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In-vitro study: Immunomodulatory and cytotoxicity effects of ethanolic leave extracts of *Aegle marmelos* and *Ficus benghalensis*

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ABSTRACT

Objective: To assess the immunomodulatory and antibacterial properties of the coastal trees *Aegle marmelos* (*A. marmelos*) and *Ficus benghalensis* (*F. benghalensis*) by *in-vitro* methods.
Methods: *A. marmelos* and *F. benghalensis* leaves were extracted with the solvents di-ethyl ether, ethanol and methanol. The extracts were investigated for antibacterial activity against human pathogenic bacteria and immunomodulatory activity against human peripheral blood mononuclear cells (PBMC), respectively.
Results: *F. benghalensis* ethanol cold extract gave maximum inhibition zones against *Escherichia coli* NCIM 2931 (B2) at 18 mm and enhanced the growth of human PBMC. It was non-toxic to human PBMC shown by the Tryphan blue dye exclusion method, sulforhodamine B and MTT assays. IR phyto-chemical analysis demonstrated the presence of the functional groups: NH, CH_2, CH_3, CO, OH, -C-C=O, and C-S. We confirmed the presence of quantifiable amounts of tannins and alkaloids with traces of phenolic compounds by thin layer chromatography analysis.
Conclusions: Leaves of *A. marmelos* and *F. benghalensis* provide both antibacterial and immunomodulatory properties.

1. Introduction

Traditional and ethnic medicines play an important role in health services around the world. About three quarters of the world population relies on plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity. Here as elsewhere were plants used since ancient times for the treatment of many ailments and diseases due to the presence of various novel and complex substances in plants. These are of different chemical composition as secondary metabolites. These compounds have properties such as wound healing, phagocytic, and anti-cellular properties with humoral immune response. Plants based immunomodulators were reported with certain valuable chemical constituents and significant results in clinical trials[1].

The isolation of antibiotic resistant bacteria has highlighted the need for reliable antimicrobial agents. It is reported that, on average, two or three antibiotics derived from microorganisms are launched every year[2]. After a slight downturn, scientists with the rise of antibiotic resistance worldwide recently realized that new antibiotic are needed. Therefore, the use of plant extracts, as well as other alternative forms of medical treatments, was obtaining much popularity in the last two decades. The major benefit of these medicinal plants is that they have fewer side effects. Therefore, immunomodulatory and antimicrobial agents from medicinal plants have become fundamental principles of several therapeutic approaches.

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The human immune system is a defense system that has evolved to protect us from invading pathogenic microorganisms, cancer and other diseases. It consists of primary lymphoid organs, secondary lymphoid tissues, immunoreactive cells and mediators which are capable of recognizing the apparently limitless variety of foreign invaders specifically or non-specifically. Such responses keep the host healthy[3].

This is being done by two interrelated activities of the immune system, *i.e.* recognition and response. The recognition could be specific or non-specific. An immune response can be functionally divided into three components: immunosupression, immunopotentiation, and immunotolerance. Immunological recognition is remarkably specific. The immune system is able to recognize subtle chemical differences that distinguish foreign molecules and the body's own cellular products. Once foreign material is recognized, the immune system enlists the participation of a variety of cells and molecules to an appropriate response, known as an effector function in order to eliminate or neutralize the foreign material. In this way the system is able to convert the initial recognition event into different effector responses, each uniquely suited to eliminate a particular type of pathogen or foreign material[4].

Under some conditions, the activation or efficacy of the immune system is reduced which is known as immunosuppression. Some parts of the immune system may have immunosuppressive effects on other parts of the immune system, and hence immunosuppression may provide an adverse reaction. This holds for instance for autoimmunity – where the immune system responds to itself by recognizing self as nonself. Immunosuppression may also be useful in conditions like transplantation. Here, to avoid rejection of transplanted foreign material, immunosuppressive drugs are given. In contrast, immunopotentiation represents an enhancement of the immune response by increasing its rate or prolonging its duration which is also useful in conditions like immunodeficiency diseases[5].

Compounds that are capable of altering the immune response are termed immunomodulators[6]. Immunomodulators change weak immune systems that are less active or that are overactive (hypersensitive), or that react on itself (autoimmunity). In addition they do boost a weak immune system, *i.e.* conditions like immunodeficiency disorders. These are also known as biological response modifiers, adaptogens, immunoaugmentors or immunorestoratives[7]. Immunomodulators could be synthetic, such as glucocorticoids, cyclosporines, tacrolimus, sirolimus, everolimus, and azathioprine[8], or natural. Natural immunomodulators are derived from terrestial and aquatic (marine and fresh water) plants, animals and microbes.

Aegle marmelos (*A. marmelos*) belonging to the plant family Rutaceae, has been used for centuries as a medicinal plant. All parts of the plant like root, bark, leaves and fruits have medicinal properties. The root is one of the ingredients of the group dasamula, used in Ayurveda[9,10]. Different parts of the tree have been reported to possess various pharmacological activity such as anti-diabetic, antiulcer, analgesic, anti-inflammatory, anti-pyretic, anti-thyroid, anti-hyperlipidemic, anticancer, and antimicrobial[11-13]. *Ficus benghalensis* (*F. benghalensis*) belongs to the family Moraceae and is known for its high medicinal values since ancient times. Various parts like aerial roots, bark, leaves, fruit, and latex serve as good medicine[14,15]. It has been reported to possess important pharmacological activities such as antidiabetic, hypolipidemic, anti-helminthic, anti-stress, antiallergic, antioxidant, anti-inflammatory, wound healing and growth promoting[16,17]. The present study has been undertaken to test the extracts of these two medicinal plants for their immunomodulatory properties on human peripheral blood mononuclear cells (PBMC) and antibacterial properties on human pathogenic bacteria (HPB).

2. Materials and methods

2.1. Collection and preprocessing of plant leaves

Fresh specimens of coastal *A. marmelos* and *F. benghalensis* were collected from Mannarpuram (78°41'12" E and 10°47'26" N) in Tiruchirappalli, Tamil Nadu, India. Voucher specimens of leaves, flowers, and roots of both species were deposited at the Department of Industrial Biotechnology, Bharathidasan University. The leaves were hand-picked and thoroughly washed with tap water followed by distilled water to remove adhering debris, associated epifauna/epiphytes, dust and other materials. After cleaning, the sample leaves were dried in the shade at room temperature for a week[18]. Dried leave material was cut into small pieces and preserved at a temperature of 28 ℃ until use.

2.2. Preparation and extraction of bioactive substances from plant leaves

The pulverized moisture free/dried leaves (*A. marmelos*) and (*F. benghalensis*) material 500 g was extracted in 1 L with the same volume of different organic solvents (v/v) that showed increasing polarity like di-ethyl ether (DEE), ethanol (C_2H_6O) and methanol (MeOH) were added to obtain a natural concentration (cold extraction method for 24 h at 0 ℃ and soaking extraction method for 24 h at (32 ± 2) ℃ room temperature. The total extract was filtered and the obtained filtrate (crude extract) was concentrated in a rotary evaporator at 40 ℃ to dryness. The dried extracts were then dissolved in phosphate buffered saline (pH 7.2), filter-sterilized by Millipore membrane filters (pore size: 0.22 μm) and stored in vials for further study.

2.3. Antimicrobial screening of HPB

Disc diffusion assay was performed using Muller–Hinton agar to assess the human pathogen strains (HPS) effect of the medicinal plant extracts (*A. marmelos* and *F. benghalensis*) in different solvents DEE, C_2H_6O and MeOH extracts (soaking and cold). HPB such as *Bacillus subtilis* NCIM 2920 (B1) (*B. subtilis*); *Micrococcus luteus* NCIM 2871

(B4) (*M. luteus*); *Staphylococcus aureus* NCIM 5021 (B8) (*S. aureus*) and *Staphylococcus epidermis* NCIM 2871 (B9) (*S. epidermis*) and negative *Escherichia coli* NCIM 2931 (B2) (*E. coli*); *Klebsiella pneumonia* NCIM 2883 (B3) (*K. pneumonia*); *Proteus mirabilis* NCIM 2241 (B5) (*P. mirabilis*); *Pseudomonas aeruginosa* NCIM 5029 (B6) (*P. aeruginosa*); *Salmonella typhimurium* NCIM 2501 (B7) (*S. typhimurium*); *Vibrio cholera* MTCC 2501 (B10) (*V. cholera*). These cultures were obtained from the Microbial Type Culture Collection (MTCC) at Chandigarh and from the National Chemical Industrial Microorganism Collections (NCIM), in Pune, India.

A sterile cotton swab was used to inoculate the standardized bacterial suspensions (test culture suspensions prepared in sterile 0.85% saline matching an optical density of 0.5 McFarland standards corresponding to 10^8 CFU/mL on the surface of agar plates for homogeneous growth. Rotary evaporator dried seaweed extract of soaking and soxhlet DEE, C_2H_6O and MeOH was dissolved in the same solvent. It was further sonicated in order to prevent the agglomeration of particles. The inoculated agar was poured into the assay plate (Petri dishes 90 mm in diameter) and allowed to cool down. Sterilized paper discs, containing the extract of the leaves (2 mg), were transferred onto these prepared Petri dishes. Oxytetracycline (Merck, Germany) was used as a positive, the solvent of each extract as a negative control. A pre-diffusion for 3 h was guaranteed and inhibition zones were measured after 24–48 h at an incubation at (37 ± 1) ℃. The inhibition zones were measured excepting the 6 mm paper disc. After incubation, the different zones of inhibition were measured with the help of HiAntibiotic ZoneScale-C. Assays were performed in triplicate and average values were presented.

2.4. Phytochemical analysis of leave extracts

All extracts from the two plants were screened for secondary metabolites like alkaloid, flavonoid, glucoside, steroid, saponin, tannin, anthraquinone, phlobatannin, terpenoid and cardiac glycoside by standard methods for various phytochemical constituents carried out using standard methods[19] as described in Table 1. The extract was characterized by subjecting the extract to UV-visible spectrophotometer (λ35, Perkin Elmer, Germany). IR absorption spectrum was seen with Fourier transformed infrared spectroscopy (FTIR) analysis (Made spectrum RX 1, Male Perkin Elmer, range 4000 cm^{-1} to 400 cm^{-1}) at CSIR-CECRI, Laboratory in Karaikudi, Tamil Nadu, India. Secondary metabolites were qualitatively analyzed by thin layer chromatography (TLC).

2.5. In-vitro bioactivity test for immunomodulatory and cytotoxicity assessment

PBMCs were separated from whole blood of a healthy donor by density gradient centrifugation[20]. The PBMCs were isolated under sterile conditions in RPMI-1640 medium. The viability of PBMCs was determined by tryphan blue dye and the viable cell count was made by using haemocytometer and light microscope (Nikon). The concentration of PBMCs was adjusted to 1×10^6 cells/mL. The cell suspension was cultured in RPMI-1640 complete media (with 10% fetal calf serum) with different concentrations of plant extracts in 96-well flat-bottomed microplates (Greiner, USA) and incubated in a CO_2 incubator (TC2323, Shel lab, USA) with 37 ℃, 5% CO_2 and 95% relative humidity. Various negative controls like plain media, complete media, vehicle control, cell, extract and positive control like a known immunomodulator phytohemagglutinin and a known cytotoxic compound. Lipopolysaccharide were also maintained.

After 24-h incubation, the toxicity of the extracts was assessed by tryphan blue dye exclusion method. The total protein content of the cells after treatment with extract were assessed by sulforhodamine B (SRB) assay[21] after 48 h using an ELISA reader (Bio-Rad). The mitochondrial enzyme dehydrogenase present in the living cells was assessed by MTT assay[22] after 72 h using an ELISA reader (Bio-Rad). Interleukin (IL)-2 was quantified after activating the mononuclear cells (PBMC) with mitogen in the presence of extracts by making use of IL-2 EASIA kit manufactured by M/s. BioSource, Belgium and supplied by M/s. Immunoshop, Chennai.

3. Results

3.1. Antimicrobial screening of HPB

The results of leave extracts from the soaking method in various polarity solvents against HPB inhibition zone (mm) were provided

Table 1
Phytochemical screening for leave (*A. marmelos* and *F. benghalensis*) extracts.

No.	Phytoconstituents	Test	Observation
1	Tannins (Braymer's test)	2 mL extract + 2 mL H_2O + 2–3 drops $FeCl_3$ (5%)	Green precipitate
2	Flavonoids	1 mL extract + 1 mL $Pb(OAc)_4$ (10%)	Yellow coloration
3	Terpenoids	2 mL extract + 2 mL $(CH_3CO)_2O$ + 2–3 drops conc. H_2SO_4	Deep red coloration
4	Saponins (Foam test)	(a) 5 mL extract + 5 mL H_2O + heat (b) 5 mL extract + olive oil (few drops)	Froth appears Emulsion forms
5	Steroids (Salkowski test)	2 mL extract + 2 mL $CHCl_3$ + 2 mL H_2SO_4 (conc.)	Reddish brown ring at the junction
6	Phlobatanins (Precipitate test)	2 mL extract + 2 mL HCl (1%) + heat	Red precipitate
7	Glycosides (Liebermann's test)	2 mL extract + 2 mL $CHCl_3$ + 2 mL CH_3COOH	Violet to blue to green coloration
8	Anthraquinones (Borntrager's test)	3 mL extract + 3 mL benzene + 5 mL NH_3 (10%)	Pinkish red to bluish violet coloration
9	Glycosides (Liebermann's test)	2 mL extract + 2 mL $CHCl_3$ + 2 mL CH_3COOH	Violet to blue to green coloration
10	Alkaloids (Hager's test)	2 mL extract + few drops of Hager's reagent	Yellow precipitate

in Tables 2–4. The *A. marmelos* C_2H_6O soaking extracts of the maximum zones of inhibition were observed against *E. coli* and *K. pneumonia* at 13 mm. The methanol soaking extract did not provide any activity. The maximum zones of inhibition were observed in the following order C_2H_6O > DEE > MeOH.

The obtained HPS inhibition zones from the cold extract method were given in Tables 5–7. The ethanol extracts of *F. benghalensis* showed a considerable inhibition zone of 16 and 15 mm against *E. coli* and *K. pneumonia*. DEE and methanol cold extract of *A. marmelos* showed a significant activity at 13 mm against *B. subtilis*.

Table 2

Antibacterial activity of DEE soaked [(32 ± 2) ˚C] leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 μg	40 μg	60 μg	80 μg	20 μg	40 μg	60 μg	80 μg
1	*B. subtilis*	-	-	-	10	-	-	-	10
2	*M. luteus*	-	-	11	11	-	-	-	-
3	*S. aureus*	-	-	-	-	-	-	-	-
4	*S. epidermis*	-	-	-	-	-	-	-	10
5	*E. coli*	-	-	10	10	-	-	10	10
6	*K. pneumoniae*	-	-	-	-	-	-	-	-
7	*P. mirabilis*	-	-	-	-	-	-	10	10
8	*P. aeruginosa*	-	-	-	-	-	-	-	-
9	*S. typhimurium*	-	-	-	-	-	-	10	11
10	*V. cholera*	-	-	10	10	-	-	11	11

Table 3

Antibacterial activity of C_2H_6O soaked [(32 ± 2) ˚C] leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 μg	40 μg	60 μg	80 μg	20 μg	40 μg	60 μg	80 μg
1	*B. subtilis*	-	-	11	11	-	-	-	10
2	*M. luteus*	-	-	10	10	-	-	-	11
3	*S. aureus*	-	-	-	10	-	-	-	10
4	*S. epidermis*	-	-	11	11	-	-	-	11
5	*E. coli*	-	10	10	13	-	10	10	10
6	*K. pneumoniae*	-	-	10	13	-	-	-	-
7	*P. mirabilis*	-	-	-	10	-	-	10	12
8	*P. aeruginosa*	-	-	-	-	-	-	-	10
9	*S. typhimurium*	-	-	-	10	-	-	-	-
10	*V. cholera*	-	-	-	10	-	-	-	-

Table 4

Antibacterial activity of MeOH soaked [(32 ± 2) ˚C] leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 μg	40 μg	60 μg	80 μg	20 μg	40 μg	60 μg	80 μg
1	*B. subtilis*	-	-	-	-	-	10	10	12
2	*M. luteus*	-	-	-	11	-	-	10	10
3	*S. aureus*	-	-	10	10	-	-	-	10
4	*S. epidermis*	-	-	-	10	-	-	-	-
5	*E. coli*	-	10	11	11	-	10	10	10
6	*K. pneumoniae*	-	-	-	10	-	-	-	10
7	*P. mirabilis*	-	-	-	11	-	-	11	12
8	*P. aeruginosa*	-	-	-	-	-	-	-	10
9	*S. typhimurium*	-	-	-	10	-	-	-	-
10	*V. cholera*	-	-	10	11	-	-	11	11

Table 5

Antibacterial activity of DEE cold (0 ˚C) soaked leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 μg	40 μg	60 μg	80 μg	20 μg	40 μg	60 μg	80 μg
1	*B. subtilis*	-	-	10	11	-	-	-	-
2	*M. luteus*	-	-	-	-	-	-	-	10
3	*S. aureus*	-	-	-	-	-	-	-	-
4	*S. epidermis*	-	-	10	11	-	-	-	-
5	*E. coli*	-	10	10	10	10	10	10	12
6	*K. pneumoniae*	-	-	-	11	-	-	-	13
7	*P. mirabilis*	-	-	10	10	-	-	-	-
8	*P. aeruginosa*	-	-	-	11	-	-	-	10
9	*S. typhimurium*	-	-	-	-	-	-	-	-
10	*V. cholera*	-	-	-	-	-	10	10	11

Table 6
Antibacterial activity of C_2H_6O cold (0 °C) soaked leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 µg	40 µg	60 µg	80 µg	20 µg	40 µg	60 µg	80 µg
1	*B. subtilis*	-	-	10	13	-	-	10	10
2	*M. luteus*	-	-	-	10	-	10	11	13
3	*S. aureus*	-	-	-	-	-	10	10	10
4	*S. epidermis*	-	-	11	12	-	-	-	12
5	*E. coli*	-	10	13	13	-	10	10	18
6	*K. pneumoniae*	-	-	-	10	-	10	10	15
7	*P. mirabilis*	-	10	10	12	-	-	10	10
8	*P. aeruginosa*	-	-	-	-	-	-	10	10
9	*S. typhimurium*	-	-	-	10	-	-	11	11
10	*V. cholera*	-	-	10	11	-	-	10	10

Table 7
Antibacterial activity of MeOH cold (0 °C) soaked leaves (*A. marmelos* and *F. benghalensis*) extract against HPS.

No.	HPS	*A. marmelos* zones of inhibition (mm)				*F. benghalensis* zones of inhibition (mm)			
		20 µg	40 µg	60 µg	80 µg	20 µg	40 µg	60 µg	80 µg
1	*B. subtilis*	-	-	10	11	-	-	10	12
2	*M. luteus*	-	-	-	-	-	-	-	13
3	*S. aureus*	-	-	-	-	11	11	12	12
4	*S. epidermis*	-	-	10	10	-	-	-	11
5	*E. coli*	-	10	11	11	-	-	12	12
6	*K. pneumoniae*	-	-	-	12	-	-	-	13
7	*P. mirabilis*	-	-	10	14	-	10	10	10
8	*P. aeruginosa*	-	-	-	10	-	-	-	10
9	*S. typhimurium*	-	-	10	10	-	11	11	12
10	*V. cholera*	-	-	11	12	10	12	12	13

Maximum inhibition zones were observed in the following order C_2H_6O > MeOH > DEE with the cold method.

3.2. Phyto-chemical analysis of leave extracts

These tests revealed the presence of various bioactive secondary metabolites which might be responsible for their antimicrobial attributes against HPS. The observations and inferences made in the phytochemical tests are presented as follows:

Tannins: A green precipitate was observed in ethanol extracts indicating the presence of tannins in both medicinal plants studied here.

Flavonoids: A yellow color was observed in all the ethanol extracts indicating thereby the presence of flavonoids in both medicinal plants.

Terpenoids: A deep red color was observed in two extracts out of six extracts.

Phlobatannins: Presence of a red precipitate in *A. marmelos* cold extract showed the presence of phlobatannins.

Anthraquinones: Absence of a pink, violet or red coloration in the ammonical layer indicated the absence of free anthraquinones in all six extracts.

Glycosides: Similarly, a colour change from violet to blue to green confirming the presence of glycosides was also observed in C_2H_6O extracts of *A. marmelos* and *F. benghalensis*.

Saponins: Persistent frothing on warming the extract did not indicate the presence of saponins in these plants. The same extract with few drops of olive oil formed a soluble emulsion, confirming the presence of saponins.

Alkaloids: A yellow colour precipitate was not observed in six extracts confirming thereby the presence of alkaloids.

Steroids: A reddish brown ring at the interface was observed only with the extract of *A. marmelos* out of six screened extracts indicating the presence of steroids only in this plant.

Cardiac glycosides: Colour change from violet to blue to green confirmed the presence of glycosides which were also observed in ethanolic leaf extracts of *A. marmelos* and *F. benghalensis*.

UV-spectrum, the C_2H_6O extract was found to have five compounds with absorption maxima ranging between 214.971 nm and 665.030 nm for *A. marmelos* and four compounds with absorption maxima ranging between 276.547 nm and 664.940 nm for *F. benghalensis* (Figure 1). The active functional compound identified by IR spectrum showed the presence of functional groups (like N-H, CH_2, CH_3, C=O, OH, -C-C=O, C-S) in *A. marmelos* and C-S, OH, C=O, CH_2 and CH_3 in *F. benghalensis* (Figure 2). Based on the antimicrobial study, the active extract was further confirmed by TLC analysis showing the presence of quantifiable amount of tannins and alkaloids with traces of phenolic compounds (Table 8 and Figure 3).

Table 8
TLC profiles of the C_2H_6O extracts.

Plant	Mobile phase	No. of spots	R_f values	Probable compounds
A. marmelos	Butanol: acetic acid: water 4:1:5	2	0.15 & 0.62	Alkaloids, tannins
F. benghalensis	Butanol: acetic acid: water 4:1:5	2	0.15 & 0.69	Saponins, tannins

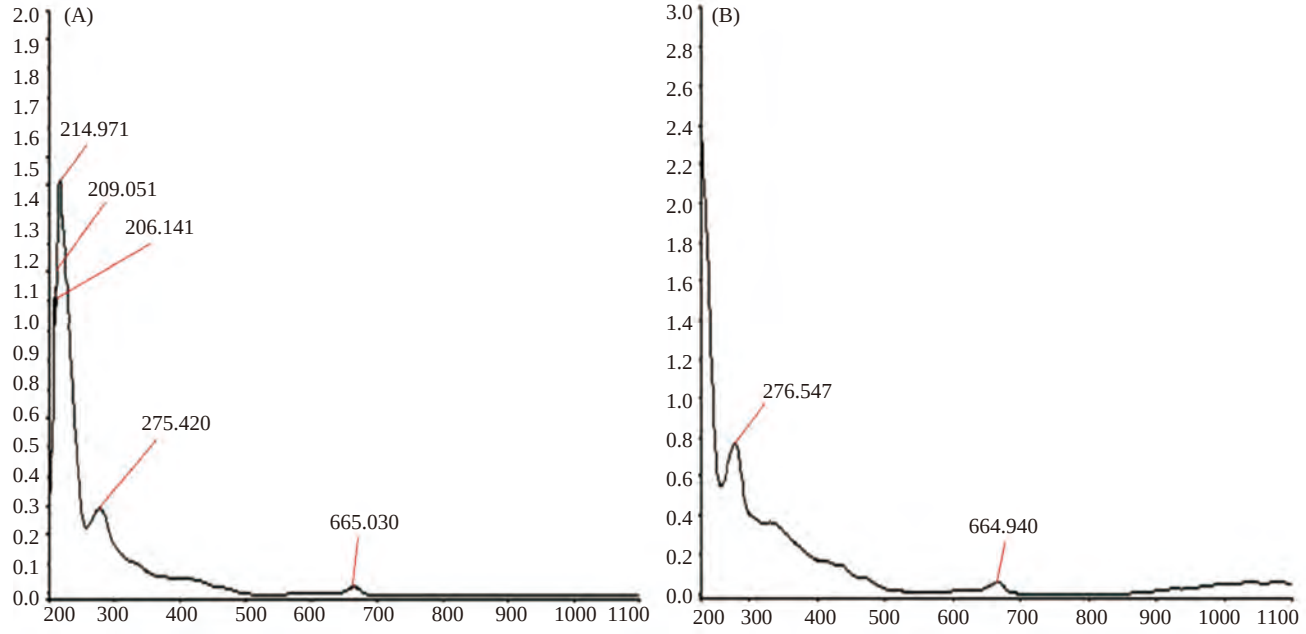
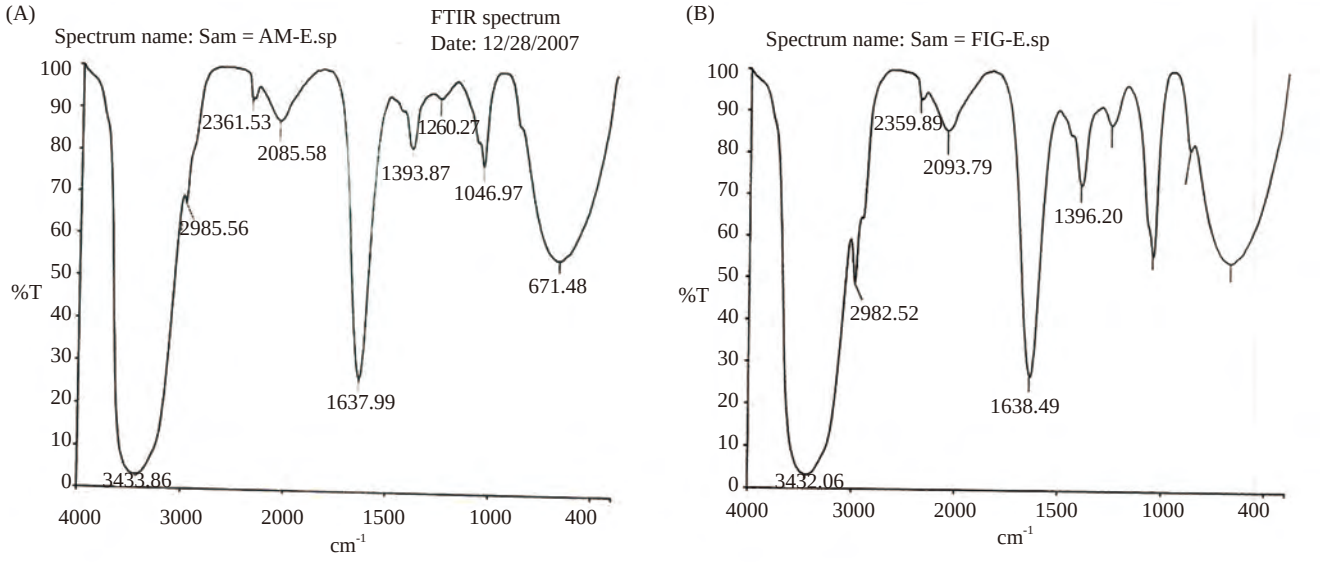


Figure 1. UV spectrum of (A) *A. marmelos* and (B) *F. benghalensis.*



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3433.86 3.16 2985.56 67.53 2361.53 92.15 2085.58 87.62 1637.99 26.85
1393.87 81.53 1260.27 93.60 1046.97 77.56 671.48 55.48

Sam = FIG-E.pk
SAM_FI~2.SP 3601 4000.00 400.00 3.17 100.00 4.00 %T 5 0 0.50
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3432.06 3.17 2982.52 48.72 2359.89 92.78 2093.79 85.33 1638.49 26.94
1396.20 72.34 1253.71 86.73 1048.48 55.48 874.46 80.83 673.90 54.17

Figure 2. FTIR spectrum of (A) *A. marmelos* and (B) *F. benghalensis*.

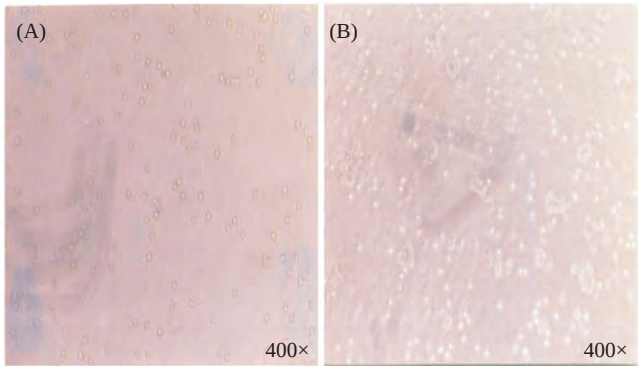

Figure 3. (A) Control-normal PBMC and (B) positive control PBMC treated with immunomodulator phytohemagglutinin.

3.3. In-vitro bioactivity test for immunomodulation and cytotoxicity assessments

The C_2H_6O extract was found to be non-toxic to human PBMC by dye exclusion method. In the sense, it neither affects the cell morphology (cytopathologic) nor the nucleus and/or the other organelles (cytotoxic) (Tables 9, 10 and Figure 4). The protein concentration of the cells after treatment with the extract was found to be more, particularly at 160 µg concentration for *A. marmelos* and there was no change in the protein content for *F. benghalensis* shown by the SRB assay (Figure 5A). There was an increase in the mitochondrial enzyme dehydrogenase of PBMC when treated with

plant extracts by MTT assay (Figure 5B). Overall results revealed that the extract was nontoxic and immunomodulatory to human PBMC by all the three assays performed. The concentration of IL-2 was found to be 160 µg concentration for *A. marmelos* and 40 µg concentrations for *F. benghalensis* (Figure 6).

Table 9

Effect of the C_2H_6O extracts on human PBMC by dye exclusion method.

Name of the plant	Nontoxic	Cytopathic	Cytotoxic	Immunomodulatory
A. marmelos	+	-	-	+
F. benghalensis	+	-	-	+

+: Yes; -: No.

Table 10

Effect of the C_2H_6O extracts on human PBMC *in-vitro* study.

Name of the plant	Dye exclusion method	SRB assay	MTT assay
A. marmelos	Nontoxic	Immunomodulatory	Immunomodulatory
F. benghalensis	Nontoxic	Immunomodulatory	Immunomodulatory

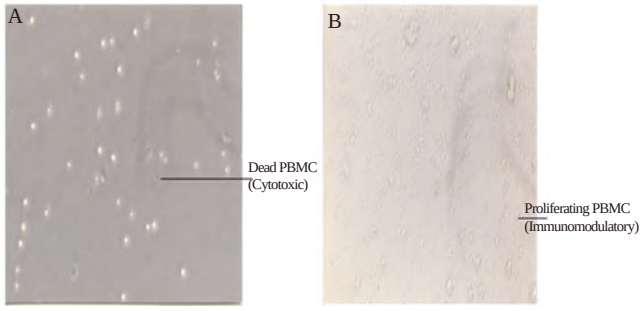

Figure 4. Ethanolic extract effects on human PBMC indicated by the dye exclusion method.

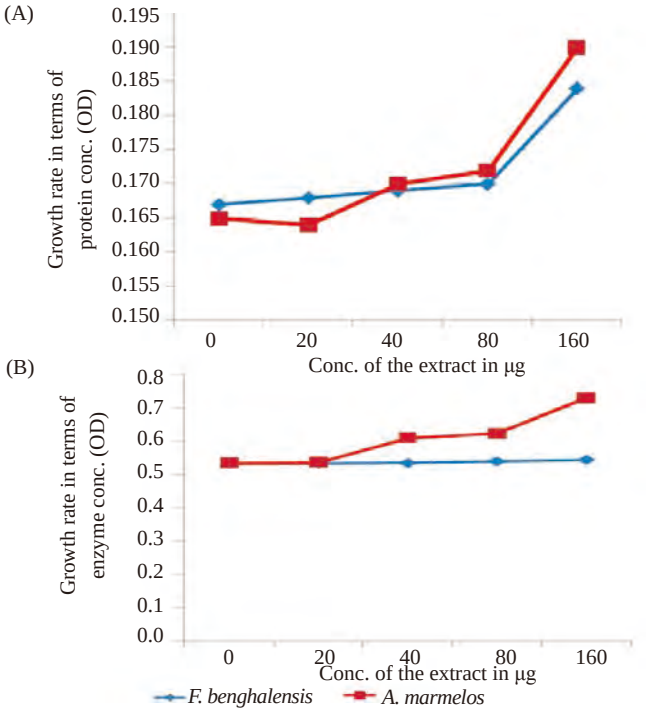

Figure 5. Effect of the extracts on human PBMC (A) SRB assay and (B) MTT assay.

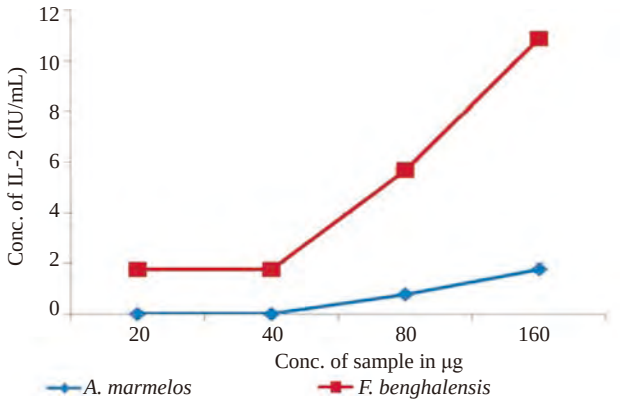

Figure 6. Effect of the extracts on human PBMC IL-2 product.

4. Discussion

In India, plants have always provided the foundation of medicinal drugs and presently they are increasingly popular throughout the world, as people focus on staying healthy with natural means in the face of chronic stress and pollution. They try to combat illness with medicines that support the body's own defense systems[23]. Recognition of the biological properties of plant products has fueled the current focus in this field, namely, the search for new drugs, antibiotics, insecticides and herbicides[24].

Available literature revealed that the leaves of *A. marmelos* are used as anti-inflammatory, expectorant, anticatarrahal, antiasthmatic, antiulcerous and ophthalmic[25]. *F. benghalensis* is traditionally used as astringent, depurative, anti-inflammatory, ophthalmic, stypic, antiarthritic, diaphoretic, antidiarrhoeic, and antihelmenthic[26]. This was also the reason for choosing these two plants in this preliminary study. The effect of immunomodulators on the immune system of the host is nonspecific and directed towards the stimulation of the defense system of the organism in general. Medicinal plants have been used to cure a number of diseases. Although the recovery might be slow, the therapeutic use of medicinal plants is becoming popular because of its ability to cure ailments without side effects[27].

A recent study reported that the methanolic extract of *A. marmelos* provides immunomodulatory potential by stimulating cellular and humoral immune mechanisms in mice[28]. In the present study, the ethanolic extract of *A. marmelos* and *F. benghalensis* was enhancing the growth of human PBMC and was nontoxic to those cells as evidenced by the dye exclusion method. SRB and MTT assays also showed that the compounds present in the extract had immunomodulatory properties. Secondary metabolites which did not participated directly in growth and development were responsible for the medicinal qualities of plants. Most of the compounds used as drugs came from vascular plants; mainly from angiosperms. Their secondary metabolic products include alkaloids, glycosides, essential oils and other organic constituents[29].

The present study revealed the presence of secondary metabolites like phlobatannins, cardiac glycosides, tannins, flavonoids, steroids, and terpenoids in the alcoholic extracts of both plants. A recent study also revealed the presence of alkaloids, saponins, flavonoids and phenolic compounds in aqueous, acetone and chloroform extracts of leaves of *A. marmelos*[30]. TLC analysis of the present study

confirmed the presence of quantifiable amounts of tannins and alkaloids with traces of phenolic compounds.

There are a number of natural (herbs, plants) agents which are used for enhancing the body's response to diseases. It is quite evident from this study that *A. marmelos* and *F. benghalensis* contain a number of phytoconstituents with applications for various therapeutic purposes. The plant or its individual parts can be used for the treatment of various disorders in human beings such as diabetes, liver toxicity, fungal infection, microbial infection, inflammation, pyrexia, antibacterial activity, immunomodulatory activity and pain relief. Still, so much work is required with *A. marmelos* and *F. benghalensis* to investigate the mechanisms of actions with respect to other therapeutic applications. Although the exact mechanisms of their physiological effects are not clear as yet, further researches in this pursuit focusing on the isolation of individual compounds from *F. benghalensis* promise to open new avenues for its use in different therapeutic approaches.

Conflict of interest statement

We declare that we have no conflict of interest.

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