Original Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CYTOTOXIC EFFECT OF *AEGLE MARMELOS* (L.) LEAVES IN HEP G2 CELL LINES- AN *INVITRO* STUDY

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ABSTRACT

Aegle marmelos, is a medicinal plant from India, used for treating cancer-related symptoms in the Indian system of medicine. Its leaf, root, bark, seed and fruits are valued highly as Ayurvedic medicine. In the present study, the active fraction of *Aegle marmelos* was found to induce apoptosis in Hep G2 cells through NFκB deactivation followed by activation of proapoptotic factor p53. To evaluate this apoptotic induction, the mRNA levels of NFκB were determined using semiquantitative RT– PCR assay. NFκB levels were significantly down-regulated in HepG2 cells following the treatment with active fraction. Similarly, incubation of HepG2 cells with active fraction resulted in increased expression of p53 mRNA levels. The activation of NFκB appears to be a very strong antiapoptotic signal which inhibits TNF α mediated apoptosis pathway. Semiquantitative RT –PCR assay in treated HepG2 cells depicted decreased levels of NFκB which reflects activation of apoptosis through TNFα mediated pathway. Increased levels of p53 mRNA reflects activation of transcription factor p53 which inturn activates Bax proteins and various other transcription factors finally leading to intrinsic apoptotic pathway. These pathways converge to a final common pathway involving the activation of caspases that cleave regulatory and structural molecules and culminate in the death of the cell. Mechanism of apoptotic induction was further evaluated through elevated levels of caspases 3 and 9 enzymes. Thusthe ability of the *Aegle marmelos* to induce apoptosis by a multi prolonged cascade of events suggests that it might be an ideal therapeutic for solid tumors of liver.

KEYWORDS : ***Aegle marmelos*, Apotosis, PCR, NFκB, p53, Caspase , HepG2, Liver cancer**

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INTRODUCTION

Cancer is now the third leading cause of death worldwide, with 7.6 million deaths estimated to have occurred in 2007.[1] By 2030, it is projected that there will be ~26 million new cancer cases per year. [2] Low and middle-income countries accounted for about 61% of cancer deaths by 2050.[3] Liver cancer is the third most common cancer in the world. There is a widespread belief that the green medicines are healthier and more harmless or safer than synthetic ones. In Siddha literature, many medicinal plants are indicated for anti cancer and one among them is *Aegle marmelos*. The medicinal properties of this plant have been described in Ayurvedic system of medicine. In fact, as per Charaka (1500 B.C) no drug has been longer or better known or appreciated by the inhabitants of India than the *Bael*.[4] Bael or Bengal quince is a deciduous sacred tree, native to India. The leaves of *Bael* are astringent, a laxative, and an expectorant and are useful in treatment of ophthalmia, deafness, inflammations, cataract, diabetes, diarrhoea, dysentery, heart palpitation and asthmatic complications. [5] Fresh aqueous and alcoholic leaf extracts of *Aegle marmelos* were reported to have a cardio tonic effects in mammals.[6] *Aegle marmelos* leaf extract has been reported to regenerate damaged pancreatic beta cells in diabetic rats and increase the activities of peroxidase in the liver tissues of isoproterenol treated rats.[7] An aqueous decoction of the leaves has been shown to possess a significant hypoglycemic effect. *Aegle marmelos* leaf extract was found to be a potential antioxidant drug, which reduces the blood sugar level in alloxan induced diabetic rats. [8] It was found to be as effective as insulin in the restoration of blood glucose and body weight to normal levels during hyperglycemic state.[9] The ethanolic extract of *Aegle marmelos* leaf possesses anti spermatogenic activity and aqueous extract of the leaf has antimotility action on spermatozoa in rats.[10] Considering the diverse medicinal properties of *Aegle marmelos*, the present study investigates the mechanism underlying the cytotoxic effect of ethanolic extract of *Aegle marmelos* leaf extract on human hepatoma cell line (HepG2), assessing its influence on the balance between pro-death pathways such as apoptotic cascade through caspases – 3 & 9, NFκB & p53. The study is mainly targeted on the regulation of apoptotic pathway in HepG2 cell line and the development of chemopreventive effect by the natural remedy on HCC.

MATERIALS AND METHODS

Chemicals

The chemicals were purchased from Sigma, USA. All other chemicals are of analytical grade.

Authentication of Plant Material

Aegle marmelos leaves were collected locally during the month of November to January from Chennai, Tamilnadu, India.The taxonomic identification of the plant material was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India. A voucher specimen is maintained in plant anatomy research centre, Chennai(PARC/2009/462).

Preparation of Aegle marmelos Ethanolic Leaf Extract (AMELE)

Air dried powder (10 g) was macerated with 100 mL of ethanol and stored for 72 hours in ice cold condition. After 72 hours the extracts were filtered through a Whatmann filter paper No. 42 (125 mm) and the organic layer was allowed to evaporate The resulted dark green extracts were concentrated using a rotary evaporator with a water bath set at 40° C. The concentrated crude extracts were lyophilized into paste (5 and 15 g respectively) and were taken for further cytotoxic and other investigation.

Cell lines and Culture

Human hepatocellular carcinoma cell lines, HepG2 cells (American Type Culture Collection [ATCC] HB-8065), obtained from National center for cell science, Pune, India was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 50 µg/ml of streptomycin.

Column Chromatography

The AMELE was subjected to column chromatography using different solvent systems. 10 g of the crude extract was subjected to column chromatography over silica gel (100-200 mesh) and the column was first eluted with 100% hexane. The polarity of mobile phase was gradually increased with ethyl acetate followed with Methanol. The fractions collected and the solvent recovered by simple distillation. All the concentrated fractions were subjected to TLC for the identification of the desired bands. The plates were visualized with iodine under UV lamp (365 nm). Fractions of *Aegle marmelos* ethanolic leaf extract (AMELE) was obtained by column fractionation.

Cytotoxicity Assay

Various fractions of AMELE obtained by column fractionation were dissolved in dimethyl sulfoxide (DMSO) (sigma, USA) and were used for the further treatment. A total of 50-60% confluent cells were treated with AMELE and their fraction for 48 hrs in complete growth medium. Cytotoxicity was assessed against the HepG2 cell line using the 3-(4, 5-dimethylthiazol-2yl)-2, 5- diphenyltetrazoliumbromide (MTT) assa. [11] 1 x 10^4 cells per plate were placed in a 96 well plate. After 48 hours incubation, cells were treated with different fractions of AMELE with different concentration of 100, 500 and 1000 µg/ml for 48 hours. Then, MTT 50µg/ml (1mg/ml in PBS) was added in each well and cells were incubated for 4 hours. The supernatant in each well was removed carefully and 100 µl of DMSO was added. Amount of formazan was determined by measuring the absorbance at 595 nm using an ELISA plate reader (EL808 ultra micro plate reader biotek laboratories, USA).

Fluorescent Microscopical Examination

To assay nuclear morphology (apoptotic nuclei), HepG2 cells were seeded in a 24 well tissue culture plate and treated with 100µg of active fraction for 24 and 48 h. After the treatment, cells were washed in phosphate buffered saline (PBS), fixed with 70% ethanol for 1 h and stained with propidium iodide (30 µg/ml in PBS) containing DNAase free RNAase A (1mg/ml) for 30min at 37 °C. The nuclear morphology of cells was

visualized by a fluorescence microscope (Olympus BH2). Fluorescent nuclei were screened for normal morphology (unaltered chromatin and apoptotic nuclei comprising those with fragmented (scattered) and condensed chromatin were counted. Photographs were taken using Kodak film Tmax 400.

DNA Fragmentation

DNA Fragmentation is a key feature of programmed cell death and also occurs in certain stages of necrosis. The cell walls are disturbed or ruptured by the addition of lysis buffer which contain 10% SDS an anionic detergent which denatures the proteins, Tris buffer is used to maintain pH of the cells, and EDTA chelates Mg^{++} and prevents the DNAase activity. Proteinase K is used to digest protein and remove the contamination. Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA and RNA during purification. It is highly-suited to this application since the enzyme is active in the presence of chemicals and denatures proteins, chelating agents such as EDTA. Proteinase K is also stable over a wide range of pH range (4-12), with optimum pH 7.5-12. Phenol a strong denaturant, denatures the Proteinase k and leaves the DNA in the supernatant.

Determination of Caspase Activity

Caspases activities were determined by chromogenic assays using caspase-3 and caspase-9 activation kits according to the manufacturer's protocol (Calbiochem, Merck). After treated with designated concentrations of compound (1 µg/ml (control), 100, 200 & 500 µg /ml), cell lysates were prepared by incubating 2×10^6 cells/ml in cell lysis buffer for 10 min on ice. Lysates were centrifuged at 10,000×*g* for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by the Bradford's method using BSA as a standard. 100–200 µg protein (cellular extracts) was diluted in 50 µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well micro titer plates with 5 µl of the 4mM p-nitroanilide (pNA) substrates, DEVD-pNA (caspase-3 activity) and Ac-LEHD-pNA (caspase -9 activity) for 2 h at 37°C. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405nm in a microtitre plate reader. Relative caspase-3 and 9 activities were calculated as a ratio of the absorbance of treated cells to untreated cells.

Reverse Transcriptase Polymerase Chain Reaction Analysis (RT-PCR)

Total RNA from cell lines were isolated using ONESTEP-RNA Reagent (Biobasic Inc.). After RNA isolation, RNA was immediately reverse transcribed with Easy Script Plus™ Reverse Transcriptase. It was a novel recombinant reverse transcriptase that exhibits much higher efficiency in the synthesis of first strand cDNA from RNA templates than traditional MMLV and AMV. It has been engineered for RNA templates with complicated secondary structure and high GC contents. For RT PCR reaction, 1-2µg of RNA was used corresponding to 1-10 µl of total RNA isolate. The primer set for GAPDH was synthesized as previously reported.[12] The PCR product along with GAPDH amplicons were run on an agarose gel, transferred to nylon membrane and blots were hybridized with specific cDNA probes for BCL2, Caspase 3 & 9. The relative densities of the spotswere normalized with corresponding GAPDH controls. Expression ratio was derived by analyzing the gel photosin software – Image J. Expression ratio was obtainedusing the formula Target Gene expression / internal control x 100.

RESULTS

Column Chromatography

AMELE was subjected to column chromatographic technique using various solvents. The column was first eluted with hexane and the polarity was gradually increased with ethyl acetate followed with acetone and methanol. A total 73 fractions were collected from the column and pooled together according to the TLC profiles. The concentrated fractions were then finally analyzed on TLC plates with different solvent systems. Then these plates were first observed under UV lamp at 365 nm, and then sprayed with iodine which showed the presence of various chemical compounds in the fractions by producing spots.

TABLE 1
YIELD OF FRACTIONS IN COLUMN CHROMATOGRAPHY

S.NO	SOLVENT USED	FRACTIONS POOLED	COLOUR	FRACTIONS OBTAINED (mg)	YIELD (%)
1.	**100% Hexane**	**1 – 6**	**Oil Green paste**	**220mg**	**3.38**
2.	**Hexane : Ethyl acetate (50:50)**	**7 – 15**	**Green paste**	**450mg**	**6.923**
3.	**100 % Ethyl acetate**	**16 – 30**	**Greeny brown paste**	**900mg**	**13.77**
4.	**Ethyl acetate : acetone (75 : 25)**	**31 – 36**	**Light brown pigment**	**700mg**	**10.71**
5.	**Ethyl acetate : acetone (50:50)**	**37 – 48**	**Oil brown paste**	**950mg**	**14.53**
6.	**100% Acetone**	**49 – 55**	**Brown cream**	**1200mg**	**18.36**
7.	**Acetone : Methanol (50 :50)**	**56 – 63**	**Brown paste**	**1200 mg**	**18.36**
8.	**100% Methanol**	**64 – 73**	**Yellow paste**	**600mg**	**9.18**
TOTAL YIELD OF ALL FRACTIONS (1 – 73)				**6.220 gms**	**95 %**

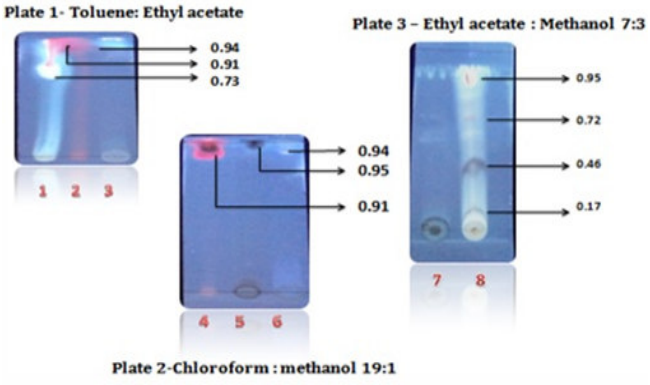


FIGURE 1
TLC of various fractions obtained from column chromatography

MTT Assay

The activities of traditional Indian medicinal plant *Aegle marmelos* leaf ethanolic extract fractions obtained from the column and were evaluated using HepG2 cell line as a model. Hepatoprotective capacity of AMELE fractions in Hep G2 cell line was studied by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay a yellow tetrazole, is reduced to purple formazan in mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. A solubilization solution (dimethyl sulfoxide, an acidified ethanol solution) was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored product can be quantified by measuring at wavelength between 500 and 600nm using a spectrophotometer.

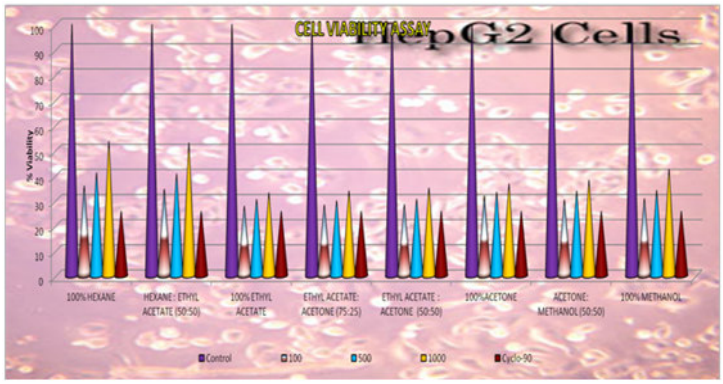


FIGURE 2
CELL VIABILITY ASSAY

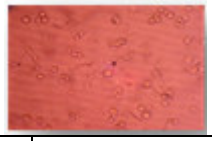
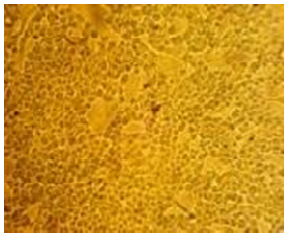
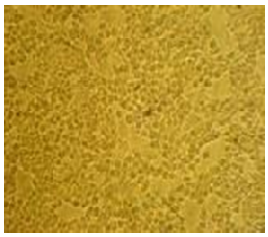
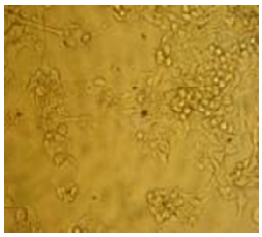
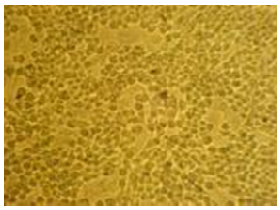
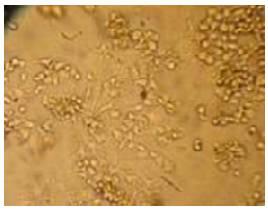
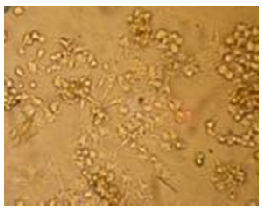
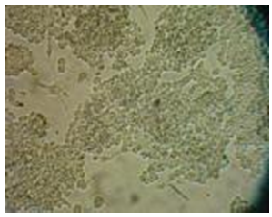
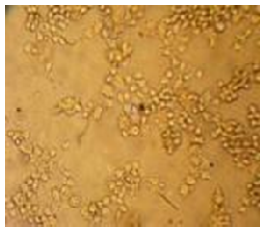
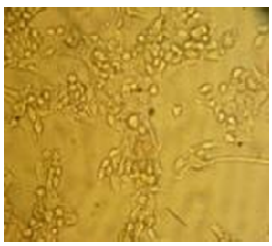
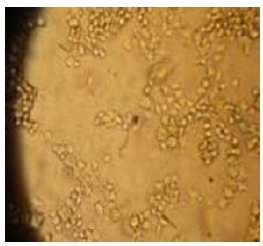
In the present study HepG2 cells were treated with each fraction of AMELE at a concentration ranging 100, 500, 1000 µg/ml for 48 hrs whereas cells incubated with DMSO were used as control. Ethyl acetate fraction had superior cytotoxic effects in a concentration dependent manner when compared to other fraction. The ethyl acetate fraction induces cell death in the Hep G2 cells which indicate its hepatoprotective effect. The hepatoprotective activity correlated considerably with the degree of treatment of the extract which induces cell death by inducing apoptosis in the target cells.[13,14] Therefore ethyl acetate fraction of AMELE (EAAMEL) was more potent than the crude leaf extract which may be due to the concentration of purified bioactive polyphenols present in the ethylacetate fraction. Figure 2 and 3 shows the inhibition of HepG2 cancer cell population by various fractions of the crude extracts of AMELE. Inhibition of cell proliferation could be the result of the induction of apoptosis and/or inhibition of growth.

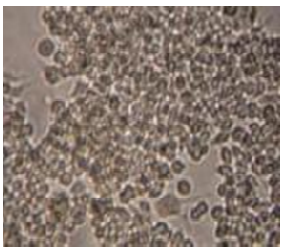
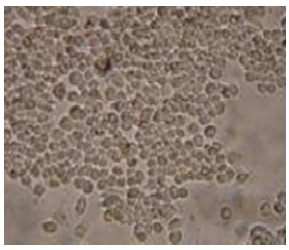
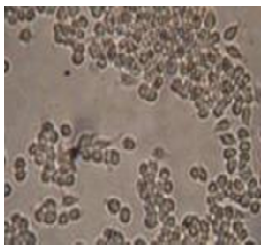
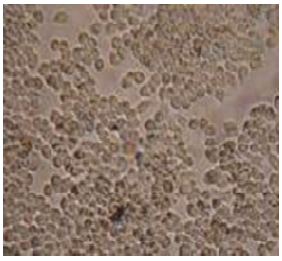
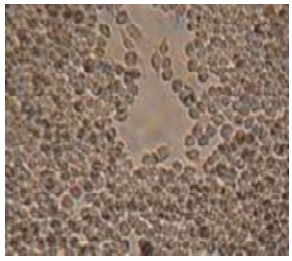
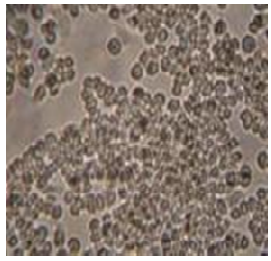
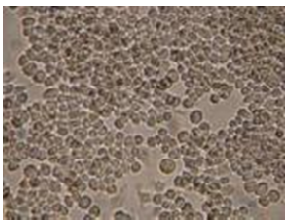
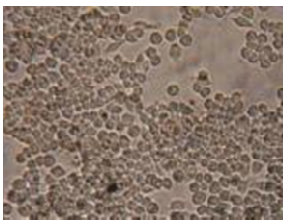
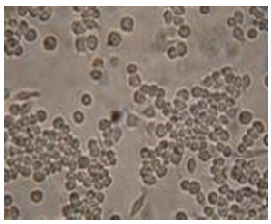
Fluorescent Microscopic study

A cell undergoing apoptosis shows a characteristic morphology such as cell shrinkage and rounding because of the breakdown of the proteinaceous cytoskeleton by caspases, the cytoplasm appears dense, the organelles appear tightly packed, Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, all these morphological changes occurs in the cell which is undergoing apoptosis.[15,16] The nuclear membrane gets destructed and the DNA inside it is fragmented in a process referred to as karyorrhexis. As a result the nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the fragmentation of DNA.[17] The cell membrane shows irregular buds known as blebs and the cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed. Based on the observation obtained in the present investigation it confirms that cell death caused by ethyl acetate fraction may be due to a programmed cell death – Apoptosis. The evidence of apoptosis was sought by

first looking for the formation of nuclear apoptotic bodies. Staining the cells with fluorescent dyes, including propidium iodide was used in evaluating the nuclear morphology of apoptotic cells. HepG2 cells in present experiment were grown in the presence of 100 µg of EAAMEL for 24 and 48 hr of incubation and analyzed by propidium iodide staining for the formation of apoptotic bodies. Figure 4 shows numerous apoptotic cells that showed nuclear condensation, formation of apoptotic bodies, giant cell formation and fragmentation.

FIGURE 3
HEP G 2 CELL VIABILITY ASSESSED WITH VARIOUS AMELE FRACTIONS OF CONCENTRATION 1000,500,100 µg

		HEP G 2 CELL CONTROL		POSITIVE CONTROL
				
1.	**100% Hexane**			
2.	**Hexane : Ethyl acetate (50:50)**			
3.	**100 % Ethyl acetate**			
4.	**Ethyl acetate : acetone (75 : 25)**			
5.	**Ethyl acetate : acetone (50:50)**			

6.	**100% Acetone**			
7.	**Acetone : Methanol (50 :50)**			
8.	**100% Methanol**			

DNA Fragmentation

DNA fragmentation which is a typical hallmark of the apoptotic cell death was analyzed in the study. The ethyl acetate fraction induces apoptosis in HepG2 cells resulting in the degradation of chromosomal DNA in to small oligonucleosomal fragments which results in fragmentation of DNA (Lane 2 and 3). The fragmentation of DNA was high at the higher concentration which is around 200 µg as observed in DNA fragmentation (Lane3), which was compared with the 1 kb ladder (Lane 1). In Case of untreated cells we observed the intact chromosomal DNA (Lane4). This interpretation confirms that cell death was caused by ethyl acetate fraction obtained from the crude AMELE. The treatment of Hep G2 cells with the ethyl acetate fraction resulted in the induction of intrinsic apoptosis activity at concentrations as low as 100 µg, which was predetermined by the MTT assay. Hep G2 cells treated with 200 µg of alcohol fraction also induces apoptosis after 48 hrs and showed typical features of DNA laddering on an agarose gel. In the figure 5, the DNA fragmentation of the HepG2 cells treated with 100 µg and 200 µg of EAAMEL is shown. Therefore, this study thus suggests that the ethyl acetate fraction at a concentration as low as 100 µg can induce nucleosomal DNA fragmentation of Hep G2 cell due to intrinsic apoptosis processes.

Molecular Players In Apoptosis - Caspases

Morphologically, apoptosis is first characterized by a change in the refractive index of the cell followed by cytoplasmic shrinkage and nuclear condensation.[22] The apoptotic cell does not provoke an inflammatory response, and only individual cells are affected by apoptosis *in vivo.* Effects of Ethyl acetate fraction of the extract on the activity of caspase 3and caspase 9 were showed in figure 6 and 7. The results indicated that 150µg/ml of ethyl acetate fraction showed significant increase of caspase 3 and caspase 9 activity when compared with the control group. The maximum activity was found at 300 µg/ml and least at 75 µg/ml. The caspase 3 activity was increased from 123.5 to 263.5 at concentration from 75 to 300 µg/ml of ethyl acetate extract. Camptothecin at concentration of 1 µg/ml could activate the activity of caspase-3 significantly at 344.5. The activity of caspase 9 was increased from 124 to 241 at concentration from 75 to 300 µg/ml of ethyl acetate extract. Camptothecin at concentration of 1 µg/ml could activate the caspase 9 significantly at 335. Caspase 9 was activated at final stage of apoptosis conditions.

Expression of NFκB & p53

Figure 8 depicts the Full length cDNA when subjected to amplification using NfκB primers (using Eppendorf Personnel Mastercycler, Germany) yielded a band at a size of 800bp.Figure 9 shows the Full length cDNA when subjected to amplification using P53 primers (using Eppendorf Personnel Mastercycler, Germany) yielded a band at a size of 700bp.Figure 10 shows the Full length cDNA when subjected to amplification using GAPDH primers (using Eppendorf Personnel Mastercycler, Germany) yielded a band at a size of 400bp.Figure 11 executes the graphical representation of expression studies.

DISCUSSION

Column Chromatography

Figure – 1 shows the TLC plates of various fractions. Based on the Rf of the spots the fractions were pooled as eight. Table 1 shows the details of the solvents used, number. of fractions pooled, color of the fraction,

fractions yield and yield % obtained from the column. All the extracts were almost pasty in nature with characteristic smell. 95 % of total extract was obtained and 5% of extract was lost by binding to silica and collection tubes. The fractions collected were concentrated.

MTT Assay

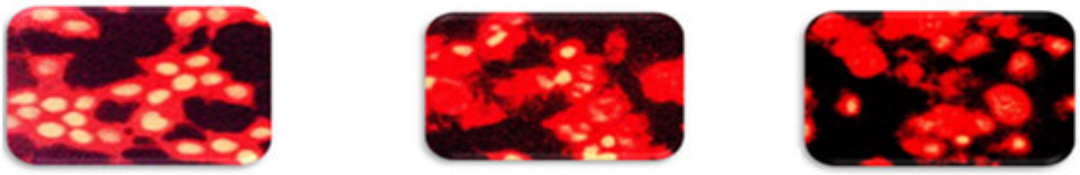
In order for a therapeutic agent to be truly effective, it should be toxic to tumor cells without harming normal cells. In the present investigation of microscopic examination of normal HepG2 are adherent, epithelial-like cells growing as monolayers and in small aggregates, appear healthy, high confluency of monolayer cells, translucent cytoplasm, cell shape rules, closely arranged with logarithmic growth. After 48 hrs treatment with AMELE extracts no change in morphology was observed at low concentration (100µg/ml) but in higher concentration which is about 1000 µg/ml changes in morphology occurs and density of Hep G2 cells were observed. Morphological assessment of treated Hep G2 with ethyl acetate fraction (100µg/ml) cells clearly indicated the play of apoptotic mechanisms leading to cell death. Morphological changes including cell shrinkage and loss of colony formation ability were observed. The treated cells appeared rounded off, shrunken and detached. 48 hrs after treatment extensive blebbing, presence of apoptotic bodies, dense cytoplasm, chromatin aggregates could also be seen in these cells. Figure 4 shows the inhibition of HepG2 cancer cell population by various fractions of the crude extracts of AMELE.

Fluorescent Microscopic Study

The result showed that there was an increase in HepG2 apoptotic cells after EAAMEL treatment for 24 and 48hr compared with the control sample. Apoptosis is the process of physiologically programmed cell death in which intrinsic pathway participates in the cell death.[18] The mechanism of apoptosis seems to depend on the stimuli (intrinsic and/or environmental such as drugs, cytotoxins, irradiation, infectious agents, etc). Apoptosis is identified as one of the most essential biological processes in eukaryotes which play a vital role in development and homeostasis, and also in several disease states.[19] The interpretation in the present study could therefore be of clinical and therapeutic significance, as well as pathophysiological research interest. This method of measuring apoptosis suggested that EAAMEL induces apoptosis in HepG2 cells in addition to cell growth inhibition. To substantiate this result, DNA fragmentation was studied by agarose gel electrophoresis.

FIGURE – 4

FLUORESCENT MICROSCOPIC PICTURES OF HEPG2 CELLS



(a) (b) (c)

(a) Control cells showing normal nuclear pattern.

(b) EAAMEL for 24 h were fixed, stained with propidium iodide clearly showing condensed and partial fragmentation red fluorescent nuclei.

(c) EAAMEL for 48 h fixed, stained with propidium iodide clearly showing nuclear condensation and apoptotic bodies, giant cell formation and fragmentation.

DNA Fragmentation

DNA fragmentation occurs in apoptotic cells that are caused by intrinsic activity which is induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180– 200 bp).[20] Caspases are indirectly involved in DNA fragmentation by activating cellular nucleases: CAD (caspase activated DNase) also called DFF40 (DNA fragmentation factor) exists in an inactive form by complexing with ICAD (inhibitor of CAD). During apoptosis caspase 3 cleaves ICAD, releasing and activating CAD which causes DNA degradation and chromatin condensation. [21] As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine whether the antiproliferative effect of *Aegle marmelos* leaves and its fractions on Hep G2 cells acts through the respective apoptosis pathway.

FIGURE - 5

DNA FRAGMENTATION

Lane 1: 1Kb DNA ladder (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp)

Lane 2: HepG2 cells treated with 100µg of EAAMELE

Lane 3: HepG2 cells treated with 200µg of EAAMELE

Lane 4: Control cells

Molecular Players in Apoptosis - Caspases

The activity of caspase 9 and caspase 3 was significantly elevated in a dose dependent manner. It was observed that 90% increase in activity of caspase 3 and caspase 9 was noticed in HepG2 liver cancer cells.The phytochemicals such as flavanoids trigger the apoptosis process through mitochondrial pathway involving activation of caspase-8, BID cleavage, release of cytochrome c, and caspase-3 activation finally leading to cell death in HepG2 cells.[23] In the present investigation, the significantly elevated activity of caspase- 9 and caspase-3 in the ethyl acetate fraction treated group further confirms that ethyl acetate fraction induces apoptosis *in vitro* by mitochondrial dysfunction.

FIGURE – 6

CASPASE 3 EXPRESSION IN HEP G2 CELL LINE

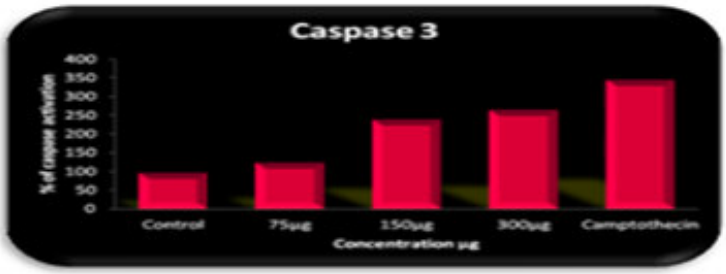
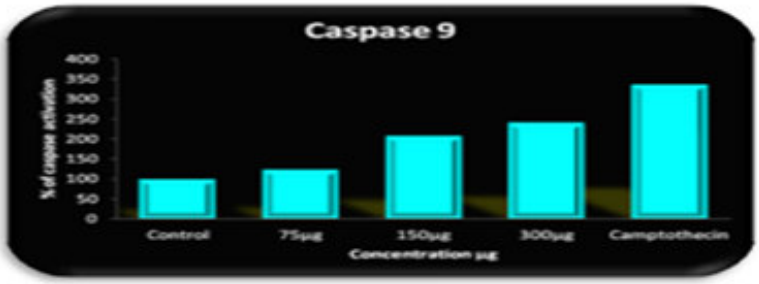


FIGURE – 7

CASPASE 9 EXPRESSION IN HEP G2 CELL LINE



Many reports have shown that plant extracts and pure compounds induce cell death in cancer cell through apoptotic mechanisms. [24,25,26,] Caspase 3 is a regularly activated death protease in mammalian cell which induce apoptosis. [27] Naturally occurring phytochemicals such as alkaloids, flavanoids and carotenoids have been reported to induce apoptosis in carcinoma cell lines by enhancing the activity of caspase 3. [28] Caspase 3 produced as an inactive zymogen which is a 32 KDa pro-caspase present in the cytoplasm, which is proteolytically cleaved at an aspartate residue to generate the cleaved fragments in the terminal, yielding a 12 KDa and 17 KDa subunit. Two subunits combine to form an active caspase 3 enzyme. This active caspase 3 have a wide range of activity over cellular substrates in which it cleaves the cellular substrates like structural proteins and DNA repair enzyme (eg: poly ADP- Ribose polymerase). It also activates endonucleases, caspase activated DNAse which may cause the DNA fragmentation. So DNA cannot be recovered back because of the inactive form of DNA repair enzyme. Finally this leads to the apoptosis of the cell. [29] In the

present study the effects of EAAMEL on caspase 3-activity in HepG2 cells and their relationship to ROS generation was studied and found to be high. As a positive control, Campothecin 1 µg/ml induced caspase 3-like activity. The extract tested induced caspase 3-like activation dose-dependently in the doses ranging from 75 µg to 300 µg/ml. These results suggested that EAAMEL induced cell death through caspase 3 pathway.

Apoptosis

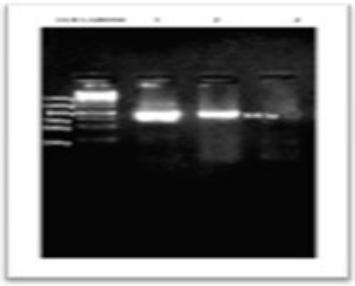
Apoptosis, or programmed cell death, was a tightly regulated mechanism where numerous proteins such as p53, NFκB, the ubiquitin proteosome system, and the P13K pathway controls cell number and proliferation as part of normal development.

Expression of NFκB & p53

NF-κB is a transcription factor that serves as a master switch for turning on certain immune and inflammatory responses. NF-κB alters cell behavior in many ways; it inhibits apoptosis, increases cell proliferation, and increases inflammatory and immune response. Recent evidence suggests that activation of NF-κB contributes to the development of several types of human cancer, including cancers of the blood and certain breast cancers. NF-KappaB (Nuclear Factor-KappaB) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The Rel/ NF-KappaB family of transcription factors are involved mainly in stress-induced, immune, and inflammatory responses. In addition, these molecules play important roles during the development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures. More recently, NF-κB family members have been implicated in neoplastic progression and the formation of neuronal synapses. NF-κB is also an important regulator in cell fate decisions, such as programmed cell death and proliferation control, and is critical in tumorigenesis.

FIGURE – 8

EXPRESSION OF NfκB



LANE 1: HepG2 cells treated with 100µg of EAAMELE

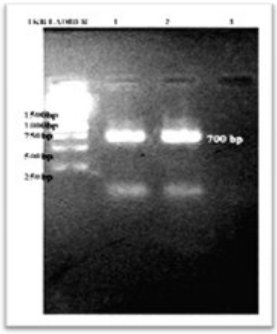
LANE 2: HepG2 cells treated with 50µg of EAAMELE

LANE 3: Control cells (Untreated cells)

p53 protein consists of an acidic N-terminus with a transactivation domain, a hydrophobic central DNA-binding core and a basic C-terminus with regulatory and oligomerisation domains. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood. In the present study, EAAMEL treatment induced the activation of the tumor suppressor protein p53. On comparing the treated cell line with untreated cells, it seems that NfκB gene was down regulated i.e., expressed at low levels than untreated cells.

FIGURE – 9

EXPRESSION OF P53



LANE 1: HepG2 cells treated with 50µg of EAAMELE

LANE 2: HepG2 cells treated with 100µg of EAAMELE

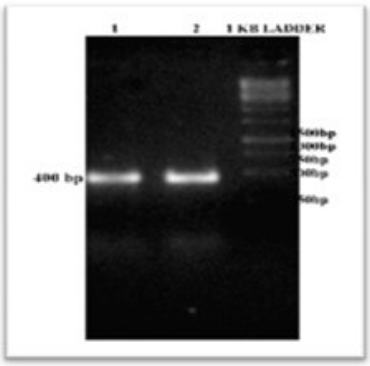
LANE 3: Control cells (Untreated cells)

On comparing the treated cell line with untreated cells, it seems that p53 gene was up regulated i.e., expressed at high levels than untreated cells. P53 mRNA levels in treated cells were 3fold increased than untreated cells.

✓ NFkB mRNA levels in treated cells were one fold decreased than untreated cells.

FIGURE – 10

EXPRESSION OF GAPDH:



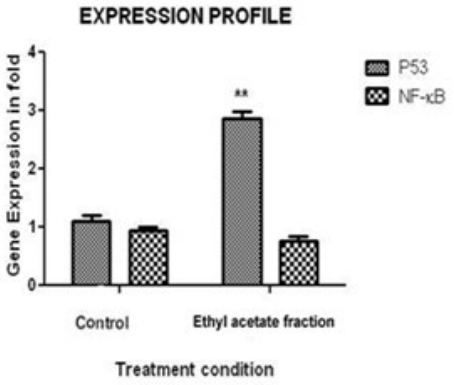
LANE 1: HepG2 cells treated with 50µg EAAMELE

LANE 2: HepG2 cells treated with 100µg EAAMELE

LANE 3 : 1 KB LADDER

FIGURE 11

GRAPHICAL REPRESENTATION OF EXPRESSION STUDIES



CONCLUSION

Aegle marmelos, is a medicinal plant from India used for treatment of cancer-related symptoms in the Indian system of medicine. The ability of cancer cells to avert the apoptotic program has been identified as one of the major mechanisms for the development of cancer. In a previous study, extracts from *A. marmelos* were found to inhibit the proliferation of multiple human tumor cell lines in in vitro assays, including the leukemic K562, erythroleukemic HEL, melanoma Colo38, and breast cancer MCF7 and MDA-MB-231 cell lines. But the exact mechanism remains unrevealed. In another study, it was shown that the ethyl acetate fraction of *A. marmelos* significantly inhibits proliferation of many cells. Subsequently, a single molecule, which was structurally characterized as HDNC, by detailed NMR and MS analysis was identified and found to inhibit tumor growth by inducing TNF-α mediated apoptosis program. The cytotoxic role of the active ethyl acetate fraction of AM was evident from the in vitro study using Hep G 2 cell lines. In this study, oxidative stress was significantly inhibited and enhanced apoptosis by upregulating the p53 and caspase 3 expressions simultaneously down regulating the NFκB. Thus, the extract increased nuclear condensation and DNA fragmentation and the ability of the active fraction from *Aegle marmelos* was potent to induce apoptosis by a multiprolonged cascade of events suggests that it might be an ideal therapeutic for many tumors of liver.

CONFLICTS OF INTEREST

Conflicts of interest declared none.

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