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GC-MS ANALYSIS, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF ETHANOL EXTRACT OF LEAVES OF *AEGLE MARMELOS* (L.) CORRÊA

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ABSTRACT

The aim of the present study was to evaluate the antioxidant activities of leaves of *Aegle marmelos* and to identify the bioactive compounds by performing GC-MS analysis resulting in the presence of volatile and semi volatile compounds. The IC_{50} of $DPPH^{\cdot}$ radical scavenging assay was 78.36µg/mL concentration respectively. Also, the IC_{50} of Phosphomolybdenum reduction and ferric reducing power assay were 41.35 and 20.58µg/mL concentration respectively. Also, total phenolic and flavonoid content were determined, in which flavonoids were found to be predominantly higher. The results of this study portray the effective antioxidant activity of *Aegle marmelos* and further studies are required to isolate the active compounds from various parts of this species and their mode of action. From the study it can be concluded that the plant might be promising as a curative for many diseases associated with free radicals.

Keywords: Free radicals, *Aegle marmelos*, Antioxidant, Dot plot, $DPPH^{\cdot}$ assay, IC_{50}, GCMS.

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INTRODUCTION

Aegle marmelos is a perennial tree, wild in the sub Himalaya tract, central and South India. This plant is commonly called as Beal in Hindi, Vilvam in Tamil and Bilva in Sanskrit. It belongs to the family Rutaceae. It is indigenous to India and is used in folk medicines. The Ayurvedic practitioners use almost all of their parts but the greatest medicinal value ascribed to its fruits. Oxidative stress is produced during normal metabolic process in the body as well as induced by the variety of environmental and chemical factors which cause generation of various reactive free radicals and subsequent damage to macromolecules like DNA, Proteins and Lipids[1].

Bael having useful medicinal properties especially as a cooling agent is a deciduous sacred tree, associated with Gods. This tree is popular in Shiva and Vishnu temples also popularly known as temple garden plant and it can be grown in every house. Its leaves are trifoliate symbolizing the Thri-murthies- Brahma, Vishnu, Shiva with spear shaped leaflets resembling trisoolam the weapon of Lord Shiva[2]. *Aegle marmelos* is a slow-growing, medium sized tree, 25 to 30 feet tall. The stem is short, thick, soft, flaking bark, and spreading, sometimes spiny branches, the lower ones drooping. There are sharp, axial one inch long spikes on this tree. The leaflets are oval or lancet shaped, 4-10 cm long, 2-5 cm wide. Leaves composed of 3 to 5 leaflets in it. The lateral leaflets are without petiole and the terminal one has a long one. The petiole is 1 to 2.5 inch long. Fruit is spherical or oval in shape with a diameter of 2 to 4 inch. Shell is thin, hard and woody in nature. It is greenish when unripe and upon ripening it turns into yellowish colour. The pulp of the fruit has 8 to 15 segments. The

pulp is yellow, soft, pasty, sweet, resinous and fragrant[3, 4].

MATERIALS AND METHODS

Collection of plant material and preparation of extracts

The leaves of *Aegle marmelos* were carefully washed with tap water followed by rinsing in distilled water and air-dried at room temperature for few hours. Then leaves were separated and taken to separate clean place and dried at room temperature for one week. Then they were ground into fine powder and sieved through fine mesh, finally stored in cool and dry place in a clean air-tight container. Extraction of leaf powder with Hexane, Ethyl acetate, Aqueous, Methanol and Ethanol was performed by direct method[5].

Antioxidant activity by dot-blot DPPH staining method

Drops of DPPH (0.4 mm) solution in methanol were loaded onto a 5 cm × 5 cm TLC plate (silica gel 60 F254; Merck) in each column and allowed to dry for 2 minutes. The first row of TLC plate was considered as control, containing only DPPH. Different extracts of *Aegle marmelos* of various concentrations was carefully loaded onto the DPPH spot in second-fifth row. The sixth row of TLC plate was considered as standard reference, where ascorbic acid was carefully loaded onto the DPPH spot[6]. Stained silica gel layer revealed a purple background with yellow or white spots at the location where radical scavenging capacity observed.

Invitro Antioxidant activity of various extracts of *Aegle marmelos*

(a) Free radical Scavenging Activity

The antioxidant activity was determined by DPPH scavenging assay[7] in which various concentrations of five different crude extracts was been pipetted out in clean test tubes. Freshly prepared DPPH (1, 1-Diphenyl-2-picryl hydrazyl) solution (1mL) was added to each tube and the samples were incubated in dark at 37°C for 20 mins and read at 517 nm. The data were expressed as the percent decrease in the absorbance compared to the control. Ascorbic acid was used as reference compound. The percentage inhibition of radical scavenging activity was calculated.

%Radical scavenging potential =

[(Control OD-Sample OD)/Control OD] X100.

(b) Phosphomolybdenum reduction assay

Total antioxidant capacity can be calculated in which various concentrations of five different extracts from the prepared sample (1mg /mL) was been pipetted out[8] and 1mL of the reagent solution was added, followed by incubation in boiling water bath at 95°C for 90mins. After cooling the sample to room temperature, the absorbance of the solution was measured at 695 nm in UV spectrophotometer. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Ascorbic acid served as standard.

% Phosphomolybdenum reduction =

[(Sample OD-Control OD)/Sample OD] X100.

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

The five different crude extracts was taken in various concentrations and was mixed with 2.5mL of phosphate buffer (0.2M, pH-6.6) and 2.5mL of potassium ferricyanide (1% w/v), and incubated at 50°C for 30 mins. Then, 2.5mL of trichloroacetic acid (10% w/v) was added to the mixture and then centrifuged at 3000 rpm for 10 mins. Finally, 2.5mL of upper layer solution was mixed with 2.5mL of distilled water and 0.5mL $FeCl_3$ (0.01% w/v) and the absorbance was measured at 700 nm [9, 10]. Ascorbic acid served as standard. Based on the antioxidant study results, one potent extract would be evaluated for further analysis.

% Ferric Reducing Potential=

[(Sample OD-Control OD)/Sample OD] X100.

Qualitative phytochemical analysis of *Aegle marmelos*

Screening of phytochemicals for *Aegle marmelos* was carried out comparatively using standardized methods[5].

Quantitative estimations of total phenol and flavonoids

Determination of total Phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds[11] with slight modifications. One hundred µL of selected extract (1mg/mL) (leaves of selected extract of *Aegle marmelos*) was mixed with 900 µL of distilled water and 1 mL of Folin-Ciocalteau reagent (1:10 diluted with distilled water). After 5 mins, 1 mL of Na_2CO_3 (20% w/v) solution was added. The mixture was then allowed to stand for 30 mins 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 leaves of selected extract of *Aegle marmelos* was determined using aluminium chloride reagent method with slight modification[12]. Five hundred µL of extract (1mg/mL) was mixed with 0.5 mL of methanol and 0.5 mL of (5% w/v) sodium nitrite solution. Then, 0.5 mL (10% w/v) aluminium chloride solution was added followed by 1 mL of 1M NaOH. The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 510 nm. The result was expressed as (µg/mg of extract) quercetin equivalent.

Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis was carried out for selected extract of *Aegle marmelos* on silica gel aluminium sheet (Merck Silica gel 60 F254)[13]. The selected extract was spotted at 0.5 mm above from the

bottom of the TLC plate. The spotted TLC plate was placed in a 100mL beaker containing solvent mixture. The chromatogram was developed and the spots were visualized under UV light at 254 nm as well as in iodine vapour. The ratio in which distinct coloured bands appeared was optimized and R_f values were calculated.

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

Screening of crude extract for antibacterial activity

Agar Well diffusion assay

Nutrient agar was prepared and poured in the sterile petri dishes and allowed to solidify. 24 hours grown bacterial pathogens were swabbed on nutrient agar plates[14]. Then, the stock crude of selected extract individually (10mg/mL) was prepared. Varying concentration (250µg, 500µg, 750µg and 1000µg) of plant extract was loaded in the wells made using cork borer. Tetracycline was used as standard. The plates were then incubated at 37ºC for 24hours. After incubation the inhibition diameter was measured using zone scale.

Identification of bioactive compounds by Gas chromatography-Mass spectrometry analysis

The presence of active compounds were been confirmed by thin layer chromatography and the compounds were identified using gas chromatography and mass spectrometry (GC-MS) method, (TSQ QUANTUM XLS). The name of the instrument is Gas Chromatography-Mass Spectrometry and the instrument made is of Thermo scientific. The software required for analytical studies is XCALIBUR (ver-2.2). The column size is of TG-5MS (30mX0.25mmX0.25um). The injector temperature and interface temperature (°C) was at 280°C.

RESULTS AND DISCUSSION

Collection and preparation of plant sample

The *Aegle marmelos* leaves were collected from shiva temple in nearby surroundings. The leaves were then separated and shade dried for ten days in a well-ventilated room at 37°C and ground to coarse powder using the mechanical grinder. After 72 hours of extraction, the individual extract supernatant was filtered by filter paper and condensed in a rotary evaporator at 50°C, which yields gummy extract. The extracted residues were weighed and re-dissolved in different solvents to yield 1mg/mL as final volume for further analysis.

Screening of radical scavenging activity by dot-blot DPPH staining method

The results of dot-blot assay showed active spots in which various concentrations of five different extracts of *Aegle marmelos* were placed in respective rows. The zone exhibiting purple colour indicates that there is no antioxidant (free radical scavenging) activity and the zone exhibiting yellow colour indicates antioxidant activity. From the results obtained (Figure 1), it is evident that ethanol extract of *Aegle marmelos* has effective antioxidant activity, when compared to other extracts, also compared as well as standard ascorbic acid.

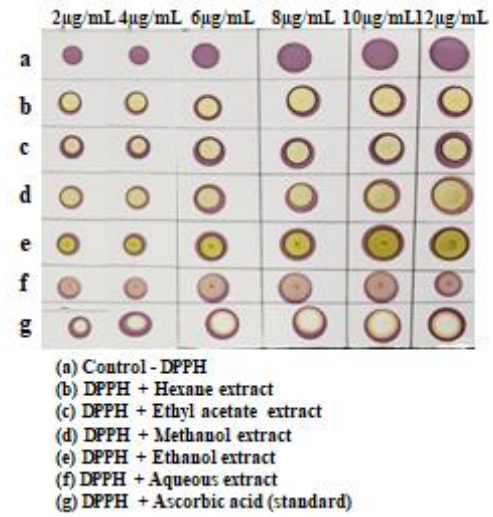


Figure 1: Dot-blot assay of different extracts of *Aegle marmelos*

The ethanol extract of *Aegle marmelos* was spotted on each row in which the colour changes from purple to yellow or white was well observed indicating potent antioxidant activity when compared to standard Ascorbic acid.

Invitro Antioxidant activity of various extracts of *Aegle marmelos*

(a) Free radical Scavenging Activity

Antioxidant molecules can quench DPPH free radicals (i.e by providing hydrogen atoms or by electron donation, via a free radical attack on the DPPH molecule) and convert them to colourless. The percentage of DPPH scavenging activity was 93.67% in ethanol fraction (Table 1) of *Aegle marmelos* when compared to other four fractions (Table 2&3). The IC_{50} value was found to be 78.36µg/mL concentration (Graph 1, 2&3) and was compared with standard (Ascorbic acid, IC_{50} value as 11.98µg/mL concentration).

The antioxidant activity for five different extracts (Methanol, Ethanol, Aqueous, Ethyl acetate and Hexane) of *Aegle marmelos* was determined by DPPH scavenging assay. The radical scavenging activity was well observed for ethanol extract in which 1,1-diphenyl-2-picryl hydrazyl was reduced to 1,1-diphenyl-2-picryl hydrazine.

Table 1: Radical scavenging activity by DPPH assay for ethanol extract of *Aegle marmelos*

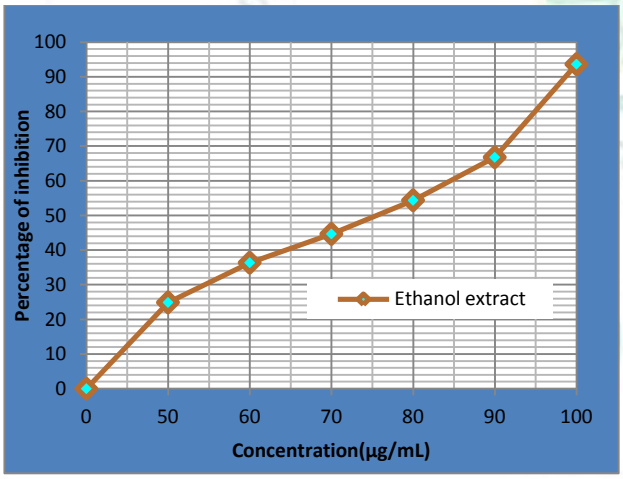
S.No	Concentration (µg/mL)	Radical scavenging activity
		Ethanol extract
1.	50	24.90
2.	60	36.36
3.	70	44.66
4.	80	54.37
5.	90	66.79
6.	100	93.67

Table 2: Radical scavenging activity by DPPH assay for ethyl acetate and aqueous extract of *Aegle marmelos*

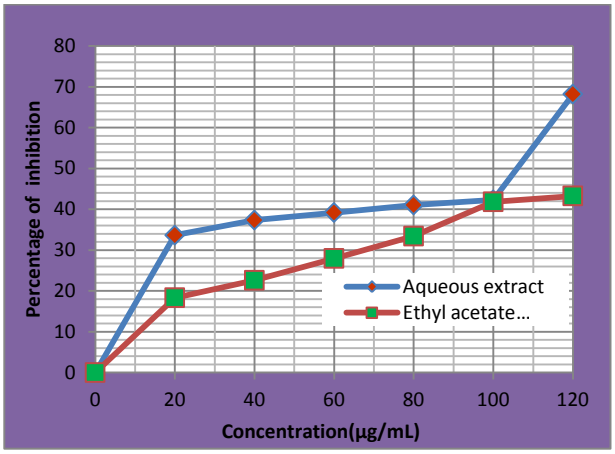
S.No	Concentration (µg/mL)	Radical scavenging activity	
		Ethyl acetate extract	Aqueous extract
1.	20	18.34	33.64
2.	40	22.60	37.34
3.	60	27.96	39.19
4.	80	33.42	41.04
5.	100	41.83	42.28
6.	120	43.27	68.20

Table 3: Radical scavenging activity by DPPH assay for hexane and methanol extract of *Aegle marmelos*

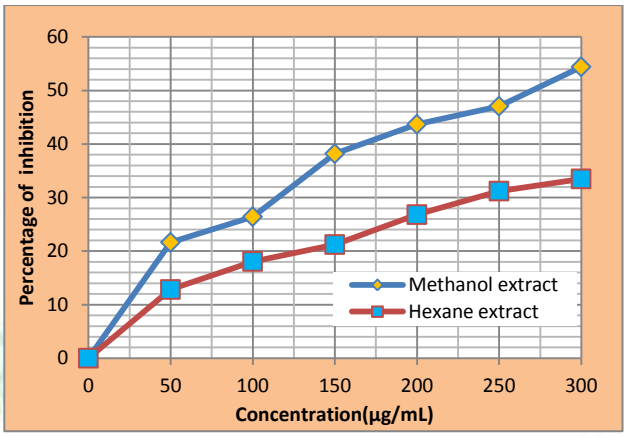
S.No	Concentration (µg/mL)	Radical scavenging activity	
		Hexane extract	Methanol extract
1.	50	12.82	21.63
2.	100	18.06	26.42
3.	150	21.22	38.19
4.	200	26.81	43.68
5.	250	31.20	47.06
6.	300	33.40	54.41



Graph 1: Radical scavenging activity by DPPH assay for ethanol extract of *Aegle marmelos*



Graph 2: Radical scavenging activity by DPPH assay for aqueous and ethyl acetate extract of *Aegle marmelos*



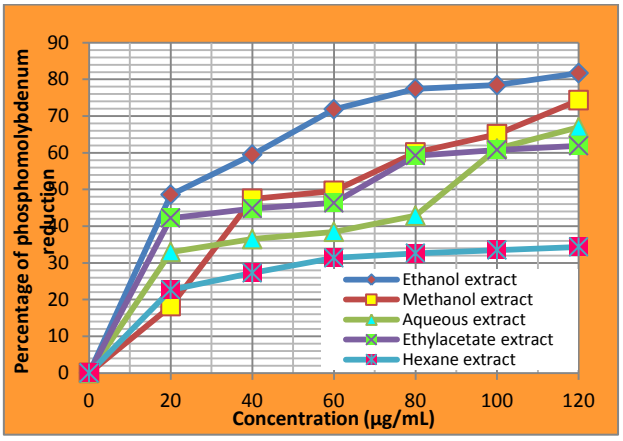
Graph 3: Radical scavenging activity by DPPH assay for hexane and methanol extract of *Aegle marmelos*

(b) Phosphomolybdenum reduction assay

The total antioxidant activity of ethanol extract of *Aegle marmelos* was measured spectrophotometrically 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 reducing ability for ethanol extract was higher as 67.83% at 120 µg/mL concentration, when compared to other extracts (Graph 4). The experiment demonstrated higher antioxidant activity the IC_{50} of 41.35µg/mL concentration (Table 4) and was compared with standard Ascorbic acid (IC_{50} = 23.28µg/mL concentration).

Table 4: Phosphomolybdenum reduction for five different extracts of *Aegle marmelos*

S.No	Concentration (µg/mL)	Percentage of phosphomolybdenum reduction				
		Hexane extract	Ethyl acetate extract	Aqueous extract	Methanol extract	Ethanol extract
1	20	10.03	19.22	38.91	31.28	41.62
2	40	13.16	23.61	43.52	42.64	48.36
3	60	19.38	29.42	46.08	44.81	53.08
4	80	24.79	34.78	52.96	47.13	55.27
5	100	29.32	36.22	54.23	52.04	64.92
6	120	34.56	42.83	57.14	55.32	67.83



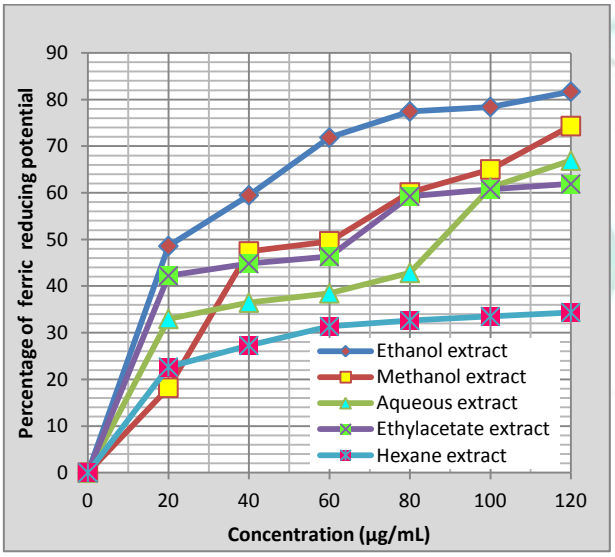
Graph 4: Phosphomolybdenum reduction for five different extracts of *Aegle marmelos*

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

Five different extracts of *Aegle marmelos* react with Potassium ferricyanide and ferric chloride there by gets reduced to Potassium ferrocyanide and ferrous chloride (Fe^{3+} to Fe^{2+}) turning to various shades of green. The reducing power of Fe^{3+} to Fe^{2+} by ethanol extract of *Aegle marmelos* was studied and showed reduction ability in a dose dependent manner (Graph 5). The maximum reduction for ethanol extract of *Aegle marmelos* was 81.70% at 120µg/mL concentration (Table 5). The IC_{50} value for ethanol extract of *Aegle marmelos* was found to be 20.58µg/mL concentration and was compared with the standard (29.11µg/mL concentration) Ascorbic acid.

Table 5: Ferric reducing activity for five different extracts of *Aegle marmelos*

S.No	Concentration (µg/mL)	Percentage of ferric reducing potential				
		Hexane extract	Ethyl acetate extract	Aqueous extract	Methanol extract	Ethanol extract
1	20	22.65	42.21	32.98	18.21	48.59
2	40	27.27	44.82	36.47	47.43	59.49
3	60	31.36	46.33	38.46	49.60	71.86
4	80	32.63	59.23	42.85	60.06	77.46
5	100	33.50	60.79	61.15	65.02	78.43
6	120	34.35	61.90	66.92	74.32	81.70



Graph 5: Ferric reducing activity for five different extracts of *Aegle marmelos*

Qualitative Phytochemical analysis

Based on the antioxidant activity results, ethanol extract was selected for further mode of research. The qualitative analysis for Ethanol extract of *Aegle marmelos* was performed based on standardized methods.

Table 6: Qualitative analysis of ethanol extract of *Aegle marmelos*

S.No	Phytochemical	Results
1.	Resins	Positive
2.	Proteins	Positive
3.	Flavonoids	Positive
4.	Phenols	Positive
5.	Terpenoids	Positive
6.	Phytosterol	Positive
7.	Saponins	Positive
8.	Alkaloids	Negative
9.	Glycosides	Negative
10.	Tannins	Negative
11.	Diterpenes	Negative
12.	Carbhohydrate	Negative

Results of Phytochemical screening of the aqueous extract revealed the presence of steroid, terpenoids, saponins, tannins, lignin, flavonoids. Alcoholic extract showed the availability of alkaloids and devoid of saponins[1]. Similarly, the phytochemical screening for Ethanol extract was performed in which the phytoconstituents such as Resins, proteins, flavonoids, phenols, terpenoids, phytosterol and saponins were present.

Quantitative estimations of total phenol and flavonoids

Determination of total phenols and flavonoids

The quantitative estimation of phenols and flavonoids were carried out in which flavonoid content was higher when compared to phenol. This proves these compounds might be responsible for potent antioxidant activity.

Table 7: Quantitative estimation of ethanol extract of *Aegle marmelos*

S.No	Phytochemical	Results(µg/mg)
1.	Phenols	9.510
2.	Flavonoids	92.43

Total phenolic content was found to be 9.510 µg/mg, flavonoid content was 92.43 µg/mg. From the results, it is significant that due to presence of higher flavonoid content, the antioxidant activities were found to be higher for different extract of *Aegle marmelos*.

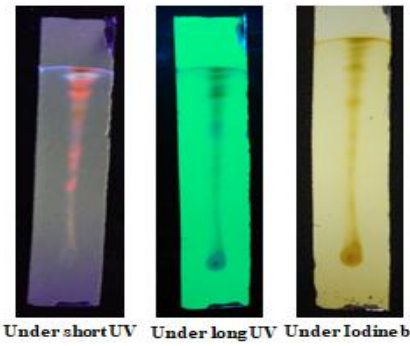


Figure.2: Compounds separation separated by Thin Layer Chromatography

Thin Layer Chromatography analysis

Thin layer chromatography analysis was carried out in the solvent system of Methanol (1mL) : Toluene (1mL). The separated compounds in TLC were showed in Figure 2.

The separated active compounds were visualized in UV light (short and long) and iodine balls. The R_f values of the separated compounds were measured and tabulated (Table 8).

Table 8: R_f values of active compounds separated by Thin Layer Chromatography from the ethanol extract of *Aegle marmelos*

	R_f values (Under Iodine balls)
Ethanol extract of *Aegle marmelos*	0.95
	0.91
	0.88
	0.82
	0.68
	0.53
	0.44
	0.35

Screening of crude extract for antibacterial activity

Agar Well diffusion assay (Eloff, 1998)

After incubation, the inhibition diameter was measured using zone scale. The maximum inhibition for ethanol extract of *Aegle marmelos* was against *P.vulgaris* (18mm), when compared to other bacterial pathogens *K.pneumoniae* (16mm), *B.subtilis* (16mm).

Table 9: Antibacterial activity of ethanol extract of *Aegle marmelos*

Test organisms	Standard	250µg/mL	500µg/mL	750µg/mL	1000µg/mL
S.aureus	19mm	14mm	14mm	14mm	15mm
K.pneumoniae	20mm	14mm	15mm	15mm	16mm
P.vulgaris	19mm	12mm	14mm	17mm	18mm
B.subtilis	20mm	12mm	13mm	15mm	16mm
S.flexneri	20mm	10mm	11mm	11mm	12mm

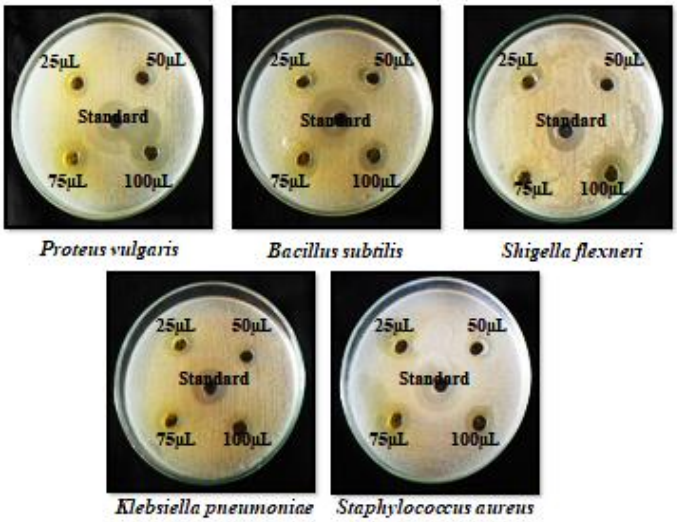


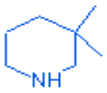
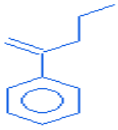
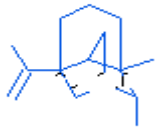
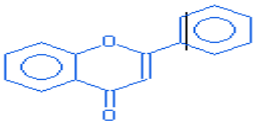
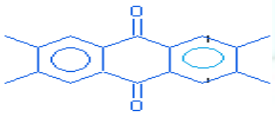
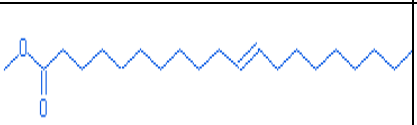
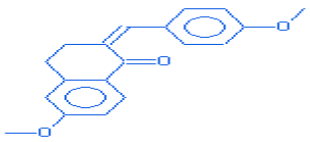
Figure 3: Antibacterial activity of ethanol extract of *Aegle marmelos* by agar well diffusion method

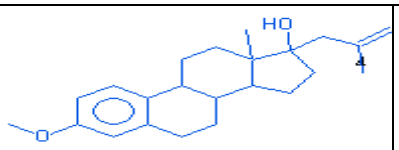
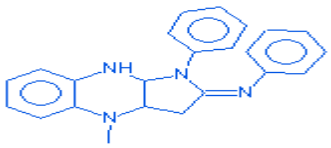
Phytochemicals such as flavonoids, phenols, sterols, terpenoids are secondary metabolites of plants that serves as defence mechanism against several microbes. In the current investigation, ethanol extract of *Aegle marmelos* possessed broad spectrum of antibiotic compounds.

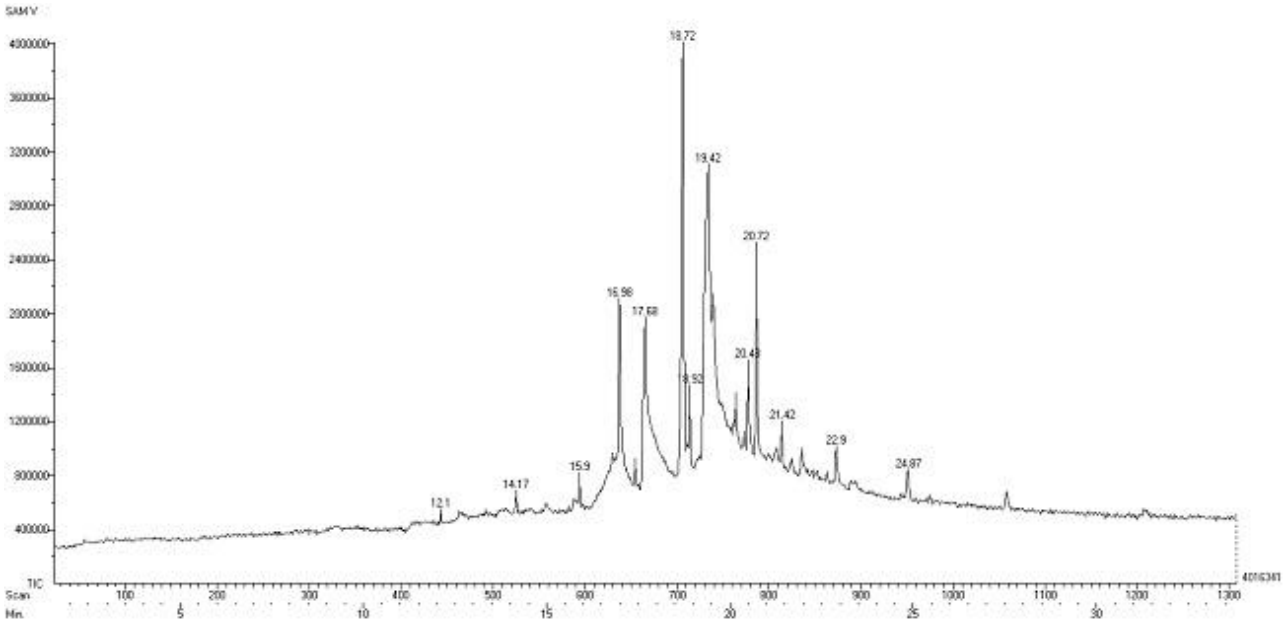
Identification of bioactive compounds by Gas chromatography-Mass spectrometry analysis:

The GCMS analysis for ethanol extract of *Aegle marmelos* revealed the presence of phyto-active compounds such as Flavone, Longifolene-12, Phytol, tetradecanoic acid exhibiting biological activities.

Table 10: GC-MS analysis of ethanol extract of *Aegle marmelos*

S.NO	COMPOUND NAME	RT	COMPOUND STRUCTURE	MOLECULAR WEIGHT	MOLECULAR FORMULA (g/mol)
1	Oleic acid	19.42		282.46	$C_{18}H_{34}O_2$
2	Piperidine,3,3-dimethyl-	12.1		113.20	$C_7H_{15}N$
3	Benzene, (1-methylene butyl)-	14.17		146.23	$C_{11}H_{14}$
4	Longifolene-12	15.9		204.35	$C_{15}H_{24}$
5	Flavone	16.98		222.24	$C_{15}H_{10}O_2$
6	Tetradecanoic acid	17.68		228.37	$C_{14}H_{28}O_2$
7	Anthraquinone, 2,3,6,7-tetra methyl-	18.72		264.28	$C_{17}H_{12}0_3$
8	Heptadecanoic acid, 16 methyl, methyl ester	18.92		298.51	$C_{19}H_{38}O_2$
9	11-Eicosenoic acid, methyl ester	20.48		324.54	$C_{21}H_{40}O_2$
10	Phytol	20.72		296.53	$C_{20}H_{40}O$
11	2H-Naphtalen-1-one,3,4-dihydro-6-methoxy-2-(4-methoxybenzylideno)	21.42		294.35	$C_{19}H_{18}O_3$

12	Estra-1,3,5(10)-trien-17a-ol,3-methoxy-17-(2-methylallyl)-	22.9		256.38	$C_{18}H_{24}O$
13	1H-Pyrrolo [2.3-b] quinoxalin-2- imine, 2,3,3a,4,9,9a-hexahydro-1, N-diphenyl	24.87		281.27	$C_{11}H_{15}N_5O_4$



Graph 6: GCMS Chromatogram of ethanol extract of *Aegle marmelos*

Table 11: Bio-activity of ethanol extract of *Aegle marmelos* from GCMS analysis

S.NO	COMPOUND NAME	PHARMACOLOGICAL ACTIVITY, REFERENCES [15-22]
1	Longifolene	Anti-feedant Antioxidant Anti-cancer Anti-inflammatory Antibacterial
2	Piperidine	Antioxidant Antimicrobial Anti-cancer
3	Phytol	Aromatic Ingredient Antinociceptive Antioxidant Antiallergic Anti-inflammatory Antimicrobial Immunostimulant
4	Flavone	Production of Reactive Oxygen Species (ROS) can be reduced by flavonoids. Relevance of plant defense mode of action is highly possible by flavonoids. Formation of oxygen radicals can be prevented by flavonoids thereby inhibiting the enzyme activity,

CONCLUSION

Different extracts of *Aegle marmelos* was evaluated for antioxidant and antibacterial activity against bacterial pathogens. Sensitivity against zone of inhibition was observed and found to be highest against `*Proteus vulgaris* 18mm as zone of inhibition. Phytochemical analysis revealed the presence of Resins, Proteins, Flavonoids, Phenols, Phytosterol, Saponins, Terpenoids in the Ethanol extract. Free radical scavenging activity by DPPH method possessed percentage of inhibition of 93.67% respectively followed by other extracts. Active compounds were identified by chromatography analysis by optimized solvent system. From the results obtained it is concluded that phytochemical present in *Aegle marmelos* of ethanol extract may be responsible for Antioxidant and antibacterial activity. GCMS analysis revealed the presence of active compounds like Piperidine, Flavone, Phytol and Longifolene as majority with various therapeutic applications.

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