

Cytotoxic and apoptotic activities of *Amorphophallus campanulatus* tuber extracts against human hepatoma cell line

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Abstract

Amorphophallus campanulatus (Roxb.) Blume belonging to the family of Araceae, is a perennial herb commonly known as elephant foot yam. Its tuber has been traditionally used for the treatment of liver diseases, abdominal tumors, piles. The aim of the present study was to evaluate the dose-dependent cytotoxic and apoptosis inducing effects of the sub fractions of *Amorphophallus campanulatus* tuber methanolic extract (ACME) namely petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MeF) on human liver cancer cell line, PLC/PRF/5. Antiproliferative effects of the sub fractions of ACME were studied by MTT assay. Apoptotic activity was assessed by 4',6-diamidino-2-phenylindole (DAPI), annexin V- fluorescein isothiocyanate (FITC) and 5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescent staining. The chemotherapeutic drug, 5-fluorouracil (5-FU) was used as positive drug control. The sub fractions of ACME were found to produce considerable cytotoxicity in human liver cancer cell line, PLC/PRF/5. In addition, the extracts were found to induce apoptosis and were substantiated by DAPI, annexin V-FITC and JC-1 fluorescent staining. A pronounced results of cytotoxic and apoptotic activities were observed in the cells treated with 5-FU and CHF, whereas, EAF and MeF treated cells exhibited a moderate result and the least effect were observed in PEF treated cells. Furthermore, these findings confirm that the sub fractions of ACME dose-dependently suppress the proliferation of PLC/PRF/5 cells by inducing apoptosis.

Keywords: *Amorphophallus campanulatus*; PLC/PRF/5; Liver cancer; DAPI; JC-1; Annexin-V

INTRODUCTION

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa (1). Hepatocellular carcinoma (HCC) accounts for about 80%-90% of all liver cancers and is the fifth most common cancer and the third leading cause of cancer death (2-3). The incidence of hepatocellular carcinoma is increasing in many countries. Each year, more than 700,000 people are diagnosed with this cancer throughout the world and accounting for more than 600,000 deaths (4). The major known risk factors for HCC include hepatitis viral infection, food additives, alcohol, aflatoxins, environmental and industrial toxic chemicals, air and water pollutants etc. (5-6). Although there are many strategies for the treatment of liver cancer, its

remedial effect remains very poor and thus several approaches are made towards its prevention. Recently, considerable research has been carried out in the search for natural or synthetic compounds as a means of chemopreventive agents against liver cancer (7-8).

The deregulated cell proliferation and suppressed cell death are postulated as the basis for neoplastic progression in almost all cancer cases (9). Apoptosis, an ordered and orchestrated cellular process that occurs in physiological and pathological conditions, may be essential for the prevention of tumor formation and its deregulation is widely believed to be involved in pathogenesis of many diseases, including cancer (10-11). Most of the chemotherapeutic agents exert their action by inducing apoptotic death to block or suppress the growth of cancer cells (12).

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Natural products are an excellent source of complex chemicals possessing a wide variety of biological activities and having great potential therapeutic value (13-15). Crude extracts or components isolated from plants are important sources to screen as apoptotic inducers. Understanding the mechanism of action of these compounds should provide useful information for their possible application in cancer therapy and also in cancer prevention (16-17).

Amorphophallus campanulatus (Roxb.) Blume belonging to the family of Araceae is a perennial herb commonly known as elephant foot yam. Its rounded tuberous root stock or corm is used as an important source of food in many parts of the world (18). *A. campanulatus* tuber has also been used traditionally for the treatment of liver diseases, abdominal tumors, abdominal pain, piles etc. Besides, the corm has been reported to possess cytotoxic, hepatoprotective, antioxidant, antibacterial and antifungal activities (19-20). In addition to the reported pharmacological properties, a previous *in vivo* experiment conducted in our laboratory demonstrated that *A. campanulatus* tuber possess anti-hepatocarcinogenic properties (unpublished data). The present study was carried out with the aim of obtaining further evidence to confirm that apoptosis is indeed a major mechanism through which *A. campanulatus* tuber mediates its anti-hepatocarcinogenic effects. Thus the present study investigated the cytotoxic and apoptotic activities of the *A. campanulatus* tuber methanolic extract (ACME) sub fractions in human liver cancer cell line, PLC/PRF/5. Moreover, the present study is a step towards the isolation and characterization of the pharmacologically active phytochemical constituents from the crude extract of *A. campanulatus* tuber.

MATERIALS AND METHODS

Chemicals

Fetal bovine serum (FBS), *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (HEPES) and 4',6-diamidino-2-phenylindole (DAPI) were supplied by Sigma Chemical Co., St. Louis, MO, USA. 5-

flourouracil (5-FU) was purchased from Biochem Pharmaceutical Industries, Mumbai, India. Dulbecco's Modified Eagle Medium (DMEM), Trypsin - EDTA and antibiotic-antimycotic were procured from Gibco, Grand Island, N.Y, USA. Cell proliferation assay kit; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was purchased from HiMedia, India. 5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Invitrogen, Carlsbad, CA, USA. Annexin V- fluorescein isothiocyanate (FITC) kit was supplied by Calbiochem, La Jolla, CA, USA. Dimethyl sulfoxide (DMSO) was obtained from Merck, Mumbai, India. All the other chemicals used were also of high purity grade.

Cell culture

PLC/PRF/5 cell line was procured from National Centre for Cell Science (NCCS), Pune, India and grown as a monolayer in DMEM containing HEPES and sodium bicarbonate supplemented with 10% FBS and 1% antibiotic-antimycotics. Cells were maintained in a tissue culture flask and kept in a humidified incubator (5% CO₂ in air at 37 °C) with a medium change every 2-3 days. When the cells reached 70-80% confluence, they were harvested with trypsin-EDTA and seeded at lower concentration into a new tissue culture flask.

Collection and extraction of the plant material

Amorphophallus campanulatus tubers were harvested from Kottayam district, Kerala, India and authenticated. A voucher specimen (SBSBRL.02) is maintained in the institute. The shade-dried tubers were powdered and subjected to Soxhlet extraction with methanol (50 g in 400 mL) and concentrated under reduced pressure using a rotary evaporator. The percentage yield of methanolic extract in our study was approximately 9.3% (w/w). The methanolic extract was then taken in a round-bottomed flask of simple condenser and further fractionated using solvents in increasing polarity, viz. petroleum ether, chloroform, ethyl acetate and methanol and the sub fractions were collected as

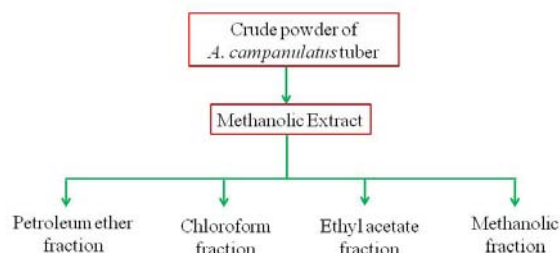


Fig. 1. Schematic diagram of the fractionation of *Amorphophallus campanulatus* tuber extract.

petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MeF) respectively, as depicted in Fig. 1.

Preparation of plant extracts and standard drug

Ten mg of PEF, CHF, EAF and MeF of ACME were dissolved in 50 μ L DMSO and made up to 1 mL with phosphate buffered saline. Subsequently, the drugs were sterilized using 0.22 μ m Durapore syringe filters (Millipore, Bedford, MA, USA) and were used as stock for further experiments. On the day of experiment, test solutions were prepared by diluting the stock solutions in DMEM to give different concentrations (100 μ g/mL and 50 μ g/mL). 5-FU, the standard control, was diluted to 50 μ g/mL and 25 μ g/mL with DMEM.

Cytotoxicity analysis

MTT assay

The cell viability was assessed by MTT assay (21), which determines the metabolically active mitochondria of intact cells. The assay was carried out using MTT cell assay kit, following the protocols described by the manufacturer's (HiMedia, India). Briefly, PLC/PRF/5 cells were seeded in 96-well plates (Greiner, Frickenhausen, Germany) with 5×10^3 cells/100 μ L and incubated for 24 h at 37 $^{\circ}$ C. The cells were then treated with PEF, CHF, EAF and MeF (100 μ g/ mL and 50 μ g/ mL), 5-FU (50 μ g/ mL and 25 μ g/ mL) and DMSO (0.1% v/v) and incubated for another 24 h at 37 $^{\circ}$ C in a 5% CO₂ atmosphere. The assay was performed by the addition of premixed MTT reagent, to a final concentration of 10% of total volume, to culture wells containing various concentrations of the test substance and incubated for further 4 h. During 4 h incubation,

living cells converted the tetrazolium component of the dye solution into a formazan product. 100 μ L of the solubilization solution (provided with the MTT assay kit) was then added to the culture wells to solubilize the formazan product and the absorbance at 570 nm was recorded using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate. Percentage inhibition was calculated using the formula,

$$\text{Percentage growth inhibition} = \frac{[(\text{Mean absorbance of the control cells}) - (\text{Mean absorbance of treated cells})]}{\text{Mean absorbance of control cells}} \times 100$$

Detection of apoptosis

The apoptotic potential of the sub fractions of ACME in PLC/PRF/5 cells were carried out by the following fluorescent staining methods: (a) 4'-6-Diamidino-2-phenylindole (DAPI) staining, (b) Annexin V- fluorescein isothiocyanate (FITC) staining and (c) 5,5',6,6' Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining.

DAPI staining assay

DAPI staining was performed according to the procedure described by Radhika *et al* (22) with minor modifications. PLC/PRF/5 cells were cultured in a 24-well tissue culture grade plate (Greiner, Germany) for 24 h. After incubation with two different concentrations (100 μ g/mL and 50 μ g/mL) of PEF, CHF, EAF and MeF for 24 h, cells were washed in phosphate buffered saline (PBS), fixed with 2% paraformaldehyde for 15 min and were treated with 0.2% triton X-100 in PBS for 15 min at room temperature. Cells after washing with PBS were stained with DAPI (1 μ g/mL) and incubated in dark for 30 min. 5-FU was used as control. The cells were then examined and photographed using a fluorescence microscope (IXL 40, Labovision, India).

Annexin V-FITC staining

Annexin V-FITC staining kit was procured from Calbiochem and the staining was carried out according to the previously described method (22). In brief, PLC/PRF/5 cells were seeded in a 12-well tissue culture grade plate (Greiner, Germany) and incubated for 24 h. After incubation, 100 μ g/mL and 50 μ g/mL of the sub fractions of ACME and 50 μ g/ mL and 25 μ g/ mL of 5-FU were further incubated for

12 h at 37 °C in a 5% CO₂ atmosphere. The cells were then washed with PBS and detached using 0.25% trypsin-EDTA solution. Subsequently, the cells were suspended with 1 X binding buffer (500 µL) containing annexin V-FITC (1.25 µL) and incubated for 15 min at room temperature in the dark. After 15 min cells were washed with 1 X binding buffer and then added with phenol red free medium and examined immediately by using the FITC filter (blue light) on a fluorescence microscope. Positive annexin V-FITC staining will appear bright apple green on the cell membrane surface.

JC-1 staining

The mitochondrial membrane potential ($\Delta\Psi_m$) was assayed using the cationic dye - JC-1 mitochondrial potential sensor (Invitrogen, USA), according to the manufacturer's directions. Briefly, PLC/PRF/5 cells were incubated for 24 h in a 24-well plates (Greiner, Germany) and the cells were treated with 100 µg/mL and 50 µg/mL of the sub fractions of ACME (PEF, CHF, EAF and MeF) and 50 µg/mL and 25 µg/mL of 5-FU for 18 h. The treated cells were washed with PBS and incubated for 30 min in 10% RPMI medium without phenol red containing JC-1 at a concentration of 2.5 µg/mL. The cells were then examined and photographed using fluorescence microscope.

Statistical analysis

Results are expressed as the Mean \pm SD and

all statistical comparisons were made by means of one way ANOVA test followed by Tukey post hoc analysis and *p*-values less than or equal to 0.05 were considered significant.

RESULTS

Cytotoxicity study

MTT assay

Cytotoxic effects of the sub fractions of ACME on PLC/PRF/5 cells were assessed by MTT assay. The results are graphically depicted in Fig. 2. PLC/PRF/5 cells were treated with 100 and 50 µg/mL of the sub fractions of ACME and the inhibition of cell proliferation was evaluated after 24 h. Percentage of cell inhibition values for 100 µg/mL of PEF, CHF, EAF and MeF were 14.9, 34.6, 27.2, and 23.9% respectively. Whereas, for 50 µg/mL of PEF, CHF, EAF and MeF, the percentage of cell inhibition values were 9.0, 20.9, 17.8 and 15.3% respectively. 5-FU, used as positive control, exhibited an inhibition of 35.6% and 23.8% when incubated with 50 µg/mL and 25 µg/mL respectively. These values of percentage inhibition of cell proliferation demonstrate the cytotoxic activity of the treated groups in the following order: 5-FU>CHF>EAF>MeF>PEF. All the treatment groups, except 50 µg/mL of PEF, exhibited significant cytotoxic effects on PLC/PRF/5 cells (*p*≤0.05) when compared to the cells treated alone with DMSO.

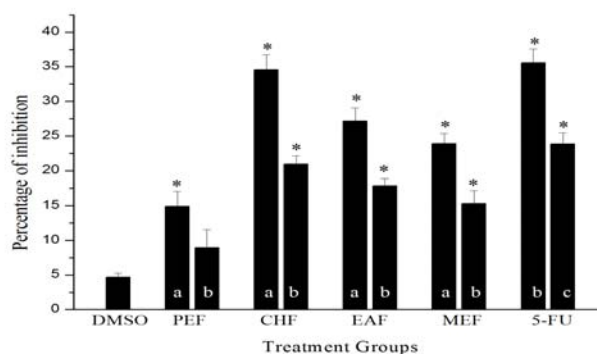


Fig. 2. Effect of PEF, CHF, EAF and MeF of *Amorphophallus campanulatus* tuber methanolic extract and 5-FU on the growth of PLC/PRF 5 cells determined by MTT assay. a; 100 µg/mL, b; 50 µg/mL, c; 25 µg/mL. Cells were treated with the sub fractions of *Amorphophallus campanulatus* tuber methanolic extract and 5-fluorouracil for 24 h and the percentage inhibition of cell proliferation was determined (n=3). **p*≤0.05 versus DMSO control. PEF; petroleum ether fraction, CHF; chloroform fraction, EAF; ethyl acetate fraction, MeF; methanolic fraction, 5FU; 5-fluorouracil.

Apoptosis assays

DAPI staining

The apoptosis inducing potential of the sub fractions of ACME and 5-FU were determined by DAPI staining. The results indicated that the number of apoptotic cells were higher in drug treated cells than untreated and DMSO controls. The changes that occurred in PLC/PRF/5 cells as a result of PEF, CHF, EAF, MeF and 5-FU treatment are represented in Fig. 3. Treatment with the sub fractions of ACME resulted in marked nuclear fragmentation and chromatin condensation which are clear indications of apoptosis. A pronounced result of apoptotic body formation and nuclear fragmentation were observed in the cells treated with 5-FU and CHF, whereas, EAF and MeF treated cells exhibited a moderate result and the least effect were observed in PEF treated cells.

Annexin V-FITC staining

Annexin V-FITC staining is an apparent marker of apoptosis. Treatment with the sub fractions of ACME and 5-FU showed a dose dependent increase in the number of cells that have taken up stain as shown in Fig. 4. Both untreated and DMSO treated group of cells displayed a very faint signal, when PLC/PRF/5 cells were stained with annexin V-FITC. Twelve h of treatment with the standard control (5-FU) resulted in the intensive staining of PLC/PRF/5 cells. Likewise, the intensity of annexin V-FITC staining were prominent in CHF treated cells indicating that

it is a potential source of apoptotic inducer in human liver cancer cell line. Whereas, the bright apple green fluorescence displayed by the EAF and MeF treated cells were modest in its intensity. It was also evident from the assay that, among the sub fractions of ACME, PEF possesses the least apoptotic activity.

JC-1 staining

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) is as an early event in apoptosis. When the cells stained with JC-1, the loss of $\Delta\Psi_m$ is indicated by the decrease of red fluorescence and the increase of green fluorescence. Eighteen h treatment of PLC/PRF/5 cells with 100 $\mu\text{g/mL}$ of CHF followed by the JC-1 staining resulted in green fluorescence in majority of cells. Cells treated with 50 $\mu\text{g/mL}$ of CHF also displayed a strong green fluorescence, indicating its potent apoptotic activity. 5-FU, positive control drug, showed green fluorescence in majority of cells in a dose dependent manner. As shown in Fig. 5, after JC-1 staining, the untreated PLC/PRF/5 cells and vehicle (DMSO) treated control cells displayed orange red fluorescence, while the EAF, MeF and PEF treated cells emitted both red orange and green fluorescence. The intensity of green fluorescence emitted from the cells treated with sub fractions of ACME was faint except in chloroform fraction. The results indicate that among the sub fraction of ACME, CHF possesses promising apoptotic potential.

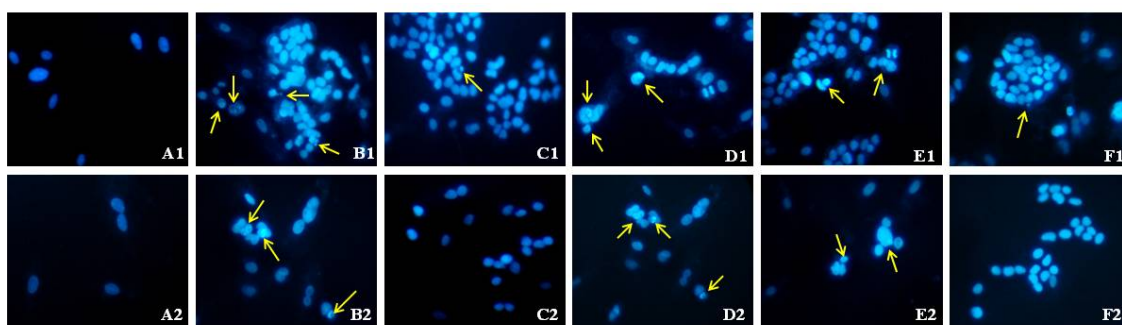


Fig. 3. Fluorescence image of PLC/PRF/5 cells treated with 4',6-diamidino-2-phenylindole (DAPI) after 24 h incubation with the sub fractions of *Amorphophallus campanulatus* tuber methanolic extract and 5-fluorouracil. Nuclear fragmentation and chromatin condensation are indicated with yellow arrows. A1; untreated cells, A2; cells treated with DMSO, B1; 5-FU (50 $\mu\text{g/mL}$), B2; 5-FU (25 $\mu\text{g/mL}$), C1; PEF (100 $\mu\text{g/mL}$), C2; PEF (50 $\mu\text{g/mL}$), D1; CHF (100 $\mu\text{g/mL}$), D2; CHF (50 $\mu\text{g/mL}$), E1; EAF (100 $\mu\text{g/mL}$), E2; EAF (50 $\mu\text{g/mL}$), F1; MeF (100 $\mu\text{g/mL}$), F2; MeF (50 $\mu\text{g/mL}$). Original magnification $\times 200$. 5FU; 5-fluorouracil, PEF; petroleum ether fraction, CHF; chloroform fraction, EAF; ethyl acetate fraction, MeF; methanolic fraction.

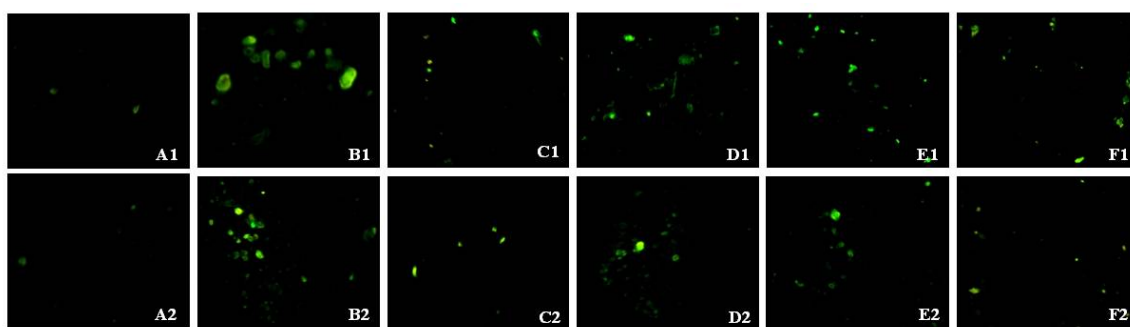


Fig. 4. Fluorescence image of PLC/PRF/5 cells treated with annexin V after 12 h incubation with the sub fractions of *Amorphophallus. campanulatus* tuber methanolic extract and 5-fluorouracil. Bright apple green fluorescence shows the annexin V- fluorescein isothiocyanate (FITC) staining on the cell membrane surface. A1; untreated cells, A2; cells treated with DMSO, B1; 5-FU (50 µg/mL), B2; 5-FU (25 µg/mL), C1; PEF (100 µg/mL), C2; PEF (50 µg/mL), D1; CHF (100 µg/mL), D2; CHF (50 µg/mL), E1; EAF (100 µg/mL), E2; EAF (50 µg/mL), F1; MeF (100 µg/mL), F2; MeF (50 µg/mL). Original magnification $\times 200$. 5FU; 5-fluorouracil, PEF; petroleum ether fraction, CHF; chloroform fraction, EAF; ethyl acetate fraction, MeF; methanolic fraction.

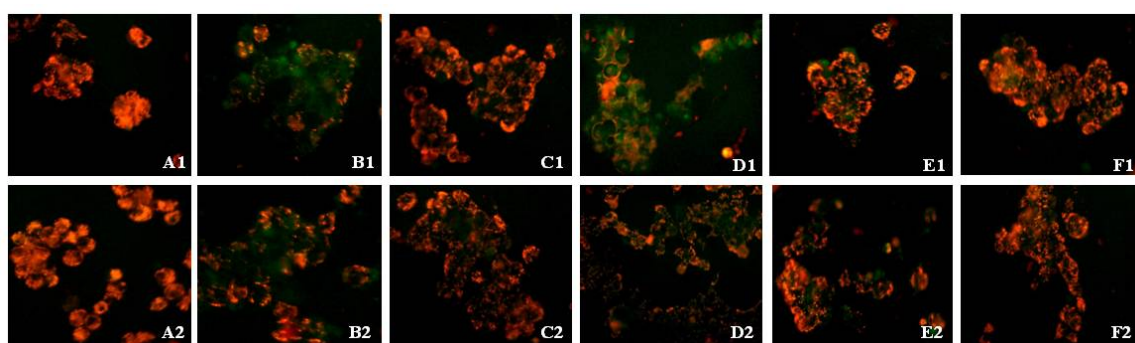


Fig. 5. Fluorescence image of PLC/PRF/5 cells treated with 5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) after 18 h incubation with the sub fractions of *Amorphophallus campanulatus* tuber methanolic extract and 5-fluorouracil. The green fluorescence indicates a decrease in mitochondrial membrane potential, an early event in apoptosis. A1; untreated cells, A2; Cells treated with DMSO, B1; 5-FU (50 µg/mL), B2; 5-FU (25 µg/mL), C1; PEF (100 µg/mL), C2; PEF (50 µg/mL), D1; CHF (100 µg/mL), D2; CHF (50 µg/mL), E1; EAF (100 µg/mL), E2; EAF (50 µg/mL), F1; MeF (100 µg/mL), F2; MeF (50 µg/mL). Original magnification $\times 200$. 5FU; 5-fluorouracil, PEF; petroleum ether fraction, CHF; chloroform fraction, EAF; ethyl acetate fraction, MeF; methanolic fraction.

DISCUSSION

Apoptosis is a process by which cells undergo programmed cell death under certain physiological or pathological conditions (23). Apoptosis and its related signaling pathways have a profound effect on the progression of cancer; therefore apoptosis is targeted in the treatment of various tumors (24-25). Many anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent the promotion and progression of tumor (26-27). Recently, researchers have

focused on screening novel anticancer drugs from plants to identify phytochemicals that could induce apoptosis.

In the present study, a dose-dependent growth inhibition observed in the drug treated cells indicates that the sub fractions of ACME are potential cytotoxic agents against human liver cancer cell line, PLC/PRF/5. Among the sub fractions of ACME, the highest cytotoxic activity was observed in CHF treated cells followed by EAF, MeF and PEF. The apoptosis inducing potential of the sub fractions of ACME, determined by DAPI staining, annexin V – FITC staining and JC-1

staining also indicate that CHF and EAF possess highest apoptotic potential than MeF and PEF. DAPI staining is a reliable apoptotic assay in chemoprevention studies and it helps to observe the apoptotic changes at DNA level (28). Whereas, the apoptotic alterations of the plasma membrane and mitochondria can be perceived through Annexin V-FITC staining and JC-1 staining respectively (29-30).

Apoptosis is initially characterized by morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (10). In the current study, the chromatin condensation and nuclear fragmentation were observed in PLC/PRF 5 cells by DAPI staining after 24 h of treatment with the sub fractions of ACME particularly with CHF and EAF. This helped in presuming that the cell death that occurred was not due to necrosis, but due to apoptosis. The result was confirmed by annexin V-FITC staining and JC-1 staining. It has been reported that loss of membrane asymmetry is an early event of apoptosis due to which phosphatidyl serine residues become exposed at the outer plasma membrane. This loss of membrane asymmetry and thereby apoptosis can be detected by utilizing the strong and specific interaction of annexin V with phosphatidyl serine (31). Annexin V binding is a major marker of apoptosis since phosphatidyl serine translocation to the cell surface precedes nuclear breakdown, DNA fragmentation and the appearance of most of apoptosis associated molecules (22). In our study, to characterize the cellular death process caused by the sub fractions of ACME and 5-FU, PLC/PRF/5 cells were treated with the drugs and binding of annexin V-FITC was detected by fluorescence microscopy. The intensive bright apple green staining on the cell membrane surface resulted after the treatment with CHF and EAF prove that these two sub fractions of ACME possess highest apoptotic potential than PEF and MeF. Furthermore, annexin V-FITC staining indicates that the apoptosis induced by the sub fractions of ACME and 5-FU on PLC/PRF/5 cells are apparently due to the loss of plasma membrane asymmetry.

JC-1 is a reliable probe for the analysis of mitochondrial transmembrane potential

changes occurring very early in apoptosis. It is a mitochondrial lipophilic dye and becomes concentrated in mitochondria in proportion to their membrane potential ($\Delta\Psi_m$); more dye becomes accumulated in mitochondria with greater $\Delta\Psi_m$ and ATP generating capacity. Therefore, fluorescence of JC-1 can be considered as an indicator of mitochondrial energy state and the dye exists as a monomer at low concentrations giving green fluorescence. At higher concentrations it forms J-aggregates giving red fluorescence. Therefore, in JC-1 staining, the apoptotic cells were identified by an increase in green fluorescence and the loss of red fluorescence (32-34). The results of JC-1 staining, observed in the present study, evidently indicate that the sub fractions of ACME are able to decrease the mitochondrial $\Delta\Psi_m$ and thereby can induce apoptosis in PLC/PRF/5 cells. Among the sub fractions of ACME selected for the present study, the green fluorescence was prominent in cells treated with chloroform fraction. The increase of green fluorescence and the loss of red fluorescence in PLC/PRF/5 cells treated with the sub fractions of ACME and 5-FU also established that the drugs exert their apoptotic potential in a dose-dependent manner. In addition, the results of the present study clearly correlate with earlier *in vitro* chemopreventive studies, which assayed the apoptotic potential of the plant extracts by using DAPI, annexin V-FITC and JC-1 fluorescent staining. The previous studies conducted in different cancer cell lines suggests that plant extracts with potential anticancer properties decreases the cell proliferation and induces early apoptotic changes such as nuclear fragmentation, flipping of phosphatidylserine of the plasma membrane and decrease of mitochondrial membrane potential (22,35).

It is reported that ACME possesses phytochemical constituents such as alkaloids, tannins, glycosides, phenols, flavonoids, saponins and carbohydrates (20). Published report also establishes the presence of betulinic acid, lupeol, stigmasterol, β -sitosterol, glucose, galactose, rhamnose and xylose in the corm of *A. campanulatus* (36). In addition to the reported phytochemicals, a previous study

conducted in our laboratory revealed the presence of four major cytotoxic/anticancer constituents in the methanolic extract of *A. campanulatus* tuber viz., cinnamaldehyde, ferulic acid, quercetin and asiatic acid (unpublished data). This in turn shows that the cytotoxic and apoptotic activity exhibited in the present study by the sub fractions of ACME might be attributed to the individual or combined activity of the identified phytochemicals.

CONCLUSION

In conclusion, this study demonstrates the antiproliferative and apoptotic activity of the sub fractions of ACME in human liver cancer cell line, PLC/PRF/5. Furthermore, the results corroborate that the sub fractions of ACME suppress cell growth by inducing apoptosis. Among the sub fractions of ACME, CHF significantly inhibited the proliferation of PLC/PRF/5 cells in a dose-dependent manner followed by EAF, MeF and PEF. Nevertheless, further studies are required to comprehend the different mechanisms by which the sub fractions of the methanolic extract of *A. campanulatus* tuber exert its cytotoxic and apoptotic effects on liver cancer cell line, PLC/PRF/5.

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