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GC-MS, PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITIES OF FRUIT PULP OF *AEGLE MARMELOS* (L.) CORRÊA

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ABSTRACT
The present work describes the phytochemical analysis, antioxidant activities of ethanol extract of the fruit pulp of *Aegle marmelos*. It is also known as bael, which is an important medicinal plant which belongs to family Rutaceae. Compounds purified from *A.marmelos* have been proven biologically active against various several major diseases like cancer, diabetes, cardiovascular diseases. The aim of the present study was to evaluate the antioxidant activities of ethanol extract of fruit pulp of *A. marmelos* and to identify the bioactive compounds by GC-MS analysis. Antioxidant activities such as DPPH radical, $ABTS^{\bullet+}$ radical cation, Fe^{3+} reducing power and phosphomolybdenum reduction assays were carried out for ethanol extract. The maximum DPPH radical and $ABTS^{\bullet+}$ radical cation scavenging activities of ethanol extract were 89.58±6.27% at 300 µg/mL and 92.19±6.45% at 30 µg/mL concentration. The IC_{50} values of $DPPH^{\cdot}$ radical and $ABTS^{\bullet+}$ radical cation scavenging activities were 84.14 µg/mL and 9.67 µg/mL concentration. The maximum reduction of Fe^{3+} and Mo^{6+} were 67.80±4.74% and 85.93±6.01% at 120 µg/mL concentrations and the IC_{50} values were 63.84 µg/mL and 21.05 µg/mL concentrations respectively.

KEYWORDS: *Aegle marmelos*, TLC, DPPH, $ABTS^{\cdot+}$, GC-MS.

INTRODUCTION
Aegle marmelos is a perenial tree, wild in the sub Himalaya tract, central and South India. It belongs to the family Rutaceae. *A. marmelos* commonly known as Bael, has been broadly used in indigenous systems of Indian medicine due to its numerous therapeutic properties.[1] It is also an important environmental protector as leaves and bark act as a sink by absorbing dust and foul and poisonous gases from surrounding atmosphere.[2] Leaves, fruit, stem, bark of this plant is used because of its medicinal properties like astringent, antidiarrheal, antipyretic, anti-inflammatory activities. The fruit contains tannic acid and volatile oil.

A. marmelos is a slow-growing, medium sized tree, up to 12-15 m tall with short trunk, thick, soft, flaking bark, and spreading, sometimes spiny branches, the lower ones drooping.[3] It is a spinous deciduous and aromatic tree, leaves are 3-5 foliate, flowers are greenish white in colour and sweet scented. Fruits are large, woody, greyish yellow; 8-15 celled and have sweet gummy orange coloured pulp. *A. marmelos* is a subtropical plant and grows up to an altitude of 1,200 m altitude from sea level. It grows well in the dry forests on hilly and plain areas. It is also known Bengal quince, golden apple, Japanese bitter orange, or wood apple, is a species of tree native to India. *A. marmelos* is a widely distributed plant and found in India, Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia, Thailand, Indonesia, Malaysia, Tibet, Sri Lanka, Java, Philippines and Fiji.

Figure 1: Habitat of *Aegle marmelos.*

MATERIALS AND METHODS

Preparation of plant extract

The fruits of *A. marmelos* were collected from IIT Madras, Chennai. The fruit was washed with tap water, rinsed with distilled water and the fruit pulp of *A. marmelos* was scrapped out from the shell. The fruit pulp was soaked in ethanol and kept it for 72 h. Then the supernatant of ethanol extract was filtered using filter paper and condensed in a hot plate at 50°C, to yield gummy extract.

Phytochemical analysis

The ethanol extract of fruit pulps of *A. marmelos* was subjected to different classes of phytoconstituents using specific standard reagents.[4,5,6]

Determination of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds [7] with slight modifications. One hundred µL of ethanol extract (1mg/mL) of fruit pulps of *A. marmelos* was mixed with 900 µL of distilled water and 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water). After 5 min, 1 mL of Na_2CO_3 (20%) solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured by UV-VIS spectrophotometer at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Determination of total flavonoids

The total flavonoid content of ethanol extract of fruits of *A. marmelos* was determined using aluminium chloride reagent method with slight modification as described by Liu et al.[8] Five hundred µL of extract (1mg/mL) was mixed with 0.5 mL of methanol and 0.5 mL of 5% sodium nitrite solution. Then, 0.5 mL 10% aluminium chloride solution was added followed by 1 mL of 1 M NaOH. The mixture was incubated for 30 min at room temperature and th absorbance was measured at 510 nm. The result was expressed as (µg/mg of extract) quercetin equivalent.

Antioxidant activities

DPPH˙ radical scavenging assay

The antioxidant activity of ethanol extract of fruit pulp of *A.marmelos* was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (50-300 µg/mL) of ethanol extract. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. One mL of methanol and 1 mL of DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis Spectrophotometer at 517 nm.[9] The percentage of inhibition was calculated using the following formula:

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] * 100$$

$ABTS^{\cdot+}$ radical cation scavenging assay

The antioxidant capacity was estimated in terms of the $ABTS^{\bullet+}$ radical cation scavenging activity.[10] $ABTS^{\bullet+}$ radical cation was obtained by reacting 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{\bullet+}$ radical cation solution was diluted with distilled water to reach an absorbance of 0.70±0.02 at 734 nm. One mL of ethanol extract of various concentrations (20-120µg/mL) was mixed with 500 µL of diluted $ABTS^{\bullet+}$ solution and the absorbance was measured after 10 min at 734 nm. The $ABTS^{\bullet+}$ radical cation scavenging activity was calculated as:

$$\%\ \text{of ABTS}^{\cdot+}\ \text{radical cation inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] * 100$$

Phosphomolybdenum reduction assay

The antioxidant capacity of ethanol extract of *A. marmelos* fruit pulp was assessed by the method of Prieto *et al*.[11] The ethanol extract with concentrations ranging from 20 to 120 µg/mL was combined with reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 95°C for 90 min. The absorbance of the coloured complex was measured at 695 nm. The percentage of inhibition was calculated using the following formula:

$$\%\ \text{of phosphomolybdenum reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} * 100$$

Ferric (Fe^{3+}) reducing power assay

The reducing power of ethanol extract of *A.marmelos* fruit determined by slightly modified method of Yen and Chen, 1995. One mL of plant extract of different concentrations (20-120 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide $[K_3Fe\ (CN)_6]$ solution. The mixtures were then incubated at 50°C for 20 min in a water bath. Five hundred µL of trichloroacetic acid (10%) was added to each mixture. Then to the mixture 200 µL of $FeCl_3$ (0.1 %) solution was added and the absorbance was measured at 700 nm.[12] The percentage of reduction was calculated using the following formula:

$$\%\ \text{of } Fe^{3+}\ \text{reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] * 100$$

Thin layer chromatography

Thin layer chromatography (TLC) was carried out for ethanol extract of *A. marmelos* fruit pulp in silica gel coated TLC aluminium sheet (Merck; 60 F254) of 5x2 cm. The ethanol extract was spotted at 0.2 mm above from the bottom of the TLC plate. The chromatogram was developed in the mixture of suitable solvent system. The spots were visualized under UV light at 254 nm. The R_f value of the fluoresced spots were recorded.[13] The ratio in which distinct bands appeared was optimized and R_f values were calculated as follows.

$$R_f \text{ value} = \left(\frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}} \right)$$

Gas chromatography–Mass Spectrometry (GC–MS)

For GC-MS analysis, the samples were injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following chromatographic conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250°C; mass range of 50-600 mass units.[14]

Identification of components

The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

RESULTS AND DISCUSSION

Phytochemical analysis

The phytochemical analysis of ethanol extract of fruit pulp of *A. marmelos* showed the presence of alkaloids, terpenoids, phenolic compounds, flavonoids, glycosides and saponins.

Table 1: Qualitative analysis of ethanol extract of fruits of *A. Marmelos*

S. No	Phytochemicals	Tests	Results
1	Alkaloids	Wagener's reagent	-
2.	Terpenoids	$CHCl_3$ + conc. H_2SO_4	+
3.	Steroids	Liebermann–Burchard test (acetic anhydride+ Con. H_2SO_4)	+
4.	Flavanoids	NaOH solution	+
5.	Phenols	$FeCl_3$ solution	+
6.	Glycosides	Sodium nitroprusside solution + Con. H_2SO_4	+
7.	Saponins	Foam test	+

Total phenols and flavonoids

The total phenols and total flavonoids were quantified in the ethanol extract of fruit pulp of *A. marmelos* seemed to be responsible for the antioxidant activity. The total phenol content was 214.7±1.85 µg/mg of GAE and the total flavonoid content was 10.90±0.99 µg/mg of QE in the extract. These results provide a comprehensive profile of the antioxidant activity of fruit pulp of *A. marmelos* with respect to the amount of phenols and flavonoids present in the ethanol extract.

Table 2: Quantitative estimations of Ethanol extract of fruits of *A. marmelos*

S. No	Phytochemical	Value (µg/mg)
1.	Phenols	214.7±1.85
2.	Flavonoids	10.90±0.99

DPPH˙ radical scavenging assay

The ability of ethanol extract fruit pulp of *A. marmelos* to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The maximum DPPH˙ radical scavenging activity was 89.58±6.27% at 300 µg/mL concentration. Ethanol extract of *A. marmelos* demonstrated high capacity for scavenging free radicals by reducing the stable DPPH (1,1-diphenyl-2- picrylhydrazyl) radical to the yellow coloured 1,1-diphenyl-2-picrylhydrazine and the reducing capacity increased with increasing concentration of the extract. The IC_{50} value was found to be 183.58 µg/mL concentration and was compared with standard (ascorbic acid, IC_{50} = 2.88 µg/mL concentration).

Table 3: DPPH radical scavenging activity of ethanol extract of *A. marmelos*

S. No.	Concentration (µg/mL)	% of inhibition
1	50	40.13±2.80
2	100	59.67±4.15
3	150	74.05±5.18
4	200	86.03±6.02
5	250	87.08±6.14
6	300	89.58±6.27

$ABTS^{\bullet+}$ radical cation scavenging assay

$ABTS^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and in the presence of the plant extract or ascorbic acid, preformed cation radical gets reduced and the remaining radical cation concentration was then quantified. The maximum $ABTS^{\bullet+}$ radical cation scavenging activity was 92.19±6.45 % at 30 µg/mL concentration. The experiment demonstrated high antioxidant activity the IC_{50} of 9.67 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 3.91 µg/mL concentration).

Table 4: $ABTS^{\bullet+}$ radical cation scavenging assay of aqueous extract of *A. marmelos*

S. No.	Concentration (µg/mL)	% of inhibition
1	5	41.46±2.90
2	10	51.70±3.61
3	15	61.95±4.33
4	20	65.12±4.55
5	25	76.58±5.36
6	30	92.19±6.45

Phosphomolybdenum reduction assay activity

The total antioxidant activity of ethanol extract fruit pulp of *A. marmelos* was measured by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum phosphomolybdenum reduction was 85.93±6.01% at 120 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50}=5.97 µg/mL concentration).

Table 5: Phosphomolybdenum reduction activity of ethanol extract of *A. marmelos.*

S. No.	Concentration (µg/mL)	Phosphomolybdenum reduction @ 695nm
		Ethanol extract
1	20	47.50±3.32
2	40	58.88±4.12
3	60	77.41±5.41
4	80	80.43±5.63
5	100	82.15±5.75
6	120	85.93±6.01

Ferric (Fe^{3+}) reducing power activity

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the ethanol extract of *A. marmelos* and the subsequent formation of ferro-ferric complex. The reduction ability increases with increase in concentration of the extract. The maximum Fe^{3+} reduction was 67.80±4.74% at 120 µg/mL concentration and was compared with the standard ascorbic acid (29.11 µg/mL concentration).

Table 6: Ferric (Fe^{3+}) reducing power activity ethanol extract of *A. marmelos*

S. No.	Concentration (µg/mL)	Fe^{3+} reducing power @ 700nm
		Ethanol extract
1	20	23.78±1.66
2	40	33.33±2.33
3	60	46.99±3.28
4	80	56.68±3.96
5	100	63.47±4.44
6	120	67.80±4.74

Thin Layer Chromatography

Thin layer chromatography analysis was carried out in the solvent system of Methanol: isopropyl alcohol with the ratio of 1:1. The separated compounds in TLC were showed in Figure.

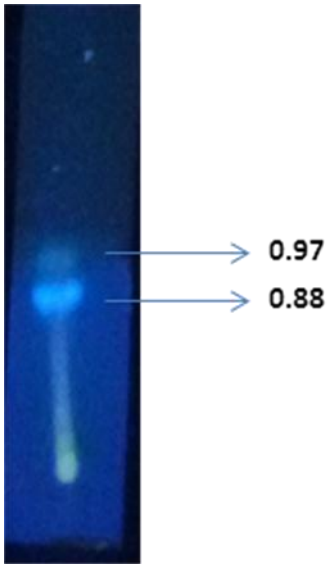


Figure 2: Compounds separated by Thin Layer Chromatography.

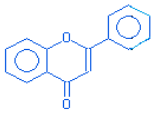
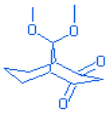
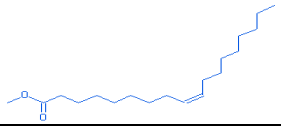
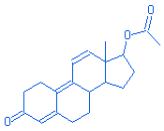
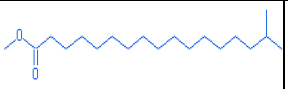
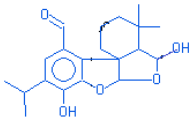
Table 7: R_f values of separated compounds by TLC from the ethanol extract of *A. marmelos*.

Spots observed	R_f Value (UV 254nm)
1	0.97
2	0.88

GC-MS analysis

GC-MS analysis was carried out for the ethanol extract of *A. marmelos* and the eluted compounds were showed in Table 8. A flavone compound (5, 7-dihydroxy-3-phenylchromen-4-one), Heptadecanoic acid,16-methyl, methyl ester possess the property of antioxidant, hypocholesterolemic lubricant and anti-androgenic.[15]

Table 8: GC-MS analysis of ethanol extract of fruit pulp of *A. marmelos*

S. No.	RT	Name	Structure	Mol. Wt g/mol	Mol. formula
1.	11.97	Hexanoic acid, 4-methylene, methyl ester		142.19	$C_8H_{14}O_2$
2.	16.1	Flavone		222.24	$C_{15}H_{10}O_2$
3.	16.75	9,9- dimethoxybicyclo(3,3,1) nona 2-4,dione		212.24	$C_{11}H_{16}O_4$
4.	17.82	Methyl oleate		296.495	$C_{19}H_{36}O_2$
5.	18.63	Trenbolone acetate		312.409	$C_{20}H_{24}O_3$
6.	18.03	Heptadecanoic acid,16-methyl,methyl ester		298.503	$C_{19}H_{38}O_2$
7.	19.27	Cariocal		346.42	$C_{20}H_{26}O_5$

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CONCLUSION

In summary, the present study showed that the ethanol extract of fruit pulp of *A. marmelos* has significant antioxidant potential and radical scavenging activities. This *in vitro* antioxidant study indicates the ethanol extract has significant natural antioxidant molecules and may helpful in preventing the oxidative stress related degenerative disease which causes damage to cells. Further molecular studies are required to find out the mechanism of action and bioactivity of various compounds present in the ethanol extract of *A. marmelos* to explore their therapeutic potential before it can be recommended for human welfare.

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