F_{4,16} = 459.62$ ,  $P < 0.0001$ ) in different extraction solvents (Fig. 2; Table 2). The flavanoid content ranged between as high as  $138.00 \pm 1.73 \text{ mg.g}^{-1}$  dry weight in hexane extracts to  $51.00 \pm 1.52 \text{ mg/g}$  dry weight in water. The decreasing order of flavanoid content in different extracts of *A. marmelos* fruits was as follows: Hexane > Methanol > Phosphate buffer > Ethanol > Water. The hexane extract had maximum flavanoid concentration. Total phenolic content in *A. marmelos* leaves and fruit has been presented in Table 1 and 2.

The phenolic content varied significantly in different leaves ( $F_{4,16} = 23127.66$ ,  $P < 0.0001$ ) and fruit extracts ( $F_{4,16} = 8137.72$ ,  $P < 0.001$ ) of *A. marmelos*. The maximum amount of phenolic compounds in leaf extracts was recorded for methanol extract (Fig. 1, Table 1). In fruit extracts the maximum phenolic extraction was achieved in hexane which was followed by methanol (Fig. 2; Table 2).

The methanol extracts of leaves and fruit of *A. marmelos* demonstrated transition metal ion chelation activity when incubated with  $\text{FeSO}_4$  at pH 7.4. The metal chelating effect of iron with test compound was evaluated by absorbance change and/or spectral shift after incubation (Fig. 3). The  $\text{Fe}^{2+}$ -*A. marmelos* leaves (AML) methanol extract complex had peaks at 211 nm and 275 nm and the  $\text{Fe}^{2+}$ -*A. marmelos* fruit (AMF) extract had peak at 222nm and 267nm.

The quantification of antioxidant capacity (AOC) by phosphomolybdenum method of the various leaves and fruit extracts of *A. marmelos* was also carried out. The antioxidant capacity of leaves extracts of *A. marmelos* was in following order: Methanol > Ethanol > Phosphate buffer > Water > Ethyl acetate (Fig. 4). The antioxidant capacity of methanol, ethanol, phosphate buffer was considerably higher as compared to the standard ascorbic acid ( $100 \mu\text{g.ml}^{-1}$ ) (Fig. 4). In fruit extracts of *A. marmelos*, the maximum reduction was recorded in hexane extract (Fig. 5). The order of antioxidant capacity of fruit extracts of *A. marmelos* was as: Hexane > Methanol > Phosphate buffer > Ethanol > Water.

The reducing power of extract was directly proportional to the concentration of extract (Fig. 6 and 7). Extracts with concentration  $50 \mu\text{g.ml}^{-1}$  and more, exhibited higher reducing power as compared to Ascorbic acid and BHT taken as standard.

The production of both Thiobarbituric reactive substances (TBARS) and MDA was inhibited by the *A. marmelos* extracts (Table 1 and 2). In leave extract of *A. marmelos*, the minimum value for TBA-MDA complex was observed in methanol extract and maximum in water extract. The ethyl acetate, ethanol and phosphate buffer extracts also showed significantly decreased malondialdehyde (MDA) levels ( $0.066 \pm 0.001$  to  $0.023 \pm 0.001$ ). Similarly fruit extracts of *A. marmelos* also exhibited significant variation

regarding formation of TBA-MDA complex ( $P < 0.0001$ ). The increasing order of MDA equivalents levels in different solvents from fruits of *A. marmelos* was as follows: Ethanol < Methanol < Hexane < Phosphate buffer < Water.

The UV-visible spectra of extracts of leaves and fruits of *A. marmelos* were also recorded. Different fraction (methanol, ethanol, diethyl ether, and ethyl acetate extracts for leaves) were prepared for the better yield of flavanoids in samples. UV-visible spectrum of standard quercetine was also recorded which gives peaks at 264.80nm, 250.80nm and 249.20nm.

Table 1: Antioxidant potential in *A. marmelos* leaves- FRAP value, Flavonoids and Total Phenolic content and MDA content of leaves extracts from *A. marmelos* prepared in different solvent. Values represent mean  $\pm$  standard error (n=3).

Extraction Medium	Ethanol	Methanol	Ethyl Acetate	Phosphate buffer	Water
<b>Plant Species</b>					
<b>FRAP value</b> ( $\mu\text{M Fe(II).L}^{-1}$ )	105.66 <sup>a</sup> $\pm$ 1.20	54.66 <sup>b</sup> $\pm$ 0.88	0.55 <sup>c</sup> $\pm$ 0.18	165.88 <sup>d</sup> $\pm$ 0.82	0.05 <sup>e</sup> $\pm$ 0.01
<b>Flavonoids</b> ( $\mu\text{g.g}^{-1}$ dry wt.)	62.20 <sup>a</sup> $\pm$ 0.11	105.16 <sup>b</sup> $\pm$ 0.21	102.96 <sup>c</sup> $\pm$ 0.08	68.46 <sup>d</sup> $\pm$ 0.08	10.13 <sup>e</sup> $\pm$ 0.08
<b>Total Phenolics content</b> ( $\mu\text{g.g}^{-1}$ dry wt.)	151.66 <sup>a</sup> $\pm$ 0.88	1311.33 <sup>b</sup> $\pm$ 1.85	38.33 <sup>c</sup> $\pm$ 1.76	55.00 <sup>c</sup> $\pm$ 1.15	46.33 <sup>c</sup> $\pm$ 0.88
<b>MDA content</b> ( $\mu\text{M.g}^{-1}$ dry wt.)	0.066 <sup>a</sup> $\pm$ 0.001	0.014 <sup>b</sup> $\pm$ 0.00	0.023 <sup>c</sup> $\pm$ 0.001	0.028 <sup>c</sup> $\pm$ 0.001	0.081 <sup>d</sup> $\pm$ 0.001

Values in the same row with similar letters in superscript indicate no significant difference according to Tukey's multiple comparison procedure at  $P < 0.05$ .

Table 2: Antioxidant potential in *A. marmelos* fruits- FRAP value, Flavonoids and Total Phenolics content and MDA content of fruit extracts from *A. marmelos* prepared in different solvent. Values represent mean  $\pm$  standard error (n=3).

Extraction Medium	Ethanol	Methanol	Hexane	Phosphate buffer	Water
<b>Plant Species</b>					
<b>FRAP value</b> ( $\mu\text{M Fe(II).L}^{-1}$ )	25.70 <sup>a</sup> $\pm$ 0.37	65.33 <sup>b</sup> $\pm$ 0.21	123.00 <sup>c</sup> $\pm$ 1.15	35.66 <sup>d</sup> $\pm$ 0.88	0.02 <sup>e</sup> $\pm$ 0.01
<b>Flavonoids</b> ( $\mu\text{g.g}^{-1}$ dry wt.)	83.33 <sup>a</sup> $\pm$ 1.33	110.33 <sup>b</sup> $\pm$ 1.45	138 <sup>c</sup> $\pm$ 1.73	91.66 <sup>d</sup> $\pm$ 1.45	51 <sup>e</sup> $\pm$ 1.52
<b>Total Phenolics content</b> ( $\mu\text{g.g}^{-1}$ dry wt.)	123.33 <sup>a</sup> $\pm$ 0.88	252.33 <sup>b</sup> $\pm$ 1.85	377.00 <sup>c</sup> $\pm$ 1.52	208.33 <sup>d</sup> $\pm$ 0.88	53.33 <sup>e</sup> $\pm$ 1.45
<b>MDA content</b> ( $\mu\text{M.g}^{-1}$ dry wt.)	0.007 <sup>a</sup> $\pm$ 0.00	0.017 <sup>b</sup> $\pm$ 0.00	0.045 <sup>c</sup> $\pm$ 0.01	0.060 <sup>d</sup> $\pm$ 0.01	0.090 <sup>e</sup> $\pm$ 0.01

Values in the same row with similar letters in superscript indicate no significant difference according to Tukey's multiple comparison procedure at  $P < 0.05$ .

Fig. 1. Comparison of DPPH Assay with total flavanoid and phenolic content in *A. marmelos* in different leaves extracts. Column represents mean value (n=3). Similar letters above bars of corresponding series represents no significant difference according to Tukey's multiple comparison procedure at  $P < 0.05$ .  $SC_{50}$  value represents the amount of extracts necessary to scavenge 50% DPPH radical in the reaction.

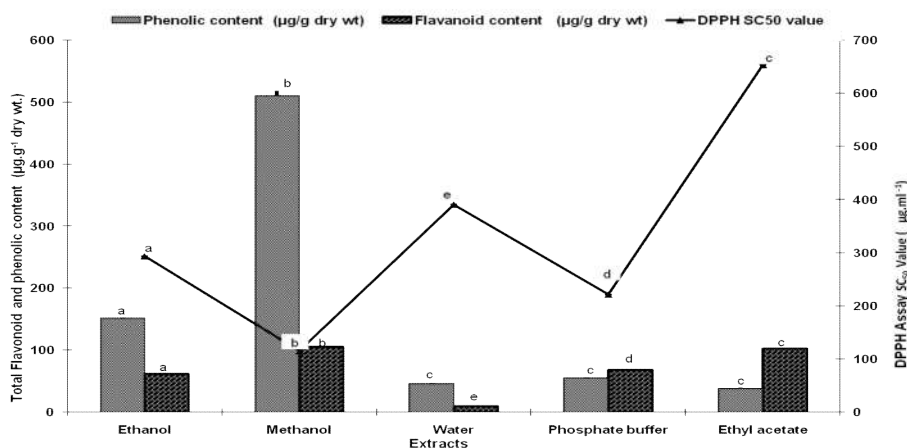


Fig. 2. Comparison of DPPH Assay with total flavanoid and phenolic content in *A. marmelos* in different fruit extracts. Column represents mean value (n=3). Similar letters above bars of corresponding series represents no significant difference according to Tukey's multiple comparison procedure at P<0.05. SC<sub>50</sub> value represents the amount of extract necessary to scavenge 50% DPPH radical in the reaction.

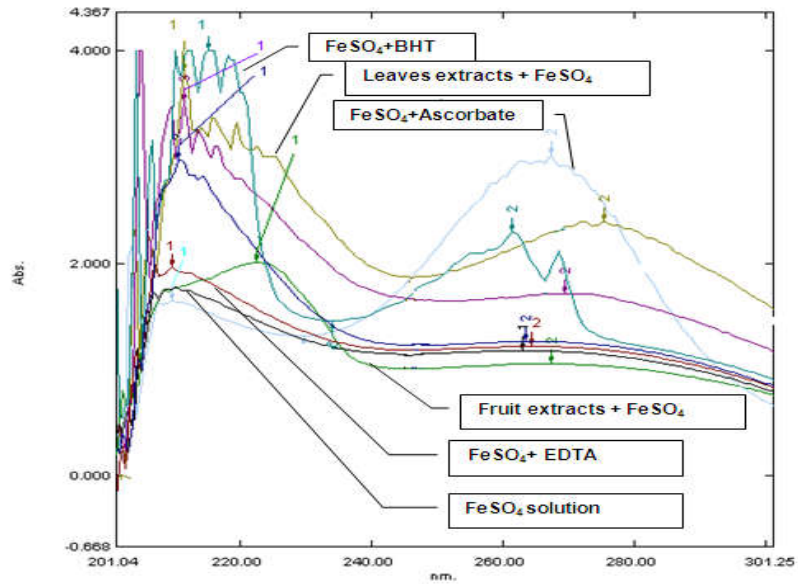
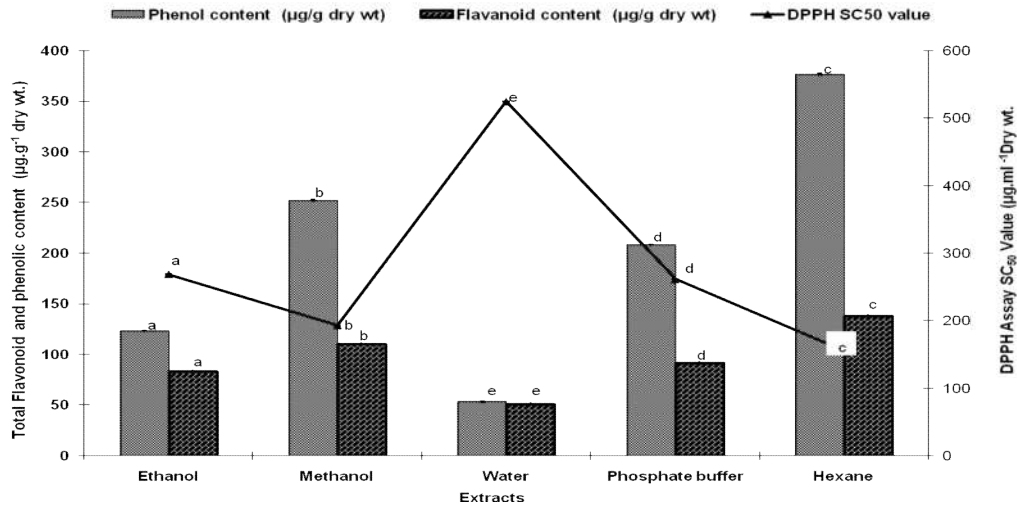


Fig. 3. Metal Chelating Effect of different extracts from *A. marmelos*

Fig. 4. Antioxidant Capacity (AOC) by phosphomolybdenum method in *A. marmelos* fruit extracts

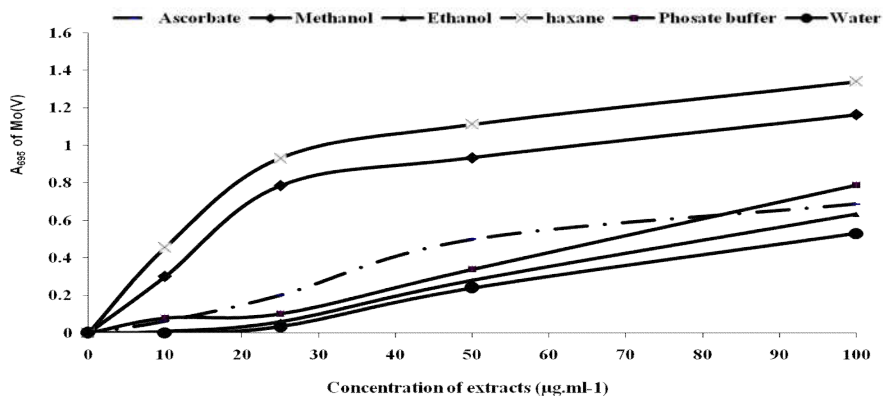


Fig. 5. Antioxidant Capacity (AOC) by phosphomolybdenum method in *A. marmelos* leaves extracts

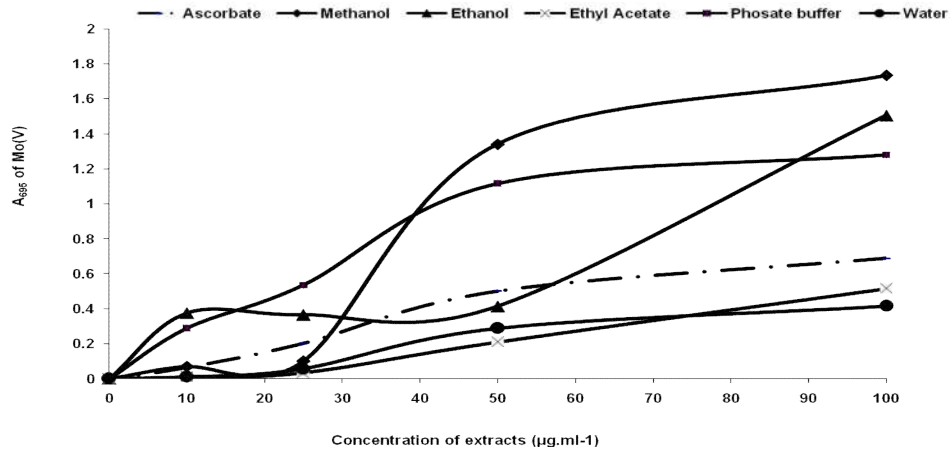


Fig. 6. Antioxidant Activity by reducing power method in *A. marmelos* leaves extracts

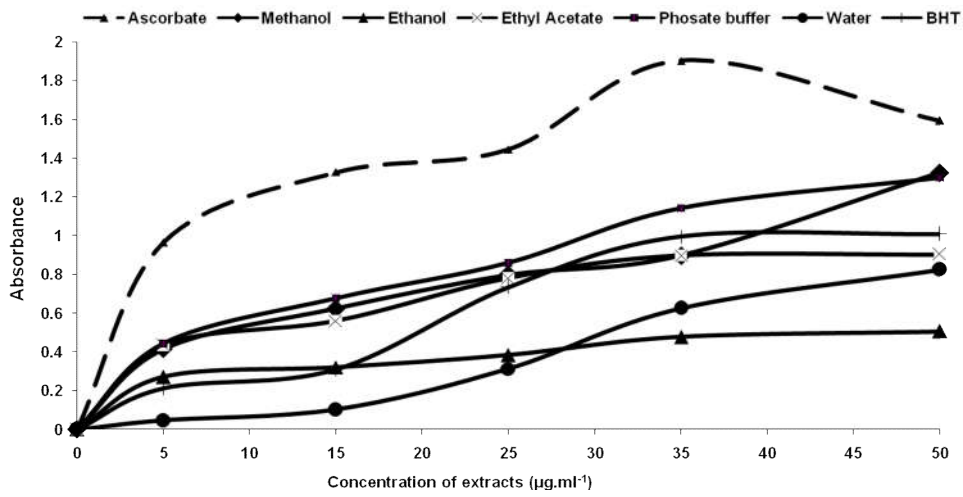
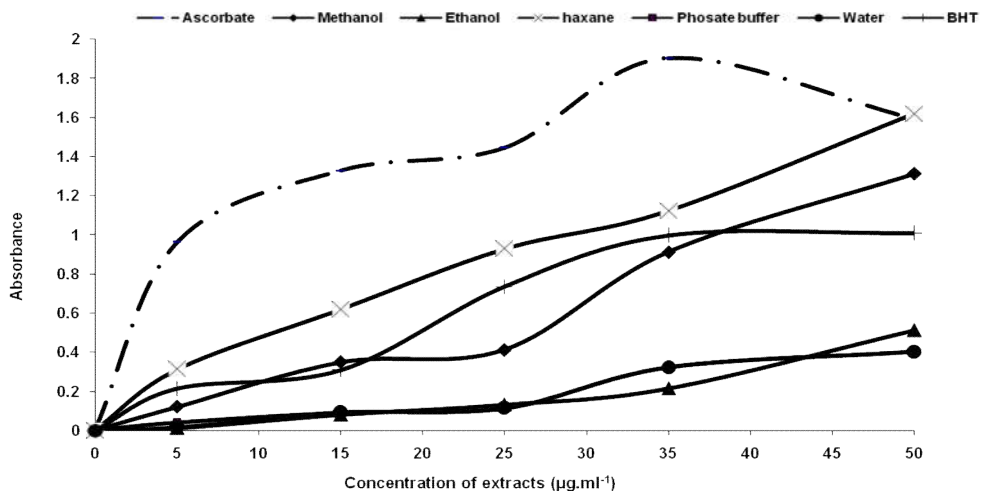


Fig. 7. Antioxidant Activity by reducing power method in *A. marmelos* fruits extracts



## DISCUSSION

Current investigation involved evaluation of anti oxidant properties of fruit and leaves of *A. marmelos* and anti-oxidant properties of extracts prepared using different solvents. Several methods used in studying anti-oxidant properties were also evaluated.

Radical scavenging assay was inversely proportional to  $SC_{50}$ . 500  $\mu\text{g.ml}^{-1}$  concentration of extracts demonstrated more than 80% scavenging activity. An increase in DPPH scavenging capacity was recorded with increment in concentration of extract used for assay. Maximum DPPH scavenging capacity recorded was up to 99%. It is suggested that an aryl hydroxyl substituent on the carbazole ring plays vital role in stabilizing thermal oxidation and rate of reaction against DPPH radical [55]. Ethanol extracts (75%) of leaves of *A. marmelos* are potent inhibitors of lipid peroxidation and effectively scavenge hydroxyl and superoxide radicals *in vitro* [45]. The results were further incorrigible by TLC screening and dot blot assay for DPPH radicle of different plant extracts at various concentrations.

FRAP assay was originally developed by Benzie and Strain [5,6] to measure reducing power in plasma, but the assay subsequently was adapted and used for the assay of antioxidants in plants. The hexane extract of fruit of *A. marmelos* caused the maximum reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored product (Table 2). The redox potential of Fe (III)-TPTZ is comparable with that of DPPH radicals. This may be attributed to the reaction conditions such as pH for these assays. Reaction at low pH results in decrement in ionization potential driving electron transfer and consequently increases the redox potential, causing a shift in the dominant reaction mechanism (20,52). Principally DPPH and FRAP can give analogous values however it has been reported that FRAP values are usually lower than DPPH values for a given series of antioxidant compounds [9,16, 40].

It is quite interesting to evaluate whether high FRAP or DPPH scavenging values correlate with the tendency of polyphenols to become pro-oxidants under certain conditions. Cao et al. (10) put forward this for some flavones and flavanones having high FRAP values. The maximum flavanoid concentration was recorded in the methanol extract of *A. marmelos* leaves that was corroborated with DPPH assay (Fig. 1; Table 1). The water and ethyl acetate extracts potentially contain non-chelating phenolic flavanoid compounds and the presence of such bound flavanoids or complexes in plant extracts does not contribute to the antioxidant capacity assays [32]. The low intensity of any radical scavenging activity by the prenylated and non-prenylated chalcones and flavanones is associated with the absence of 30, 40-dihydroxy substituent on the B-ring of such flavanoids [61]. The hexane extract had maximum flavanoid concentration in fruit extracts of *A. marmelos*. This also supported the correlation of antioxidant assay and the flavanoid content of different plant extracts further.

Investigations regarding phenolic and flavanoid compounds in leaves and fruits of *A. marmelos* have been carried out in some early studies also [19]. Influence of secondary metabolite milieu on concentration of phenolic compounds of various plant tissues have been extensively explored [7,8]. A direct correlation between DPPH radical scavenging assay (RSA) values and total phenolic levels has been established suggesting that phenolic compounds were likely to be contributing to the RSA of these extracts [31]. The identical phenolic levels do not necessarily correspond to the same antioxidant responses (Fig.1 and 2). The response of phenolics in the Folin-Ciocalteu assay also depends on their chemical structure [2]. It has been a matter of debate that what is being detected in total antioxidant capacity assays e.g. only phenols, or phenols plus reducing agents plus possibly metal chelators [17, 48].

Metal ions caused a spectral shift and absorbance change of  $\text{FeSO}_4$  (Fig. 3). The  $\text{FeSO}_4$  solution had peaks at 263 nm while the  $\text{Fe}^{2+}$ -EDTA complex has peaks at 209 nm and 264 nm. Generally, compounds with structures containing two or more of the following functional groups, -OH, -SH, -COOH,  $-\text{PO}_3\text{H}_2$ ,  $-\text{NR}_2$ , -S- and -O- in a favorable structure-function configuration will have chelation activity [27]. Molecules including organic acids, flavanoids like quercetin and rutin have been recorded for their ability to chelate transition metal ions [1,27].

The quantification of antioxidant capacity (AOC) by phosphomolybdenum method of the various leaves and fruit extracts of *A. marmelos* was further supplemented the results of DPPH and FRAP assay as methanol extract of leaves and hexane extract of fruit showed maximum activity.

Similar to antioxidant activity, the reducing power of different extracts was also concentration dependent in leaves and fruits extracts of *A. marmelos*. The hexane extract of *A. marmelos* showed maximum reducing power activity. Similar activity was reported in *Palmaria palmata* where reducing activity in fresh fruit extracts was comparable to the values recorded in present study [33].

The efficacy of the *A. marmelos* extracts to inhibit oxidation of the linoleic acid emulsion is a reflection of the complexity of the extract composition (i.e. aqueous vs. hydrophobic nature of extract or polarity of extracts) as well as potential interactions between the extract and emulsion components (i.e. oil: water or lipid: air interfaces [24]. These interactions form the basis of whether or not the "polar paradox" can be observed in the present study. The polar paradox occurs in emulsions when non-polar (Hexane extracts



of fruit of *A. marmelos*) compounds have strong antioxidant activity in an emulsion due to the concentration of the antioxidant at the lipid: air interface, thereby ensuring strong protection of the emulsion against oxidation [24]. Conversely, polar compounds (water extract of leaves of both the plants) exhibit weak antioxidant activity in emulsions due to the dilution of these compounds in the aqueous phase. Moreover, the opposite antioxidant profile is observed in bulk lipid or oil systems [24]. These phenomena no doubt help to explain the variability in the efficacy of different leaves and fruits extracts of *A. marmelos* to prevent lipid oxidation *in vitro*.

In the leaves and fruit extracts of *A. marmelos*, different absorption peaks ( $\lambda$  max in nm) for flavones (255-275; 350-385), flavones (275-295; 255-285), isoflavanones (250-270), auronones (385-415), chalcones (370-395) especially Butein-4'-glucoside at 450 nm and anthocynin (475-545) were recorded (28). Presence of these auronones and chalcones could be correlated with lesser activity of water and ethyl acetate extracts obtained during DPPH, FRAP and other antioxidant activity assays results. The position and relative intensities of these maxima gives information on the nature of the flavanoid and its oxygenation pattern; variation within these ranges will depend on the hydroxylation pattern and on the degree of substitution of the hydroxyls (30,47). For example, quercetin 7-glycoside had  $k_{max}$ : 254 and 370 nm, while quercetin 3-glycoside had  $k_{max}$ : 254 and 354 nm. Moreover, apigenin detected in *A. marmelos* extract, also having the same molecular weight, were identified on the basis of their UV spectra. The UV spectrum of apigenin 7-glycoside is characterized by the presence of two maxima at 262 and 333 nm while flavanones exhibited a very strong maxima at 285 nm (Band II) and a small peak or shoulder at 320–330 nm (Band I) [2].

## CONCLUSION

*A. marmelos* which is an important medicinal plant has an effective antioxidant potential also and thus is a good natural antioxidants source. The use of more than one method to evaluate lipid oxidation and antioxidant activity is prudential in determination of the efficacy of the leaves and fruit extracts of *A. marmelos*. Present investigation indicated flavanoids to be the major antioxidants in *A. marmelos*. UV-spectra of different extracts support that the presence of chalcones and other bound flavanoids also influence the obtained values for determination of antioxidant potential. Specific studies on detailed chemistry of antioxidant compounds present in *A. marmelos* remains an area with scope for research.

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