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IN VITRO EVALUATION OF FREE RADICAL SCAVENGING ACTIVITY AND GC-MS ANALYSIS OF BIO-ACTIVE COMPOUNDS IN ETHANOL EXTRACT OF *AEGLE MARMELOS* LEAVES

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ABSTRACT
The present study aims to evaluate free radical scavenging activity present in the ethanolic leaf extracts of *Aegle marmelos*. Further the extracts were subjected to GC-MS for the identification of bioactive components present in the *Aegle marmelos* leaves. GC-MS analysis in the ethanol extract of *Aegle marmelos* leaves was done by using National Institute Standard and Technology (NIST) database 2005 to identify the compounds present. Nineteen chemical constituents were identified. The results showed that the leaves containing a wide range of phyto constituents which could be subjugated for the development of plant based innovative drugs which may help to give protection from various diseases.

KEYWORDS: *Aegle marmelos,* Free radicals, GC-MS.

INTRODUCTION

Medicinal plants have been used by mankind for its therapeutic value since time immemorial. The plant based traditional medicine systems continue to play a vital role in health care, with about 80% of the world's population relying mainly on traditional medicines for their primary health benefits.[1] Phytochemical constituents derived from plants are the basic source for the establishment of several pharmaceutical industries the constituents is playing a significant role in the identification of crude drugs. The medicinal value of plants lies in primary and secondary metabolites that produce a distinct physiological action on the human body.[2]

Aegle marmelos, commonly known as Bael, is a spiny tree belonging to the family Rutaceae. It is an indigenous tree found in Iran, India, Myanmar, Pakistan, Bangladesh and most of Southeast Asian countries.[3] The Bael fruit pulp contains many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants which may protect us against chronic diseases.[4]

A detailed investigation of *Aegle marmelos* tree have been reported to have medicinal uses but that of chemical components of leaves has not been well-documented and hence there is need for analysing the ethanolic *Aegle marmelos* leaf extract for separation and identification of the bioactive chemical compounds by using GC-MS analysis technique and free radical scavenging activity

MATERIALS AND METHODS

Aegle marmelos plant leaves were collected from Ponmala, Malappuram district in Kerala, India in the month of January 2022. The leave sample were cleaned with clean water and allowed dry in shade to protect from direct sunlight at room temperature. After ten days, the dried leaves were cut into small pieces and powdered and stored for further uses.

Preparation of Extracts:
The fresh leaves was air-dried to constant weight, pulverized in a grinder and stored in an air-tight container for further use. Exactly 250 g of plant material was extracted in ethanol for 4 days with occasional shaking. Ethanol used is of analytical grade. The separated extracts were then filtered through Whatman's No. 1 filter paper and the ethanol filtrate was separately concentrated to dryness in vacuum dryer using a rotary evaporator to remove the ethanol. The extract was lyophilized to obtain a dry powder extract. The samples were used for free radical scavenging property

FREE RADICAL SCAVENGING ASSAY

- Hydrogen peroxide scavenging activity
- Superoxide radical scavenging activity

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the ethanol extracts was determined according to the earlier described method. The hydrogen peroxide scavenging of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanins, tannins and flavonoids which can donate electrons to hydrogen peroxide, thus neutralizing it to water.[5] 1.4 ml of each extract at various concentrations (0.0–50.0 μg/ml) in distilled water was added to 0.6 ml of the hydrogen peroxide solution (40 mM in phosphate buffer pH 7.4). The absorbance of mixture was noted at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide solution. The results were compared with ascorbic acid (control) as μg/g dry weight. All experiments were repeated three times. Percentage of hydrogen peroxide scavenging by the extracts and standard was calculated by following formula

% scavenged of hydrogen peroxide = (Ao − A1)/ Ao × 100

Where Ao is the absorbance of the control and A1 the absorbance of the mixture containing either the extract or standard

Superoxide radical scavenging activity

Superoxide radicals were generated by method[6] with slight modification. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue formazan (NBT2+). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of Extract in the reaction mixture was 20 μg/ml. The total volume of the reactant mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT was 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reactant was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated O_2. This reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. Extract was added to the reaction mixture, in which O_2. Was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by using the following formula: O_2. - scavenging effect (%) = (1 – As Ac) × 100 where, AC is the absorbance of the l-ascorbic acid and AS is the absorbance of ethanolic extracts of *Aegle marmelos* leaves.

GC – MS ANALYSIS

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30mm×0.25mm I.D ×1 μ M df, composed of 100% Dimethyl polysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 μ l was employed (split ratio of10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5seconds and fragments from 45 to 450 Da[7].

RESULTS AND DISCUSSION

Hydrogen peroxide scavenging assay

Table-1: Hydrogen peroxide assay. (Values are average of triplicate experiment and are represented as mean ± SE).

Concentration (μg/ml)	ASCORBATE (%)	CRUDE EXTRACT (%)
50	13±0.87	46±1.45
100	33±1.37	53±1.95
150	46±1.38	68±1.26
200	60±2.54	82±1.59
250	80±2.12	95±2.60
Hydrogen peroxide IC_{50} value =131.35 μg/ml		

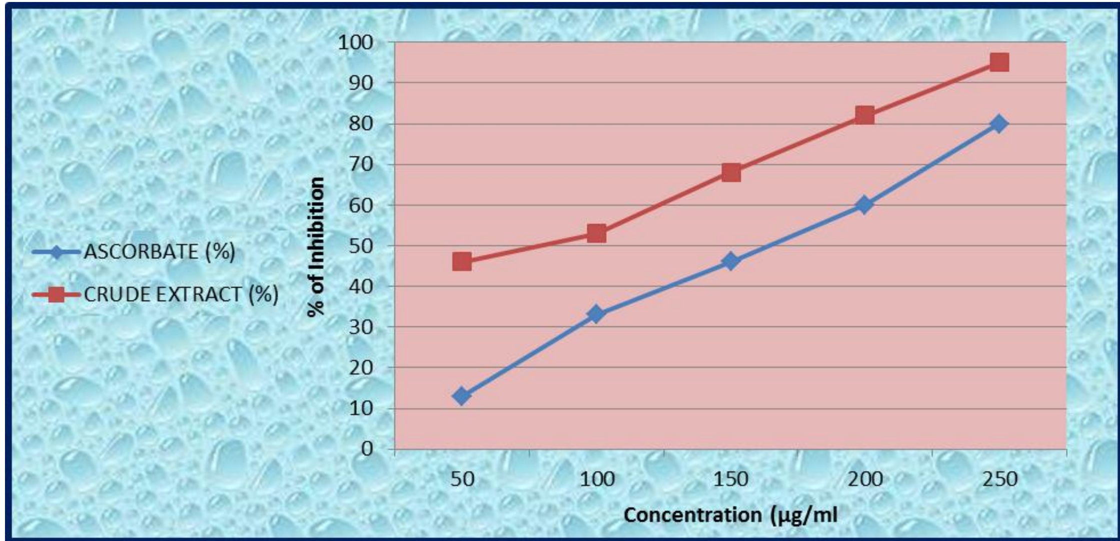

Fig-1: Graphical representation of Hydrogen peroxide assay.

In this study, *Aegle marmelos* leaf crude was taken different 50, 100, 150, 200 and 250µg/ml concentration, produced a dose depended scavenging of hydrogen peroxide. The activity of *Aegle marmelos* leaf crude was compared with standard ascorbic acid and the maximum scavenging effects of hydrogen peroxide were obtained at 84% of inhibition in 250µg/ml and the IC_{50} values were found to be 131.35µg/ml.

Superoxide radical scavenging assay

Table-2: Superoxide radical assay. (Values are average of triplicate experiment and are represented as mean ± SE).

Concentration (µg/ml)	ASCORBATE (%)	CRUDE EXTRACT (%)
50	13±0.32	46±0.67
100	33±0.56	53±0.82
150	46±1.92	68±0.91
200	60±0.28	82±0.75
250	80±0.87	95±0.39
Superoxide Radical IC_{50} value =163.96 µg/ml		

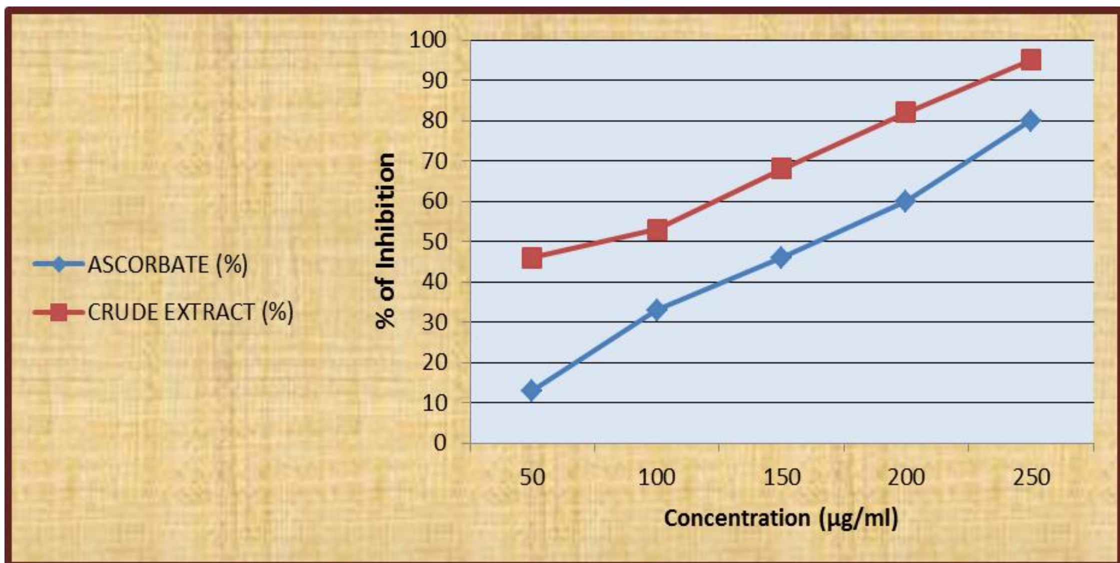

Fig-2: Graphical representation of Superoxide radical assay.

Radicals of superoxide anions pose harmful effects on cellular components and act as precursor of more reactive oxidative species including hydroxyl and singlet oxygen radicals. Superoxide radical scavenging activity of *Aegle marmelos* was investigated to clarify the mechanism of antioxidant system. The inhibition of O^{2-}

production was found to be concentration dependant and the percentage inhibition for ethanolic leaf extract was found to be consistent. The highest inhibition was exerted by the standard ascorbic acid with Superoxide Radical IC_{50} value =163.96 µg/ml.

GC-MS Analysis

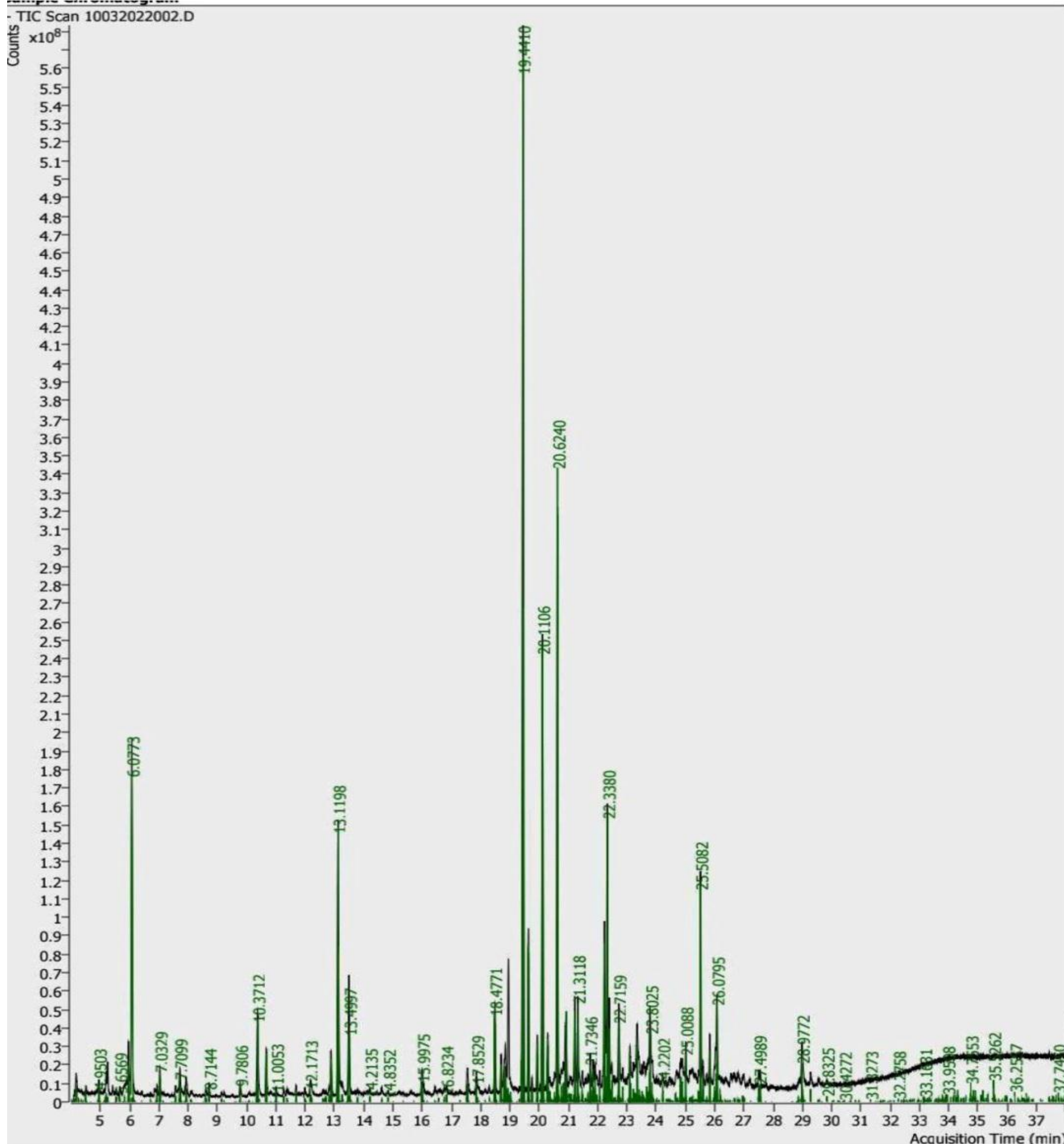

Fig-3: Graphical representation of GC-MS analysis.

Table-3: Highest peak value compounds of GC-MS analysis.

S.No	RT(min)	Name of Compound	Molecular formula	Molecular weight	Peak area
1.	19.4410	Caryophyllene	$C_{15}H_{24}$	204.36	12.16
2.	20.6240	1-oxo-1-phenyl-2-(-phenylisopropylimino)propane	$C_{18}H_{19}NO$	265.3	1.23
3.	20.1106	Santolina triene	$C_{10}H_{16}$	136.24	1.49
4.	6.0773	5-Amino-2-methyl-2H-tetrazole	$C_2H_5N_5$	99.10	2.89
5.	22.3380	Acetamide, N-(3,4-dichlorophenyl)-N-hydroxy-	$C_8H_7C_{12}NO_2$	220.5	1.75
6.	13.1198	Benzenemethanol, .alpha.,.alpha.,4-trimethyl-	$C_{10}H_{14}O$	150.22	3.69
7.	25.5082	Neophytadiene	$C_{20}H_{38}$	278.516	1.84
8.	26.0795	5-Nonadecen-1-ol	$C_{19}H_{38}O$	282.5	2.60
9.	21.3118	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-	$C_{15}H_{24}$	204.35	1.85

		dimethyl1-(1-methylethyl)-, (1S-cis)-			
10.	18.4771	Methanamine, N-(5-phenyl-1,3,4-oxadiazol-2-yl)methyl-	$C_{10}H_{11}N_3O$	189.22	4.59
11.	10.3712	1-Methyl-2-phenylcyclopropane	$C_{10}H_{12}$	132.20	5.25
12.	22.7159	1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	$C_{15}H_{24}O$	220.35	1.37
13.	23.8025	-Isopropyl-4,10-dimethylenecyclodec-5- enol	$C_{15}H_{24}O$	220.35	1.47
14.	28.9772	Phytol	$C_{20}H_{40}O$	296.5	2.49
15.	25.0088	Heptacosane	$C_{27}H_{56}$	380.7	1.56
16.	21.7346	7,11,15-Trimethyl-3-methylene-hexadeca1,6,10,14-tetraene	$C_{20}H_{32}$	272.47	2.40
17.	7.0329	Benzaldehyde	C_7H_6O	106.12	9.84
18.	12.1713	Benzene,1-ethenyl-4-methoxy-	$C_6H_{12}O_3$	132.16	3.39
19.	15.9975	2-Cyclohexen-1-one, 4-ethyl-3,4-dimethyl-	$C_{10}H_{16}O$	152.23	2.53

The GC-MS analysis revealed the presence of 19 compounds from ethanolic extract of *Aegle marmelos*. The major constituents were Caryophyllene ($C_{15}H_{24}$) with peak area of 12.16 and Molecular weight of 204.36.

Caryophyllene ($C_{15}H_{24}$) Pharmacological Classification

Anti-inflammatory agents that are non-steroidal in nature. In addition to anti-inflammatory actions, they have analgesic, antipyretic, and platelet-inhibitory actions. They act by blocking the synthesis of prostaglandins by inhibiting cyclooxygenase, which converts arachidonic acid to cyclic endoperoxides, precursors of prostaglandins. Inhibition of prostaglandin synthesis accounts for their analgesic, antipyretic, and platelet-inhibitory actions; other mechanisms may contribute to their anti-inflammatory effects.

CONCLUSION

The current study established that the ethanolic extract of *Aegle marmelos* leaves acquired potential free radical scavenging which leads to be a favorable in prevention of various oxidative stress-related diseases; hence, it is essential for identifying the phytocompounds to identify their pharmacological properties. Nineteen phytoconstituents have been identified from ethanolic extract by GC-MS analysis. Identification of these compounds in the plant serves as the basis in determining the possible medicinal benefits of the plant leading to further biological and phytopharmaceutical studies.

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