Research Paper

Pharmacognostic Standardisation and Antiproliferative Activity of *Aegle marmelos* (L.) Correa Leaves in Various Human Cancer Cell Lines

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Therapeutic management of cancer is a great clinical challenge and alternative medicines are being extensively explored to have integrated approach to cure cancer. *Aegle marmelos* (L.) Correa (Rutaceae) is known for its hypoglycaemic, radioprotective, antidiarrhoeal and many other pharmacological activities. The present study is designed to carryout pharmacognostic standardisation and evaluation of antiproliferative activity of the leaf extracts *Aegle marmelos* (L.) Correa (Rutaceae) and the chromatographic fractions of the most active extract. Hexane, petroleum ether, chloroform and ethanol extracts of the shade dried leaves were prepared by soxhelation and antiproliferative activity was assessed using human cancer cell lines of lung (A-549), colon (CoLo-05), ovary (IGR-OV-1), prostrate (PC3), leukaemia (THP-1) and breast (MCF-7) cancer. Bioactivity-derived fractionation was carried out for most active extract by column chromatography. The phytochemical studies indicated alkaloids, anthraquinones, terpenoids in the alcohol, chloroform extracts and tannins, terpenoids, reducing sugars in the petroleum ether and hexane extracts. Ethanol extract showed maximum inhibition in colon and breast carcinoma cell lines at a dose of 100 µg/ml. Column chromatography of the ethanol extract yielded five fractions. Out of this, fractions 2, 4 and 5 showed significant inhibition in leukaemia cell line with IC_{50} of 12.5, 86.2 and >100 µg/ml for fractions 2, 4 and 5, respectively. High-performance thin layer chromatography of the fraction 2 revealed imperatorin as one of the major phytoconstituents. Among the different extracts investigated, ethanol extract exhibited significant antiproliferative activity and its fraction 2 containing furanocoumarin imperatorin showed antiproliferative activity against leukaemia cell line with IC_{50} of 12.5 µg/ml.

Key words: Alternative medicine, high-pressure thin layer chromatography, imperatorin, phytochemical, rutaceae

Natural products have played a vital role in prevention and mitigation of human diseases for centuries. Due to their large structural diversity, the natural products have emerged as appealing sources of useful leads for the discovery of new drugs; their potential is further enhanced with the present sophisticated techniques for isolation, identification, structure elucidation and combinatorial synthesis[1]. The World Health Organisation (WHO) has described guidelines for the standardisation of medicinal plants with regard to their macroscopic and microscopic description[2]. Several studies have suggested that the pharmacognostic details of a plant may provide useful criteria in identification and authentication of plant drugs[3,4].

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Aegle marmelos (L.) Correa (Rutaceae) commonly called as '*Bael*' is indigenous to India and is cultivated throughout the Indian subcontinent. Leaves, fruits, stem and roots of *A. marmelos* have been used in ethnomedicine for a variety of purposes, such as astringent, antidiarrhoeal, antidysentery, demulcent, antipyretic, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom[5,6]. *A. marmelos* has been reported to be useful for diabetes mellitus[7] and its complications[8]. It has also been claimed to be useful in treating pain, fever and inflammation[9].

All the parts of the plant leaf, root, bark and fruits are reported to have hypoglycaemic properties[7,10,11]. Linear furanocoumarin present in *A. marmelos* has been reported to protect rat myocardium against lipidperoxidation and membrane damage during experimental myocardial injury[12]. Studies have also

revealed the plant to possess immunomodulatory[13] and radioprotective activity[14]. The stem bark of the plant has been reported to have anticancer activity[15]. The present study was undertaken to study microscopic features of *A. marmelos* leaves, establish pharmacognostic and physicochemical parameters of *A. marmelos* leaves followed by *in vitro* antiproliferative activity using human cancer cell lines.

MATERIALS AND METHODS

Fresh leaves of *A marmelos* were collected from the botanical garden of Guru Nanak Dev University, Amritsar in the months of March-April, authenticated at Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar and a voucher specimen is deposited in the herbarium of the same department (SR./Bot.Sci./0350). Healthy plants were carefully selected for study.

Microscopy of plant leaves:
The leaves were removed from the plant and fixed in formalin acetic acid solution (formalin:acetic acid:70% ethanol in ratio of 0.5:0.5:9). After 24 h, the specimens were dehydrated with tertiary butyl alcohol (TBA) in a graded manner. Infiltration was carried by gradual addition of paraffin wax till TBA solution attained supersaturation. The specimens were cast into paraffin blocks and cut into sections of 10-12 µm thickness with the help of rotary microtome[16]. After dewaxing, the sections were stained with toluidine blue, safranin, fast green and iodine-rich potassium iodide[17,18].

Paradermal sections of the leaf were cleared with 5% sodium hydroxide and epidermal peeling was carried out employing Jeffery's maceration fluid for studying the morphology of stomata, pattern of venation and trichomes[17]. Glycerine mounted temporary preparations were used. For microscopy of powdered leaves the material was cleared with sodium hydroxide and mounted in glycerine after staining. The photomicrographs were taken with Nikon labphoto 2 microscopic unit. Bright field was used for normal observations and polarised light was employed for studying structures such as lignified cells. These structures exhibit birefringent property and appear bright against the dark background. The magnifications are indicated by the scale bars. Leaves were shade dried, powdered and passed through a 40 mesh sieve. The powder was stored in air tight container till further use.

Physicochemical and phytochemical analysis:
The ash values were calculated in accordance with the WHO guidelines for standardisation of medicinal plants. The effect of different reagents on leaf powder under normal and ultraviolet (UV) light was investigated and the tests for heavy metals, pesticide residues and microbial contamination were carried out as per the WHO guidelines. Preliminary phytochemical analysis of the crude extracts was done by following the standard tests for the various phytoconstituents such as alkaloids, anthraquinones, flavonoids, glycosides and proteins, reducing sugars, steroids, tannins and terpenoids[19]. The extracts were made by soxhelation of 100 g of the freshly dried powder with hexane, petroleum ether, chloroform and ethanol for 16-18 h to obtain 3.43, 1.6, 3.92 and 9.6% w/w residue, respectively. The ethanol extract was subjected to column chromatography over silica gel in hexane: ethyl acetate in varying ratios as eluent to collect five fractions AME-1, AME-2, AME-3, AME-4 and AME-5. The most active fraction (AME-2) was subjected to further characterisation by high-performance thin layer chromatography (HPTLC) on precoated silica hexane:ethyl acetate plates (20×20, Merck, Germany) using Linomet-IV applicator and Camag TLC Scanner-III with WinCATS 4 software. Aliquots of standard and fraction were applied on TLC plates using toluene:ether (1:1 saturated with 10% acetic acid) taking imperatorin as the standard.

Antiproliferative studies:
In vitro cytotoxicity evaluation of the extracts and the chromatographic fractions of the most active extract was carried out using different human cancer cell lines including lung (A-549), colon (CoLo-205), ovary (IGR-OV-1), prostate (PC-3), leukaemia (THP-1) and breast (MCF-7) cancer. The human cancer cell lines procured from National Cancer Institute, Frederick, USA were used in the present study. Briefly, cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2 mM glutamine, pH 7.4, supplemented with 10% foetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37°, 5% CO_2, 90% RH). The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% in phosphate buffered saline, pH 7.4 containing 0.02% EDTA). Cells with viability

of more than 98%, as determined by tryptan blue exclusion, were used for determination of cytotoxicity. The cell suspension of 1×10^5 cells/ml was prepared in complete growth medium. The extracts were used in a concentration of 100 µg/ml prepared in DMSO. *In vitro* cytotoxicity was determined using 96-well tissue culture plates. The 100 µl of cell suspension was added to each well of the 96-well tissue culture plates. The cells were allowed to grow in CO_2 incubator for 24 h; after which test materials were added in complete growth medium. The plates were incubated further for 48 h and then the growth was arrested by gently layering trichloroacetic acid over the medium. The plates were again incubated at 4° for 1 h. The supernatant was discarded and plates washed with distilled water and air dried. Cell growth was measured by staining with sulforhodamine B[20]. The adsorbed dye was dissolved in Tris-HCl buffer and absorbance recorded on ELISA reader at 540 nm. Adriamycin, 5-florouracil, paclitaxel and mitomycin C were used as positive controls for breast, colon, lung and prostate cancer, respectively.

RESULTS AND DISCUSSION

The leaf is dorsiventral, smooth and even. The midrib is prominent, projecting both on the adaxial and abaxial sides. The adaxial part of the midrib has broad, blunt, conical hump (fig. 1a), the abaxial part is broadly semicircular and about 2 mm in vertical plane and 2.2 mm in horizontal plane along the basal part. The midrib has a thin epidermal layer of small cubical thick walled cells. The ground tissue consists of angular or circular compact parenchyma cells. The cells towards the periphery are smaller measuring 50 µm wide and those towards the centre are wider and 170 µm in diameter. Circular, wide secretory cavities are sparsely distributed in the ground parenchyma and have lysigenous secretion of volatile compounds. A fully grown cavity is 250 µm wide. The vascular system consists of a planoconvex cylinder, which consists of lower bowl-shaped part and upper flat plate. The cylinder has several parallel rows of short as well as long lines of xylem elements, which are wide, angular and thick walled. Phloem occurs in prominent masses on the outer part of the xylem cylinder (fig. 1b and c). The lamina is 550 µm thick and has bilateral symmetry. The adaxial epidermis has rectangular, or square, thick walled epidermal cells measuring 40 µm in thickness. The abaxial epidermis is comparatively thin and the cells are cylindrical or rectangular and the layer is stomatiferous. The mesophyll tissue is differentiated into adaxial zone of palisade cells and abaxial zone of spongy parenchyma. The palisade zone has two layers of short, wide compact cells and is 100 µm in height. The spongy parenchyma cells are spherical and lobed. Secretory cavities containing dark amorphous contents, 250 µm wide, are common in the mesophyll tissue and occur either in the spongy mesophyll (fig. 1b) or in the palisade zone (fig. 1c).

Fragments of epidermal layer with angular, polyhedral epidermal cells with narrow lumen, thin and straight walls are seen in the powder (fig. 2). Cyclocytic stomata, with each stoma having a ring of four cells encircling the guard cells or a second ring of

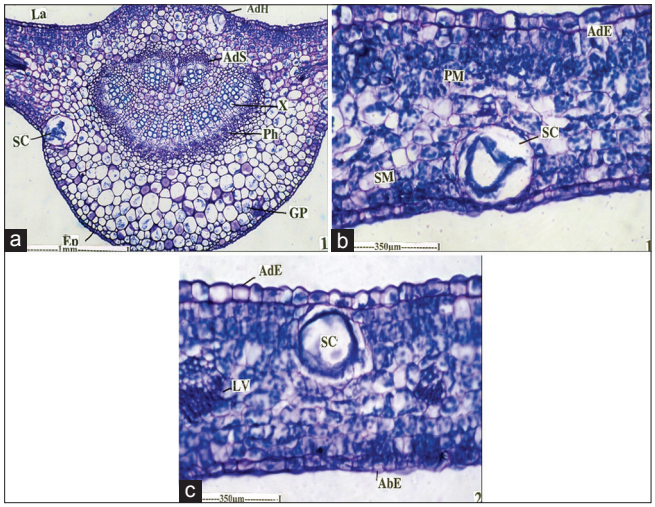

Fig. 1: Leaf microscopy.
(a) T.S. of leaf through midrib with lamina. [AdH- adaxial hump, AdS- adaxial strand, Ep- epidermis, GP- ground parenchyma, La- lamina, Ph- phloem, SC- Secretory cavity, X- Xylem]. 1 (b) Anatomy of the lamina T.S. of lamina showing abaxial secretory cavity. 1 (c) T.S. of lamina showing adaxial secretory cavity [AbE- abaxial epidermis, AdE- adaxial epidermis, LV- lateral vein, PM- palisade mesophyll, SC- secretory cavity, SM- spongy mesophyll].

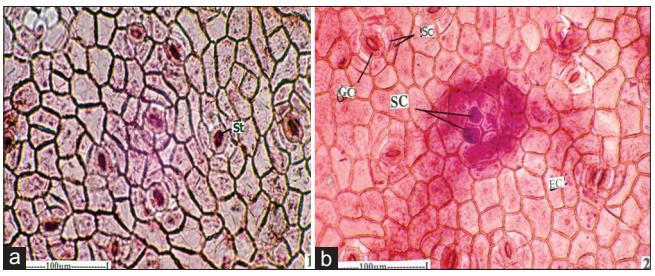

Fig. 2: Stomatal microscopy.
(a) Fragment of abaxial epidermis with stomata {stained with sudan-III}. (b) Abaxial epidermis with stomata and secretory cavity {stained with safranin}. [EC- epidermal cells, GC- guard cell, Sc- Subsidiary cells, SC- secretory cavity, St- stoma].

subsidiary cells, are seen on the epidermis (fig. 2). Some of the epidermal cells are secretory cells, which produce aromatic oil compounds and contain dense cytoplasm that stains deeply (fig. 2b). Non-glandular epidermal trichomes, 150-200 µm long and 10 µm thick, unicellular, unbranched, curved or less commonly straight having thick walls, wide lumen and smooth surface are common in the powder and occur mostly along the veins of the leaf fragments (fig. 2a-c).

Changes of the leaf powder fluorescence with different reagents under normal and UV light were observed and are reported in Table 1. The ash values revealed that the leaves of *A. marmelos* were not more than 9.0% w/w in total ash, not more than 2.52% w/w in acid insoluble ash and not more than 6.32% w/w in water soluble ash. The content of heavy metals like lead and cadmium was found to be 2.86 and 0.09 ppm respectively, which is within the permissible limits (not more than (NMT) 10 ppm for lead; NMT 0.3 ppm for cadmium), while mercury and arsenic were not detected. Pesticide residues were also not detected. The microbial count was within the permissible limits (10^5 CFU/g for bacteria; 1000 CFU/g for fungi) while pathogenic microbes such as *Escherichia coli, Salmonella, Staphylococcus aureus and Pseudomonas aeruginosa* were absent (Table 2). The ethanol extract was found to contain alkaloids, flavonoids, proteins, tannins and terpenoids. The phytoconstituents in other extracts were relatively less varied (Table 3). The HPTLC of the most active fraction (AME-2) confirmed the presence of furanocoumarin imperatorin (fig. 4). The content of imperatorin from standard curve was found to be 0.1367% w/w.

The effect of the various extracts on different cancer cell lines indicated that ethanol and chloroform extracts had significant anticancer activity against some cancer cell lines. The ethanol extract was found to be the most active and it showed 69% inhibition in both colon and breast carcinoma cell lines,

TABLE 1: FLUORESCENT ANALYSIS OF *AEGLE MARMELOS* LEAF POWDER

Solvent	UV short	UV long	Visible
Drug powder	Green	Green	Pale brown
Conc. sulphuric acid	Black	Greenish brown	Dark brown
Conc. HCl	Dark green	Black	Green
Conc. nitric acid	Olive green	Black	Light brown
Glacial acetic acid	Green	Reddish brown	Bottle green
Methanol	Green	Pink	Green
Ethanol	Yellowish green	Dark pink	Light green
Chloroform	Greenish brown	Yellowish pink	Green
Pet. Ether	Green	Pale brown	Green
Water	Green	Blackish brown	Dull green
10% NaOH	Green	Blackish green	Dull green
5% Iodine	Brownish black	Black	Dark brown
Picric acid	Dark green	Reddish black	Pale green
Ammonia solution	Dark green	Yellowish green	Pale green

UV=Ultraviolet

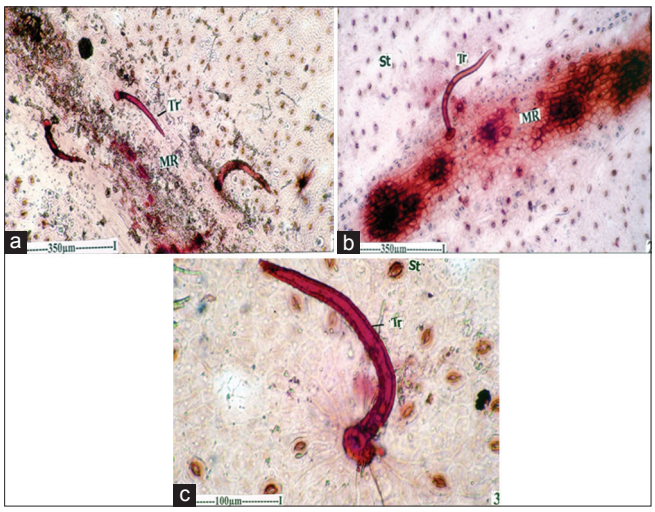

Fig. 3: Trichome morphology.
(a) Non-glandular covering trichomes on the midrib portion of the leaf fragment. (b) A covering trichome under low magnification. (c) A covering trichome (enlarged). [MR- midrib, St- stoma, Tr- trichome].

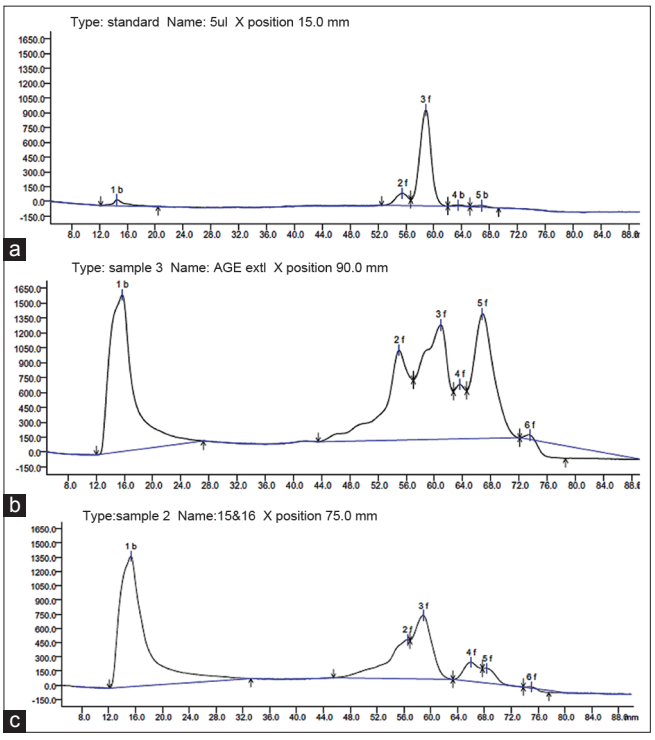

Fig. 4: Comparative HPTLC profiles of standard, imperatorin (a); leaf extract (b) and AME-2 (c).

which were comparable to the standard drug, that is adriamycin while inhibition in lung cancer cell lines was relatively less (42%). The chloroform extract showed 68% and 55% inhibition in breast and colon cancer cell lines, respectively. The petroleum ether extract and hexane extract showed negligible anti-cancer activity in all cancer cell lines (fig. 5). The various fractions of the ethanol extract were studied for anticancer activity and of the various fractions AME-1, AME-2, AME-3, AME-4 and AME-5 showed anticancer activity (fig. 6). The IC_{50} values were studied for fractions AME-2, AME-4 and AME-5 and were found to be 12.5, 86.2 and >100 µg/ml, respectively, in leukaemia cell line (THP-1).

Determination of pharmacognostic parameters of the leaves of *A. marmelos* is useful for setting standards for identification and authentication of the crude drug since many of these factors are relatively constant for a plant. *A. marmelos* is an important traditional plant and a study of the microscopic features of the leaves can be useful in distinguishing the crude drug from adulterants and foreign matter. The ash value, pesticide residue and microbial testing of the crude drug are important to check contamination. Chemotherapeutic agents remain the mainstay in the treatment of a wide variety of cancers but the therapy is paradoxical in the sense that on one hand these agents destroy the cancerous cells while on the other they precipitate toxicity in many other organs with actively dividing cells such as the bone marrow, the reproductive system, the skin or precipitate secondary tumours. Such side effects can sometimes prove fatal even before the tumour itself. The complex metabolic effects like nausea, vomiting, lack of appetite lead to morbidity and malaise associated with chronic ailments, especially cancer, have been termed as cachexia[21]. Recently, complementary and alternative

TABLE 2: PHYSICOCHEMICAL ANALYSES OF THE LEAVES OF *A. MARMELOS*

Parameter	Content
Total ash	9.0%
Acid insoluble ash	2.52%
Water soluble ash	6.32%
Water soluble extract	9.2%
Ethanol soluble extract	2.0%
Heavy metals	
Lead	2.86 ppm
Cadmium	0.09 ppm
Mercury	ND*
Arsenic	ND
Total pesticide residues	ND
Total bacterial count	112 CFU/g
E. coli	Absent
Salmonella	Absent
S. aureus	Absent
P. aerugenosa	Absent
Total fungal count	Absent

*ND=Not detected, CFU=colony forming units

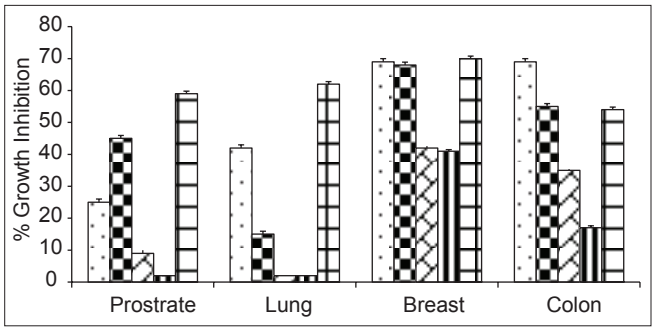

Fig. 5: Effect of the various leaf extracts on the human cancer cell lines.
Effect of the various leaf extracts (100 µg/ml) on the human cancer cell lines. Standard drug prostate cancer cell line mitomycin-c (20 µM), lung cancer paclitaxel (10 µM), breast cancer is adriamycin (10 µM) and colon cancer is 5-florouracil (10 µM). ▫ Ethanol ◪ chloroform ◩ pet. ether ◘ hexane ▤ standard.

TABLE 3: PHYTOCHEMICAL ANALYSIS OF EXTRACTS OF *A. MARMELOS*

Phytoconstituent	Water	Alcoholic	Chloroform	Hexane	Pet. ether
Alkaloids	+	+	+	-	-
Anthraquinones	-	-	-	-	-
Flavonoids	-	+	-	-	-
Glycosides	+	+	-	-	-
Proteins	+	-	+	-	-
Reducing sugar	+	+	+	+	+
Coumarins	-	+	+	-	-
Steroids	-	-	+	-	-
Tannins	+	+	+	-	+
Terpenoids	-	+	+	+	+

+=Present, -=absent

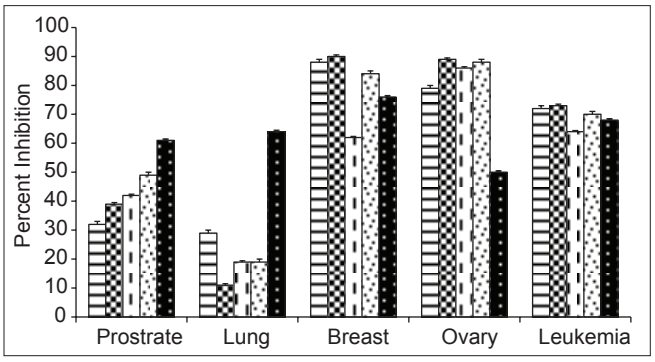

Fig. 6: Effect of the various fractions of ethanol extract on the human cancer cell lines.
Effect of the various fractions of ethanol extract (100 µg/ml) on the human cancer cell lines. Standard drug prostate cancer cell line mitomycin- c (20 µM), lung cancer is aclitaxel (10 µM), breast cancer is adriamycin (10 µM) and colon cancer is 5-florouracil (10 µM). ▤ Fraction 1 ◪ Fraction 2 ◫ Fraction 3 ◰ Fraction 4 ◙ Fraction 5.

medicine is being explored to find therapeutic options with improved efficacy and minimum the morbidity. Plants are also being explored as biological response modifiers that may work synergistically as adjuncts in cancer chemotherapy and decrease the side effects of chemotherapeutic agents and at the same time enhance the effectiveness of these agents[22]. Also, because the plants have a wide variety of phytoconstituents, some phytochemicals serve as tumoricidal while others serve to decrease cancer cachexia by stimulating appetite and nourishing the normal cells[23].

In the present investigation, the ethanol extract of *A. marmelos* leaves was found to possess significant antiproliferative activity against human cancer cell lines and the most potent fraction isolated (AME-2) showed an IC_{50} of 12.5 µg/ml. Also HPTLC analysis of the fraction revealed the presence of linear furanocoumarin imperatorin. Imperatorin has been previously documented to have anticancer activity in human promyelocytic leukaemia cells through a variety of mechanisms such as promoting apoptotic activity and prevention of oxidant damage[24]. Imperatorin has been postulated to promote apoptosis in cancer cells by stimulating both the extrinsic death receptor pathway and the intrinsic mitochondrial pathway[25]. Studies have postulated that imperatorin down-regulates the antiapoptotic Bcl-2 and activates caspase-9 and caspase-3 both of which are pro-apototic in cancer cells[24]. Other mechanisms that have been proposed to contribute to the anticancer activity of imperatorin include inhibition of formation of reactive oxygen species and preventing the activation of oncogenes[26]. The antiproliferative activity of AME-2 may therefore be attributed to the presence of imperatorin and is mediated through multiple mechanisms and target sites including induction of apoptosis, antioxidant effect and inhibition of oncogenes.

The various histological features of the leaves of *A. marmelos* were studied and the phytochemical studies indicated the presence of alkaloids, anthraquinones, coumarins, glycosides, tannins and triterpenoids in the ethanol extract of the leaves of the plant, which was found be most active against the cancer cell lines used in the study. The fractions AME-1, AME-2, AME-3, AME-4 and AME-5 were obtained after column chromatography and out of these AME-2, AME-4 and AME-5 indicated promising anticancer activity. IC_{50} values for AME-2, AME-4 and AME-5 were found to be 12.5, 86.2 and >100 µM, respectively, in leukaemia cell line (THP-1). HPTLC of AME-2 revealed a prominent peak similar to imperatorin, which is well documented for anticancer activity.

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