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ANTI-MICROBIAL AND ANTI-CANCER ACTIVITY OF *AEGLE MARMELOS* AND GAS CHROMATOGRAPHY COUPLED SPECTROMETRY ANALYSIS OF THEIR CHEMICAL CONSTITUENTS

Revathi Seemaisamy [1], Lukmanul Hakkim Faruck [2, 3], Sampath Gattu [1], Rameshkumar Neelamegam [4], Hamid A Bakshi [5], Luay Rashan [2], Mohammed Al-Buloshi [6], Sidgi Syed Anwar Abdo Hasson [6] and Kayalvizhi Nagarajan [* 1]

Department of Zoology [1], Periyar University, Salem - 636011, Tamil Nadu, India.
Research Center [2], Department of Mathematics and Sciences [3], College of Arts and Applied Sciences, Dhofar University, Salalah, Oman.
Department of Environmental Biotechnology [4], Insect Molecular Biology Laboratory, Bharathidasan University, Tiruchirappalli - 620024, Tamil Nadu, India.
Department of Pharmacy [5], School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield, United Kingdom.
Immunology Division [6], Department of Microbiology & Immunology, College of Medicine and Health Sciences, Sultan Qaboo's University, Oman.

Keywords:

Aegle marmelos, Phytochemical investigation, Antimicrobial activity, Anticancer activity, GC-MS analysis

Correspondence to Author:
Nagarajan Kayalvizhi

Department of Zoology,
Periyar University, Salem - 636011,
Tamil Nadu, India.

E-mail: kayalvizhinagarajan@gmail.com

ABSTRACT: In this study, we investigated anti-cancer and antimicrobial activity of *Aegle marmelos* leaf extracts and their chemical profile characterized by gas chromatography coupled mass spectrometry (GC-MS). *A. marmelos* leaves were extracted with acetone, methanol, ethanol, and chloroform. Presence of phenolic compounds was identified in these extracts by qualitative analysis. All the extracts were subjected for anti-bacterial activity against the different strains of bacteria (*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Bacillus ariyabattai, Bacillus megaterium, Pseudomonas putida, Klebsiella pneumonia, Serratia marcescens, and Escherichia coli*). It is noteworthy that acetone extract elicited maximum growth inhibition on *Serratia marcescens*. Based on profound anti bacterial activity, acetone and methanol extract of *A. marmelos* were checked for cytotoxicity against MDA-MB-231, HEp-2 and vero cells. MDA-MB-231 cells were more sensitive to acetone extract of *A. marmelos* with an IC_{50} value of 79.62 µg/ml where as HEp-2 cells are more sensitive to methanol extract of *A. marmelos* with an IC_{50} value of 47.08 µg/ml. Vero cells withstand 24 h treatment of both extract, and it is evidenced that both acetone and methanol extract of *A. marmelos* exhibited chemo sensitive property towards cancer cells. GCMS analysis was performed to characterize the active principles of acetone and methanol extracts of *A. marmelos*. GC MS data revealed the presence of ten major components. Overall, both acetone and methanol extract of *A. marmelos* found to be promising anti antibacterial and anti-cancer agent however the active principle of these should be isolated and characterized before reaching a concrete scientific conclusion.

INTRODUCTION: Nature has wealthy medicinal resources which can cure the ailment of humanity.

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Traditional medicine becomes an integral part of patient care and around 80% of the world's population depends wholly or partially on traditional medicine for its primary health care needs [1]. Phytochemicals obtained from medicinal plants became sources for getting novel drugs in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles

and lead compounds in synthetic drugs [2]. The Ayurvedic remedies, which are both preventive and therapeutic, are mostly of plant origin [3]. Preclinical studies with experimental animals have shown that many of the commonly used Ayurvedic plants are effective and have the potential to be of human use in future [3].

Aegle marmelos (L.) Correa (*A. marmelos*), commonly known as Bael belonging to the family Rutaceae, has been widely used in indigenous systems of Indian medicine due to its various medicinal properties. *A. marmelos* is native to Northern India, but widely found throughout the Indian Peninsula and in Ceylon, Burma, Bangladesh, Thailand and Indo-China [4]. It is a medium to large sized deciduous glabrous, an armed tree with the axillary and 2.5 cm long alternate, trifoliate leaves, short flower, and globular fruits [4]. According to Charaka (1500 BC), the high priest of Ayurveda, Bael is one of the most important medicinal plants in Ayurveda, which has been in existence for a long time and is extensively used by inhabitants of India [5]. A sweet drink prepared from the ripe fruit pulp of Bael is supposed to be effective against bacillary dysentery. The unripe fruits of Bael are reported to be useful in treating diarrhea, dysentery with spells of constipation, and stomach aches [5]. The roots are also one of the important ingredients of the Ayurvedic traditional drug Dashamula, a panacea for colitis, dysentery, diarrhea, flatulence, and fever [5]. The leaves are supposed to reduce bowel complaints, bleeding piles, dropsy (edema), diarrhea, and dysentery [5].

The essential oil and extracts obtained from wild varieties of *A. marmelos* reported for several biological activities such as antidiarrhoeal, Radiation protection, anticancer, Antipyretic, ulcer healing, chemoprevention, antigenotoxic agent, diuretic, anti-inflammatory, and anti-fertility [6]. However antibacterial and anticancer activity of the different solvent extract of *A. marmelos* is not yet reported. Solvents with different polarity can extract different bioactive compounds with diverse combinations from plants. Further, this could pave the way to achieve synergistic bioactive active efficacy of plant extracts. To address this issue in this study *A. marmelos* leaves extracted with acetone, methanol, ethanol, and chloroform and extracts were subjected for antibacterial activity against gram positive and gram negative bacteria and anti cancer activity.

MATERIALS AND METHODS:

Plant Materials: The fresh leaves of *A. marmelos* were collected from Foundation for Revitalization of Local Health Traditions, Bangalore, India (Latitude 12.9715987; Longitude 77.5945627). The voucher specimen (no. 2332) stored in the department of Botany, Periyar University, Salem.

Bacterial Cultures: The microbial strains were obtained from the IMTECH, Chandigarh India. The experiment Gram-positive bacteria's, like *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 441), *Bacillus megaterium* (MTCC 441), *Bacillus cereus* (MTCC 6840), *Bacillus arya-bhattai* (MTCC 14579), and Gram-negative bacteria such as *Pseudomonas putida* (MTCC 1194), *Escherichia coli* (MTCC 1302), *Klebsiella pneumonia* (MTCC 4727), *Serratia marcescens* (MTCC 4822) were grown in a nutrient broth at 37°C for 12 h.

Preparation of Plant Leaves Extracts: The fresh leaves of *A. marmelos* were collected from Foundation for Revitalization of Local Health Traditions, Bangalore, India and the fresh leaves were washed in running tap water, after it allowed to shade dried for 10 days and made into a fine powder of 40 meshes in size using the laboratory mill, 100 g of powder was filled in the thimble and extracted successively with 70% acetone, methanol, ethanol and chloroform taken in a clean flat-bottomed glass container and soaked for 72 h at room temperature. The container was sealed and kept into shaking chamber at three days for accompanying occasional shaking. Then the whole mixture was underwent a coarse filtration by a piece of clean, white cotton material. The extract was filtered through Whatmann no.1 filter paper, and it removes all unextractable matters, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated via dry air. The extracts were stored in sterile bottles at room temperature for further use.

Qualitative Analysis of Phytochemicals: Major constituents of plant extracts were screened qualitatively as per standard procedures described

by [7]. Major constituents like alkaloids, tannins, terpenoids, saponins, anthrao quinones, coumarins, carbohydrates, and steroids were analyzed.

GC-MS Analysis: GC-MS analysis of various crude organic extracts of *A. marmelos* leaves was performed on a Perkin Elmer Clarus 600 GC System, fitted with a Rtx-5MS capillary column (30 m 0.25 mm inner diameter, 0.25 μm film thickness; maximum temperature, 350°C), coupled to a Perkin Elmer Clarus 600°C MS. Ultra-high purity helium gas. The GC-MS analysis of both extracts was performed using GC-MS Perkin Elmer GC Model clarus 680, Mass Spectrometer clarus 600 (EI) Using Software Turbo Mass ver. 5.4.2 Library ver NIST-2008 SHIMADZU MS 2010 instrument equipped with AB in wax column (60 × 0.25 mm, film thickness 0.25 μm). Initially, the oven temperature was maintained at 60 °C for 2 min, ramp 10 °C/min and the temperature was gradually increased up to 300 °C hold 6 min, and 1μl of the sample was injected for analysis. Helium was carrier gas; the flow rate of helium gas was 1.2 ml/min. The sample injector and mass transfer line temperature were set at 24 °C and 250 °C and the split ratio is 10 throughout the experiment periods. The ionization mass spectroscopic analysis was done with 70 eV. Mass spectra were recorded across the range of 40 to 1000 m/z for 30 min.

Identification of components was based on a comparison of their mass spectra with those of Wiley and NIST libraries [8]. The identification and characterization of compounds in various crude extracts were based on GC-MS retention time. The mass spectra were computer matched with those of standards available in mass spectrum libraries.

Agar Well Diffusion Assay: The agar well diffusion method as adopted earlier (Perez and Bezique, 1990) briefly 0.1 ml of diluted inoculums (10^{-5} CFU/ml) of test organisms was swabbed in the agar plates. Wells of 8 mm diameter were punched into the agar medium and filled with 100 μl (150 mg/ml) of each plant extract. Plant crude extracts were diluted in the range of 10^{-1}, 10^{-2}, 10^{-3} and 100 μl from the stock solution of tested extracts concentration of 50 mg/ml was added in the plates, solvent blank to which the test bacteria were sensitive. The plates were incubated for 24 h at 37 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism [9].

Time-Kill Assay: The time-kill assay was performed by the broth macro dilution technique [10]. The extracts were incorporated into 50 μl of nutrient broth, were used for control medium untreated culture at of Luria broth at different hours, with the extract and the respective alone at the test organism to a final inoculums density of approximately 10^{-5} CFU/ml. Immediately after inoculation, aliquots (100 μl) of the negative control 18 h culture flasks were taken, respectively while in case of Gram-positive the combination of plant extract and range from at the 3, 6, 9, 12, 15, 18, 21, and 24 h time intervals, Death time was to determine the rate of bactericidal activity. Acetone extract of *A. marmelos* leaves was evaluated for the bactericidal activity effect against *Serratia marcescens*. The culture was harvested and added in the Luria broth for 3 ml in 27 test tube were taken and adding the 5% bacterial inoculums of 18 h fresh culture. The culture was incubated at different time intervals in orbital shaker 120 rpm for 24 h by measuring the optical density (OD) at 640 nm.

Cell Lines and Culture Method: MDA-MB-231, HEp-2, and Vero cells were purchased from ATCC, USA these cells were cultured in DMEM, MEM and RPMI 1640 medium respectively with 10% fetal bovine serum and 1% antibiotics (Penicillin / Streptomycin) and maintained in humidified cell incubator at 37 °C and 5% CO_2.

Drug Preparation: Stock solution of ethanol and methanol extracts of *A. marmelos* was prepared in dimethylsulfoxide (DMSO). Different concentrations of (25, 50, 75 and 100 μg/ml) of extracts were prepared.

3- (4, 5- dimethyl thiazolyl- 2)- 2, 5-diphenyl-tetrazolium bromide (MTT) assay: MDA-MB-231, HEp-2, and Vero cells ($1X10^5$/well) were seeded in 96 well plates (100 μl/well) and allowed to adhere firmly overnight in DMEM, MEM and RPMI 1640 medium respectively with 10% FBS. Then cells were treated with different concentration of freshly prepared extracts for 24 h. Then the medium was removed, and cells were incubated with MTT reagent (5 mg/ml) for 4 h and violet

crystals dissolved in DMSO and absorbance was read at 540/690 nm. The absorbance of control (without treatment) was considered as 100% cell survival. Doxorubicin was used as positive control.

Statistical Evaluation: Data presented as mean ± SD of two duplicates of three independent experiments. Experimental data were evaluated by students't' test and one or two-way analysis of variance (ANOVA). A significant difference between each set of data was considered at the confidence level of $p<0.05$ and $p<0.001$.

RESULTS:

Phytochemical Analysis: Qualitative phytochemical analysis revealed that active phenolic compounds such as alkaloids, flavonoids, tannins, and saponins were present in acetone, methanol, ethanol and chloroform extracts of *A. marmelos* **Table 1**.

TABLE 1: PHYTOCHEMICAL SCREENING OF *AEGLE MARMELOS* LEAVES EXTRACT USING VARIOUS SOLVENT

Phytochemicals constituents	Test	Various solvent extracts			
		AE	ME	EE	CE
Alkaloids	Test for alkaloids	+	+	+	-
Terpenoids	Test for Terpenoids	+	-	+	+
Flavonoids	Test for Flavonoids	+	+	-	+
	Test for alkaline	+	+	+	-
Saponins	Test for saponins	+	+	+	+
Tannins	Test for $FeCl_3$	+	+	+	+
Proteins	Test for millons	+	+	+	+
	Ninhydrin test	-	+	-	-
Sugar	Fehlings test	-	-	-	+
	Benedicts test	-	+	-	+
	Molichs test	-	+	-	-
	Iodine test	-	+	-	+
	Sugar test	-	+	-	-
Steroids	Test for glycosides	-	+	+	+
	Test for steroids	+	+	-	-
	Test for salkowskis	+	+	+	-
Glycosides	Keller-Kilani test	+	+	+	+
Phlobatannins	Test for phlobatannins	+	+	+	+
Quinones	Test for Quinones	+	+	+	+
Coumerin	Test for Coumerin	+	+	+	+

Agar-Well Diffusion Assay: *A. marmelos* leaves extracts inhibited the growth of all the tested bacteria and results were summarized in **Fig. 1**. The maximum zone of inhibition was observed in acetone extract treated *Serratia marcescens* (30.45 ± 0.2) followed by *S. aureus* (20.15 ± 0.1), *P. putida* (12.3 ± 0.1), *B. ariyabattai* (20.4 ± 0.3), *B. subtilis* (20.4 ± 0.2), *B. megaterium* (15.51 ± 0.1), *B. cereus* (20.4 ± 0.3) and *E. coli* (10.3 ± 0.3) whereas methanolic extract *A. marmelos* showed moderate activity against *S. aureus* (12.0 ± 0.1), *B. subtilis* (10. 0 ± 0.1), *S. marcescens* (9.9 ± 0.1), *B. ariyabattai* (13.3± 0.5), *B. megaterium* (11.2 ± 0.1), *P. putida* (8.4 ± 0.5) and *E. coli* (13.5 ± 0.2). Ethanolic and chloroform extracts were showed least antibacterial activity compared to acetone, and methanol extracts **Fig. 1**.

Time-Kill Assay: Since *S. marcescens* was most sensitive to acetone extract of *A. marmelos* and to address their growth inhibition pattern, time-dependent bactericidal activity of acetone extract of *A. marmelos* against *S. marcescens* was evaluated. The result indicated that the acetone extract of *A. marmelos* induced growth inhibition with respective to time duration **Fig. 2**.

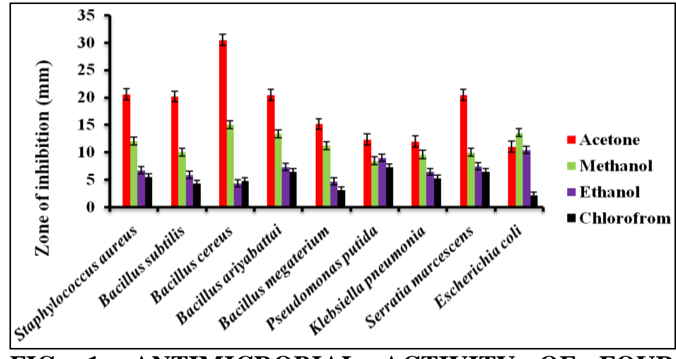

FIG. 1: ANTIMICROBIAL ACTIVITY OF FOUR EXTRACT FROM *A. MARMELOS*

Data presented as mean ± SD of triplicates of three independent experiments

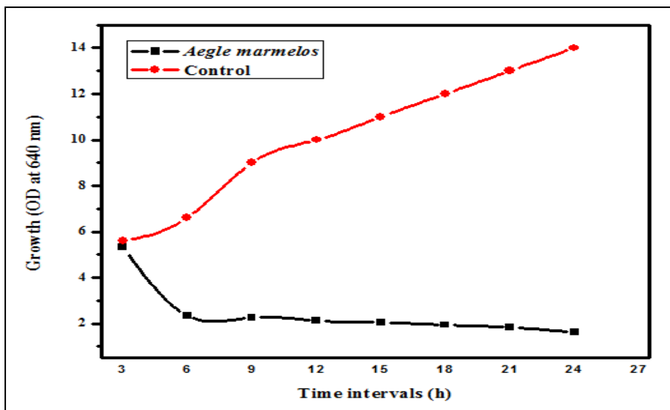

FIG. 2: *IN-VITRO* TIME-DEPENDENT KILLING ASSESSMENT OF THE CRUDE ACETONE EXTRACT OF *A. MARMELOS* AGAINST *SERRATIA MARCESCENS*

Cytotoxicity of Acetone and Methanol Extract of *A. marmelos* on MDA-MB-231, HEp-2, and Vero Cells: We tested the antiproliferative potential of the acetone and methanol extract of *A. marmelos* on MDA-MB-231, HEp-2 and vero cells (normal). We found that *A. marmelos* derived active principles elicited dose-dependent cell death on both MDA-MB-231 and HEp-2 cells after 24 h of treatment **Fig. 3** and **4**. **Table 2** depicts the IC_{50} value of *A. marmelos* methanol and acetone extract on MDA-MB-231 cells (79.62 and 61.79 µg/ml respectively) and HEp-2 cells (>100 and 47.08 µg/ml respectively). Interestingly, both methanol and acetone extract of *A. marmelos* did not exhibit significant cytotoxicity on normal Vero cells.

Further, both MDA-MB-231 and HEp-2 cells lost their adherence and morphology and were found to be necrotic based on their size and shape after 24 h of treatment with different concentrations of extract (data not shown). Conversely, the normal Vero cells withstood *A. marmelos* treatment, indicating that acetone and methanol extracts of *A. marmelos* have a specific chemo-sensitization property towards tumor cells.

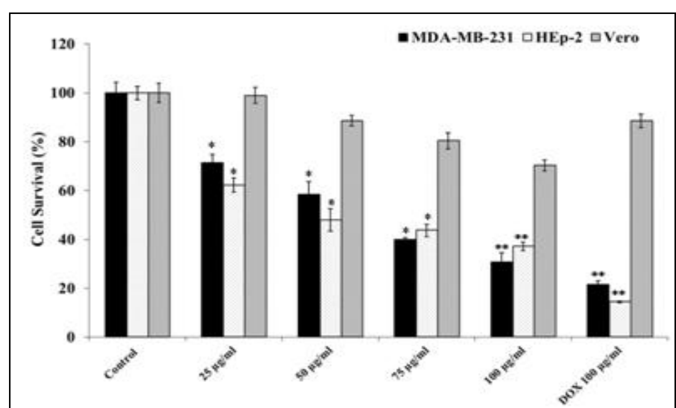

FIG. 3: CYTO-TOXICITY OF ACETONE EXTRACT OF *A. MARMELOS* ON MDA-MB-231, HEp-2 AND VERO CELLS. Values are presented as mean ± SD of four duplicates of three independent experiments. Asterisks indicates the significant difference compare to control (*: P < 0.05, **: P < 0.001)

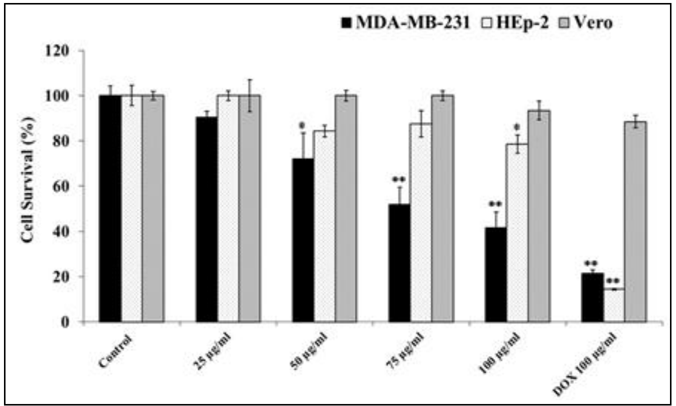

FIG. 4: CYTOTOXICITY OF METHANOL EXTRACT OF *A. MARMELOS* ON MDA-MB-231, HEp-2 AND VERO CELLS. Values are presented as mean ± SD of four duplicates of three independent experiments. Asterisks indicates the significant difference compare to control (*: P < 0.05, **: P < 0.001)

TABLE 2: IC_{50} VALUES OF METHANOL AND ETHANOL EXTRACT OF *A. MARMELOS* ON DIFFERENT CELL LINES

		IC_{50} (µ g/ml)		
		MDA-MB-231	**Hep-2**	**Vero**
A. marmelos	Acetone extract	79.62	>100	>100
A. marmelos	Methanol extract	61.79	47.08	>100

GCMS Analysis of *A. marmelos* Acetone and Methanol Extract: Based on the GC-MS chromatograms **Fig. 5** and **6**, acetone and methanol extract of *A. marmelos* have major components like benzoic acid, 4-[1-oxo-2-(1-pyrrolidinyl) ethyl] amino-, methyl ester, (RT: 10.457), 2, 2'-Dipiperidine (RT: 10.567), Diphenylmethane (RT: 12.027) Nonanoic acid (RT: 13.438). Oleic acid, trimethylsilyl ester (RT: 17.794), 10-bromo-decanoic acid, ethyl ester (RT: 17.924), Phytol (RT: 19.020), docosa-8, 14-diyn-cis-1, 22-diol, bis (trimethylsilyl) ether (RT: 28.499), Benzene, 1, 1', 1''-(1-ethanyl-2-ylidene) tris (RT: 29.159), and acetamide, N-[4-(chlorodifluoromethoxy) phenyl]-2-pyrrolidin-1-yl (RT: 15.639). **Table 3** illustrated respective compound structures.

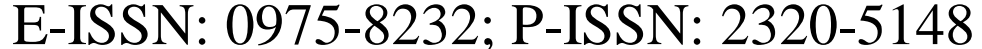

FIG. 5: GC-MS CHROMATOGRAM OF ACETONE EXTRACT OF *A. MARMELOS*

FIG. 6: GC–MS CHROMATOGRAM OF METHANOL EXTRACT OF *A. MARMELOS*

TABLE 3: PHYTOCOMPONENTS IDENTIFIED IN THE ACETONE AND METHANOL EXTRACT OF *A. MARMELOS* BY GC-MS

RT	Compound name	Molecular weight	Molecular formula	Compound structure
10.457	Benzoic acid, 4-[1-oxo-2-(1-pyrrolidinyl)ethyl]amino-, methyl ester	262	$C_{14}H_{18}O_3N_2$	
10.567	2,2'-dipiperidine	168	$C_{10}H_{20}N_2$	

12.027	Diphenylmethane	168	$C_{13}H_{12}$	
13.438	Nonanoic acid	158	$C_9H_{18}O_2$	
17.794	Oleic acid, trimethylsilyl ester	354	$C_{21}H_{42}S_2SI$	
17.924	10-bromodecanoic acid, ethyl ester	278	$C_{12}H_2O_2BR$	
19.020	Phytol	296	$C_{20}H_{40}O_2$	
28.499	Docosa-8,14-diyn-cis-1,22-diol, bis (trimethylsilyl) ether	478	$C_{28}H_{54}O_2SI_2$	
29.159	Benzene, 1,1',1''-(1-ethanyl-2-ylidene)tris-	258	$C_{20}H_{18}$	
11.392	Acetamide, n-[4-(chlorodifluoromethoxy)phenyl]-2-pyrrolidin-1-ylo	304	$C_{13}H_{15}O_2N_2CLF_2$	

DISCUSSION: Traditional use of plants as medicines provide the basis for indicating which plant-based formulation useful for specific therapy. Finding a drug from natural sources is always encouraged by world health organization due to its less toxic nature. Emerging drug resistance of infectious bacteria and cancer cells is remain resolvable challenges, and it demands the need of new formulation which can overcome clinical hurdles. In this study, we tested antibacterial and anticancer activity of *A. marmelos* extracts. Agar well dilution method was employed to assess the antibacterial activity of extracts. It is well reported that gram-positive bacteria are more sensitive than gram-negative bacteria to plant-based organic extracts [11].

In our study, all the tested bacteria are sensitive to extracts. Interestingly, *Serratia marcescens* was found to be more sensitive while *Klebsiella pneumonia* was found to be less sensitive to plant organic extract than other organisms. The antimicrobial action might be due to the presence of compounds like terpenoids, tannins, deoxy sugars, saponins, phenolic compounds, and flavonoids. The antibacterial activity principles or chemical constituents can be effectively extracted only through the organic solvent medium [12]. Previously extract of *A. marmelos* has shown antimicrobial activity against *Vibrio cholerae, Salmonella typhimurium, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus* [13].

Similarly, aqueous, acetone and the petroleum ether extract of *A. marmelos* leaves were found to be effective against *Bacillus coagulans, Bacillus subtilis, Bacillus thuringiensis, Pseudomonas aeruginosa* and *Staphylococcus aureus* [6]. Noticeably presence of terpenes, phenolic compounds of *A. marmelos* exhibited antibacterial and antifungal activity [4]. *In-vitro* time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent which is a reliable method for determining tolerance [10]. In this study, we found time-dependent growth inhibition of *S. marcescens* upon acetone extract treatment. After 24 h, the growth pattern of *S. marcescens* has been declined significantly and indicates that bactericidal efficacy of *A. marmelos*.

Further, we found the significant anti-cancer potential of *A. marmelos* extracts (methanol and acetone) on tumor cells such as (MDA-MB-231 and HEp-2) by sparing normal Vero cells. As per the guidelines of the National Cancer Institute, USA, we set 24 h as a treatment period and since this time point is widely used for initial drug screening. A limitation of our study is that we did not include respective normal cells of tumor tissue

origin; instead, we used vero cells as a normal control for the *in-vitro* MTT assay. Vero cells are fibroblasts derived from the adult African monkey kidney [14] fibroblastic cells are known to be associated with every tissue. Also, fibroblast cells support tissue architecture and play a pivotal role in drug response.

Some *in-vitro* anti-cancer studies used vero cells as normal controls to screening crude extracts. Chemosensitive property of acetone and methanol extract of *A. marmelos* towards tumor cells is not clear. However, it is attributed that synergistic mixture of compounds present in the extracts could induce irreparable DNA damage or induced apoptosis in cancer cells. The molecular mechanism of *A. marmelos* extracts induced cancer cell death should be studied in detail.

CONCLUSION: In conclusion, acetone and methanol extracts of *A. marmelos* showed significant antimicrobial activity against tested microorganisms. *S. marcescens* was the most sensitive among tested organism and had the high zone of inhibition in all the four extracts of *A. marmelos.* The phytochemical analysis of acetone, methanol, ethanol and chloroform extracts of *A. marmelos* leaves revealed that presence of biologically active phytochemicals.

Also we found the significant anti-cancer potential of *A. marmelos* extracts (methanol and acetone) on tumor cells such as (MDA-MB-231 and HEp-2) by sparing normal vero cells. Isolation and characterization of active principles from *A. marmelos* extract are warranted to define antimicrobial and anti-cancer formulation.

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CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest.

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