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ORIGINAL ARTICLE

Nontargeted Analysis and Cancer Cells Cytotoxicity of *Aegle marmelos* Correa Ex Roxb.

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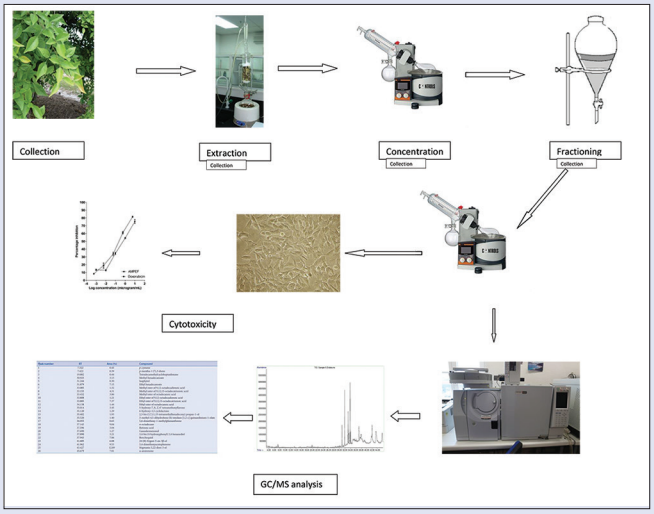
ABSTRACT

Background: *Aegle marmelos* belongs to the family Rutaceae, a medium-sized perennial tree which grows in subtropical and tropical parts of India and South-East Asia. Studies have reported that leaf extracts of *A. marmelos* have anticancer, cardiotonic, antidyslipidemic, and hypoglycemic effects and were ethnologically used for the treatment of dropsy, opthalmitis, ulcers, beri beri, and cholera. **Objective:** In the present study, we have carried out nontargeted analysis of the leaf extract of *A. marmelos* Correa ex Roxb., by gas chromatography-mass spectrometry (GC/MS) and estimated the inhibitory concentration (IC_{50}) in human cancer cell lines using MCF-7, H-460, and SF-268. **Materials and Methods:** Dried leaves of *A. marmelos* were extracted with 50% aqueous alcohol using Soxhlet apparatus. The dried extract was suspended in water and re-extracted with petroleum ether and the fraction was named as AMPEF (petroleum ether fraction of Aegle marmelos leaf extract); the dry yield of the AMPEF was found to be 7.56% (w/w). Nontargeted GC/MS analysis of AMPEF was performed using Shimadzu QP 2000 GC equipped with ULBON-HR-5 capillary column and mass spectrometer as detector. The cancer cell lines were obtained from the National Centre for Cell Science, Pune. *In vitro* cytotoxic activity of AMPEF on cancer cell lines was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. **Results:** GC/MS analysis of the AMPEF enabled the identification of 26 compounds, and IC_{50} of AMPEF in MCF-7, H-460, and SF-268 was found to be 0.53 ± 0.10, 0.65 ± 0.05, and 0.18 ± 0.01 µg/ml, respectively. **Conclusion:** The AMPEF was found to be a potential cytotoxic agent against the used cell lines.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, anticancer, chromatography-mass spectrometry, H-460, MCF-7, SF-268

SUMMARY

- The leaves of Aegle marmelos contain methyl ester of palmitic acid, methyl ester of linolenic acid, stearic acid, stigmasterol and ergost-5-en3-ol
- The petroleum ether fraction of the leaf extract of Aegle marmelos contains stigmasterol which has shown antiproliferative, apoptosis and chemopreventive potential
- The petroleum ether fraction of the leaf extract of Aegle marmelos has shown cytotoxic effects in breast cancer, lung cancer and glial cancer cell lines in low doses.



Abbreviations used: AMPEF: Petroleum ether fraction of leaf extract of Aegle marmelos; DMBA: 7,12-Dimethylbenz[a]anthracene; IC: Inhibitory concentration; MTT:3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; NIST: National Institute of Standards and Technology; RT: Retention time; TIC: Total ion chromatogram.

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INTRODUCTION

Analysis, characterization, and profiling of the various constituents of plants or extracts thereof can be easily carried out using hyphenated techniques such as gas chromatography-mass spectrometry (GC/MS), liquid chromatography-mass spectrometry (LC/MS), and high-performance liquid chromatography (HPLC)-high-pressure counter current chromatography. A general chromatographic profile of any sample could be obtained with the assistance of these hyphenated techniques which tell us about the relative or absolute quantity of all constituents of the extract.[1]

It is estimated that only 5% of the compounds have been characterized out of more than 200,000 compounds which were obtained from the plant kingdom.[2] Both targeted and nontargeted analyses could be done with the assistance of GC/MS. Relatively limited number of predefined compounds, such as biological amines, are investigated, in a targeted analysis.[3] In targeted analysis approach, methods are adjusted in such a way that signals from selective constituents are emphasized, while rest of the signals are neglected.[2] This helps in increasing the accuracy and precision of the method followed in characterization and identification of the constituents which could be further increased by utilizing stable

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isotope labeled internal standards. But then again, standards with stable isotope are significantly expensive and are commercially available only for a few selected compounds.[1] While, in case of nontargeted analysis, all constituents of the extract which show peaks above a certain intensity can be characterized on the basis of their GC retention indices and mass spectral patterns. It has been proved that most of the constituents of the extract in a nontargeted analysis may not be identified completely. However, their comparative measurement could be obtained reliably even though their structure is not known.[4]

Conventional GC/MS employs the electron ionization technique to generate and measure only abundantly available positively charged ions. Here, constant energy of 70 eV is provided to obtain a reproducible mass spectrum by fragmentation of the parent ion. Due to the use of constant energy, investigators can share libraries of such spectra as reference spectra with each other, and several commercial libraries are also available for reference and interpretation.[5]

Aegle marmelos belongs to the family Rutaceae, a medium-sized perennial tree that grows in tropical and subtropical regions of South-East Asia and India and popularly known as Bilwa or Bael in India. The tree is considered as divine and its parts are utilized in various alternative systems of medicine such as Ayurveda, Siddha, and Unani for the treatment of various diseases such as chronic diarrhea, dyspepsia, and dysentery.[6] More than 100 secondary metabolites have been isolated from various parts of the tree and studies have reported diverse pharmacological effects of these metabolites against many ailments such as hyperglycemia, dyslipidemia, cardiac arrhythmia, cancer, malaria, and gastroduodenal disorders.[7-12]

Cancer is considered one of the prominent causes of death worldwide. More than 82 lakh people died in the year 2012 alone because of cancer. As per the World Health Organization, lung, liver, and stomach cancers are the top three death-causing cancers during the above-mentioned period killing 15.9, 7.45, and 7.23 lakh people, respectively.[13] Furthermore, it is estimated that there will be 70% increase in the number of cancer incidence by 2035.[14] Hutchinson and Kirk have reported that attrition rate is 95% in case of new anticancer drugs during the Phase II of clinical trials which is significantly more than the attrition rate of new drugs for cardiovascular diseases.[15] In addition, there are problems of drug resistance, severe side effects, tumor recurrence, metastasis, etc., which further delay the successful treatment of cancer.[16] Therefore, there is a need to find new drugs for the treatment and management of cancer which is not a single disease but a diverse collection of diseases which require multifarious approach along with cytotoxic drugs, adjuvant therapy, radiation therapy, hormonal therapy, and monoclonal antibody therapy.

In this study, nontargeted analysis of extract of leaves of *A. marmelos* using GC/MS was carried out. The IC_{50} of the extract was estimated in three human tumor cell lines, namely MCF-7 (breast adenocarcinoma), H-460 (non-small cell lung cancer), and SF-268 (anaplastic astrocytoma).

MATERIALS AND METHODS

Collection and preparation of plant material

The leaves of *A. marmelos* were collected during November–December from the local herbal gardens and were cleaned and aerial dried for 20–25 days and then powdered for extraction. The plant was authenticated by Dr. Sunita Garg, Chief Scientist, Raw Materials Herbarium and Museum at CSIR-NISCAIR, New Delhi, India, and a specimen was deposited at the museum.

Extraction of plant material

Accurately weighed 350 g of dried leaves of Bael was powdered and extracted with 50% (v/v) aqueous alcohol using Soxhlet apparatus for 7–8 h. The extract was filtered through Whatman filter paper Grade 1, and the filtrate was concentrated in a rotary evaporator below 40°C. The percentage yield of the extract was 20.63% (w/w). About 67 g of the dried extract was resuspended in distilled water and extracted with petroleum ether in a separating funnel. The petroleum ether extract was arbitrarily labeled as AMPEF. The AMPEF was concentrated in a rotary evaporator below 40°C and stored at 4°C until further use.

Chromatography-mass spectrometry analysis of AMPEF

Nontargeted GC/MS analysis of AMPEF was performed using a Shimadzu QP 2000 GC (Shimadzu, Japan), equipped with a ULBON-HR-5 (Shinwa Chemical Industries Ltd) capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) and a mass spectrometer as detector. Helium was used as a carrier gas at a flow rate of 1 mL/min. Column temperature was initially 60°C for 3 min, then gradually increased to 250°C at 5°C/min, and then to 280°C at 10°C/min and kept at 280°C for 11 min. For GC/MS detection, an electron ionization system was used with ionization energy of 70 eV. Diluted samples (1.0 μL) were injected automatically in splitless mode. Injector and detector temperatures were set at 250°C and 280°C, respectively.

Cell culture

Three human cancer cell lines, namely MCF-7, H-460, and SF-268, were obtained from the National Centre for Cell Science, Pune. These were grown as monolayer and routinely maintained in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), and a combination of antibiotics penicillin at 100 U/mL and streptomycin at a concentration of 100 μg/mL at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity study

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, a colorimetric assay developed by Mosmann, 1983, was used to evaluate cell vitality.[13] Approximately, 1×10^4 cells per well were seeded on 96-well plate in RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS, and antibiotics, and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. Later, the medium was replaced with fresh medium containing different concentrations of AMPEF (0.001, 0.01, 0.1, 1.0, and 10 μg/ml). Stock solution was prepared by dissolving AMPEF in dimethyl sulfoxide (DMSO) followed by filtration through syringe filters (pore diameter 0.2 μm). Stock solution was diluted in RPMI-1640 medium to obtain required concentrations for the assay. The concentration of DMSO was kept below 0.1% in all experiments. After 48 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, media was replaced with fresh media and 20 μL of MTT solution (2 mg/mL in PBS) was added to each well and incubated for another 4 h. The media was replaced with 100 μL of DMSO and the plate was shaken for 15 min to solubilize the crystals of MTT formazan. The optical density for each well was determined using enzyme-linked immunosorbent assay reader at 550 nm. The experiments were repeated at least three times and each reading was taken in triplicate. The effect of AMPEF on the proliferation of cancer cells was expressed as the percent of cytotoxicity, using the following formula:

Percent of cytotoxicity = $(A_{550}$ control $- A_{550\ sample})/A_{550}$ control $\times 100$

Statistical analysis

All data were expressed as mean ± standard deviation.

RESULTS

Extraction yield

The extraction yield for AMPEF was 1.56% (w/w).

Chromatography-mass spectrometry analysis of high-performance anion exchange

Gas chromatography-mass spectroscopy analysis was carried out on petroleum ether fraction of aqueous-alcoholic extract of leaves of *A. marmelos* (AMPEF). The total ion chromatogram (TIC) of AMPEF showing the GC/MS profile of the compounds identified is depicted in Figure 1. The peaks in the TIC were integrated and were compared with the spectrum of components stored in the NIST GC/MS library. The GC/MS analysis of the AMPEF enabled the identification of 26 constituents of which mostly are steroidal compounds as per library reference. Detailed tabulations of GC/MS analysis of AMPEF are given in Table 1.

Cytotoxicity study

Cytotoxicity studies of AMPEF were carried out by the MTT assay using MCF-7, H-460, and SF-268 cells treated with varying concentrations of the extract/positive control (doxorubicin) for 48 h. As shown in Table 2, AMPEF was cytotoxic to the three cancer cell lines tested and inhibited proliferation of the cells in a dose-dependent manner. AMPEF showed considerable cytotoxic activity in MCF-7, H-460, and SF-268 cancer cell lines [Figures 2-4]. The IC_{50} of AMPEF in MCF-7, H-460, and SF-268 cancer cell lines was found to be 0.53 ± 0.10, 0.65 ± 0.05, and 0.18 ± 0.01 μg/mL, respectively [Table 2].

DISCUSSION

The separation of different secondary metabolites present in a plant extract can be achieved with separation techniques such as LC, GC, and capillary electrophoresis (CE). Each technique has its pros and cons as HPLC and UPLC have wide adaptation capacity whereas GC and CE have high resolution. These separation techniques are combined with detector(s) which are based on different working principles such as photo diode array, nuclear magnetic resonance (NMR), infrared (IR), and MS for ascertaining the presence of a particular constituent in an extract. NMR-based detector is convenient for highly abundant polar secondary metabolites while detector based on MS is a much better

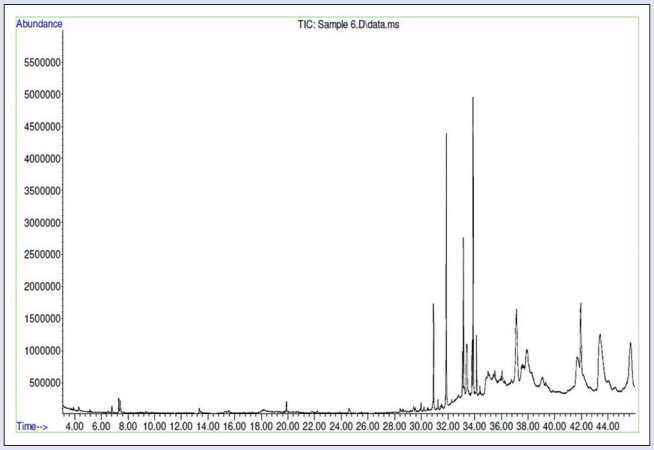

Figure 1: Total ion chromatogram of AMPEF

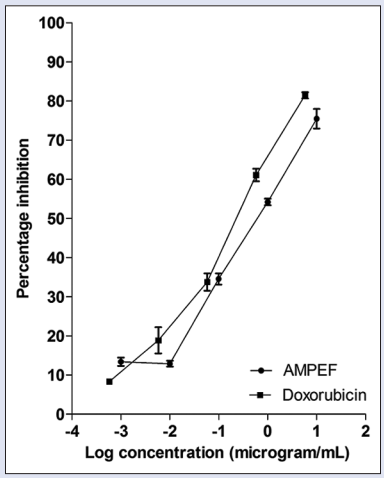

Figure 2: Cytotoxicity of AMPEF in MCF-7 cancer cell line

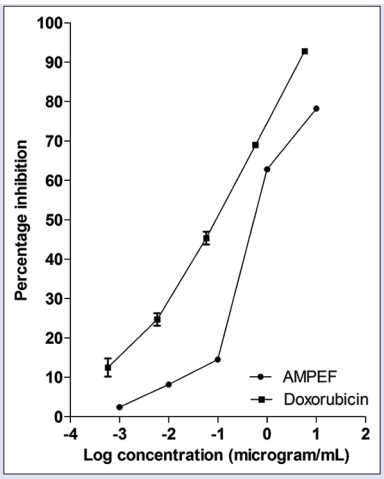

Figure 3: Cytotoxicity of AMPEF in H-460 cancer cell line

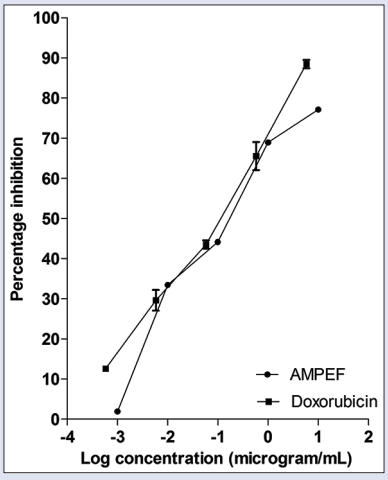

Figure 4: Cytotoxicity of AMPEF in SF-268 cancer cell line

Table 1: Gas chromatography/mass spectrometry analysis of AMPEF

Peak number	RT	Area (%)	Compound
1	7.322	0.43	*p*-cymene
2	7.422	0.39	*p*-mentha-1 (7),3-diene
3	19.882	0.44	Tetradecamethylcycloheptasiloxane
4	30.925	3.13	Methyl hexadecanoate
5	31.244	0.30	Isophytol
6	31.879	7.15	Ethyl hexadecanoate
7	33.085	1.32	Methyl ester of 9,12-octadecadienoic acid
8	33.155	4.31	Methyl ester of 9,12,15-octadecatrienoic acid
9	33.432	3.86	Methyl ester of octadecanoic acid
10	33.808	1.21	Ethyl ester of 9,12-octadecadienoic acid
11	33.885	7.37	Ethyl ester of 9,12,15-octadecatrienoic acid
12	34.138	1.44	Ethyl ester of octadecanoic acid
13	35.014	3.45	5-hydroxy-7, 8, 2', 6'-tetramethoxyflavone
14	35.120	1.29	6-hydroxy-3,5 cyclolactone
15	35.402	1.93	2,3-bis (3,7,11,15-tetramethylhexadecoxy) propan-1-ol
16	35.520	1.40	3-methyl-4,5-dihydrobenz (h) imidazo [1,2-c] quinazolinium-1-olate
17	36.055	0.65	5,6-dimethoxy-1-methylphenanthrene
18	37.143	9.04	*n*-octadecane
19	37.596	3.04	Retinoic acid
20	37.690	1.27	Ganodermenonol
21	37.890	3.21	3,4-bis (4-hydroxyphenyl) 3,4-hexanediol
22	37.943	7.06	Benchequiol
23	41.685	6.08	24 (R)-Ergost-5-en-3β-ol
24	41.962	9.53	3,4-dimethoxyacetophenone
25	43.427	12.89	Stigmasta-5,22-dien-3-ol
26	45.679	7.81	α-amirenone

Table 2: *In vitro* cytotoxicity of AMPEF (*n*=3)

Cell lines	IC50 (μg/mL)	
	AMPEF	Doxorubicin
MCF-7	0.53±0.10	0.18±0.03
H-460	0.65±0.05	0.07±0.02
SF-268	0.18±0.01	0.08±0.01

option for nonpolar or semipolar constituents that are present in low concentrations in an extract.[17] The use of GC/MS technique for the separation and identification of constituents of plant extract is common these days and GC/MS data systems, now available, have integrated electron ionization mass spectral reference libraries. The electron ionization mass spectral library of NIST/EPA/NIH provides spectra of over 200,000 compounds for reference.[18] Keskes *et al.* analyzed the hexane extract of *Juniperus phoenicea* L. (Cupressaceae) and reported the presence of 32 constituents in the extract along with the antioxidant activity of the extract.[19] In another study conducted by Hussein *et al.*, methanolic extract of dried gall of *Quercus infectoria* was analyzed by GC/MS which led to the identification of 11 constituents in the extract, and the functional groups present in the constituents of the extract were established by Fourier transform-IR technique.[20]

The GC/MS analysis of AMPEF was performed using ULBON-HR-5 capillary column, and 26 compounds were identified [Table 1], with typical TICs of the extract shown in Figure 1. The analysis enabled the identification of 26 constituents present, belonging to different chemical subclasses, in the extract. AMPEF was found to contain steroidal constituents.

Mujeeb *et al.* confirmed the presence of methyl ester of hexadecanoic acid (palmitic acid, methyl ester), methyl ester of 9,12,15-octadecatrienoic acid (linolenic acid, methyl ester), octadecanoic acid (stearic acid), stigmasta-5,22-dien-3-ol (stigmasterol), and erogost-5-en-3-ol in the extract of leaves of *A. marmelos* with the help of GC/MS technique.[21] Our data also suggested the presence of these constituents in the petroleum ether fraction of aqueous-alcoholic extract of leaves of *A. marmelos*. Previous studies have shown the presence of *p*-cymene in the fruit and stearic acid, palmitic acid, and linolenic acid in the oil obtained from the seeds of *A. marmelos*.[22,23] Our analysis indicated that the presence of these compounds is not limited only to the fruits and seeds but these are also present in the leaves. The diverse phytoconstituents present in different parts of Bael tree might be responsible for the assorted medicinal benefits of the plant.

Like cholesterol in animals, plants have sterols or phytosterols which exist in several different forms, for example, β-sitosterol, campesterol, stigmasterol, and cycloartenol, and the most abundant phytosterol is β-sitosterol.[24] Studies have proved the antiproliferative potential of apoptosis of phytosterols against breast, prostate, and leukemic cancer cells.[25,26] Kim *et al.* have shown that stigmasterol induces apoptosis in hepatocarcinoma (HepG2) cells by upregulating the expression of pro-apoptotic genes Bax and p53, by downregulating the expression of antiapoptotic gene Bcl-2 and by activating caspase 8 and 9-mediated apoptotic pathway.[27] Stigmasterol has been proved to possess chemopreventive effects in DMBA-induced skin cancer in mice.[28] Therefore, it has been proved that stigmasterol is a significant phytosterol which has cytotoxic and antiproliferative potential among the various phytosterols that exist in the plant kingdom.

Bael has been proved to possess cytotoxic activity in ovarian cancer (A 2780) cell lines, in Dalton's lymphoma ascites and in Ehrlich ascite carcinoma.[29-31] In 2008, Subramaniam *et al.* have shown the anticancer activity of 1-hydroxyl-5,7-dimethoxy-2-napthelene carboxaldehyde extracted from Bael in colon cancer cell lines (HCT-116) and in alveolar epithelial carcinoma cells (Hep-2).[32] In this study, AMPEF has shown cytotoxicity against MCF-7, H 460, and SF-268 cancer cell lines at low concentration. This could be attributed to the presence of stigmasterol in AMPEF which had been already proved to exhibit anticancer effects. Our findings further support the information available regarding the potential use of *A. marmelos* for exploring anticancer constituents.

CONCLUSION

The present study analyzes the constituents of petroleum ether fraction of aqueous-alcoholic extract of *A. marmelos* and to ascertain the cytotoxicity of the fraction on three cancer cell lines, i.e., MCF-7, H-460, and SF-268. According to the data obtained, the fraction was found to be cytotoxic in the three cancer cell lines at low doses. This study further supports the existing information concerning cytotoxicity of *A. marmelos*. Further studies are required in other cancer cell lines and *in vivo* cancer modalities to confer anticancer activity of the various constituents of *A. marmelos*. In addition, there is a need to pinpoint the actual active constituent of the fraction and identification of such constituent(s) which will further help in revealing the mechanism by which AMPEF exerted the cytotoxic effects.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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