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Potential Anticancer Natural Product Against Human Lung Cancer Cells

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Abstract: PM 701 has potential anticancer activity and it caused selective programmed cell death in lung cancer cells (A549) as described previously, this further study is aimed to present the cytotoxic effect of the lyophilized PM 701 and its fraction. Lyophilized PM 701 appears to have cytotoxic activity due to its in vitro inhibition of the proliferation of lung cancer cells. The bio-guided extraction of PM 701 gives fraction PMF (150 mg g^{-1} of lyophilized PM 701). PMF was able to inhibit significantly the proliferation of A549 cells without affecting the normal, HFS cells. Chromatographic fractionation of PMF produced seven compounds, where the subfraction PMEK (108.7 mg g^{-1} of PMF) was the most effective one with high anti-proliferative activity against A549 cells. However, the subfraction PMFK is under extensive work in order to isolate the pure bio-active compound (s) in nano-scale levels as a novel pharmaceutical composition.

Key words: PM 701, lung cancer cells, human foreskin, PMF, PMFK

INTRODUCTION

Cancers are uncontrolled cell proliferations that result from the accumulation of genetic changes in cells endowed with proliferative potential. After a variable latency period during which they are clinically silent, the malignant cells progress to aggressive invasive and metastatic stages with tumor formation and wide-spread dissemination throughout the body (Feng, 2006; Coufal *et al*., 2007). Despite significant advances achieved in cancer treatment, which has relied on surgery, chemotherapy, radiotherapy, hormone therapy and more recently immunotherapy, cancers still a cause of pain and death in our world (He and Liu, 2007). The management of malignancies in humans still constitutes a major challenge for contemporary medicine (Widodo *et al*., 2007). Chemotherapy very often causes severe side effects, which are in part a consequence of destruction of normal cells (Khorshid and Moshref, 2006). It was revealed that commonly used anticancer approaches cause significant toxicity in body systems and is responsible for harmful side effects (Moshref *et al*., 2006; Stierle *et al*., 2006; Veluri *et al*., 2006). It is of crucial importance that anticancer drugs display antiproliferative activity in tumor cells without affecting normal tissues. Therefore, taking all the above-mentioned evidences into account, the development of novel approaches and effective anticancer strategies is critically needed and eagerly being pursued (Sporn, 1996; Vijayakumar and Hellman, 1997; Chabner and Roberts, 2005).

PM 701 is a natural liquid product which is clean, sterile and it is free of toxicity as proved by our research team in previous work (Khorshid *et al*., 2005; Khorshid, 2008). It has been proven that PM701 had anti-proliferative and apoptotic efficacy which targeted only the cancer cells. Conversely, it does not affect normal healthy cells (Khorshid *et al*., 2005; Khorshid and Moshref, 2006; Moshref *et al*., 2006); this implies that PM701 may have a selectively killing effect on cancer cells with a reparative effect on normal dividing cells. It was effective in limiting of metastasic spreading effect of leukemia cells in animal models (Moshref *et al*., 2006).

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Therefore, this study is directed for the further examination of lyophilized PM701 and it's active fractions on the growth of cancer and normal cells.

MATERIALS AND METHODS

This study was conducted in 2007- 2008 at Tissue Culture Unit, King Fahd Medical Research Center, King Abdul Aziz University in Jeddah, Saudi Arabia.

Preparation of PM 701 Powder (Lyophilized Form)

Lyophilized PM701 was prepared according to the method of Remington (2006) with slight modification.

Ninety gram of PM701 was added to microcrystalline cellulose (10 g), mix, lyophilized and kept under vacuum for 24 h at room temperature in a desiccator over calcium chloride. PM701 concentration was estimated from the weight of dry powder.

Extraction of PM 701

Water suspension of PM 701 was shacked with different solvents, such as $CHCl_3$, ethyl acetate, n-butanol or with methyl alcohol. The best solvent for extraction was found to be methyl alcohol which coded fraction PMF, 150 mg g^{-1} of lyophilized PM701.

Fractionation of PMF

PMF fractionation was performed using classic column chromatography on silica gel (Ferguson *et al*., 2004). This yielded to seven subfractions (Table 1), whereas G7 that coded PMEK (108.7 mg g^{-1} of PMF) was the most effective one.

Cell Lines

Human non small lung cancer (A549), Human Fore Skin (HFS) and Vero (Kidney, African green monkey, Cercopithecus aethiops) cell lines have been obtained from American Type Cultural Collection (ATCC) and were available in the cell bank of Tissue Culture Unit, King Fahd Medical Research Center (KFMRC), Jeddah, KSA.

Treatment with Lyophilized PM 701 or It's Fractions

Cytotoxicity assays were performed using short incubation for only 24 hr in serials dilutions of examined drug (1-10 µg) that were used to estimate the IC_{50} concentration. The long incubation for more than 72 h, with a concentration lower or higher than IC_{50} concentration, was also used to confirm the cytotoxicity effect. The cells were maintained 24 h in MEM containing 10% heat-inactivated FCS (Gibco Laboratories, Cergy Pontoise, France), 50 µg mL^{-1} geneticin (G418), 300 µg mL^{-1} glutamine, 0.25 µg mL^{-1} fungizone, 100 µg mL^{-1} streptomycin and 100 units mL^{-1} penicillin G. All cell lines were grown as monolayers at 37°C in a humidified 5% CO_2 incubator. The cells were harvested with 0.5 g L^{-1} trypsin (Gibco Laboratories) and 0.2 g L^{-1} EDTA (Gibco Laboratories) for 3 min.

Table 1: PMF subfractions and their weight in each gram of PMF

Subfractions of PMF	Amount of subfractions in each gram of PMF
G1	165.3 (mg)
G2	32 (mg)
G3	0.68 (g)
G4	194 (mg)
G5	270 (mg)
G6	193.3 (mg)
G7	108.7 (mg)

Serial dilutions of lyophilized PM 701 or PMF or PMFK were prepared and supplied to the normal and cancer cells (treated), after the selected time of treatment, the medium was aspirated. Then cells washed with PBS, trypsinized and counted by two methods counter coulter and Hemocytometer using trypan blue dye exclusion test (Pollared and Walker, 1998; Khorshid, 2005).

RESULTS

IC_{50} Estimation (Short Incubation)

This experiment was preformed to estimate the IC_{50} concentration of all tested agent.

Effect of Lyophilised PM701 on A549 Cells

The cytotoxic effect of lyophilized PM 701 was studied by incubated lung cancer cells A549 for 24 h in MEM media with serial concentration of the drug. PM 701 inhibited the proliferation of cancer cells and the IC_{50} was about 4 µg of PM701 mL^{-1} (Table 2).

Effect of PMF on A549 Cells

Treatment of A549 with serial concentrations of PMF fraction in incubated media showed inhibition of the cell proliferation with IC_{50} near the concentration 2.5 µg of PMF mL^{-1} (Table 3).

Effect of PMFK on A549 Cells

The serial concentrations of PMFK treatment in incubated media inhibited the proliferation of A549 cells and the IC_{50} was determined near the concentration 5 µg of PMFK mL^{-1} (Table 4).

Long Incubation

In vitro Experiment Using Lyophilized PM 701

PM 701 treatment caused the inhibition of cell proliferation of A549 cancer cells compared with non-treated cells. The inhibition of cell growth was increased by prolonging the time of incubation

Table 2: The effect of lyophilized PM701 on the growth of A549 cancer cells after 24 h of incubation comparing with non treated cancer cells

Conc. (µg mL^{-1}) (X)	N	Mean (Y)	SD	SE
Control	3	0.270	0.000	0.000
1	3	0.256	1.527	8.819
7.5	3	0.230	1.000	5.774
10	3	0.226	2.886	1.667

Table 3: The effect of PMF on the growth of A549 cancer cells after 24 h of incubation comparing with non treated cancer cells

Conc. (µg mL^{-1}) (X)	N	Mean (Y)	SD	SE
Control	3	5.233	0.839	0.484
2.5	3	4.000	0.700	0.404
5	3	3.367	0.702	0.406
7.5	3	3.133	0.611	0.353
10	3	3.033	0.902	0.521

Table 4: The effect of PMFK on the growth of A549 cancer cells after 24 h of incubation comparing with non treated cancer cells

Conc. (µg mL^{-1}) (X)	N	Mean (Y)	SD	SE
Control	3	1.200	0.000	0.000
5	3	1.000	0.173	1.000
7.5	3	0.838	0.153	8.819
10	3	0.733	5.700	3.333

(Fig. 1, 2). There were 65 and 76% decrease in cell proliferation after 24 and 48 h of incubation, respectively. Furthermore the count of the normal cells (Vero) shows normal progress, when incubated in media containing PM 701 (Fig. 3).

In vitro Experiment Using the Fraction PMF (G)

Exposure of cell line A549 cells to fraction PMF showed a decrease in the cell number compared with the number of untreated control cells (Fig. 4, 5). At the same time, the number of the normal cells (HFS) did not influence by PMF treatment (Fig. 6).

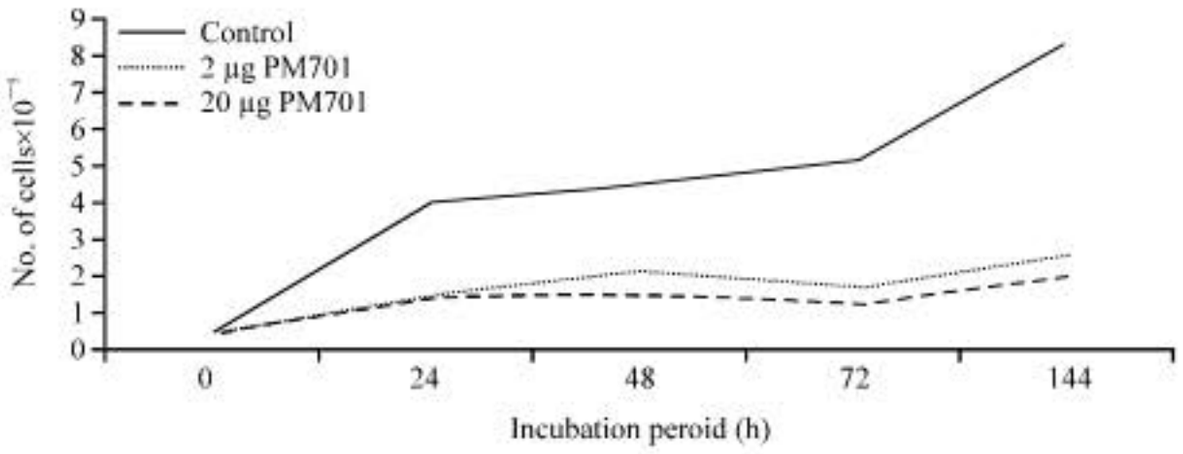

Fig. 1: The effect of lyophilized PM 701 on the proliferation of cancer cells after different incubation periods comparing with non treated cancer cells (control)

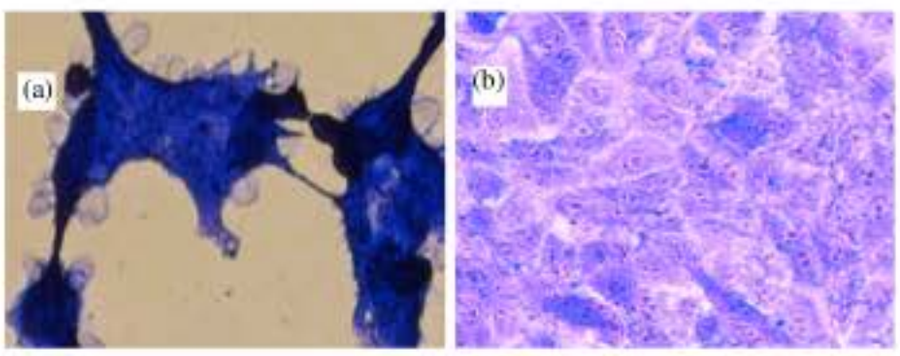

Fig. 2: The effect of lyophilized PM701 on the cell morphology of human lung carcinoma cell line, A549. A549 cells imaged (40x) after incubation for 24 h, fixed and stained with Coomassie blue: (a) in PM 701, note the damage of cells as compared with the control cells and (b) that were incubated in normal MEM media

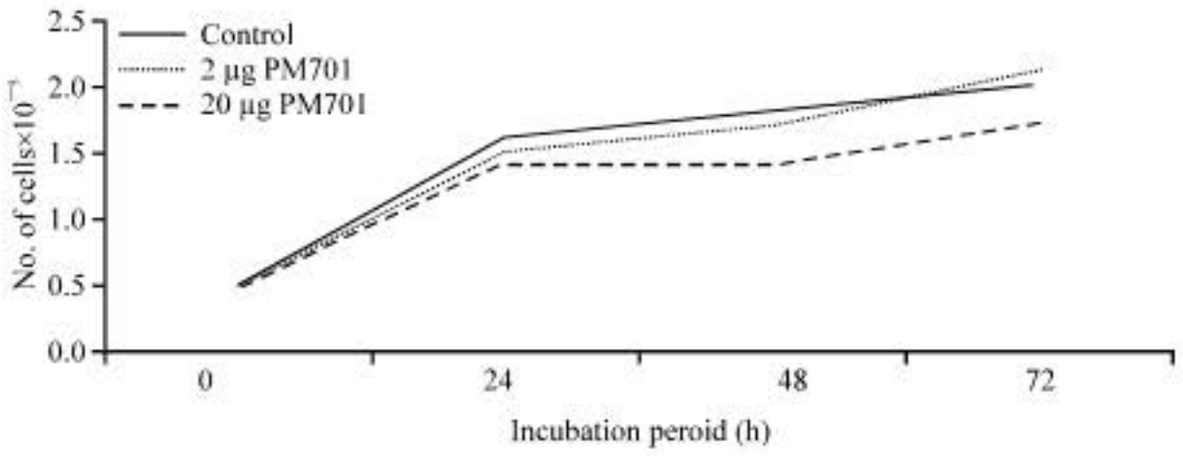

Fig. 3: The effect of lyophilized PM 701 on the growth of normal cells after different incubation periods comparing with non treated normal cells

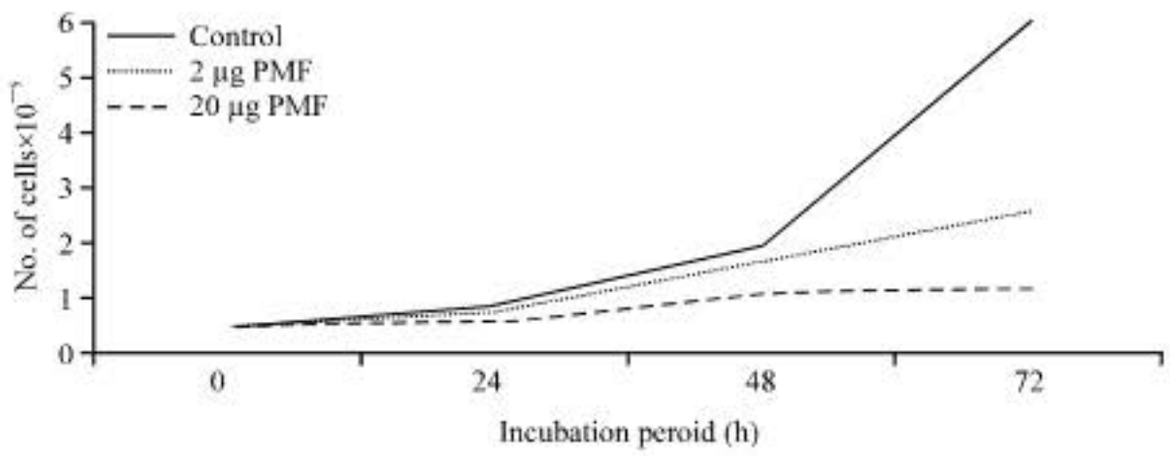

Fig. 4: The effect of PMF on A549 cells after different incubation periods comparing with non treated cancer cells (control)

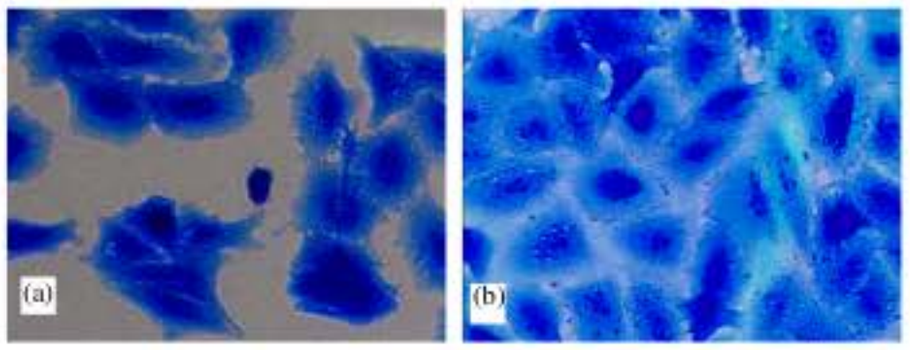

Fig. 5: The effect of PMF fraction on the cell morphology of human lung carcinoma cell line, A549, (a) treated cells, note that cancer cells lose the communication between them which character the living cells in culture and (b) non treated cells (40x)

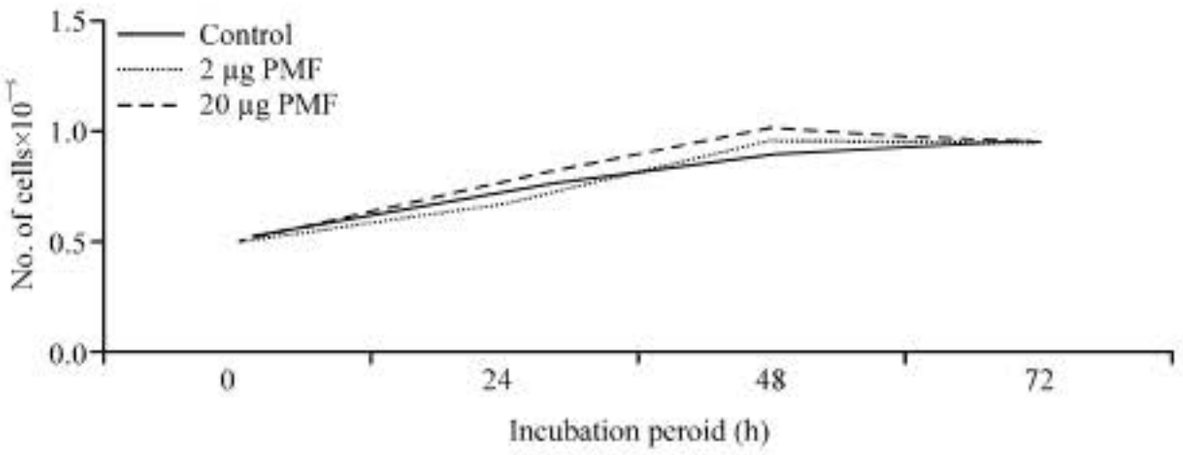

Fig. 6: The effect of PMF treatment on the growth of normal cells (HFS) after different incubation periods comparing with non treated normal cells (control)

There was 80% inhibition of the growth of cancer cells after the incubation for 72 h in media with 2 µg mL^{-1} of PMF, whereas there was more growth of normal cells obtained after 72 h of incubation with 20 µg mL^{-1} of PMF. These results showed that PMF isolated from PM701 has a potent selective antiproliferative activity and may be partially responsible for the antiproliferative activities of whole PM701.

Subfraction PMFK Induced Growth Inhibition in A549 Cells

Different periods of incubation with subfraction PMFK showed a decrease in the proliferation of A549 cells with maximum activity obtained at 72 h of treatment. There were 58 and 64% inhibition of cell growth after 72 h of incubation with 2 µg mL^{-1} and 20 µg mL^{-1} PMFK,

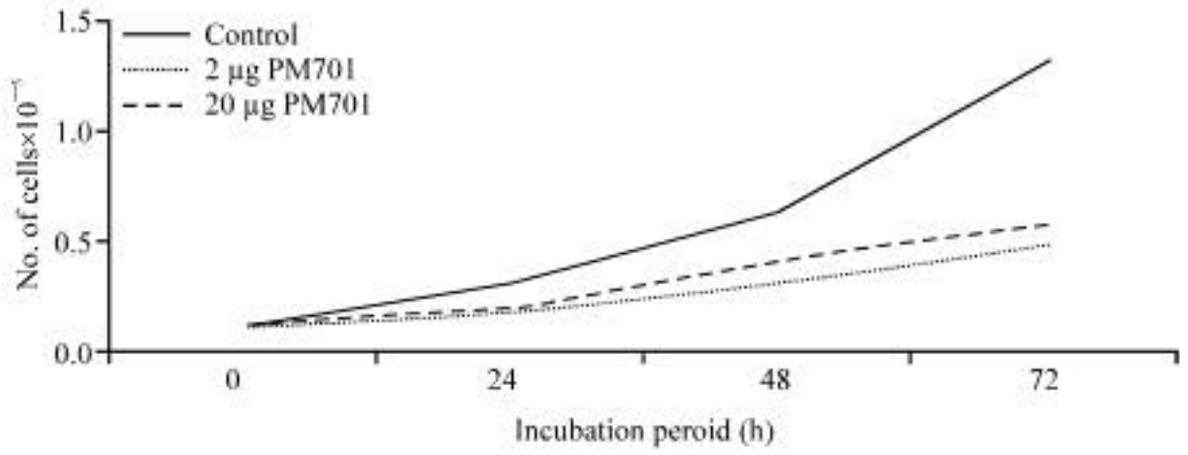

Fig. 7: The effect of PMFK on A549 lung cancer cells after different incubation periods comparing with non treated cancer cells (Control)

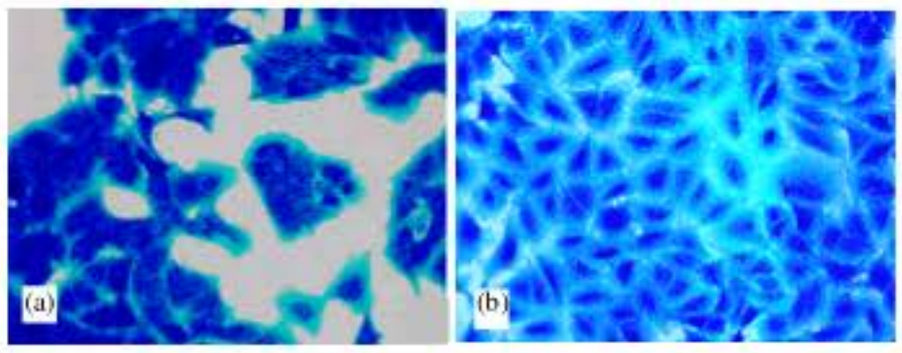

Fig. 8: The effect of subfraction PMFK on the cell morphology (20x) of human lung non small cancer cells A549, (a) treated cells and (b) non treated cells

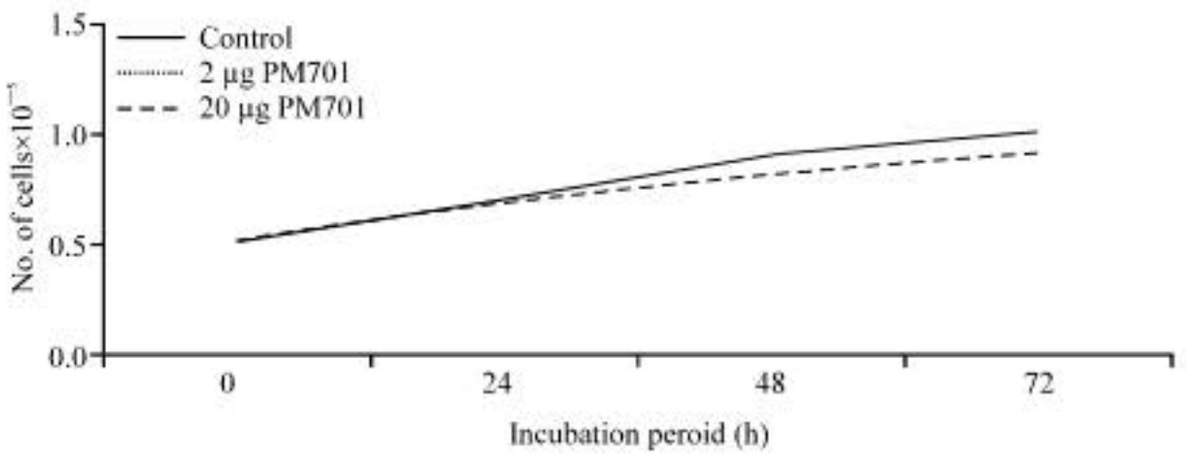

Fig. 9: The effect of PMFK on normal cells after different incubation periods comparing with non treated normal cells

respectively (Fig. 7, 8). Furthermore the normal cells (HFS) number shows normal progress, when incubated in PMFK in different incubated periods (Fig. 9).

DISCUSSION

According to NCI recommendations, anticancer drug candidates must demonstrate their anti-proliferative effect and anticancer specificity on *in vitro* cell lines models, before *in vivo*, pre-clinical and clinical development (http://imaging.cancer.gov).

The relationship between apoptosis, cancer and anticancer agents has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve alteration of the normal apoptotic pathways and the apoptotic susceptibility of the

anticancer agent is a critical determinant of therapeutic efficacy (He and Liu, 2007). Recently (data under publication), our research team has proved that PM701 exerted antiproliferative action and growth inhibition effect in diverse cultured human cancer cells including lung cancer and leukemia by inducing apoptosis. The dying cells exhibited the morphological hallmarks and biochemical features that characterize apoptosis, as shown by loss of cell viability, chromatin condensation and reducing metabolic activity using MTT test (data under publication). Apoptosis provides a number of clues and evidences with respect to effective anticancer therapy and many chemotherapeutic agents reportedly exert their anticancer effects by inducing apoptosis in cancer cells (Cheng *et al*., 2004). In this study, we have analyzed the anti-proliferative and apoptotic effects of the lyophilized PM701, on lung cancer cell line, A549. Lyophilized PM701 induced cell death in an apoptotic pathway in cancer cells.

The bio-active fraction G, which is coded PMF has recently been isolated from PM701 with cytotoxic properties against cancer cells. We observed that PMF was the most highly cytotoxic bio-active fraction against A549 human lung cancer cells more than other fractions and was not cytotoxic to human normal cells (as shown in the results) suggesting that PMF can act selectively on cancer cells while does not affecting normal cells.

This observation made the PMF bio-active fraction attractive for further subfractionation in order to isolate other bio-active subfraction G7, which is coded PMFK, where it was cytotoxic to A549 cells and safe to human normal cells at concentrations 2 and 20 µg, suggesting cytotoxic activity and selectivity of PMFK. The observed bioactivity of these fractions PMF and PMFK, as determined by their cytotoxicity against cancer cells, also validates our previous identification of a whole PM701 as potential ideal cytotoxic in previous studies (Khorshid and Mosheref, 2006).

In conclusion, PMFK exhibits an antiproliferative effect by induction of apoptosis on human lung cancer cells. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential of PMFK as an agent of cytotoxic therapy in human lung cancer cells. However, further investigation of its in vitro activity on diverse human carcinoma cell lines and in vivo cancer-bearing mice or rats are also necessary to elaborate and exploit this nascent promise.

CONCLUSION

PM701 is increasingly being considered as a source of selective cytotoxic agent. The bioassay-guided fractionation were undertaken to isolate our new bioactive constituents PMF that contributed to this cytotoxic activity and exhibited a strong cytotoxic effect against human lung cancer cell line A549 by 80% after 72 h without any harmful effect on human foreskin cell line HFS. PMF shown to block A549 cells growth and induced them to undergo apoptosis in a dose-independent manner. PMF subfractionation led to isolate our new subfraction PMFK, which also demonstrated antiproliferative activity on A549 cells in a dose-independent manner.

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