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

ABSTRACT

*Aegle marmelos*L. belongs to the family Rutaceae,is also known as bael,Bengal quince,golden apple, Japanese bitter orange, stone apple or wood apple a moderate sized aromatic tree.It is mainly found in tropical and sub tropical region. The antioxidant activities of leaves are due to the presence of phenolics and flavonoids contents and act as as anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective, and vasodilatory agents. The current study aims to investigate the phytochemical constituents, GCMS analysis, antioxidant potential, total phenolic & flavonoid contents, and antioxidant potential of *A. racemosa* leaf extracts. In existing study we carried out a well systematic record of phytoconstituents in a quantitative manner in aqueous and methanolic extracts of *Aegle.marmelos.* In TLC total 5 spots were present in the methanolic extract with 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, whereas antioxidant activity was dose dependent. Many biologically active components were present as analysed by GCMS. 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. Belongs to the Rutaceae family, is a perenial tree, found wildly in the sub Himalaya tract, central and South India. Commonly it is called as Bael in Hindi, Vilvam in Tamil and Bilva in Sanskrit. Its leaves used as a devotional purpose for the worship of Lord Shiva. It is indigenous to India and is used in folk medicines. Plants are the good sources for the discovery of pharmaceutical compounds and medicines. Natural products could be potential drugs for humans or live stock species and also these products and their analogues can act as intermediates for synthesis of useful drugs [1].

The traditional medicinal methods, especially the use of medicinal plants, still play a vital roleto cover the basic health needs in the developing countries. The medicinal value of these plantslies in some chemical active substances (phytoconstituents) that produce a definitephysiological action on the human body. The most important of these bioactive constituents ofplants are alkaloids, tannin, flavonoid and phenolic compounds (Akindele and Adyeyemi,2007) [2].carotenodis, ascorbic acid, tocopherol and Polyphenols [3-5].Natural antioxidants haven't cause health problems that may arise from the use of synthetic antioxidants which have side effects [6]. Antioxidant substances block the action of free radicals. Free radicals generated in aerobic metabolism are involved in a series of regulatory processes such as cell proliferation, apoptosis, and gene expression. When generated in excess, free radicals can counteract the defense capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA, and enzymes. Oxidative stress results in cytotoxic compounds occurrence (malonyl dialdehyde, 4-hydroxynonenal) and alters the oxidant-antioxidant balance (redox homeostasis) that characterizes normal cell functioning [7-9]. Oxidative stress-induced pathology includes 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 that has antioxidant activity. Polyphenols and flavonoids are known to have chemopreventive activity as ROS scavenger [21-22]. Reactive oxygen species (ROS) play a role in various pathological processes including cancer, aging, and atherosclerosis [23]. Chronic disease can be reduced by ROS scavenging. Some studies suggest that increased intake of foods containing polyphenols and flavonoids can reduce oxidative stress, inflammation, tumor and coronary heart disease [22] [24-25]. Antioxidant function in reducing DNA damage, lowering lipid peroxidation, maintaining the immune

system and inhibit the malignant transformation of cells [26]. It has been found out that plant having polyphenolic compounds such as flavonoids posses' antioxidant activity (Cook and Samman, 1996) [27]. According to prior studies the biological actions of many compounds are related to their antioxidant activity. In this present investigation, phytochemical screening of methanolic extracts of leaves of *A. marmelos*was revealed for the presence of some phytoconstituents, thin layer chromatography, total phenolic and flavonoids contents hence the present study was designed to evaluate antioxidant activity of *A. marmelos.*

In the abstract, both aqueous and methanolic extracts of Aegle marmelos are mentioned as part of the work plan. However, the introduction only refers to "methanolic extracts of leaves of A. marmelos." Additionally, the Materials and Methods section only reports on methanolic extracts. This inconsistency leaves it unclear whether the study focused on both extracts or just one.

2. MATERIALS AND METHODS

2.1.Plant Material

Leaves sample of *A. marmelos* was collected from the Bundelkhand University campus, Jhansi, identified and authenticated 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). After thoroughly washing with tap water followed by de-ionized water, kept in the dark for drying at room temperature. And finally crushed with the help of mixer grinder, and stored for the further extraction purpose.

Plant identification (voucher) number?

2.2.Extraction Procedure

Leaves sample was extracted by two methods -

- Aqueous extraction and
- Methanolic extraction

Aqueous Extract

Different amount of powdered sample i.e. 5 gm and 10 gm with equal amount (100 ml) of de-ionized water were extracted in the water bath for 1 hour at 90°C. After 1 hour filtered the extract with 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 filtered and evaporated to dryness in a water bath at temperature 40 °C and stored in air tight bottles.

2.3.Phytochemical Analysis

Additions of some modifications, the presence or absence of secondary metabolites were carried out by [28-30].

What phytochemical tests were conducted? Which photochemical constituents (secondary metabolites) were examined? More details on the procedures used are needed.

2.4.Total antioxidant capacity (TAC) by Phosphomolybdenum assay

For the total antioxidant activity phosphomolybdenum assay was used carried out by Prieto et al [31] with some of modifications. Different (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) concentrations of the test sample was combined with 2 mL of reagent solution. Reaction mixture was incubated at 95°C for 90 min. 80 % Methanol was used in the place of extract for the blank tube with same ratio. As same as test samples ascorbic acid with various concentrations in methanol was used as a standard. After cooling at room temperature measured the absorbance at 695nm with the help of multi plate reader. Finally draw the calibration curve with the respect of ascorbic acid.

What setup was used for incubation? Additionally, details regarding the aqueous extract study are missing.

2.5.Thin layer chromatography

Based on *in vitro* results, TLC plates coated with silica gel-G of 0.2 mm thickness was used for the testing of methanolic extract of *A. marmelos*leaves. Here we used a solvent mixture (Butanol- acetic acid-water) at the ratio of 2:1:1 v/v as described by somewhere else. Spotting the methanolic extract above 4mm from the base of the plate, spots migrate with the solvent mixture on the silica coated plates by the capillary action. Fully developed silica coated plate was air dried followed by heating for 20-25 minutes. The plate was sprayed with 0.2% freshly prepared ninhydrin solution to detect the bands.

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

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

2.6.Total Phenolic Content (TPC)

The total phenolic content was determined by using the Folin- Ciocalteu method [32]. 100 µl of various concentrations were mixed with 500 µl of water followed by adding 100 µl of Folin-Ciocalteu reagent, this mixture was allowed to stand for 6 minutes. Now add the 7% sodium carbonate and distilled water were 1ml and 500 µl respectively to the reaction mixture tubes. All the tubes were incubated for 90 minutes at room temperature. The absorbance was measured at 760 nm. Same as the extract various concentration of gallic acid was used as a standard. Calculate the total phenolic content with the respect of gallic acid equivalents (mg GAE/g). All the experiments were performed in duplicates.

2.7.Total Flavonoid Content (TFC)

The total flavonoids content of the methanolic extract of *A. marmelos*leaves was done by the aluminium chloride complex forming assay carried out by Piyanete*et al.* [33]. Flavonoid content was determined as quercetin equivalent (mg QE/g) because it was used as a standard. 100 µl of the quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% sodium nitrate and allowed to stand for 6 minutes. Then 150 µl of 10% Aluminium chloride solution was added and allowed to stand for 5 minutes after which 200 µl solution of 1M Sodium hydroxide was added sequentially. The absorbance of this reaction mixture was recorded at 510 nm. All the procedures were performed in duplicates.

2.8.Nitric oxide radical scavenging assay

Free radicals generated from sodium nitroprusside (SNP) were measured according to the earlier described method [34] 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. Add Griess reagent followed by rest for 10 minutes at room temperature. Ascorbic acid was used as a standard. The absorbance was measured at 560 nm using a UV-Vis microplate reader.

2.9.Superoxide anion scavenging assay

The total antioxidant capacity of methanolic extract was based on the reduction of NBT according to a previously reported method [35] with some modification. The 1-mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), PMS (60µM), NBT (156µM), and

various concentrations of sample solution. 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 positive control.

2.10.(GC-MS) Analysis

On a Perkin Elmer Turbo Mass Spectrophotometer with a Perkin Elmer autosampler XLGC, GC-MS analysis of methanolic extract of *A. marmelous* leaves was performed. The column was a Perkin Elmer Elite - 5 capillary column with a film thickness of 0.25 mm and a length of 30 m. It was made of 95 percent dimethylpolysiloxane. At a flow rate of 0.5 ml/min, carrier gas helium (99.999 percent) was used as the carrier gas. As an injection length, a 1 l sample was used. The inlet temperature of the GC was maintained at 250 °C, with a programmed oven temperature of 110 °C (isothermal for 2 min), followed by a 10 °C/min increase to 200 °C, followed by a 5 °C/min increase to 280 °C, and a 5 °C/min increase to 280 °C, with a 5 °C/min isothermal at 280 °C. The GC took 30 minutes to run. The temperature of the MS transfer line is kept at 200°C, while the source temperature is kept at 180°C. The GCMS analysis was carried out using electron impact ionisation at 70eV, and Total Ion Count was used for data 60 Sharma and Kumar evaluation of compound detection and quantification (TIC). 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.

3. RESULTS

Screening of the aqueous and methanolic extracts shows the presence of different secondary metabolites as determined by biochemical tests (Table 1). The presence or absence of these secondary metabolites depends on the solvent used as well as the qualitative detection methods. Secondary metabolites are essential for humans and animals alike. Two different concentrations i.e. 5 & 10 gm of plant material were used for aqueous extraction and it was observed that there are not many differences in the presence or absence of secondary metabolites. Further, aqueous and methanolic extract are equally important for the determination of secondary metabolites. The thin layer chromatography of methanolic extract of *A.marmelos*leaves shows the presence of total 5 spots with their unique Rf values 0.31, 0.59, 0.63, 0.71 and 0.79 respectively (Fig. 1). Here we used alanine amino acid as a standard and its Rf value was 0.69.

Table1:Qualitativephytochemicalanalysisoftheaqueousandmethanolicextractsof*A.marmelous*leaves

S No.	PhytochemicalTest	AqueousExtract		Methanolic Extract
		10 gm	5 gm	
1.	**Alkaloids**			
	Mayer'sW	+ ve	+ ve	+ ve
	agner's	+ ve	-ve	+ve
	Hager's	+ ve	+ ve	+ ve
2.	**Carbohydrates**			
	Molisch	- ve	+ ve	- ve
	Barfoed's	- ve	+ ve	- ve
3.	**ReducingSugars**			
	Fehling'sB	+ ve	+ ve	+ ve
	enedict's	+ ve	+ ve	+ ve
4.	**Flavonoids**			
	AlkalineReagent	+ 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			
	ChlorideLead	+ ve	+ ve	+ ve
	Acetate	+ ve	+ ve	+ ve
	Diluteiodinesolution	+ ve	+ ve	+ ve
7.	**Saponin**			
	Froth	-ve	-ve	+ve
8.	**Protein& A.A.**			
	Ninhydrin	+ ve	+ ve	+ve
	Biuret	-ve	-ve	-ve
9.	**Triterpenoids&Steroids**			
	Salkowski's	-ve	-ve	+ ve
10.	**Hydrolysabletannin**	+ 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

The percent inhibition of methanolic extract was found to be 64.40% at a concentration of 1000 mg/ml whereas ascorbic acid, on the other hand, had a scavenging activity of 91.0% at the same concentration (Fig. 2). As a result, the antioxidant potential is due to the phenolic and flavonoid material. The mean values of total phenolic and flavonoid contents were 33 ± 7.62 mg GAE/g and 307.8 ± 130.12 mg QE/g respectively(Table 2).

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	150	50
500	128	120	40
250	112	90	27
125	392	60	15
62.5	784	30	
Mean ± SD	**307.8 ± 130.12**	**Mean ± SD**	**33± 7.62**

Values of standard used are missing in table 2

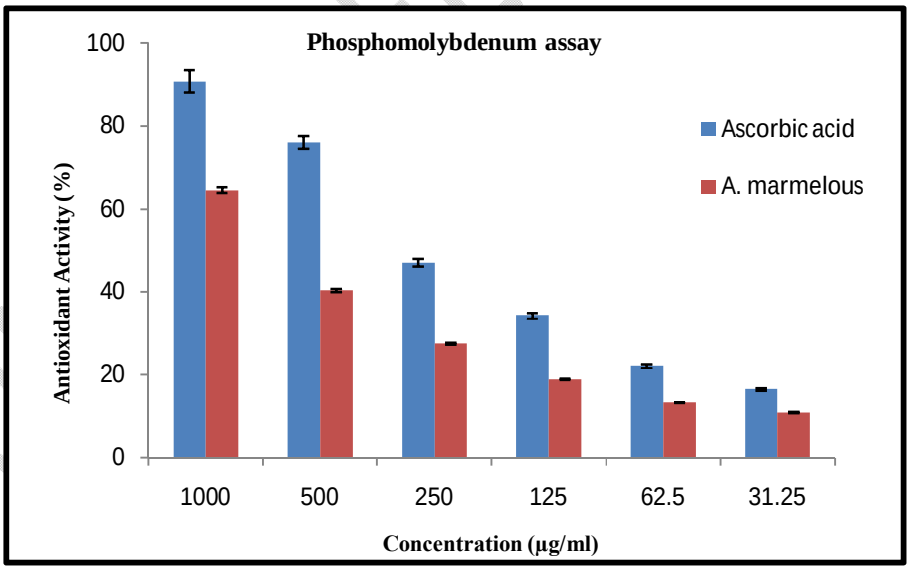

Figure2:TheAntioxidantactivity ofmethanolicextractof*A.marmelous*leaves

The nitric oxide and superoxide radical scavenging behaviour of the methanolic extract of *A. marmelous* leaves were dose dependent (Fig.3 and 4). The active principle analyzed by GCMS with molecular weight, molecular formula, peak area %, retention time,

and composition of the bioactive components of methanolic extract of *A. racemosa* leaves (Table 3). The GC-MS chromatogram of the identified compounds is shown in (Fig. 5).

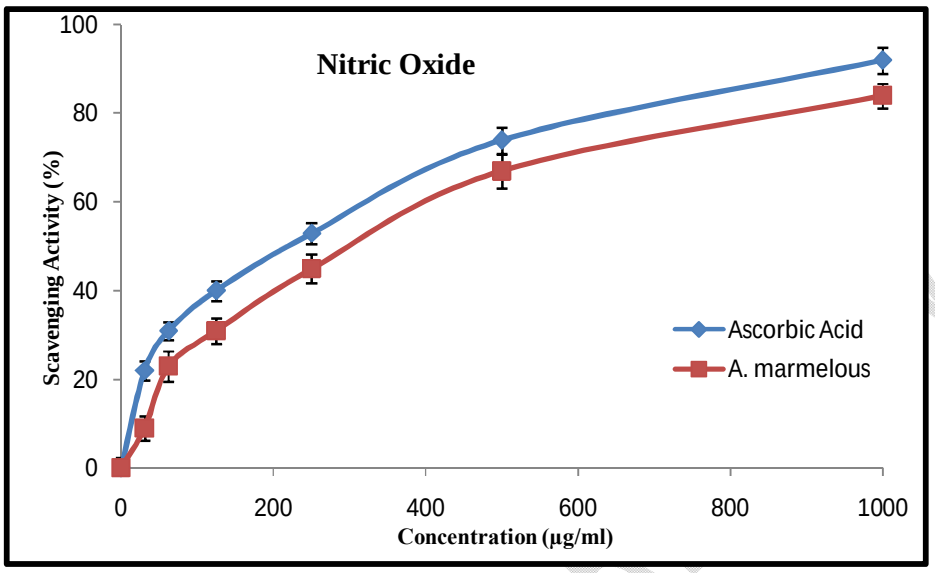

Figure3:Nitricoxidescavengingactivityof methanolicextractof *A.marmelous*leaves

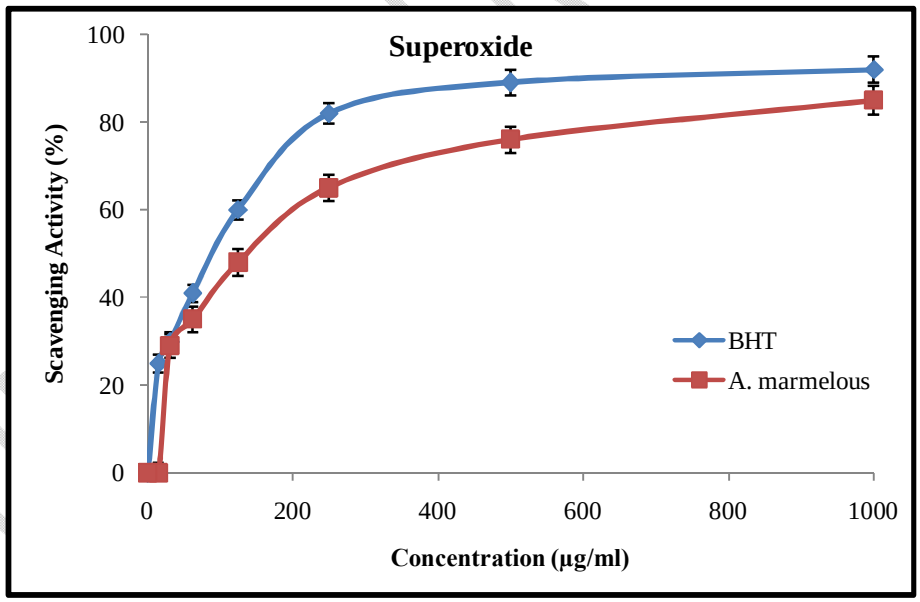

Figure 4: Thesuperoxideradicalscavengingactivityofmethanolicextractof *A.marmelous*leaves

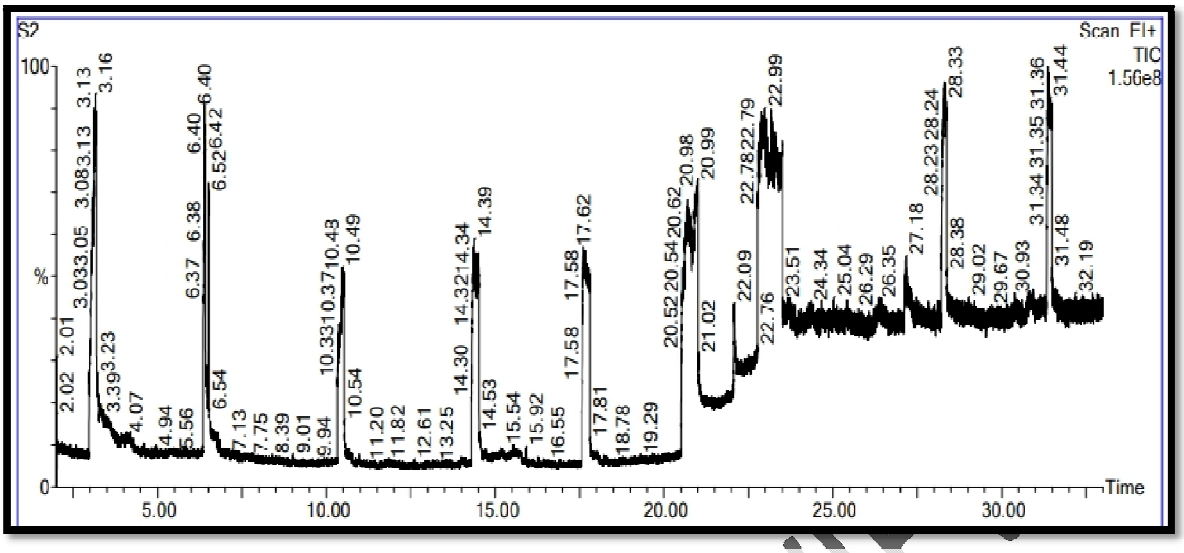

Figure 5: Total Ion Chromatogram of methanolic extract of *A.marmelous*leaves

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 [40]
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 [41]
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 [42]
4.	28.321	3.040	Lutein	$C_{40}H_{56}O_2$	568.9g /mol	An antioxidant Plant metabolite Antiviral activity [43]
5.	28.321	3.040	Rhodopin	$C_{40}H_{58}O$	554.9g /mol	Bacterial metabolite [44]

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

Many biologically active components were observed. 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-, tereoisomer (3.502 %), Lutein (3.040 %), Rhodopin (3.040 %), Hyocholic acid (2.468 %) and Hematoporphyrin (1.311 %)shown different biological activities like Antioxidant, anticancerous, antimalerial and antileishmanial activities etc.

4. DISCUSSION

*A. marmelos*belongs to rutaceae family have traditionally used for many ailments and the nutritional benefits also reported. Thin-layer chromatography (TLC) is a highly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction. It also permits the optimization of the solvent system for a given separation problem. In comparison with column chromatography, it only requires

small quantity of the compound and is much faster as well. A thin layer chromatography uses a thin, uniform layer of silica gel or alumina coated onto a piece of glass, metal or rigid plastic The mobile phase is a suitable liquid solvent or mixture of solvents [36]. The antioxidant activity of bael leaf was screened by using phosphomolybdenum assay. It is well that plant flavonoids and phenolics in general, are highly effective free radical scavenging and antioxidants. The phenolic compounds have been recognized as antioxidant agents, which act as free radical oxidation terminators [37] and have been known to show medicinal activity as well as for exhibiting physiological functions [38]. It has been reported that compounds such as the flavonoids, which contain hydroxyls, are responsible for the radical scavenging effects of most plants [39].Leaves of *A. marmelos*were found to contain, phenolic compounds, flavonoids, fats, triterpenoids, glycosides, carbohydrate and alkaloids as their chemical constituents.

In the discussion section, only general points about the planned work are addressed. The results of the current study are not discussed in detail, and specific values are lacking. It is important to correlate these results with existing research to justify the findings obtained.

5. CONCLUSION

On the basis of results obtained it can be concluded that *Aegle marmelos*leaves possess some phytoconstituents and potent antioxidant activities, It concludes that their presence and absence are solvent dependent and also depend upon methods. 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. Further. The present study also suggests the use of these medicinal plants may be exploited for health supplements. 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 requested from the corresponding author.

CONSENT AND ETHICAL APPROVAL

Not applicable

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