Chapter 6

IDENTIFICATION OF THE ACTIVE CYTOTOXIC AND APOPTOTIC FRACTION OF *AMORPHOPHALLUS CAMPANULATUS* (ROXB.) BL. TUBER USING HUMAN HEPATOMA, PLC/PRF/5 CELLS AND COLON CANCER CELL LINE, HCT-15
Chapter 6 (A)

Cytotoxic and apoptotic activities of *Amorphophallus campanulatus* (Roxb.) Bl. tuber extracts against human hepatoma, PLC/PRF/5 cells
6A.1. INTRODUCTION

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa (Qian and Ling, 2004). 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 (El-Serag and Rudolph, 2007; Marra et al., 2011). 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 (American Cancer Society, 2012). Major risk factors for liver cancer include hepatitis viral infection, food additives, alcohol, aflatoxins, environmental and industrial toxic chemicals, air and water pollutants, etc (Farazi and DePinho, 2006; Jemal et al., 2007). Although there are many strategies for the treatment of liver cancer, the poor treatment success make HCC one of the leading causes of death. As HCC is difficult to treat 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 (Marrero, 2005; Al-Rejaie et al., 2009).

In almost all instances, deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression (Evan and Vousden, 2001). 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 (Thompson, 1995; Wong, 2011). Most of the chemotherapeutic agents exert their action by inducing apoptotic death
to block or suppress the growth of cancer cells (Lee and Hong, 2010). Natural products are an excellent source of complex chemicals possessing a wide variety of biological activities and having great potential therapeutic value (Chiu et al., 2006; Deng et al., 2006). 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 (Taraphdar et al., 2001; Lin et al., 2007).

*Amorphophallus campanulatus* tuber has been traditionally used for the treatment of liver diseases. Besides, the corm has been reported to possess cytotoxic and antioxidant activities (Khan et al., 2007). In addition to the reported pharmacological effects, our previous investigations demonstrated that *A. campanulatus* tuber possesses antioxidant, hepatoprotective and anticancer properties, particularly against *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma. 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 *Amorphophallus campanulatus* tuber methanolic extract (ACME) sub fractions in human liver cancer cell line, PLC/PRF/5. Moreover, the cytotoxic and apoptotic assays of the sub fractions of ACME, is a step towards the identification and isolation of the biologically active compounds from the extract.
6A.2. MATERIALS AND METHODS

6A.2.1. Chemicals

Fetal bovine serum (FBS), N-2-Hydroxyethylpiperazine-N-2-ethane-sulphonic acid (HEPES) and 4′,6-diamidino-2-phenylindole (DAPI) were procured from 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) and antibiotic-antimycotic were purchased from Gibco, Grand Island, N.Y, USA. Cell Proliferation Assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide, [MTT]) was purchased from HiMedia, India. 5,5’,6,6’ Tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Invitrogen, Carlsbad, CA, USA. Annexin V- fluorescein isothiocyanate (FITC) kit was supplied by Calbiochem, La Jolla, CA, USA. All the other chemicals used were also of high purity grade.

6A.2.2. 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 (ethylene diamine tetra acetate) and seeded into a new tissue culture flask.
6A.2.3. Collection and preparation of plant extracts and its sub fractions

The shade-dried tubers of *A. campanulatus* 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, as depicted in figure 6.1.

![Figure 6.1. Schematic diagram of the fractionation of *A. campanulatus* tuber extract](image)

6A.2.4. Preparation of drugs

10 mg of petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) of the *A. campanulatus* tuber methanolic extract (ACME) were dissolved in 50 µL DMSO and made up to 1 mL with phosphate buffered saline. Subsequently, the drugs were sterilized using 0.22 µm 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 µg/mL
and 50 µg/mL). 5-flourouracil, the standard drug control, was diluted to 50 µg/mL
and 25 µg/mL with DMEM.

6A.2.5. Cytotoxicity study

6A.2.5.1. MTT assay

6A.2.6. Detection of apoptosis

6A.2.6.1. DAPI staining assay

6A.2.6.2. Annexin V-FITC staining

6A.2.6.3. JC-1 staining

(Detailed protocols are given in chapter 2, section 2.2.11. In vitro cytotoxic and
apoptotic assays)

6A.2.7. Statistical analysis

Results are expressed as mean ± standard deviation (SD). 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 0.05were considered significant.

6A.3. RESULTS

6A.3.1. Cytotoxicity study

6A.3.1.1. 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 figure 6.2.
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. PEF, CHF, EAF and
MEF exerted cytotoxic effects on PLC/PRF/5 cells with percentage of cell inhibition
values 14.9, 34.6, 27.2, and 23.9% for 100 µg/mL and 9.0, 20.9, 17.8 and 15.3% for 50 µg/mL respectively. 5-flourouracil, 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 ($p \leq 0.05$) when compared to the cells treated alone with DMSO.

![Figure 6.2. Effect of petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) of ACME and 5-fluorouracil on the growth of PLC/PRF 5 cells determined by MTT assay](image)

Cells were treated with the sub fractions of ACME (100 and 50 µg/mL) and 5-fluorouracil (50 and 25 µg/mL) for 24 hrs and the percentage inhibition of cell proliferation was determined. ($n = 3$). * $p \leq 0.05$ versus DMSO control.
6A.3.2. Apoptosis assays

6A.3.2.1. DAPI staining

The apoptosis inducing potential of the sub fractions of ACME and 5-FU were determined by DAPI staining. The changes that occurred in PLC/PRF/5 cells as a result of PEF, CHF, EAF, MEF and 5-FU treatment are represented in figure 6.3. The results indicated that the number of apoptotic cells were higher in drug treated cells than untreated and DMSO controls. 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.

6A.3.2.2. 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 figure 6.4. 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 hours 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.
6A.3.2.3. **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 hours treatment of PLC/PRF/5 cells with 100 $\mu$g/mL of CHF followed by the JC-1 staining resulted in green fluorescence in majority of cells. Cells treated with 50 $\mu$g/mL of CHF also displayed a strong green fluorescence, indicating its potent apoptotic activity. 5-fluorouracil, positive control drug, showed green fluorescence in majority of cells in a dose dependent manner. As shown in figure 6.5, 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 possess promising apoptotic potential.
Figure 6.3. Fluorescence image of PLC/PRF 5 cells treated with DAPI after 24 h incubation with the sub fractions of *A. 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 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 200×.
Figure 6.4. Fluorescence image of PLC/PRF 5 cells treated with Annexin V after 12 h incubation with the subfractons of *A. campanulatus* tuber methanolic extract and 5-fluorouracil.

Bright apple green fluorescence shows the Annexin V-FITC staining on the cell membrane surface, a marker of early-stage apoptosis.

**A**1 - Untreated cells; **A**2 - Cells treated with DMSO; **B**1 - 5-FU (50 µg/mL); **B**2 - 5-FU (25 µg/mL); **C**1 - PEF (100 µg/mL); **C**2 - PEF (50 µg/mL); **D**1 - CHF (100 µg/mL); **D**2 - CHF (50 µg/mL); **E**1 - EAF (100 µg/mL); **E**2 - EAF (50 µg/mL); **F**1 - MEF (100 µg/mL); **F**2 - MEF (50 µg/mL). Original magnification 200×.
Figure 6.5. Fluorescence image of PLC/PRF 5 cells treated with JC-1 after 18 h incubation with the sub fractions of *A. 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 200×.
6A.4. DISCUSSION

Apoptosis is a process by which cells undergo programmed cell death under certain physiological or pathological conditions (Qin et al., 2010). 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 (Lowe and Lin, 2000; Hsu et al., 2004). Many anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent the promotion and progression of tumor (Surh, 2003; Xu et al., 2009). Recently, researchers have focused on screening novel anticancer drugs from plants to identify phytochemicals that could induce apoptosis.

In the present study, the apoptosis inducing potential of the sub fractions of ACME were determined by DAPI, Annexin V – FITC and JC-1 staining. The initial characteristics of apoptosis such as chromatin condensation and nuclear fragmentation can be observed by DAPI staining. Likewise, Annexin V binding is a major marker of apoptosis because it detects the loss of membrane asymmetry – an early event in apoptosis. JC-1 also detects the early stage apoptosis by identifying the mitochondrial transmembrane potential changes.

The cytotoxicity induced by the sub fractions of ACME on PLC/PRF 5 cells were assessed using MTT assay. A dose-dependent growth inhibition observed in the drug treated cells indicates that the sub fractions of ACME possess potential cytotoxic activity 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.

Apoptosis is initially characterized by morphological features such as cell shrinkage, membrane blabbing, chromatin condensation and nuclear fragmentation (Thompson, 1995). 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 (van Engeland et al., 1998). 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 (Radhika et al., 2010). In this 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 (Smiley et al., 1991; Salvioli et al., 1997; Savitskiy et al., 2003). 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 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. Whereas, EAF and MEF treated cells exhibited a moderate result and the least effect were observed in PEF treated cells. 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.
Chapter 6 (B)

Cytotoxic and apoptotic activities of *Amorphophallus campanulatus* (Roxb.) Bl. tuber extracts against human colon carcinoma cell line HCT-15
6B.1. INTRODUCTION

Colon cancer arises due to diverse genetic and epigenetic changes in the colonic epithelium. Its development is often characterized at an early stage by hyperproliferation of the epithelium leading to the formation of adenomas. This is mainly a consequence of dysregulated cell cycle control and/or suppressed apoptosis as usually observed in colon cancers (Gryfe et al., 1997; Potten 1997). In addition, several studies have reported that loss of control of apoptosis results in cancer initiation and progression and that many new treatment strategies targeting apoptosis are feasible and may be used in the treatment of various types of cancer (Tu et al., 1996; Vitale-Cross et al., 2004; Tian et al., 2007; Wong, 2011).

Nowadays, large numbers of natural compounds have been identified that are pharmacologically highly effective against cancerous cells. Block et al. (1992) reported that the consumption of a diet rich in phytochemicals can reduce the risk of cancer. Fruits and vegetables, which contain a diverse range of phytochemicals, are suggested to have properties important to the prevention of cancer. It includes antioxidant, anti-inflammatory and antiproliferative activities as well as modulatory effects on subcellular signaling pathways, induction of cell cycle arrest, and apoptosis (Aviram et al., 2000; Kaplan et al., 2001; Afaq et al., 2005).

Among the various possible experimental models of cancer, human cancer cell lines have been the most widely used. They have retained the hallmarks of cancer cells namely self sufficiency in growth control, insensitivity to antigrowth signals, escape from checkpoints (apoptosis), genetic instability and invasiveness (van Staveren et al., 2009). HCT-15 cells, used in the present study, are one of the human adenocarcinoma cell lines extensively used in colon cancer studies. These
cells were established from a colorectal cancer after surgical resection before the chemotherapeutic treatment of a tumor (Tompkins et al., 1974).

*Amorphophallus campanulatus* tuber has been traditionally used for the treatment of abdominal tumors (Warrier et al., 1994). Our previous investigations demonstrated that *A. campanulatus* tuber possesses antioxidant, hepatoprotective and anticancer properties, particularly against 1, 2-dimethylhydrazine (DMH) induced colon carcinogenesis. In the present study, our aim was to investigate the dose-dependent cytotoxic and apoptosis inducing effect of the subfractions of *Amorphophallus campanulatus* tuber methanolic extract (ACME) viz. petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) in colon cancer cell line, HCT-15. The results were compared with 5-fluorouracil (5-FU), a pyrimidine analog used in the treatment of colorectal cancer (Mohapatra et al., 2011).

**6B.2. MATERIALS AND METHODS**

**6B.2.1. Chemicals**

Fetal bovine serum (FBS), *N*-2-Hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (HEPES) and 4′,6-diamidino-2-phenylindole (DAPI) were procured from Sigma Chemical Co., St. Louis, MO, USA. 5-flourouracil (5-FU) was purchased from Biochem Pharmaceutical Industries, Mumbai, India. RPMI Medium and antibiotic-antimycotic were purchased from Gibco, Grand Island, N.Y, USA. All the other chemicals used were also of high purity grade.

**6B.2.2. Cell culture**

HCT-15 cell line was procured from National Centre for Cell Science (NCCS), Pune, India and grown as a monolayer in RPMI (Rosewell Park Memorial
Institute) medium 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 (ethylene diamine tetra acetate) and seeded into a new tissue culture flask.

6B.2.3. Preparation of plant extracts and its sub fractions

The shade-dried tubers of *A. campanulatus* 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 petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) respectively.

6B.2.4. Preparation of drugs

10 mg of PEF, CHF, EAF and MEF of ACME were dissolved in 50 µL DMSO and made up to 1 mL with phosphate buffered saline. Subsequently, the drugs were sterilized using 0.22 µm 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 RPMI medium to give different concentrations (100 µg/mL and 50 µg/mL). 5-flourouracil, the standard control, was diluted to 50 µg/mL and 25 µg/mL with RPMI medium.
6B.2.5. Cytotoxicity study

6B.2.5.1. MTT assay

6B.2.6. Detection of apoptosis

6B.2.6.1. DAPI staining assay

6B.2.6.2. Annexin V-FITC staining

6B.2.6.3. JC-1 staining

(Detailed protocols are given in chapter 2, section 2.2.11. In vitro cytotoxic and apoptotic assays)

6B.2.7. Liquid chromatography-mass spectrometry (LC-MS) analysis of CHF

The chloroform fraction (CHF) of the methanolic extract of A. campanulatus tuber was analyzed using LC-MS 2010A instrument (Shimadzu, Kyoto, Japan). 10 µl of the filtered sample was injected to the manual injector using a Microsyringe (1-20µl, Shimadzu). The mobile phase used was Water: Methanol (50:50) in an isocratic mode. The column and pump used were Reverse Phase C-18 (25 cm X 2.5 mm) (phenomenex) and SPD 10 AVP-RD respectively. The separated compounds were then ionized using Electrospray Ionisation method (ESI). The flow rate was maintained to 2.0 ml/min with a temperature of 25°C and spectral data were collected at 254 nm. Mass analysis was performed in the range 50-1000 m/z, under both positive and negative ion mode. The class V P integration software was used for the data analysis. The constituents of the CHF were identified by referring the LC-MS library, Metwin 2009 (version 2.1).
6B.2.8. Statistical analysis

Results were expressed as mean ± standard deviation (SD). 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.

6B.3. RESULTS

6B.3.1. Cytotoxicity study

6B.3.1.1. MTT assay

The results of dose dependent cytotoxic evaluation of the sub fractions of ACME on HCT-15 cells are graphically shown in figure 6.6. The cells were treated with 100 and 50 µg/mL of the sub fractions of ACME and the inhibition of cell proliferation was assessed after 24 h. PEF, CHF, EAF and MEF exerted cytotoxic effects on HCT-15 cells with percentage of cell inhibition values 14.0, 32.5, 28.1, and 24.0% for 100 µg/mL and 8.5, 19.6, 18.6 and 16.2% for 50 µg/mL respectively. 5-flourouracil, used as positive control, showed an inhibition of 34.8% and 24.3% 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 HCT-15 cells ($p \leq 0.05$) when compared to the cells treated alone with DMSO.
Figure 6.6. Effect of petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) of ACME and 5-fluorouracil on the growth of HCT-15 cells determined by MTT assay

Cells were treated with the subfractions of ACME (100 and 50 µg/mL) and 5-fluorouracil (50 and 25 µg/mL) for 24 hrs and the percentage inhibition of cell proliferation was determined. (n = 3). * p ≤ 0.05 versus DMSO control.

6B.3.2. Apoptosis assays

6B.3.2.1. DAPI staining

The results of DAPI staining indicated that the number of apoptotic cells were higher in drug treated cells than untreated and DMSO controls. The changes that occurred in cells as a result of PEF, CHF, EAF, MEF and 5-FU treatment are shown in figure 6.7. After DAPI staining, HCT-15 cells treated with the drugs showed 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.
6B.3.2.2. Annexin V-FITC staining

Staining with Annexin V-FITC is an apparent marker of apoptosis. Both untreated and DMSO alone treated group of cells displayed a very faint signal, when HCT-15 cells were stained with Annexin V-FITC. 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 figure 6.8. Twelve hours of treatment with the standard drug control (5-FU) resulted in the intensive staining of HCT-15 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 colon 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.

6B.3.2.3. 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 HCT-15 cells with 100 $\mu$g/mL of CHF followed by the JC-1 staining resulted in green fluorescence in majority of cells. Cells treated with 50 $\mu$g/mL of CHF and 100 $\mu$g/mL of EAF also displayed a strong green fluorescence, indicating its potent apoptotic activity. 5-fluorouracil, the positive control, showed green fluorescence in majority of cells in a dose dependent manner. MEF and PEF treated cells exhibited both red orange and green fluorescence. Whereas the untreated cells and vehicle treated control cells showed orange red fluorescence only (Figure 6.9).
6B.3.3. LC-MS analysis of CHF

The constituent of CHF identified using LC-MS analysis that possesses potent cytotoxic and anticancer property was ferulic acid (Molecular mass 194.19). The mass spectrum of CHF by LC-MS is given in figure 6.10
Figure 6.7. Fluorescence image of HCT-15 cells treated with DAPI after 24 h incubation with the sub fractions of *A. campanulatus* tuber methanolic extract and 5-fluorouracil.

Nuclear fragmentation and chromatin condensation are indicated with red arrows.

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 200×.
Figure 6.8. Fluorescence image of HCT-15 cells treated with Annexin V after 12 h incubation with the sub fractions of A. campanulatus tuber methanolic extract and 5-fluorouracil.

Bright apple green fluorescence shows the Annexin V-FITC staining on the cell membrane surface, a marker of early-stage 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 200×.
Figure 6.9. Fluorescence image of HCT-15 cells treated with JC-1 after 18 h incubation with the sub fractions of *A. 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 200×.
Figure 6.10. Mass spectrum of CHF by LC-MS analysis

(A). Mass spectrum of positive ionization. (B). Mass spectrum of negative ionization
6B.4. DISCUSSION

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. In cancer, there is a loss of balance between cell division and cell death and cells that should have died did not receive the signals to do so. Defects along apoptotic pathways play a crucial role in carcinogenesis (Wong, 2011). Although many treatment strategies that target apoptosis are introduced, the interests in the search for natural compounds with potential apoptotic activity are still in high. In the present work, we have studied the cytotoxic and apoptotic potential of the subfractions of ACME on human colon carcinoma cell line HCT-15.

MTT assay is an established method of determining viable cell number in proliferation and cytotoxicity studies (Sylvester, 2011). In the present study, cytotoxic effect of the subfractions of ACME on HCT-15 cells were determined based on reduction of the yellow colored water soluble tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) to formazan crystals. Mitochondrial dehydrogenase produced by live cells reduces MTT to blue formazan product, which reflects the normal function of mitochondria and cell viability (Lau et al., 2004). A dose-dependent reduction of MTT activity (or color change from yellow to purple) observed in 5-FU and extracts treated cells indicated their cytotoxic potential against HCT-15 cells. Among the subfractions of ACME, the highest activity was observed in CHF treated cells followed by EAF, MEF and PEF.

Apoptosis, a form of programmed cell death, is characterized by cell shrinkage, chromatin condensation; inter nucleosomal DNA fragmentation, and the
formation of apoptotic bodies. Apoptosis and its related signaling pathways have a profound effect on the progression of cancer; therefore, the induction of apoptosis is a desirable goal for the prevention of cancer. Apoptosis has emerged as an important mechanism by which dietary compounds may exhibit chemopreventive potential (Song et al., 2012; Ramesh and Alshatwi, 2013). Salomons et al. (1999) also reported that anticancer drugs exert their antitumor effect against cancer cells by inducing apoptosis. Thus, in the present investigation, it is hypothesized that the subfractions of ACME may exert its cytotoxic activity on HCT-15 cells by inducing apoptosis. We tested this hypothesis and found that the cytotoxic effect of the subfractions of ACME is coupled with apoptosis. The morphological changes that occur in apoptotic cells, induced by the drugs, were perceived through DAPI staining. This demonstrated that the treatment with 5-FU, CHF and EAF mainly resulted in apoptotic body formation, chromatin condensation and nuclear fragmentation. It clearly indicates their apoptotic potential against colon cancer particularly on HCT-15 cells.

In healthy cells, phospholipids are asymmetrically distributed, with the anionic phospholipid phosphatidylserine normally confined to the cytoplasmic face of the plasma membrane (Balasubramanian and Schroit, 2003). It has been reported that loss of membrane asymmetry is an early and critical event of apoptosis due to which phosphatidylserine 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 phosphatidylserine (van Engeland et al., 1998). Phosphatidylserine translocation to the cell surface precedes nuclear breakdown, DNA fragmentation, and the appearance of most
apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis. In the present study, to characterize the cellular death process caused by the sub fractions of ACME and 5-FU, HCT-15 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 confirm that these two sub fractions of ACME possess highest apoptotic potential than PEF and MEF. Annexin V-FITC staining also indicates that the apoptosis induced by the sub fractions of ACME and 5-FU on HCT-15 cells are apparently due to the loss of plasma membrane asymmetry.

Mitochondria represent key organelles for the cell survival, and their role in programmed cell death is known since several years (Lugli et al., 2005). 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. J-aggregates are multimers of JC-1 formed inside intact mitochondria and their formation and fluorescence responds linearly to increase in mitochondrial membrane potential. Therefore, in JC-1 staining, the apoptotic cells were identified by an increase in green fluorescence and the loss of red fluorescence (Smiley et al., 1991; Salvioli et al., 1997; Savitskiy et al., 2003). The results of JC-1 staining, observed in the
present study, obviously indicate that the sub fractions of ACME are capable to
decrease the mitochondrial $\Delta \Psi_m$ and thereby can induce apoptosis in HCT-15 cells.
Among the sub fractions of ACME, the green fluorescence was prominent in cells
treated with CHF and EAF. The increase of green fluorescence and the loss of red
fluorescence in HCT-15 cells treated with 5-FU, CHF and EAF also demonstrated
that the drugs exert their apoptotic potential in a dose dependent manner.

Many studies revealed the anticancer properties of plant based foods in cell
culture and animal models. The phytochemical constituents present in the plant
based foods are mainly responsible for their apoptotic activity (Surh, 2003). *A. campanulatus*
tuber, evaluated for the apoptotic activity in the present study, is
mainly consumed as food in many parts of the world. The cytotoxic and apoptotic
activity exhibited by the extracts of *A. campanulatus* tuber might be attributed to the
presence of the already identified pharmacologically active phytochemical
constituents such as Cinnamaldehyde, Ferulic acid, Retinol, Quercetin and Asiatic
acid. Whereas, the major anticancer compound identified by the LC-MS analysis of
the most promising chloroform fraction was ferulic acid. Janicke et al. (2011)
reported that the phenolic compound ferulic acid possess antiproliferative effect
against colon cancer cell lines.

In conclusion, this study demonstrates the antiproliferative and apoptotic
activity of the sub fractions of ACME in human colon carcinoma cell line, HCT-15.
Among the sub fractions of ACME, CHF significantly inhibited the proliferation of
HCT-15 cells in a dose-dependent manner. Whereas, EAF and MEF treated cells
exhibited a moderate result and the least effect were observed in PEF treated cells.
The inhibitory effect of natural bioactive substances in carcinogenesis and tumor
growth may be through two main mechanisms: modifying redox status and interference with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis) (Kampa et al., 2007). Apoptosis has been reported to play an important role in the elimination of seriously damaged cells or tumor cells by chemopreventive or chemotherapeutic agents (Galati et al., 2000). Therefore, apoptosis-inducing natural products including the CHF, identified in the present investigation, can be explored as novel target for anticancer drug development. However, further studies are needed 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 colon cancer cell line, HCT-15.