***In vitro* Investigation of Phytoconstituents, GC-MS, TLC, Antioxidant activity, Total Phenolic & Flavonoid contents from *Aeglemarmelos* L(Bael) leaves extract**

ABSTRACT

The Rutaceae family includes *Aegle marmelos*L., a moderately sized aromatic tree that is also known as Bael, Bengal quince, golden apple, Japanese bitter orange, stone apple, or wood apple. It is mostly found in tropical or subtropical regions. Fever, seminal weakness, nausea, vomiting, swellings, diarrhea, dyspepsia, and intermittent fever are the most common ailments that bael leaves are used to treat. Because of their high phenolic and flavonoid content, leaves have anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective, and vasodilatory properties. Therefore, the aim of the present study was to determine the phytochemical components, antioxidant capacity, GCMS analysis, total phenolic & flavonoid contents in the methanolic extracts of *A. marmelos* leaves.Various secondary metabolites were observed in the aqueous and methanolic *A. marmelos* leaf extracts. TLC analysis of methanolic extracts revealed five distinct spots having different Rf values.Total phenolic content was found to be 33 ± 7.62 mg GAE/g, flavonoid content was 307.8 ± 130.12 mg QE/g. The highest antioxidant activity of methanolic extract of *A. marmelous*leaves was obtained at 1000 µg/ml conc. and it was dose dependent. Many biologically active compounds were present as analysed by GC-MS. The highest peak area of Perylo[1,12-def]-1,3-dioxepin-5,11-dione, 6,12-dihydroxy-8,9-bis(2-hydroxypropyl)-7,10-dimethoxy-, stereoisomer (3.502%) was observed.

Keywords: *A. marmelos,* Ascorbic acid, Antioxidant Activity, GC-MS, Total Phenolic content, Total Flavonoid Content

1. **INTRODUCTION**

*Aegle marmelos*L., a perennial tree belonging to the Rutaceae family, is widely distributed in sub-Himalayan region, central and southern India. It is commonly called as Vilvam in Tamil, Bilva in Sanskrit, and Bael in Hindi. Its leaves are utilized in spiritual practices to honor Lord Shiva. It is widespread to India and is utilized in folk medicines. Plants are excellent sources for the investigation of pharmaceutical compounds and drugs. Natural products and their analogues can serve as intermediates in the manufacture of beneficial pharmaceuticals, and they may also have therapeutic value for humans or live stock species [1].

In developing nations, traditional medical practices mostly the use of medicinal plantsremain essential for providing basic healthcare needs. The therapeutic usefulness of these plants is because of the presence of active chemical components (phytoconstituents) that leads to physiological action on the human body.Alkaloids, tannin, flavonoids, and phenolic compounds are the most significant of these bioactive components of plants [2], along with carotenoids, ascorbic acid, tocopherol, and polyphenols [3-5].

The use of synthetic antioxidants leads many adverse effects while natural antioxidants are safe with no side effects [6]. Antioxidant substances prevent damages caused by free radicals. Apoptosis, gene expression, and cell proliferation are just a few of the regulatory mechanisms that are impacted by free radicals produced during aerobic metabolism. Excessive generation of free radicals may disrupt the antioxidant defencesystems, damaging vital biomolecules (such as membrane lipids, cell proteins, carbohydrates, DNA, and enzymes). Moreover, oxidative stress causes the occurrence of cytotoxic compounds and modifies the oxidant-antioxidant balance (redox homeostasis) that characterizes normal cell functioning [7-9]. Pathologies caused by oxidative stress include cancer [10–11], cardiovascular disease [12], neural disorders [13], Alzheimer’s disease [14], mild cognitive impairment [15], Parkinson’s disease [16], alcohol-induced liver disease [17], ulcerative colitis [18], atherosclerosis [19], and aging [20]. Polyphenols, flavonoids, vitamin C and Vitamin E are phytochemicals in foods that have been known as a natural antioxidant and shows antioxidant activity. Polyphenols and flavonoids act as reactive oxygen species (ROS) scavenger and have chemopreventive activity [21-22]. Ageing, atherosclerosis, and cancer are only a few of the pathological processes that are impacted by ROS [23]. Thus, scavenging ROS can minimize chronic illness. It has been reported that taking foods high in polyphenols and flavonoids contents mayreduces the inflammation, oxidative stress, tumors, and coronary heart disease [22,24–25]. Antioxidant functions in reducing DNA damage,

lowering lipid peroxidation, supporting the immune system and inhibit the malignant transformation of cells [26]. Plants that contain polyphenolic chemicalssuch as flavonoidshave antioxidant potential [27]. According to prior studies, the biological actions of many compounds are related to their antioxidant activity.In this present investigation, phytochemical screening of aqueous and methanolic extracts of leaves of *A. marmelos*was evaluated and biologically important compounds were identified by GC-MS analysis. Thin Layer Chromatography, total phenolic & flavonoids contents, and antioxidant activity of *A. marmelos*leaveswas determined.

2. MATERIALS AND METHODS

2.1. Plant Materials

Leaves sample of *A. marmelos* was collected from the Bundelkhand University campus, Jhansi and was identified by Dr. J. C. Arya Research Officer, (Botany) from the Central Ayurveda Research Institute under the Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Gwalior Road, Jhansi (Uttar Pradesh). The leaves were thoroughly washed with tap water followed by de-ionized water. After washing, it was kept in the dark for drying at room temperature and finally crushed with the help of mixer &grinder. The extraction was performed with the stored plant materials.

2.2 Extraction Procedure

Leaves sample was extracted by two methods -

- Aqueous extraction and
- Methanolic extraction

Aqueous Extract

In the water bath, different amounts of powdered sample i.e. 5 and 10 gmswere extracted for one hour at 90°C with 100 ml of de-ionized water.After 1 hour, the extract was filtered and stored at 4°C for the further tests.

Methanolic Extract

The powdered leaves were percolated using 80% of methanol in the Soxhlet apparatus at 60-65°C. This extract was evaporated to dryness in a water bath at 40 °C temperature and stored in air tight bottles.

2.3. Phytochemical Analysis

The presence or absence of secondary metabolites was carried out as described previously [28-30]. The alkaloids, carbohydrates, reducing sugars, flavonoids, glycosides,

tannin, saponin, protein, triterpenoids & steroids were determined in the aqueous and methanolic extracts of leaves of *A. marmelos.* More detail procedures are mentioned elsewhere [28].

2.4. Total antioxidant capacity (TAC) by Phosphomolybdenum assay

The total antioxidant activity was measured using the phosphomolybdenum test [31]. Two milliliters of the reagent solution were mixed with differentconc. of the test sample (1000-31.25 µg/ml). The reaction mixture was incubated for 90 minutes at 95°C in an oven-cum-incubator. For the blank tube, the extract was replaced with 80% methanol in the same ratio. Ascorbic acid was used as a reference control.The samples were cooled at room temperature and the absorbance taken at 695 nm wavelength using a multiplate reader.

2.5.Thin layer chromatography

Methanolic extract of *A. marmelos* leaves was tested on TLC plates coated with 0.2 mm thick silica gel-G. The solvent mixture that we employed in this instance was butanol, acetic acid, and water at a 2:1:1 v/v ratio, as previously mentioned. Spots move with the solvent mixture on the silica-coated plates due to capillary action, detecting the methanolic extract over 4 mm from the plate base. A fully developed silica coated plate was heated for 20-25 minutes after being allowed to air dry. To observe the bands, 0.2% freshly prepared ninhydrin solution was sprayed across the plate.

These spots were expressed by its retention factor (Rf).

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

2.6. Total Phenolic Content (TPC)

The Folin-Ciocalteumethod [32] was used for the determinationof TPC. 100 µl of different concentrations extracts was mixed with 500 µl of water and 100 µl of Folin-Ciocalteu reagent. The mixture was incubated for six minutes. Further, 500 500 µl of distilled water and 1 ml of 7% sodium carbonate was added to the reaction mixture. The absorbance was taken after ninety minutes at 760 nm spectrophotometrically. The gallic acid equivalents (mg GAE/g) were used to calculate the TPC. Each test was carried out in triplicates.

2.7.Total Flavonoid Content (TFC)

The aluminum chloride complex formation assay, which was performed by Piyanete et al. [33], was used to determine the TFC of the methanolic extract of *A. marmelos* leaves.

The quercetin was utilized as a reference, the flavonoid content was calculated as mg QE/g. After mixing 100 µl of the quercetin dilution with 500 µl of distilled water and 100 µl of 5% sodium nitrate, the mixture was left to stand for 6 minutes. Subsequently, 200 µl of 1M NaOH solution was added after 150 µl of a 10% $AlCl_3$ solution had been added and incubated for 5 minutes. This reaction mixture's absorbance was measured at 510 nm. All the procedures were performed in triplicates.

2.8.Nitric oxide radical scavenging assay

Free radicals generated from sodium nitroprusside (SNP) were measured according to the earlier described method [31] with some modifications. Different concentration of reaction mixture containing SNP (15 mM) in PBS (pH 7.3) with and without sample, incubated at 25°C for 210 mins. After adding the Griess reagent, the reaction mixture was incubated for 10 minutes at room temperature.The standard used was ascorbic acid. The absorbance was measured at 560 nm using a UV-Vis microplate reader.

2.9. Superoxide anion scavenging assay

Based on a previously described method [34] with minor modifications, the reduction of NBT determined the total antioxidant capacity of methanolic extract. Phosphate buffer (20 mM, pH 7.4), PMS (60µM), NBT (156µM), and different conc. of extract were all included in the 1-mL reaction mixture. After incubation for 5 min at 25°C temperature, the absorbance was taken at 560 nm against an appropriate blank solution. BHT was used as reference control.

2.10. (GC-MS) Analysis

The GC-MS analysis of the methanolic extract of *A. marmelous* leaves was carried out on a Perkin Elmer Turbo Mass Spectrophotometer using a Perkin Elmer autosampler XLGC. The column was a Perkin Elmer Elite - 5 capillary columns with a film thickness of 0.25 mm and a length of 30 m. It was made of 95 % dimethylpolysiloxane. At a flow rate of 0.5 ml/min, helium (99.999 percent) was used as the carrier gas. An injection length of one liter was employed. The GC's inlet temperature was kept at 250°C, and it had a programmed oven temperature of 110°C (isothermal for two minutes). After that, it increased by 10°C/min to 200°C, then by 5°C/min to 280°C, and finally by 5°C/min to 280°C, with a 5°C/min isothermal at 280°C. The GC ran in 30 minutes. The source temperature is maintained at 180°C, and the temperature of the MS transfer line is maintained at 200°C. Total Ion Count was utilized for data 60 Sharma and Kumar evaluation of compound detection and quantification (TIC), and electron impact ionization at 70eV was used for the GC-MS analysis. The spectrum of the components was compared to the known components stored in

the GC-MS library. For peak area measurement and data processing, Turbo-Mass OCPTVS-Demo SPL programme 19 was used.

2.11.Statistical analysis:

The results were shown as mean ± SEM. The student's t test (paired) was used at the 5% level to determine the significance of the observed differences. The comparisons were done between reference controls and methanolic leaves extracts of *A. marmelous.*

3. RESULTS

Both the humans and animals require secondary metabolites. The biochemical tests showed (Table 1) the presence of different secondary metabolites in the aqueous and methanolic extracts. The results depend on the solvent as well as the qualitative detection methods used. It was observed that there are not many differences in the presence or absence of secondary metabolites when 5 & 10 gm of plant material were used for aqueous extraction. For the determination of secondary metabolites, aqueous and methanolic extract are equally important.The total 5 spots with different Rf values (0.31, 0.59, 0.63, 0.71 and 0.79) were detected by TLC in the methanolic extract of *A.marmelos*leaves(Fig. 1). Here we used alanine amino acid as a standard and its Rf value was 0.69.

Table1: Qualitative phytochemical analysis of the aqueous and methanolic extracts of *A. marmelous*leaves

S No.	Phytochemical Tests	Aqueous Extract		Methanolic Extract
		10 gm	5 gm	
1.	**Alkaloids**			
	Mayer's	+ ve	+ ve	+ ve
	Wagner's	+ ve	- ve	+ve
	Hager's	+ ve	+ ve	+ ve
2.	**Carbohydrates**			
	Molisch	- ve	+ ve	- ve
	Barfoed's	- ve	+ ve	- ve
3.	**Reducing Sugars**			
	Fehling's	+ ve	+ ve	+ ve
	Benedict's	+ ve	+ ve	+ ve
4.	**Flavonoids**			
	Alkaline Reagent	+ ve	-ve	-ve
	Lead Acetate	+ ve	+ ve	+ ve
5.	**Glycosides**			
	Borntrager's	+ ve	+ ve	+ ve
	Legal's	- ve	- ve	-ve
	Keller-killiani	- ve	- ve	+ ve
6.	**Tannin & phenolic**			
	Ferric Chloride	+ ve	+ ve	+ ve
	Lead Acetate	+ ve	+ ve	+ ve
	Dilute iodine solution	+ ve	+ ve	+ ve

7.	**Saponin** Froth	-ve	-ve	+ve
8.	**Protein & A.A.** Ninhydrin Biuret	+ ve -ve	+ ve -ve	+ve -ve
9.	**Triterpenoids & Steroids** Salkowski's	-ve	-ve	+ ve
10.	**Hydrolysable tannin**	+ ve	+ ve	+ ve

(+) indicates presence while, (–) indicates the absence of the components

Figure 1.TLC plate showing spots having different Rf values (0.79, 0.71, 0.63, 0.59 & 0.31) of methanolic extract of *A. marmelous*leaves

Ascorbic acid showed 91.0% scavenging activity whereas methanolic extract was found to be 64.40% at a concentration of 1000 mg/ml (Fig. 2).Significant differences have been observed in the antioxidant potential between the ascorbic acid (reference control) and methanolic extract of *A. marmelous* (p=0.00985).The antioxidant potential observed was due to the presence of phenolic and flavonoid contents. The mean values of TPC and TFC were 33 ± 7.62 mg GAE/g and 307.8 ± 130.12 mg QE/g respectively(Table 2).

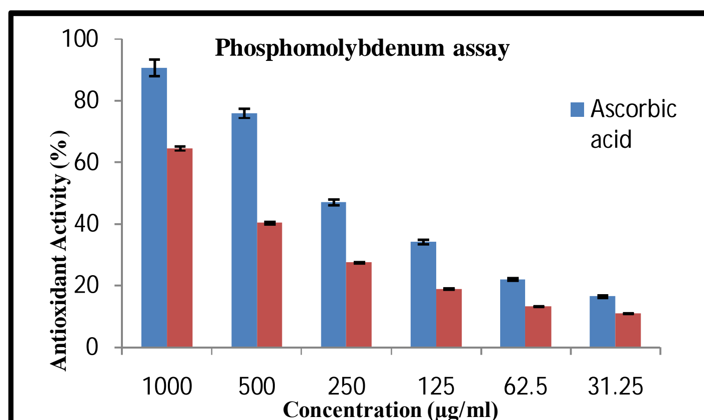

Figure 2: The Antioxidant activity of methanolic extract of *A. marmelous*leaves

Table 2. Total Flavonoid & Phenolic Content of methanolic extract of *A. marmelous*leaves

Total Flavonoid Content (mgQE/g)		Total Phenolic Content (mgGAE/g)	
Conc. (µg/ml)	***A. marmelous***	**Conc. (µg/ml)**	***A. marmelous***
1000	123±13.5	150	50±5.3
500	128±15.6	120	40±4.1
250	112±11.7	90	27±2.4
125	392±21.6	60	15±2.2
62.5	784±26.8	30	
Mean ± SD	**307.8 ± 130.12**	**Mean ± SD**	**33± 7.62**

The dose dependent NO and superoxide radical scavenging activity of the methanolic extract of *A. marmelous* leaves were observed (Fig.3 and 4). Significant differences have been observed in the NO and superoxide radical scavenging activity between the ascorbic acid (reference control) & methanolic extract of *A. marmelous,* respectively (p=0.000161; p=0.036419). The active principle analyzed by GC-MS with MW, MF, peak area %, RT, and composition of the bioactive components of methanolic extract of *A. marmelous* leaves (Table 3). The GC-MS chromatogram of the identified compounds is shown in (Fig. 5).

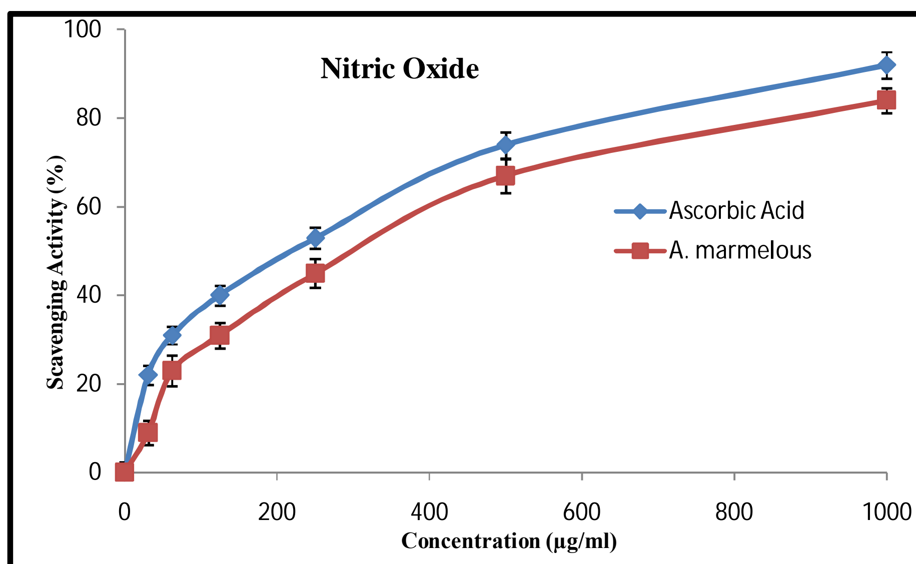

Figure 3: Nitric oxide scavenging activity of methanolic extract of *A. marmelous*leaves

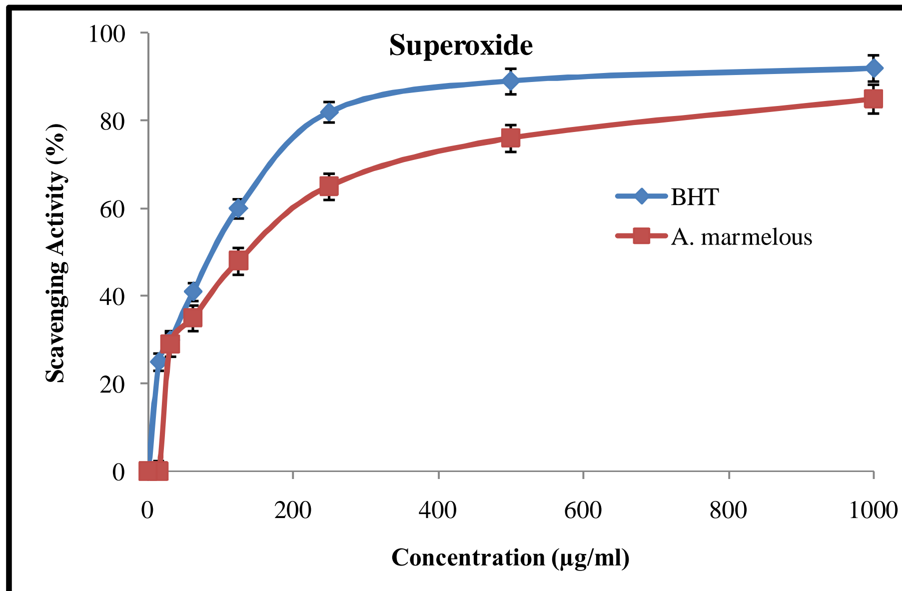

Figure 4: The superoxide radical scavenging activity of methanolic extract of A. *marmelous*leaves

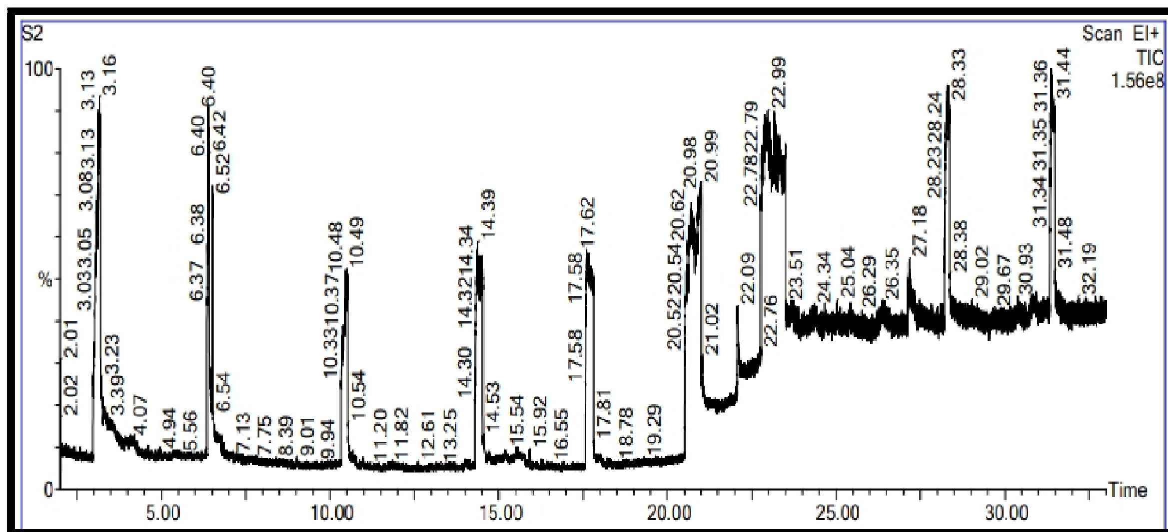

Figure 5: Total Ion Chromatogram of methanolic extract of *A.marmelous*leaves

Table 3: Compounds identified by GC-MS analysis of methanolic extract of *A. marmelous*leaves

Sr. No	RT	PP %	Compound name	M.F.	M.W.	Biological Activities**
1.	20.610	1.311	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	598.7g /mol	Phototherapy of malignant neoplasms [35]
2.	20.751	2.468	Hyocholic acid	$C_{24}H_{40}O_5$	408.6g /mol	Mouse metabolite, a human urinary metabolite and a rat metabolite Antiplasmodial activity antitrypanosomal activity [36]
3.	23.178	3.502	Perylo[1,12-def]-1,3-dioxepin-5,11-dione, 6,12-dihydroxy-8,9-bis(2-hydroxypropyl)-7,10-dimethoxy-, stereoisomer	$C_{29}H_{26}O_{10}$	534.5g /mol	Antimalarial Antileishmanial [37]
4.	28.321	3.040	Lutein	$C_{40}H_{56}O_2$	568.9g /mol	An antioxidant Plant metabolite Antiviral activity [38]
5.	28.321	3.040	Rhodopin	$C_{40}H_{58}O$	554.9g /mol	Bacterial metabolite [39]

**** Biological Activities Based on the PubChem Database**

Many components that are biologically active were observed. The area with highest peak of Perylo[1,12-def]-1,3-dioxepin-5,11-dione,6,12-dihydroxy-8,9-bis(2-hydroxypropyl)-7,10-dimethoxy-, tereoisomer (3.502 %), Lutein (3.040 %), Rhodopin (3.040 %), Hyocholic acid (2.468 %) and Hematoporphyrin (1.311 %) shown different biological activities like Antioxidant, anticancerous, antileishmanial and antimalerial activities etc.

4. DISCUSSION

*A. marmelos*have been traditionally used for many ailments and the nutritional benefits are also reported. The best solvents for extracting this plant's metabolites are methanol and water, with ethanol coming in second [40]. The phytochemistry of *A. marmelos* has been thoroughly investigated, and it has been discovered that the plant contains a wide range of physiologically active substances. *A. marmelos* contains a number of important

phytochemicals, such as: Alkaloids which are well-known for their medicinal effects.Marmelosin, aegeline,and marmesinare among the alkaloids that have been found in leaves &roots of *A. marmelos* [41]. *A. marmelos* fruit contain abundance of tannin, and studies have indicated that tannins have potent anti-inflammatory and antioxidant properties. Our results also showed the presence of tannins in the aqueousand methanolic leaf extracts. Flavonoids are a class of chemicals that have antioxidant, anti-cancer, and anti-inflammatory properties. Roots and leaves of *A. marmelos* contain flavonoids, which are recognized for their anti-cancer, antioxidant, andanti-inflammatory properties [42]. *A. marmelos* has been shown to contain terpenoids, some of which have been demonstrated to have antibacterial &antifungal properties. Saponins are also present in the leaves and fruit of *A. marmelos*, which are recognized for their ability to foam and emulsify. Studies have demonstrated the anti-inflammatory &antinociceptive effects of several of these chemicals [43]. Glycosides are well-known for their medicinal properties and have been shown to have antinociceptive and anti-inflammatory activities[44].

In synthetic chemistry, TLC is widely used method for component identification, purity analysis, and progress of reaction. Additionally, it enables the solvent system to be optimized for a specific separation problem. It is considerably faster than column chromatography and just needs a minimal amount of a compound. A thin, homogeneous coating of silica gel or alumina is applied to a piece of glass, metal, or rigid plastic in TLC. A suitable liquid solvent or solvent mixture serves as the mobile phase [45].

Antioxidants are very important in preventing human illnesses by acting as scavengers of free radicals, pro-oxidant metal complexes, reducing agents, and quenchers of singlet oxygen production [46]. Because of their ability to scavenge, antioxidants are able to manage certain conditions, including as AIDS, cancer, heart disease, neurological disorders, and cataracts [47].Scientific knowledge regarding the antioxidant abilities of many plants is currently insufficient, particularly those less commonly used in food and medicine. Thus, evaluating these characteristics continues to be a worthwhile and fascinating endeavor, especially when it comes to discovering fresh sources of functional foods, natural antioxidants, and neutraceuticals. It has been suggested that synthetic antioxidants such as gallic acid esters, BHT (butylated hydroxyl toluene), tertiary butylated hydroxy quinone, and BHA (butylated hydroxy anisole) are carcinogenic. As a result, there are strict limitations on their use, and replacing them with antioxidants that occur naturally is recommended. These synthetic antioxidants likewise exhibit minimal antioxidant activity and poor solubility [48]. Finding a more potent antioxidant with less adverse effects from natural sources is still

necessary. It has been shown that plants containing flavonoids exhibit antioxidant effects [49].

Using the phosphomolybdenum assay, bael leaf's antioxidant activity was evaluated. It is widely known that plant flavonoids and phenolics in general are potent antioxidants and scavengers of free radicals [50,51]. The majority of plants' capacity to scavenge radicals has been attributed to substances like flavonoids, which include hydroxyls [52]. Leaves of *A. marmelos*were found to contain, phenolic compounds, flavonoids, fats, triterpenoids, glycosides, carbohydrate and alkaloids as their chemical constituents. Thus, the current investigation has confirmed that the leaves of remedial plants may be a rich source of antioxidants. The substantial amount of phenolic hydroxyl groups that phenolics possess may account for their potent ability to scavenge free radicals.

5. CONCLUSION

The findings indicate that *Aegle marmelos* leaves have many phytoconstituents and strong antioxidant properties, and that their presence or absence depends on the solvent and the methodology used. The present study reveals that methanolic extract contains more phytochemicals compare to aqueous extract, it is enough excited to explore more information about this medicinal plant, therefore it inhibits production of free radicals. GC-MS analysis revealed many compounds *viz.* Hematoporphyrin, Hyocholic acid, Lutein Rhodopin, etc. with known biological activities. In addition, the current study suggests the possibility of manufacturing health supplements using these medicinal plants. In many respects, *A. marmelos*is a unique source of drugs. Each and every part of this plant treat like a medicine and used by human beings and for their cattles also.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author(s) thus declare that during the drafting or editing of manuscripts, NO generative AI tools, such as large language models (ChatGPT, COPILOT, etc.), or text-to-image generators, were employed.

DATA AVAILABILITY STATEMENT

Data set supporting this manuscript is included within the manuscript. Any further information should be requested from the corresponding author.

CONSENT AND ETHICAL APPROVAL

Not applicable

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