

Quantification of Bcl-2/Bax genes in A549 Lung Cancer Cell Lines Treated with Heptamethoxy Flavones

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Abstract

Introduction: Heptamethoxy flavones (HMF) isolated from *Sphaeranthus amaranthoides* can bring inhibitory result on the growth of lung adenocarcinoma cells. However, very few studies have demonstrated a relative effect of the HMF on A549 cells. This study aims to investigate the underlying molecular mechanisms involved in the therapeutic activity of HMF in human lung adenocarcinoma cells. **Materials and Methods:** HMF was isolated from *S. amaranthoides* using column chromatography, and the structure was elucidated using nuclear magnetic resonance studies. The cytotoxicity was assessed using MTT assay, and the morphological change was observed by performing dual staining (ethidium bromide/acridine orange). The integrity of nuclear membrane was investigated using propidium iodide staining, and the apoptosis proteome was also assessed. The levels of proliferative and apoptotic genes were quantified using quantitative polymerase chain reaction. Finally, apoptosis was confirmed with the cell cycle analysis using flow cytometry. **Results:** The cells were maintained in the exponential phase by optical cell count, and HMF showed the half maximal inhibitory concentration value of 83.67 µg/mL. Morphological changes of cell and the nucleus proved the apoptosis with evidence of blebbing and chromatin condensation. The cell cycle analysis includes an accumulation of the cells in G1–G0 and less number of cells at other stages of cell cycle. The enhanced levels of apoptotic gene (caspase-3 and Bax with GAPDH) expression and lowered levels of Bcl-2 (proliferative gene) expression were triggered through intrinsic and extrinsic pathways during apoptosis. **Interpretation and Conclusion:** It is concluded that the HMF isolated from *S. amaranthoides* was induced apoptosis through Bcl-2/Bax signaling pathways with the involvement of caspase-3 at G1–G0 cell cycle arrest.

Key words: A549, apoptosis, blebbing, caspase, heptamethoxy flavone, *Sphaeranthus amaranthoides*

INTRODUCTION

Lung cancer is the major public health problem and is one of the leading causes of death in the developing countries until 2030. Although advanced therapeutic strategies are available, poor diagnosis of lung cancer showed a survival rate of only 15%. Surgery is the most effective treatment option for the patients diagnosed at a later stage, only 30% of them are suitable for therapeutic resection. Therefore, the alternative options are chemotherapy and radiotherapy. Clinical evidence showed that these alternative options are associated with serious side effects with poor efficacy.^[1] According to the studies on gefitinib treatment, the effective rate is 8.9–69% in an advanced stage in lung cancer.^[2] Thus, these factors made the urgent need for the new therapeutic agents with the lower side effects.

Dietary phytochemical compounds and medicinal plants are the important sources of novel bioactive compounds as cytotoxic agents. Among them, flavonoids could play an important role in the anticancer activity. Thus, the effects of flavonoids have been observed on different organs. Around the world, the medicinal plants provide a diversity of medicines for cancer treatment. Noteworthy, the search for the bioactive compounds will not only end with the plants but also microbes and marine organisms proved to be rich sources of chemically diverse cytotoxic

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compounds.^[3] *Sphaeranthus amaranthoides* has a proven pharmacological activity, such as rosmarinic acid isolated showed a protective effect on developing Zebrafish embryos.^[4] *S. amaranthoides* is a small and decumbent herb, with stem rooting, pubescent, and exploited hair leaves palmately 3-foliolate. Features of the herb: Annual with spreading branches are not winged of 8–12", leaves of 2–4", linear, oblong narrowed at the base. This plant is well known for its medicinal value for the treatment of eczema, blood disorder, stomach worms, and filarial fever and also known as a reducer of kapha, vata, and piles. It is also known to cure the skin diseases. *S. amaranthoides* belongs to plant kingdom, dicotyledon class, gamopetalae subclass, inferae series, asterales order, and Asteraceae (Compositae) family. It is widely found as a weed in the paddy fields of southern India and grows well in Himachal Pradesh in extreme conditions.

There is a growing interest in herbal medicines due to their effectiveness, minimal side effects in clinical experience, and relatively low cost. Herbal drugs or their biological active compounds are still unexploited. Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Natural remedies from medicinal plant are considered to be an effective and alternative treatment for lung cancer. Hence, here we focus to study the protective role of heptamethoxy flavone (HMF) from *S. amaranthoides* in lung cancer cell lines.

MATERIALS AND METHODS

The method column chromatography used to isolate and confirmed using thin-layer chromatography (TLC). 10 and 11 fractions were collected based on the TLC result. For these two fractions, nuclear magnetic resonance (NMR) studies were performed and identified the presence of HMF.^[5]

Cell growth and toxicity assay of drug

Human lung cancer cell line A549 and Vero cell lines were procured from National Centre for Cell Science, Pune, India, with the passage number of 29. Cells were maintained in Dulbecco's modified Eagle's medium and enriched with 10% fetal bovine serum (FBS), with 100 µg/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C in the incubator. Cells were grown in 75 cm² culture flasks, and after a few passages, cells were seeded for experiments. A 0.25% trypsin-EDTA solution was added on obtaining the 70–80% confluence growth was observed by inverted microscope, and the culture solution was replaced every 2 days. On the 2nd day, cells were exposed to various concentrations (80 and 160 µg/mL) of HMF and cultured at a temperature of 37°C with 5% CO₂ for 24 h.

Viability of cell

The cytotoxicity was measured by colorimetric assay (MTT assay). The A549 cells were seeded in 96-well titer plates at the optical density of 5×10^4 cells/well and incubated for 24 h to get the attachment. The proliferation test was based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. After 24 h incubation, the medium was replaced with 100 µL of fresh medium and HMF at different concentrations (10–200 µg/well) and incubated for 24 h. Untreated cells served as control and received only 0.1% dimethyl sulfoxide in which the fraction was prepared. At the end of treatment period, medium from control and HMF-treated cells was discarded, and 50 µL of MTT (5 mg/mL phosphate buffered saline [PBS]) was added to each well. Cells were then incubated for 4 h at 37°C in CO₂ incubator. At the end of the incubation period, the cell viability was determined using an ELISA reader (Bio-Rad). The optical density used was 570 nm. Optical density of each sample was compared with control optical density, and graphs were plotted. Each experiment was repeated 3 times.

Assay of nuclear morphology

Ethidium bromide (EB)/acridine orange (AO) staining was carried out to identify the morphology of the nucleus in the apoptotic cells by the method of Xu *et al.*,^[6] with slight modifications. The cells were seeded in a 96-well plate with 5×10^4 cells/well in 0.6 mL medium and covered with sterile coverslip then incubated. After incubation, the medium was removed and replaced with fresh medium plus 10% FBS, 20 and 40 µg/mL of HMF, and 80 and 160 µg/mL (selected based on the half maximal inhibitory concentration [IC₅₀]) HMF for 24 h. After the incubation period, the coverslip with monolayer cells was inverted on the glass slide with 20 µg of 100 µL of dye mixture (1:1) of EB and AO at room temperature. The culture medium was aspirated from each well, and cells were gently rinsed twice with PBS and incubated in a binding buffer with 100 µL of dye mixture (1:1) of EB and AO at room temperature. The fluorescence was read on a fluorescence microscope (Olympus Co., Japan). The percentage of apoptotic cells was determined by the equation: Percentage of apoptotic cells = (total number of apoptotic cells/total number of cells counted) × 100.

Apoptotic assay

Apoptosis was measured with Annexin V/propidium iodide (PI) dual dye kit. The cells were maintained in the logarithmic growth phase in a density of 5×10^4 in six-well plates and allowed to grow at 37°C in a humidified CO₂ incubator until they are 70–80% confluent. On the following day, HMF in a concentration of 80 and 160 µg/mL was added to these tissue culture plates. Then, the cell culture was allowed at

37°C in a humidified 5% CO₂ for 24 h. The culture medium was aspirated slowly, and the cells were rinsed with 1 mL of pre-cooled 1× PBS twice, and then cells were collected. The cells were incubated with 1 mL of PI (Annexin V/PI dual dye kit) in the dark at 37°C for 1 h and nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy. The percentage of cells in the different phases of cell cycle was analyzed.

Distribution of cell cycle

The determination of cell cycle distribution was done by staining the DNA with PI. The logarithmic phase cells in six-well plates with the concentration of 3–5 × 10⁴/well and incubated at 37°C in a humidified 5% CO₂ for 24 h. On the next day, two different concentrations (80 and 160 µg/mL) of HMF were added to the six-well tissue culture plates. After culturing in a cell culture condition, the medium was aspirated gently from the culture plates, and the cells were washed with 1 mL of pre-cooled 1× PBS. The cells were incubated with 1 mL of PI for 1 h. The cell cycle distribution was analyzed by flow cytometry (FCM, Becton–Dickinson, San Jose, CA, USA), and the percentage of cells in the different phases (G₀/G₁) of the cell cycle was analyzed by Flow Jo 7.6 software and represented graphically.

Apoptotic markers assay using quantitative-polymerase chain reaction (Q-PCR)

The A549 lung cancer cells were grown in half confluence in a six-well plate, and the different concentrations of HMF were added and kept for 12 h. Precise primers target gene, and GAPDH genes (reference gene) were designed based on sequence data from the Ensembl database (<http://www.ensembl.org>) [Figure 1]. Blast search was performed to confirm the total genes specificity of the nucleotide sequences chosen for the primers against dbEST and nr (the non-redundant set of GenBank, EMBL, and DDBJ database sequences). The amplification of contaminating genomic DNA was inhibited using one of the two primers at the junction between two exons. The total RNA was isolated (using TRNzol A+kit) and converted to cDNA using kits (Promega, Madison, WI, USA). The primer sequences were obtained from IDT. Q-PCR was then carried out with an SYBR Green PCR Master Mix kit (Promega) according to the manufacturer's instructions. Housekeeping gene (GAPDH) was used as an internal control, and the fold-change was calculated using 2^{-ΔΔCT}.

Statistical analysis

Data were expressed as mean ± standard error of the mean and analyzed by Tukey's test to determine the significance of differences between groups. *P* < 0.05, 0.01, and/or 0.001 was considered to be significant.

RESULTS

HMF

The isolated HMF from *S. amaranthoides* NMR studies was already published³ and structure was analyzed [Figure 2].

Preparing the microtiter plates of cell culture and growth kinetics

The cytotoxicity experiment initiated by ensuring the exponential phase of cell growth and maintained throughout the assay.

During cytotoxicity experiments, maintaining the cells at the exponential phase and cell count are necessary to study the further assay. Hence, to initiate the cytotoxicity experiments, the cell count was maintained at the exponential phase for the complete duration of the assay and an optical density at the end of the experiment in a range of 1.5–2.0 [Figure 3].^[7]

Cytotoxicity assay: Proliferation inhibition in human A549 lung adenocarcinoma cells.

The results for cytotoxicity assay in normal Vero cell line are shown in Figure 4. The non-toxic dose of the HMF in Vero cell line showed that the percentage with regard to the viability of cells was found to be 97% at a concentration of 160 µg/mL. With this, the toxicity of the HMF was found. A549 cells were with fusiform or polygon with well grown, and good amount of cytoplasm was observed in the control group when compared with the HMF-treated group. The

S.No	GENE	Primer Sequence	Size (bp)	Accession no
1	CASPASE 3	Forward 5' CTGAGGAACCGCTTTGTG3' Reverse 5' CCACCAGCCAATGAGAAG3'	116	NM_131877.3
2	GAPDH	Forward 5' ATACACGGAGCACCAGGTTG3' Reverse 5' GGTACATACACGGTTGCTG 3'	155	NM_001115114.1
3	BCL2	Forward 5' CTGCTGAGGAAGATGACAC3' Reverse 5' GGCTATCAGGCATTAGAG3'	116	NM_001030253.2
4	BAX	Forward 5' GTGTATGAGCGTGTTCGT3' Reverse 5' GAGTCGGCTGAAGATTAGAG3'	187	AF231015.1

Figure 1: Reverse and forward primer sequence for quantitative polymerase chain reaction

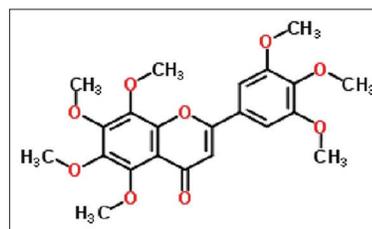


Figure 2: Heptamethoxy flavones structure

change in the morphology and number of cells was obviously seen with the HMF treatment at the concentrations of 10–200 $\mu\text{g}/\text{mL}$ for 24 h. After 24 h of treatment, the number of cells was decreased, the morphology and size were changed with week adherence. The IC_{50} of HMF was found as 83.67 $\mu\text{g}/\text{mL}$. With this concentration of the HMF, the number of dead cells was increased, and morphology of the cell was changed. The changes in the morphology in control and HMF treatment groups at various concentrations are shown in Figure 5.

Observation of cellular morphology change using dual staining assay

Since in dual staining assay, AO is an imperative dye and could stain nuclear DNA in the cells with intact plasma membrane and EB could only stain cells which lost the membrane integrity. The control group cells are green in color indicating them as live cells. Hence, live cells will be in uniform green color, early apoptotic cells will be densely stained as green-yellow fragments, whereas late apoptotic cells will stain densely in orange color and show orange fragments, the third type of cells which are necrotic cells completely visible in bright orange color. There was no condensed chromatin found in necrotic cells by AO/EB dual staining. From the current results, it was very clear that after the treatment with HMF

in a concentration of 20–160 $\mu\text{g}/\text{mL}$ for 24 h, the nuclear morphological changes were analyzed. Figure 6a shows the green live A549 cells are with normal nuclear morphology. The early apoptotic cells were with yellow-green dots, and the bright green early apoptotic cells with the nuclear margination and chromatin condensation were observed in A549 cell nuclei in the experimental group when treated with HMF (20 and 40 $\mu\text{g}/\text{mL}$). The orange-colored apoptotic cells showed that the condensed chromatin fragmentation and apoptotic bodies could be seen when treated with HMF. The results suggested that HMF is able to induce apoptosis in lung adenocarcinoma cell lines. The apoptotic cell percentage is significantly low when compared with HMF, whereas the 80 and 160 $\mu\text{g}/\text{mL}$ of HMF [Figure 6b-e] showed a drastic increase in apoptotic cells ($P < 0.001$) to 69 and 84%, respectively [Figure 7].

Apoptosis induced by HMF

Apoptosis was further confirmed by analyzing the nuclear morphology in A549 lung adenocarcinoma cell lines. The HMF-treated cells were less in number, flatter, enlarged, and with vacuolated cytoplasm [Figure 8b and c]. This was a concentration-dependent event. The fragmented nuclei were with intense fluorescence and membrane blebs [Figure 8a-c].

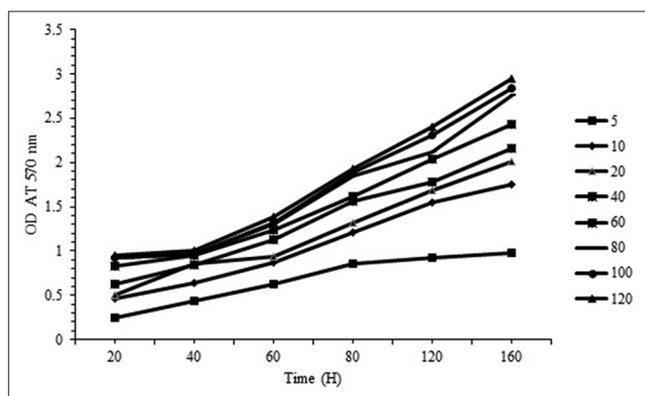


Figure 3: Growth kinetics

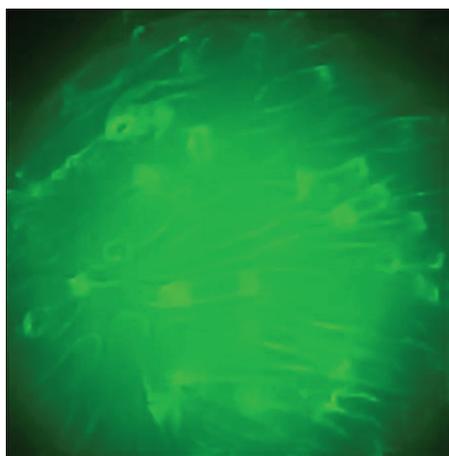


Figure 4: Vero cell line treated with heptamethoxy flavones

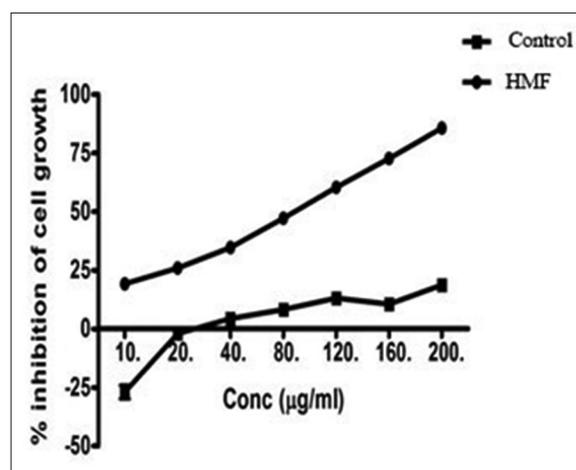


Figure 5: Cytotoxicity of heptamethoxy flavones compared with control against A549 lung adenocarcinoma cells

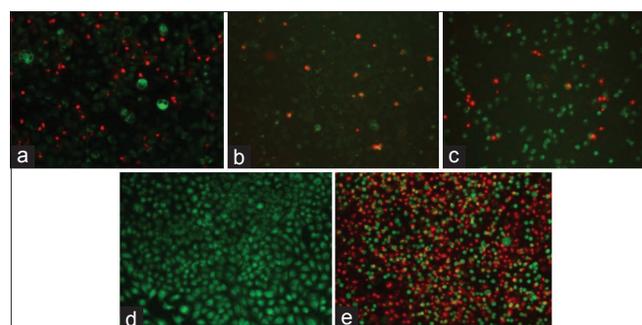


Figure 6: (a-e) Effect of heptamethoxy flavones on the morphology of lung cancer cell line A549 using acridine orange/ethidium bromide staining (dual staining)

The percentage of apoptotic nuclei was increased enormously ($P < 0.001$) to 70 and 89% after treating with 80 and 160 $\mu\text{g}/\text{mL}$ of the HMF, respectively [Figure 9].

HMF induced cell cycle arrest in G0/G1 phase

Figure 10 shows the proportion of accumulation of cells in G1 as the concentration of HMF increases. The percentage of lung adenocarcinoma cells in G1 stage was 20% at the concentration of 160 $\mu\text{g}/\text{mL}$ and the cell accumulation was 33% when compared with control ($P > 0.05$). When we plotted cells in sub- G0–G1 phase against the concentration of the HMF showed a sigmoid behavior [Figure 10a-c].

Q-PCR

Quantification of expressed pro-apoptotic and proliferation genes was calculated after normalizing with GAPDH gene. We noticed a significant difference in the expression of Bax, Bcl-2, and caspase-3 in A549 cells exposed to HMF at the concentration of 80 and 160 $\mu\text{g}/\text{mL}$ for 24 h. The lowered levels of the Bcl-2 [Figure 11c] gene were observed with the increase in the concentration of HMF. On the other hand, the levels of caspase-3 and Bax are significantly raised. These increased levels are correlated with the Facs analysis and specify the reduced proliferating cells. Our results showed that HMF enhanced the fold change of the Bax and caspase-3 when normalized with the GAPDH [Figure 11a and b] genes in A549 cell lines with time and concentration of HMF.

DISCUSSION

Lung cancer is a leading cause of cancer-related death worldwide.^[8] The anticancer activity of alkaloids from *S. amaranthoides*^[9] was proved earlier.

However, this study made an attempt to find the anticancer effects of the HMF at purified flavonoid through suppress cell proliferation, inducing cell apoptosis, and affecting cell cycle gene expression. According to the previous reports, flavonoids inhibit the growth of RD cell lines *in vitro*^[10] and possess anti-tumorigenic activities. The HMF isolated from *S. amaranthoides* showed significant inhibition of cell growth in A549 cell lines by inducing the apoptosis of cancer cells. Hence, this study demonstrates that HMF induces cytotoxicity in A549 lung cancer cell lines. The IC_{50} of HMF was 83.67 $\mu\text{g}/\text{mL}$. The annexin V and PI negative staining do not distinguish between the apoptotic death and necrotic pathway died, the dead cells will stain with both annexin V and PI. Moreover, apoptosis is measured over time, cells were tracked from annexin V and PI negative (viable cells), annexin V positive and PI negative (indicates early apoptosis and cells with membrane integrity), and finally to annexin V and positive PI (shows end-stage apoptosis and death). In this study, the passage of cells through these stages by HMF

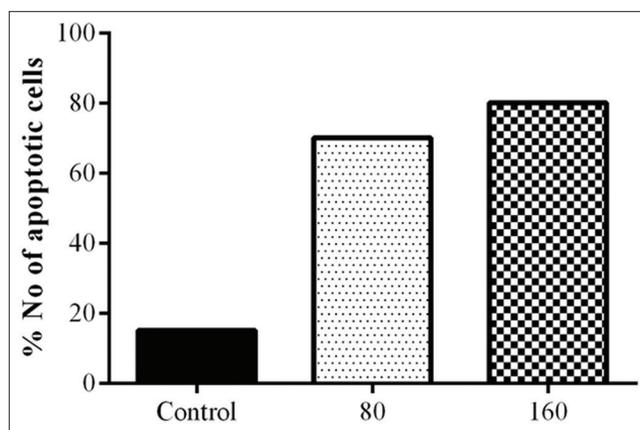


Figure 7: Bar graph showing the percentage number of apoptotic cells in heptamethoxy flavones nuclear fragmentation assay

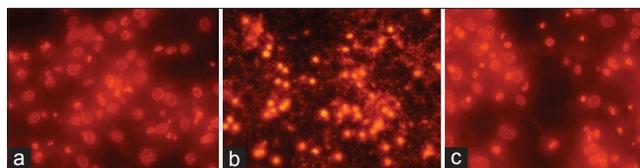


Figure 8: Propidium iodide staining assay of heptamethoxy flavones (HMF) and their morphological changes in the nuclei. (a) Control without treatment (b) HMF (80 $\mu\text{g}/\text{mL}$) (c) HMF (160 $\mu\text{g}/\text{mL}$)

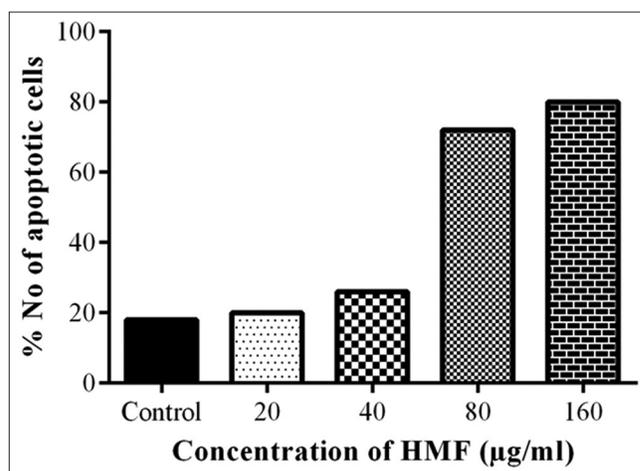


Figure 9: Percentage of apoptotic nuclei in lung cancer cell lines with heptamethoxy flavones

suggests that apoptosis and apoptotic cell percentage increase when compared with the normal cells.

The cell cycle arrest is dependent on the time and dose of HMF. The growth inhibition rate in A549 cell follows two mechanisms: (1) Inducing cell apoptosis and (2) blocking the cell cycle. Like most of the flavonoids, HMF may also induce cell apoptosis through enhancing the caspase-3 gene and Bax. Caspase is proteolytic enzymes produced in the cells of living organisms. In this study, the expression of mRNA of caspase-3 and Bcl-2 was upregulated. The results proved that

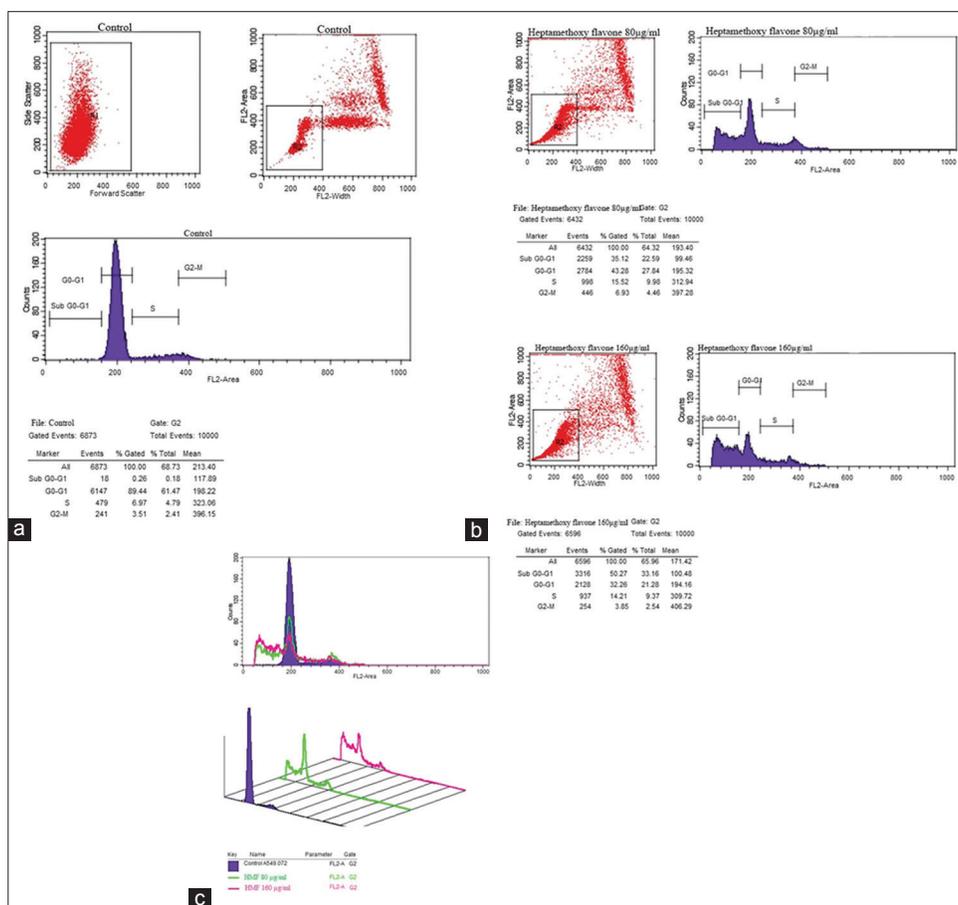


Figure 10: Flow cytometry (a) control (b) heptamethoxy flavones (c) cell count

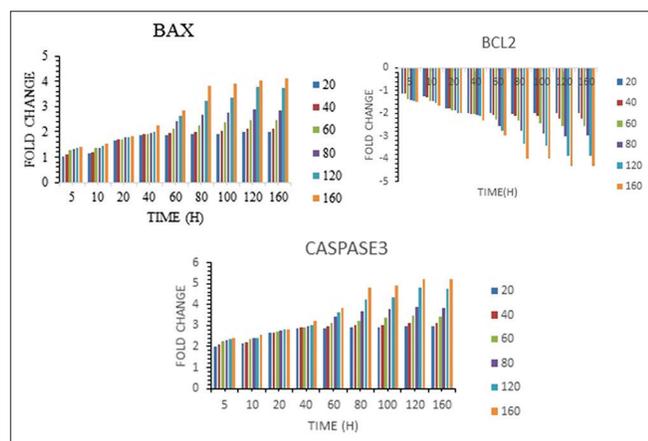


Figure 11: Quantitative polymerase chain reaction gene expression analysis of apoptotic and proliferative gene when treated with heptamethoxy flavones

HMF may induce apoptosis both at mRNA expression level and by influencing the cell cycle by increasing the percentage of cells in G0/G1 stage, which made the cells difficult to enter next phase and reduce the S phase and G2/M phase. Therefore, the mitosis division is abnormal; cyclin D proteins are cell cycle regulatory proteins may play an important role in altering the cell cycle. The HMF found to be more toxic for cancer cells than normal cell lines. The findings from

this study suggest that HMF may be a novel and attractive therapeutic compound for cancer treatment.

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