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Antioxidant, anticancer, antibacterial activities and GC-MS analysis of aqueous extract of pulps of *Aegle marmelos* (L.) Correa

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ABSTRACT

Aegle marmelos (Bael) is an important medicinal plant which belongs to family Rutaceae. Leaves, fruit, stem, bark of this plant is used because of its medicinal properties like astringent, antidiarrheal, antipyretic, anti-inflammatory activities. 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, anticancer and antibacterial activities of aqueous extract of *A. marmelos* and to identify the bioactive compounds by GC-MS analysis. Antioxidant activities such as DPPH radical, $ABTS^{\cdot+}$ radical cation, Fe^{3+} reducing power and phosphomolybdenum reduction assays were carried out for aqueous extract. The maximum DPPH radical and $ABTS^{\cdot+}$ radical cation scavenging activities were 60.70±4.24% at 300 µg/mL and 95.77±6.70% at 30 µg/mL concentrations. The IC_{50} values of $DPPH^{\cdot}$ radical and $ABTS^{\cdot+}$ radical cation scavenging activities were 183.58 µg/mL and 10.81 µg/mL concentration. The maximum reduction of Fe^{3+} and Mo^{6+} were 52.05±3.64% and 87.81±6.19% at 120 µg/mL concentration and the IC_{50} values were 102.83 µg/mL and 32.76 µg/mL concentration. The anticancer activity was carried out by MTT assay method, which showed the IC_{50} value of 47.92 µg/mL concentration.

Keywords: *Aegle marmelos*, DPPH, $ABTS^{\cdot+}$, MTT assay, antibacterial, GC-MS.

INTRODUCTION

Aegle marmelos commonly known as Bael belonging to the family Rutaceae, has been broadly used in indigenous systems of Indian medicine due to its numerous therapeutic properties [1]. *A. marmelos* is native to North India, but widely found throughout the Indian Peninsula and in Burma, Ceylon, Thailand, Myanmar and Bangladesh. Forests on hills and Rajasthan is suitable climate for this tree. It is also known Bengal quince, golden apple, Japanese bitter orange, or wood apple, is a species of tree native to India, Nepal, the Andaman and Nicobar Islands and Myanmar [2]. It is present in Sri Lanka, Thailand and Malaysia as a naturalized species. The fruit contains tannic acid and volatile oil. It is a spinous deciduous and aromatic tree with long, strong and axillary spines. This tree grows up to 18 m in height and thickness of tree is about 3-4ft [3]. Leaves are 3-5 foliate, leaflets are ovate and have typical aroma. 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. Seeds are compressed and numerous found in aromatic pulp. It is also distributed in other countries such as Pakistan, Nepal, Philippines, Fiji, Cambodia and Thailand.

Taxonomy

Domain: Eukaryota
Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Dicotyledonae
Subclass: Rosidae
Order: Sapindales
Family: Rutaceae
Genus: *Aegle*
Species: *Aegle marmelos*

Figure 1: Fruits of *Aegle marmelos*

MATERIALS AND METHODS

Collection of Plant material

The fruits of *A. marmelos* were collected from IIT Madras, Chennai. The fruit was washed with tap water and rinsed with distilled water.

Plant material and extract preparation

The fruit pulp of *A. marmelos* was scrapped and boiled with distilled water in a cookware. Then the supernatant of aqueous extract was filtered by filter paper and condensed in a hot plate at 50°C, to get gummy extract.

Qualitative phytochemical analysis

The aqueous extract of fruit pulps of *A. marmelos* was subjected to preliminary phytochemical screening using standard methods [4]. The aqueous extract was screened for different classes of phytoconstituents using specific standard reagents [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 aqueous 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 aqueous solution of Na_2CO_3 (20%) 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 aqueous extract of fruits of *A. marmelos* was determined using aluminium chloride colorimetric method with slight modification as described by Liu et al [8]. One mL of extract (1mg/mL) was mixed with 0.5 mL of 5% sodium nitrite solution and incubated for 5 min at room temperature. Then, 0.5 mL 10% aluminium chloride solution was added and incubated for further 5 min at room temperature followed by 1 mL of 1 M NaOH solution was added. The total volume was made up to 5 mL with distilled water. Absorbance was measured at 510 nm using spectrophotometer. The result was expressed as (µg/mg of extract) quercetin equivalent.

In vitro antioxidant assays

DPPH˙ radical scavenging assay

The antioxidant activity of aqueous extract of *A.marmelos* fruit 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 plant extracts. The mixture was then allowed to stand for 30 min incubation in dark. Distilled water was used as the reference standard. One mL methanol and 1 mL 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 following the procedure described by Re *et al*. ABTS$^{\bullet+}$ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70±0.02 at 730 nm. After the addition aqueous extracts of varying concentrations to 1 mL of diluted ABTS$^{\bullet+}$ solution, the absorbance was measured after 10 min [10]. The ABTS$^{\bullet+}$ radical-scavenging activity of the samples was expressed 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 the aqueous extract of *A. marmelos* fruit assessed as described by Prieto *et al*. The plant 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 90ºC for 90 min. The absorbance of the coloured complex was measured at 695 nm. Distilled water was used as standard reference [11]. The percentage of inhibition was calculated using the following formula:

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

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

The reducing power of aqueous extract of *A. marmelos* fruit determined by slightly modified method of Yen and Chen, 1995. One mL of plant extract of different concentrations (20 - 1200 µg/mL) was

mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1 %). The mixtures were then incubated at 50°C for 20 min. One mL of trichloroacetic acid (10 %) was added to each mixture. Then to the mixture 1 ml of $FeCl_3$ (0.1 %) was added and the absorbance was measured at 700 nm using Spectrophotometer [12]. Distilled water was used as the standard reference. The percentage of inhibition was calculated using the following formula:

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

Anticancer activity on MCF7 (human breast cancer) cell line

MTT assay method

Cell viability was measured with the conventional MTT reduction assay method as described Mossman [13] with slight modification. Briefly, MCF7 cells were seeded at the density of 5×10^3 cells/well in 96-well plates for 24 h, in 200 µL of RPMI with 10% FBS. Then the culture supernatant was removed and RPMI containing various concentrations (3.125-100 µg/mL) of pulp extract was added and incubated for 48 h. After treatment the cells were incubated with 10 µL of MTT (5 mg/mL) at 37°C for 4 h and then with DMSO at room temperature for 1 h. The plates were read at 595 nm on a scanning multi-well spectrophotometer.

Antibacterial activity

Microbial strains

The microorganisms of Gram negative strains such as *Escherichia coli*, *Klebsiella pneumonia* and *Shigella flexneri* as well as Gram positive strains such as *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* were used for the evaluation of antibacterial activity.

Reference and control

Tetracycline was chosen as the standard reference for bacteria. The controls consist of solidifying agar onto which was solvent, and the test compounds were soluble in it.

Aseptic conditions

The aseptic chamber which consist of a wooden box (1.3m x 1.6m x 0.6m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from lamp).

Nutrient broth agar medium

Nutrient broth agar medium was prepared(peptone-5 g; yeast extract-3 g; NaCl-5 g; distilled water-100 mL; pH-7±0.2; agar-20 g) according to the standard methods and was suspended in 200 mL of distilled water in a 500 mL conical flask, stirred, boiled to dissolve and then autoclaved at 15 lbs and at 121ºC for 15 minutes. The hot medium was poured in sterile petriplates which were kept in the aseptic Laminar chamber. The medium was allowed to solidify for 15 min [14].

Determination of antibacterial potential of the extracts was carried out using the agar well diffusion method. The solidified nutrient agar in the petriplates was inoculated by dispensing the inoculum using sterilized cotton swabs and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The control, extract and standard were then poured into each well of desirable concentrations. Tetracycline was used as the standard with the concentration of 25 µg. All the plates containing sample loaded wells were incubated for 24 h at 37°C. After the incubation period, zone of inhibition in each plate, for each concentration of extract and standard were measured by calculating the diameter of zone of inhibition.

Thin layer chromatography

Thin layer chromatography (TLC) was carried out for the aqueous extract of *A. marmelos* fruit in Merck TLC aluminium sheets, silica gel 60 F254 (20 x 20 cm), preloaded plates. The extract was spotted at 0.3 mm above from the bottom of the TLC plate. The chromatogram was developed in a mixture of suitable solvent system. The spots were visualized with UV light at 356 nm. The R_f values of the coloured spots were recorded [15]. The ratio in which distinct bands appeared was optimized and R_f values were calculated.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

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 [16].

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

Table 1: Qualitative analysis of aqueous 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.	Flavanoids	NaOH solution	+
4.	Phenols	$FeCl_3$ solution	+
5.	Glycosides	Sodium nitroprusside solution + Con. H_2SO_4	+
6.	Saponins	Foam test	+

Total phenols and flavonoids

Total phenols and flavonoids were quantified in the aqueous extract of fruit pulp of *A. marmelos* showed to be responsible for the antioxidant activity. The total phenol content was 343.00±1.33 µg/mg of GAE and total flavonoid content was 21.92±1.38 µg/mg of QE in the extract. These results provide a comprehensive profile of the antioxidant activity of fruits of *A. marmelos* with respect to their phenols and flavonoids content.

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

S. No	Phytochemicals	Value (µg/mg)
1.	Phenols	343.00 ±1.33
2.	Flavonoids	21.92 ±1.38

DPPH$^{\cdot}$ radical scavenging assay

The ability of aqueous extract of *A. marmelos* to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The maximum DPPH$^{\cdot}$ radical scavenging activity was 60.70% at 300 µg/mL concentration. Aqueous 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 (IC_{50} = 11.98 µg/mL concentration) ascorbic acid.

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

S. No.	Concentration (µg/mL)	% of inhibition (Aq.)
1	50	18.28±1.27
2	100	41.05±2.87
3	150	45.13±3.15
4	200	54.47±3.81
5	250	58.94±4.12
6	300	60.70±4.24

ABTS$^{\cdot+}$ 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 95.77±6.70% at 30 µg/mL concentration. The experiment demonstrated high antioxidant activity the IC_{50} of 10.81 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 4.21 µg/mL concentration).

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

S. No.	Concentration (µg/mL)	% of inhibition (Aq.)
1	5	29.00±2.03
2	10	46.22±3.23
3	15	61.63±4.31
4	20	71.30±4.99
5	25	84.28±5.89
6	30	95.77±6.70

Phosphomolybdenum reduction assay activity

The total antioxidant activity of aqueous extract 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 87.81±6.14% at 120 µg/mL concentration. It was compared with the standard ascorbic acid.

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

S. No.	Concentration (µg/mL)	Phosphomolybdenum reduction @ 695nm
		Aqueous extract
1	20	24.71±1.72
2	40	61.04±4.27
3	60	65.46±4.58
4	80	72.76±5.09
5	100	78.99±5.52
6	120	87.81±6.14

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

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the aqueous 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 52.05±3.46% at 120 µg/mL concentration and was compared with the standard ascorbic acid.

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

S. No.	Concentration (µg/mL)	Fe^{3+} reducing power @ 700nm
		Aqueous extract
1	20	13.50±0.94
2	40	38.12±2.66
3	60	44.77±3.13
4	80	47.66±3.33
5	100	48.62±3.40
6	120	52.05±3.64

Anticancer Activity

Cancer chemoprevention with natural phytochemical compounds is an emerging strategy to prevent, delay or cure cancer [17]. The MTT assay is a colorimetric assay for assessing cell metabolic activity. The MTT *in vitro* cell proliferation assay is one of the most widely used assays for evaluating preliminary anticancer activity of synthetic derivatives, natural products and natural product extracts [18]. This assay gives an indication of whole cell cytotoxicity; however, to determine the exact molecular target further assays need to be perform kinase enzyme activity that play a key role in a number of physiological processes and their inhibitors have been found to exhibit anticancer activity against various human cancer cell lines. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan [19]. The formazan is then solubilized, and the concentration was determined by optical density at 570 nm. The maximum MCF7 cell death was 66.51±4.65% at 100 µg/mL concentration of aqueous extract of fruit pulp of *A. marmelos* and the IC_{50} was 47.92 µg/mL concentration.

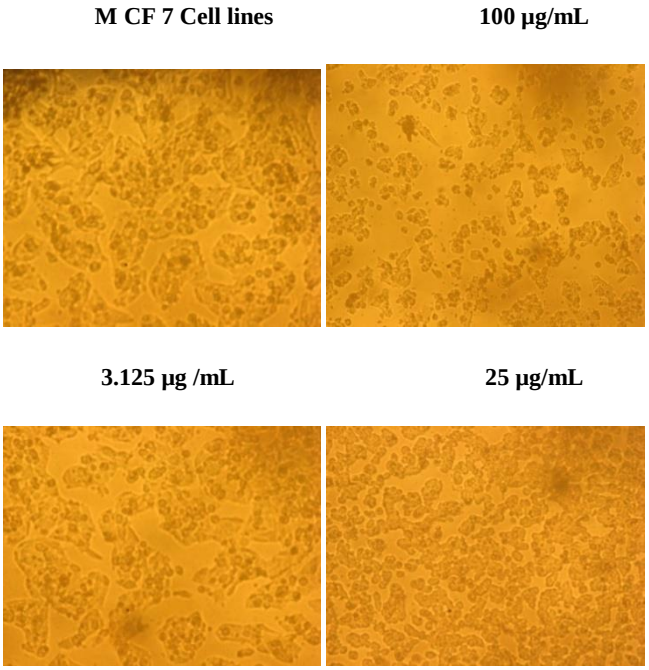


Figure 2: Anticancer activity of fruit pulp of *Aegle marmelos* on MCF7 cell.

Table 7: Cytotoxic activity of aqueous extract of *A. marmelos*

S. No.	Concentration (µg/mL)	% of cell death
		Aqueous extract
1	3.125	17.94±1.26
2	6.25	22.66±1.58
3	12.5	30.76±2.15
4	25	41.63±2.91
5	50	52.16±3.65
6	100	66.51±4.65

Antimicrobial Analysis

The aqueous extract of fruit pulp of *A. marmelos* was investigated for antibacterial activity against microorganism including Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Shigella flexneri*). The antibacterial sensitivity of the crude extract and their potency were assessed quantitatively by measuring the diameter of clear zone in cultures in petriplates.

The antibacterial activity of these extracts could be correlated as due to the presence of secondary metabolites such as flavonoids, phenolic compounds, terpenoids, tannin and alkaloids that adversely affect the growth and metabolism of microbes.

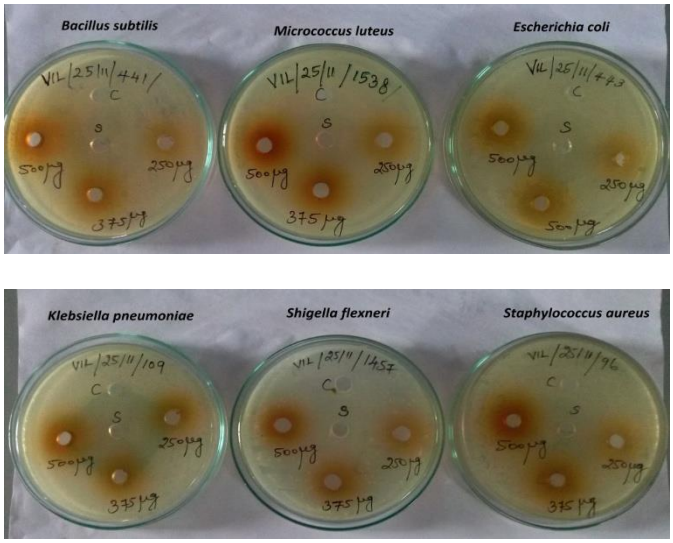


Figure 3: Antimicrobial activity of *A. marmelos*

Table 8: Antimicrobial activity of aqueous extract of fruit pulp of *A. marmelos*

S. No	Organisms	Zone of inhibition mm			Standard (Tetracycline)
		250 µg	375 µg	500 µg	
1	*Bacillus subtilis*	12	14	16	20
2	*Micrococcus luteus*	13	16	18	22
3	*Escherichia coli*	12	14	16	24
4	*Klebsiella pneumoniae*	10	12	14	34
5	*Shigella flexneri*	11	12	13	20
6	*Staphylococcus aureus*	10	12	14	20

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.

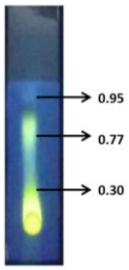


Figure 3: Compounds separated by Thin Layer Chromatography.

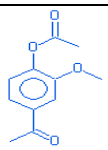
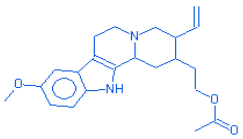
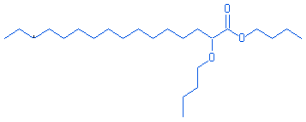
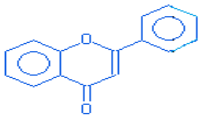
Table 9: R_f values of Aqueous extract compounds separated by Thin Layer Chromatography

Spots observed	Rf Value (UV 254nm)
1	0.95
2	0.77
3	0.30

GC-MS analysis

GC-MS analysis was carried out for the aqueous extract of *A. marmelos* and the eluted compounds were showed in Table 10. A flavone compound (2-phenylchromen-4-one) Corynan-17-ol,18,19-didehydro,10-methoxy, acetate was eluted by GC-MS which is a potential G-protein-coupled receptor kinase inhibitor, Lipid metabolism regulator and Growth hormone agonist.

Table 10: Active compounds identified in aqueous extract of *A. marmelos*

S. No	RT	Name	Structure	Mol. Wt g/mol	Mol. Formula
1.	16.22	4-Acetoxy-3-methoxyacetophenone		208.211	$C_{11}H_{12}O$
2.	17.27	Undecanoic acid,10-methyl, methyl ester		214.344	$C_{13}H_{26}O_2$
3.	19.03	Phytol		296.53	$C_{20}H_{40}O$
4.	20.42	Corynan-17-ol,18,19-didehydro,10-methoxy,acetate(ester)		368.477	$C_{22}H_{28}N_2O_3$
5.	21.28	Hexadecanoic acid, 2-butoxy-,butyl ester		384.00	$C_{24}H_{48}O_3$
6.	18.3	Flavone		222.24	$C_{15}H_{10}O_2$

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The authors are thankful to SAIF, IIT Madras for helping GCMS analysis.

CONCLUSION

The results of the present study indicate that aqueous extract of fruits of *A. marmelos* has significant antioxidant activities to reduce harmful effect of radicals. Further molecular studies are required to find out the mechanism of action of bioactive compounds present in *A. marmelos* against cancer cell before it can be recommended for any practical widespread use of the plant. The results of various experiments conducted in the present study provide promising guideline regarding the potential uses of *A. marmelos* as an anticancer agent.

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