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A Novel Poly Herbal Formulation Induces Apoptotic Cell Death in Prostate and Colorectal Carcinoma Cells

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ABSTRACT

Cancer metastasis is refractory to most forms of chemotherapy. Conventional and alternative treatment/herbal remedies have been developed to target metastatic cancer cells in Asian countries. In this study, we investigated the effects of poly herbal formulation (PHF) [mixture of *Rhus succedanea* (stem), *Rheum emodi* (resin) and *Gardenia gummifera* (resin) extracts] on human prostate (PC-3) and colorectal (HCT116) carcinoma cells. PHF significantly exhibited anti-proliferation of PC-3 and HCT116 cells as compared to above individual extracts with an IC₅₀ values of 0.365 µg/ml and 0.374 µg/ml respectively. The growth inhibition observed by clonogenic assay in PHF treated PC-3 and HCT116 cells was further investigated and found to induce apoptosis as evidenced by DNA fragmentation. In a mechanistic approach, we found that PHF inhibited cancer cell growth by causing cell cycle arrest at subG0/G1 of PC-3 and G2/M of both HCT116 and PC-3 cells that blocks cell proliferation and induces apoptosis. Hence, our results suggest that PHF has a potential to be developed as a therapeutic agent against prostate and colon cancer cells.

KEY WORDS: Apoptosis, antiproliferation, Colorectal, Prostate and cell cycle.

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INTRODUCTION

Prostate cancer is the most common cancer amongst males in developed countries^{1, 2, 3}. Prostate and Colon cancer has been linked to high dietary fat intake and lack of adequate dietary fiber. For complete eradication of prostate and colon cancer, surgical resection is enforced^{2, 4}, but the metastatic disease is refractory to most forms of chemotherapy. However, over 50% of patients with metastatic or locally advanced disease experience local recurrence or develop distant metastases after potential curative surgery⁵. Further, drug toxicity and resistance on chemotherapeutic agents make a struggle to treat cancer. For this reason, nontoxic dietary phytotherapy has been considered as a preventative and/or inhibitory method against cancer cells^{6, 7}. Cancer commences due to the imbalance between cell proliferation and apoptosis pathway^{8, 9}. Apoptosis is a term used to illustrate the terminal morphological and biochemical events seen in programmed cell death¹⁰. There is a clinical need to develop novel treatment strategies exploiting the antiproliferative activity of both conventional and alternative drugs/medicinal plants targeting many levels of regulation in cellular growth that induce the apoptotic process^{2, 9}.

Antiproliferative and antitumor effects of an herbal preparation termed PC-SPES, which is a refined powder of eight different medicinal plants. PC-SPES administered as a food supplement caused a dramatic decrease in prostate specific antigen levels in some prostate cancer patients with advanced disease². PC-SPES, an herbal mixture, is made up of eight chinese herbs (*Isatis indigotica*, *Glycyrrhiza glabra*, *Panax pseudoginseng*, *Rabdosia rubescens*, *Dendranthema morifolium*, *Scutellaria baicalensis*, *Ganoderma lucidum* and *Saw palmetto*). This mixture contains eight recognized and active anti-neoplastic compounds. PC-SPES had been widely used for prostate cancer and PC-SPESII has been used in a phase I trial for prostate cancer^{2, 3}.

The EquiguardTM is a dietary supplement comprised of standardized extracts from nine herbs, *In-vitro* study of EquiguardTM significantly reduced cancer cell growth, induced apoptosis, suppressed expression of the androgen receptor (AR) and lowered intracellular and secreted prostate specific antigen (PSA), and almost completely abolished colony forming abilities of prostate cancer cells⁶.

Hwangryunhaedok-tang (HRT; Oren-gedoku-to as Japanese name) is one of famous traditional herbal medicine being used in Asian countries, which contains four kinds of herb including *Coptis japonica*, *Scutellaria baicalensis*, *Phellodendron amurense*, and *Gardenia jasminoides*. HRT has been clinically used in Korea for the treatment of defervescence, detoxication, and inflammation for a long time, and a lot of studies have reported its various biological effects. They demonstrated that it has

antihypertension, antioxidation, anti-inflammation, superior mesenteric, liver and cardiovascular protections, and anticancer effects¹¹.

Our previous study revealed that *Solanum indicum*, *Rhus succedanea*, *Rheum emodi* and *Gardenia gummifera* extracts exhibited an anti-proliferation of different five human cancer cells¹². The present study was carried out with an aim of understanding the synergistic effect of poly herbal formulation (PHF) comprising of three constituent herbs (*Rhus succedanea*, *Rheum emodi* and *Gardenia gummifera*) on apoptosis in cancer cells. The potential apoptotic process mediated antiproliferative activity of the PHF in colorectal (HCT116) and prostate cancer (PC-3) was evaluated. Antiproliferative activity of PHF through apoptosis was substantiated by cytotoxicity, DNA fragmentation; inhibition of survival colonies (clonogenic assay) and cell cycle arrest at subG0/G1 and G2/M phase.

MATERIALS AND METHODS

Reagents and Chemicals

Roswell park memorial institute medium (RPMI-1640), Fetal bovine serum (FBS), penicillin (1000 U/ml) and streptomycin (100 µg/ml) were purchased from Gibco, Invitrogen Life Technologies, Inc, CA, USA. 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), RibonucleaseA (RNase A), proteinase K, ethylene diamine tetra acetic acid (EDTA), dimethyl sulfoxide (DMSO), ethidium bromide (EtBr) and other chemicals from Sigma (MO, USA). Propidium iodide (PI) and FITC Annexin V Apoptosis Detection kit II was obtained from BD Pharmingen™ (San Diego, CA, USA).

Plant material collection

Plants materials of *Rhus succedanea* (Stem), *Rheum emodi* (Resin) and *Gardenia gummifera* (Resin/sticks) were collected from the Amruth Kesari Depot, Bengaluru.

Extraction of plant material

The air-dried and powdered plant materials (20g of each) were extracted with 250 ml methanol (CH₃OH) by Soxhlet apparatus¹². The crude extracts were filtered and the filtrate evaporated using a rotary evaporator. The dissolved constituents were further dried under pressurized vacuum conditions. Stock solutions were prepared by dissolving the dried residue in dimethyl sulphoxide (DMSO). Extract solutions were stored at -20°C until use.

Preparation of poly herbal formulation [PHF]

A mixture of equal proportions of *Rhus succedanea* (Stem), *Rheum emodi* (Resin) and *Gardenia gummifera* (Resin/sticks) was prepared.

Cells and Culture

Human prostate carcinoma (PC-3) and colorectal carcinoma (HCT116) cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) media supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a 5% CO₂ humidified incubator.

Cytotoxicity assay

Inhibition of cell proliferation was determined using a MTT assay. Briefly, PC-3 and HCT116 cells (5×10^4 /well) were seeded in a 96-well culture plates with RPMI-1640 media and incubated at 37°C for overnight to adhere, and then plant extracts and PHF were treated (0 – 64 µg/ml and 0 – 2 µg/ml respectively) in serum free RPMI media and incubated for 24 h. Untreated ‘control’ cells were incubated with DMSO at final concentration of < 1%. After incubation, a 10 ml aliquot of MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated in the dark at 37°C for 4 h. The medium was removed, formazan precipitates were dissolved with dimethyl sulfoxide (DMSO), and then optical density was measured at 570 nm using a microplate reader (ELx 800, Bio-Tek Instruments Inc., Winooski, VT, USA). The percentage inhibition was determined using the formula: % Inhibition = 100 - (optical density of sample/optical density of control) × 100. IC₅₀ values were calculated as the concentrations that show 50 % inhibition of proliferation on any tested cell^{13, 14, 15, 16}.

Clonogenic survival assay

PC-3 and HCT-116 cells were plated at a density of 500 cells/well in a 12-well plate in RPMI-1640 culture medium containing 10 % FBS for 24 h. Then, the cells were treated with PHF (0 – 2 µg/ml) in a serum free RPMI-1640 media. After 24 h treatment and once in 5 days, the medium was replaced by adding fresh media. On the 21 days to produce colonies of >50 cells/colony, media was removed from the dishes and washed once with ice-cold PBS. The colonies were stained with 1ml of 0.5 % crystal violet in 80 % methanol for 30 min on a rocking platform. The dishes were rinsed three times

with PBS and air-dried, and the colonies were counted. Finally pictures were taken at 4x by using magnus inverted microscope (Olympus, Japan).

DNA Fragmentation Assay

To investigate the apoptotic effect of PHF, we checked the appearance of oligonucleosomal DNA fragmentation by agarose gel electrophoresis. Briefly, PC-3 and HCT116 cells (2×10^5 /well) were seeded in a 12-well culture plates with RPMI-1640 media and incubated at 37°C for overnight to adhere, and then PHF was treated (0 - 2 µg/ml) in serum free RPMI media and incubated for 24 h. After treatment, adherent and detached cells were harvested and washed with phosphate buffered saline (1X PBS), then cells were lysed with a lysis buffer composed of 50 mM Tris-HCl, 10 mM ethylene diamine tetra acetic acid (EDTA)-4Na and 0.5 % sodium-*N*-lauroyl sarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/ml RNase A at 50°C for 30 min and then further incubated in the lysis buffer containing 0.33 mg/ml proteinase K at 50°C for 30 min. Equal amount of DNA was electrophoresed on 2.0 % agarose gel. Gels were stained with 0.5 mg/ml ethidium bromide for 15 min and visualized under UV light.

Cell cycle analysis

Cells were seeded at a density of 5×10^5 cells/well of 12-well culture plate and allowed to adhere overnight. After incubation with PHF (0 – 2 µg/ml) for 24 h, cells were harvested, washed twice with cold PBS, fixed with ice-cold 70 % ethanol, and kept at 4°C for 24 h. After centrifugation to remove ethanol, cells were washed twice with PBS and then intracellular DNA was labeled with 0.5 ml of cold propidium iodide (PI) solution [0.1 % Triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNase A, 50 mg/ml PI in PBS] at room temperature for 1 h in the dark. The percentage of cells in various stages of cell cycle was measured in FACS Calibur flow cytometry using Cell Quest Pro software (Becton Dickinson, San Jose, CA) and analyzed using Flow Jo 7.5.5 (Tree Star Ashland OR).

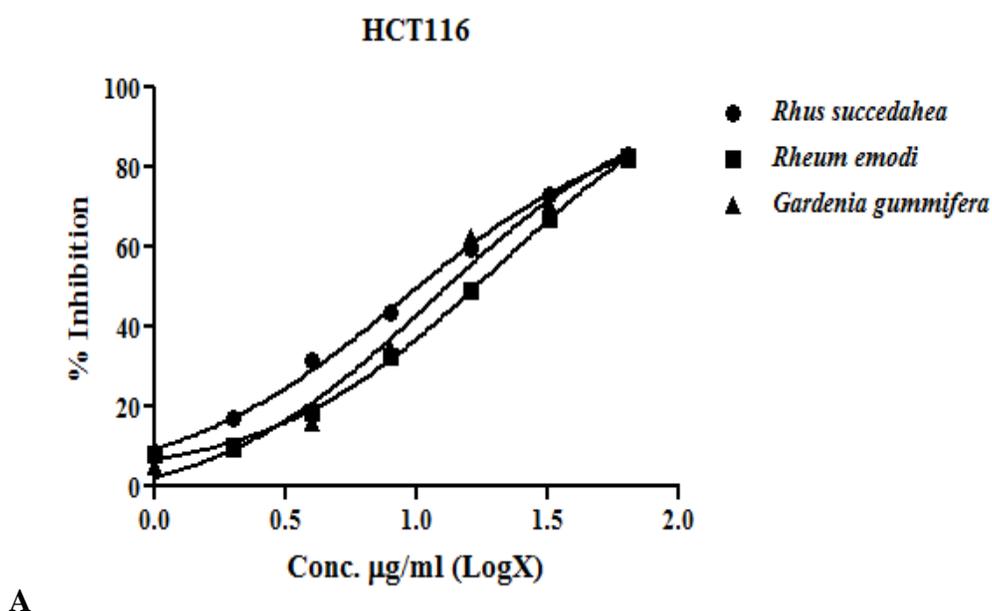
Statistical Analysis

All the assays were performed in triplicates of two independent experiments. IC₅₀ values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoidal dose response curve (variable) and computed using GraphPad Prism 5 (Graphpad, San Diego, CA, USA)^{17, 18}.

RESULTS

Cytotoxicity assay

A preliminary assessment of the comparative antiproliferative effect of *Extract of R succedanea*, *R emodi* and *G gummifera* and those mixtures called as PHF on HCT116 and PC-3 cells were studied and its inhibitory effects on cell growth were determined by MTT assay, which measures the metabolically live cells based on their mitochondrial dehydrogenase activity. HCT116 and PC-3 cells were treated with various concentrations of above individual extracts and PHF (0 – 64 $\mu\text{g/mL}$ and 0-2 $\mu\text{g/mL}$ respectively) for 24 h and their growth inhibition was determined. The above individual extracts exhibited significant growth inhibition in a dose dependent manner in both HCT116 and PC-3 cells. The IC_{50} values of *R succedanea*, *R emodi* and *G gummifera* are 11.69, 11.09 and 23.25 $\mu\text{g/ml}$ on PC-3 cells (Fig. 1A). Similarly the IC_{50} values of *R succedanea*, *R emodi* and *G gummifera* are 8.72, 19.89 and 11.51 $\mu\text{g/ml}$ on HCT116 cells (Fig. 1B). Interestingly, very less concentration of PHF (< 2 $\mu\text{g/ml}$) exhibited significant growth inhibition in a dose dependent manner with IC_{50} values of 0.365 $\mu\text{g/ml}$ and 0.374 $\mu\text{g/ml}$ on PC-3 and HCT116 cells respectively (Fig. 1C). These results suggested that PHF shows synergistic growth inhibitory activity as compared to individual extracts.



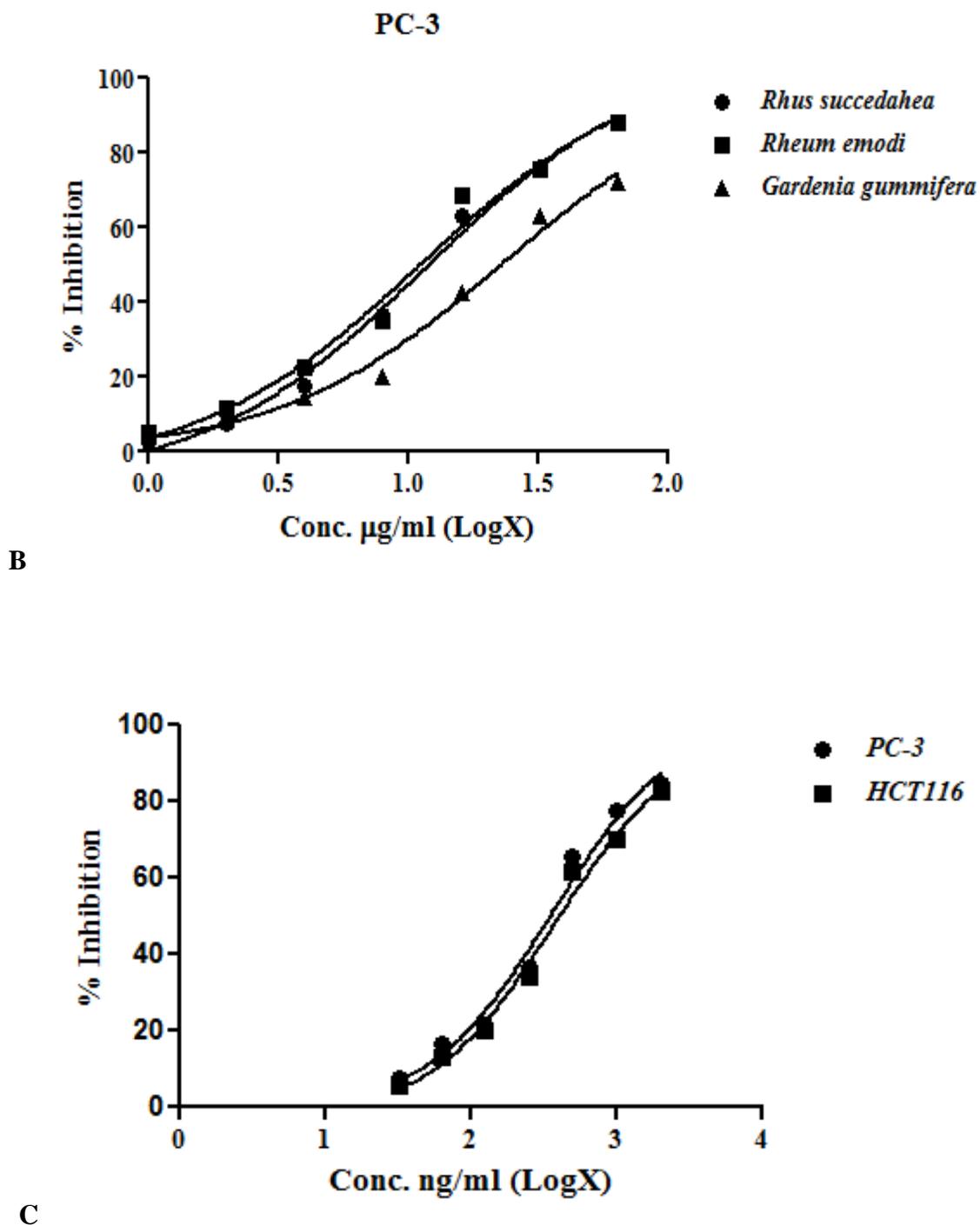


Figure 1 Cytotoxic effect of *Rhus succedanea*, *Rheum emodi*, *Gardenia gummifera* (A and B) and its mixture PHF (C) on a prostate (PC-3) and colorectal cancer (HCT116) cells.

Clonogenic survival assay

The synergistic effect of PHF on the clonogenic property of HCT116 and PC-3 cells were evaluated. Our data showed that the clonogenic ability of HCT116 and PC-3 cells were inhibited in the presence of 0.5, 1.0 and 2.0 µg/ml of PHF (Figure 2A and B). Maximum reduction of clonogenic ability obtained with 0.5, 1.0 and 2.0 µg/ml of PHF were 99, 99 and 100% (*p 0.01) respectively (Figure 2C). PHF significantly inhibited the cancer growth as compare to control of above cells at the lowest concentration (0.5 µg/ml).

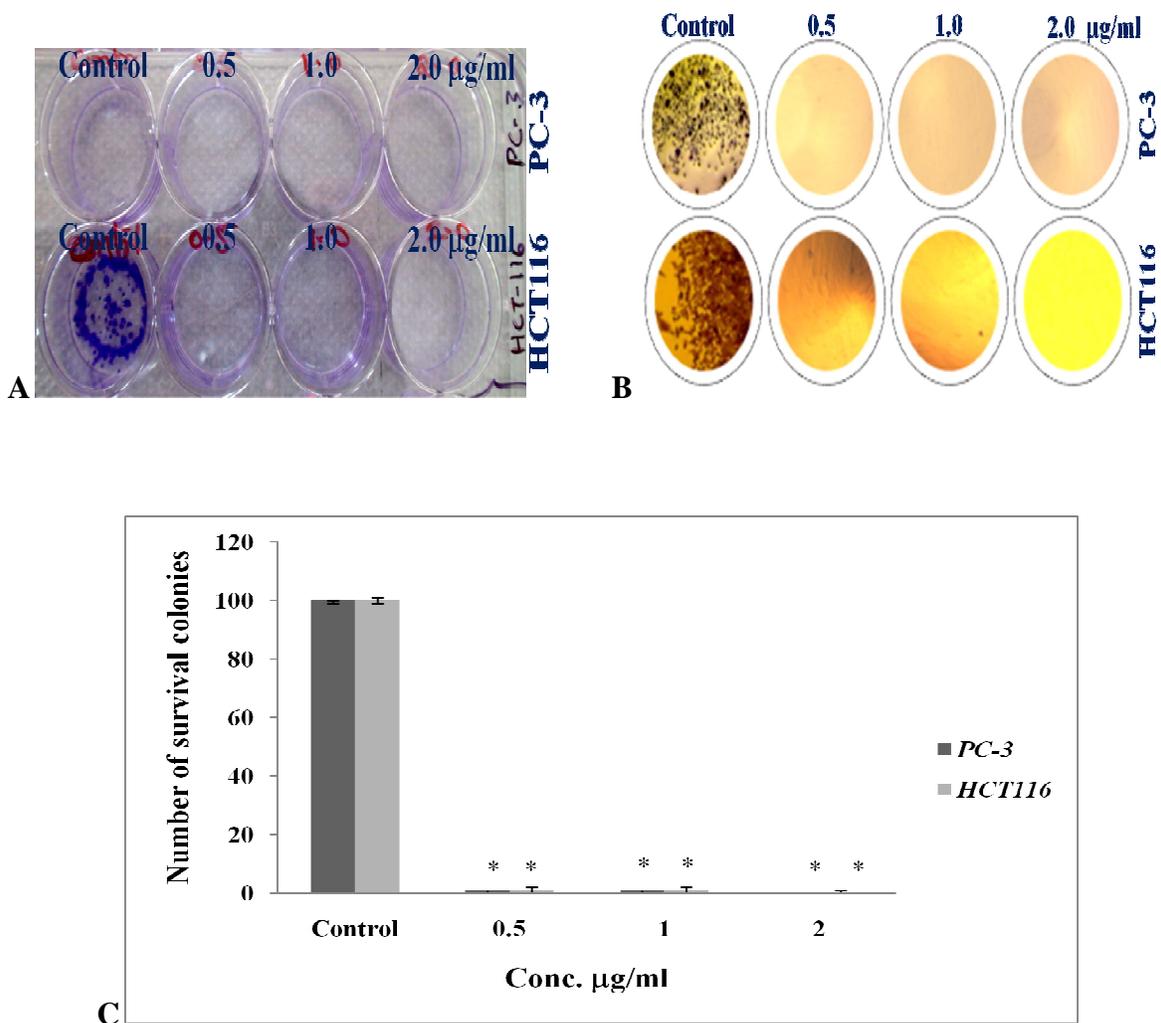


Fig 2 Clonogenic assay shows the long-term effects of PHF on human cancer cells. Data are presented as mean ± standard deviation. *P < 0.01, vs control group.

DNA Fragmentation Assay

For further confirmation of apoptotic cell death by DNA damage, DNA fragmentation assay was carried out. A DNA agarose gel electrophoresis demonstrated that both the HCT116 and PC-3 cells displayed smear or a typical DNA fragmentation pattern for apoptosis after treatment with 1 and 2 $\mu\text{g/ml}$ of PHF in the cultured media (Fig.3). No DNA fragmentation was observed in both HCT116 and PC-3 control cells (Fig. 3). This finding establishes the apoptosis inducing abilities of the PHF in both the treated cell lines.

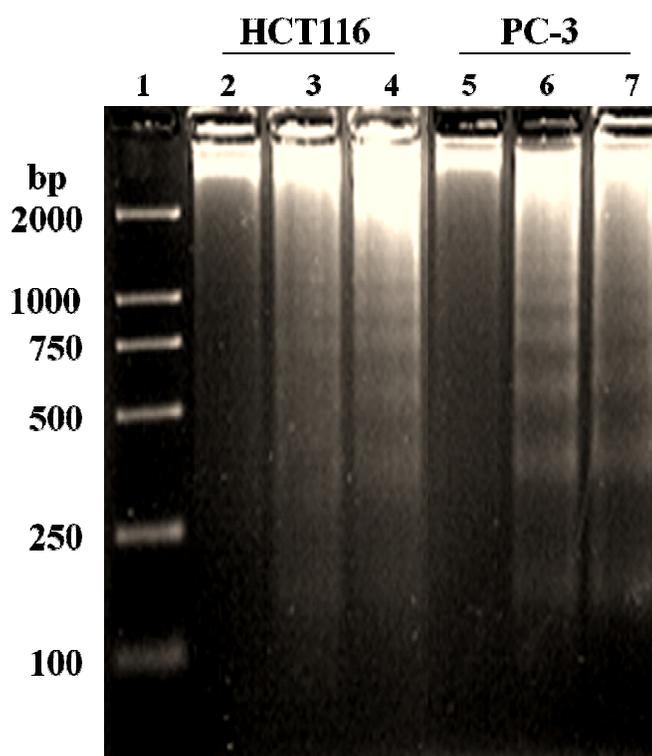
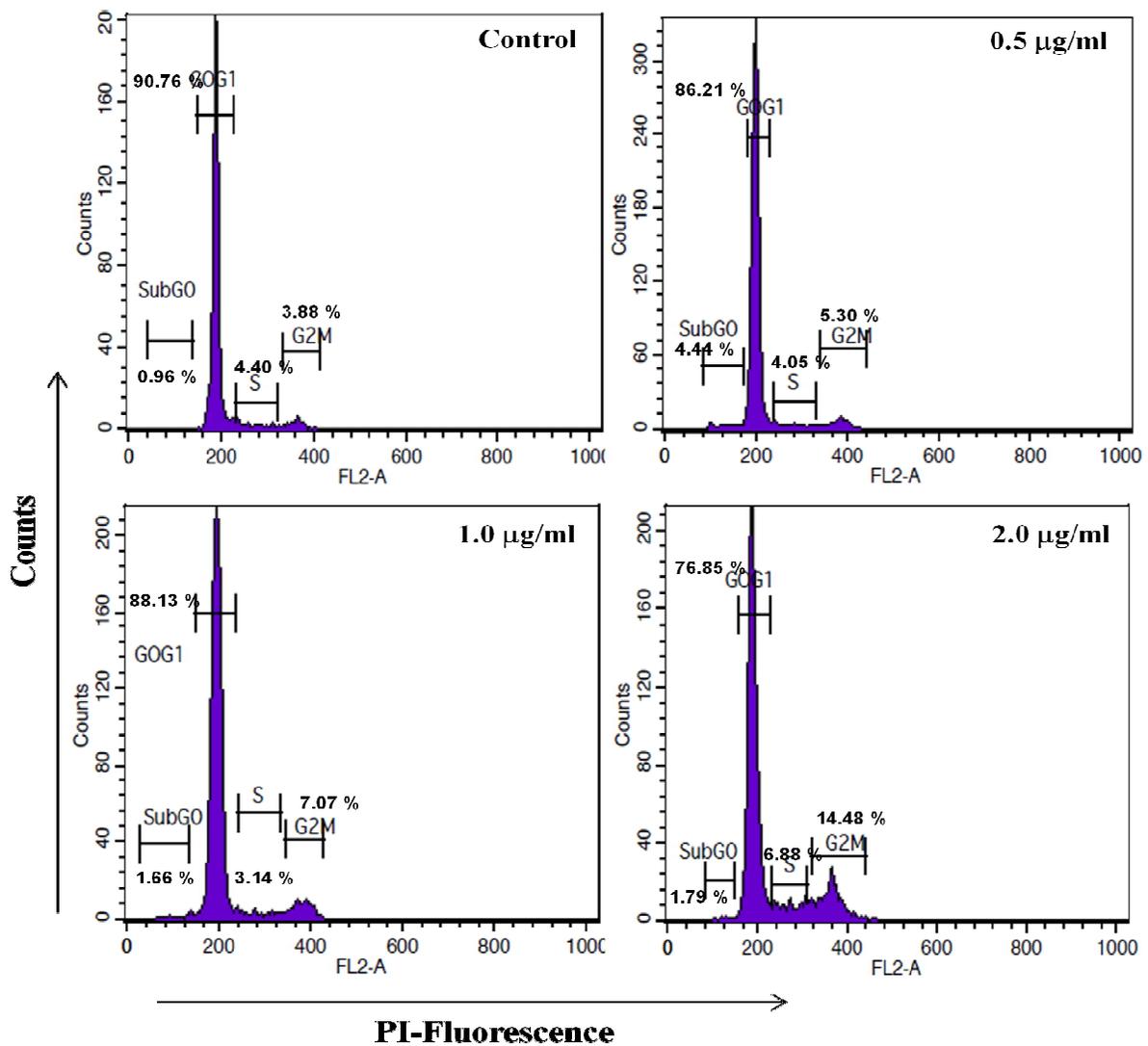


Fig 3 PHF induced HCT116 and PC-3 cell apoptosis measured by DNA fragmentation assay. A typical DNA fragmentation pattern is visible in lanes 3, 4, 6 and 7 only and not in lanes 2 and 5 control.

Cell cycle analysis

The phase distribution in cell cycle was analyzed by flow cytometry with PI staining. The percentage of cells in subG0/G1, G0/G1, S and G2/M phase, respectively are shown in Fig 4A and 4B. HCT116 cells treated with various concentrations of PHF had an increased percentage of apoptotic cells (i.e., cells arrest in the G2/M phase). The percentage of apoptotic cells after 24 h were 5.3, 7.07 and 14.48 % when treated with 0.5, 1.0 and 2.0 $\mu\text{g/ml}$ of PHF respectively, as compared to the 3.88 % in control cells (Fig.

4A). Interestingly PC-3 cells treated with various concentrations of PHF had an increased percentage of apoptotic cells (cells arrest) in both the SubG0/G1 and G2/M phase. The percentage of apoptotic cells after 24 h were 4.81, 5.39 and 75.36 % in SubG0/G1 phase and the percentage of apoptotic cells were 16.27, 22.43 and 20.43 % in G2/M phase when treated with 0.5, 1.0 and 2.0 $\mu\text{g/ml}$ of PHF respectively, as compared to the 2.38 % (SubG0/G1 phase) and 13.63 % (G2/M phase) respectively in control cells (Fig. 4B). These data are consistent and correlate with the results from the MTT assay. The results indicated that cell death in both HCT116 and PC-3 cells were triggered through apoptosis.



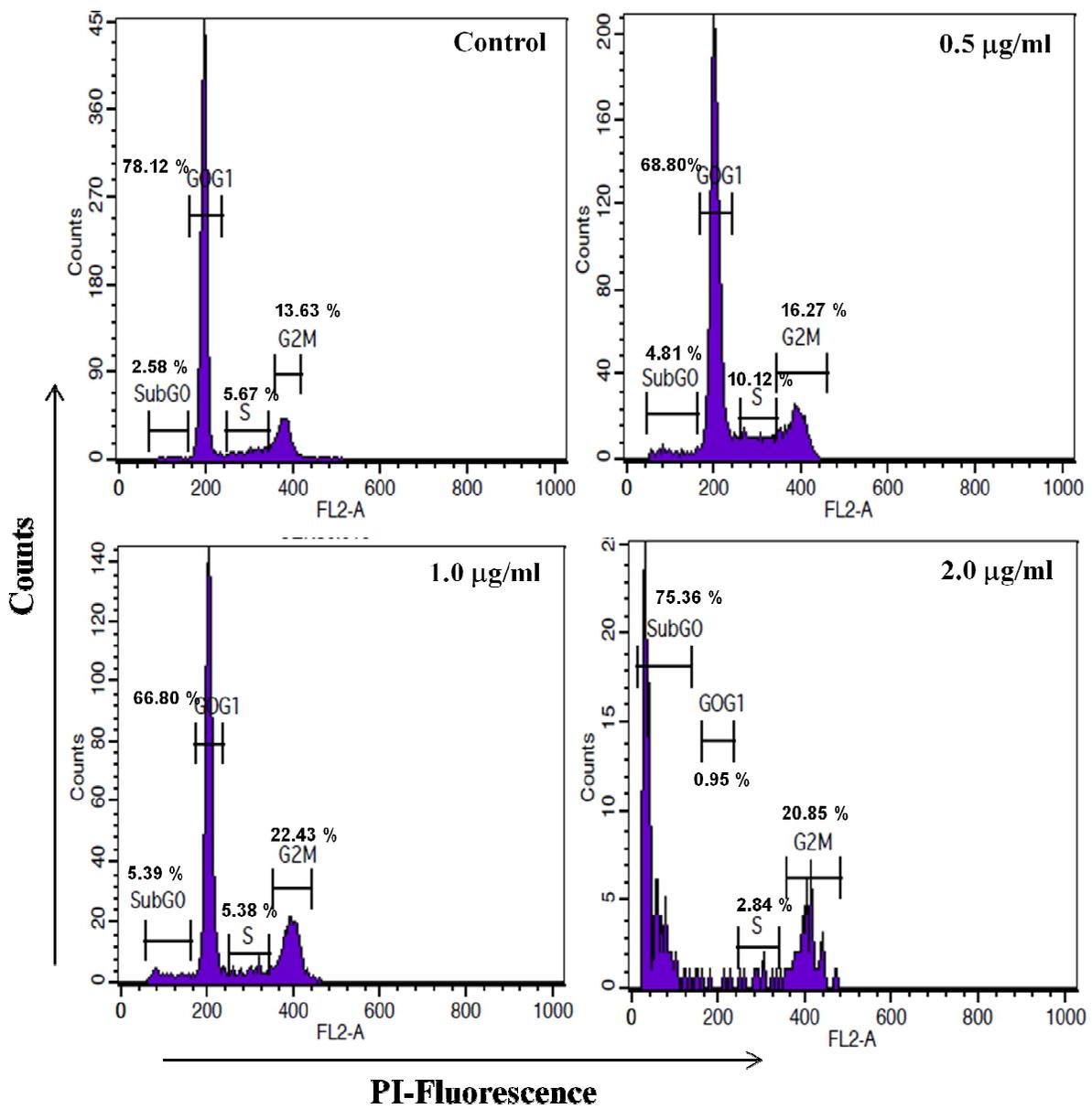


Figure 4: Effect of PHF on cell cycle progression in HCT116 (A) and PC-3 (B) cells.

DISCUSSION

In this study, we examined and provide the mechanistic evidence for novel PHF comprising *R. succedanea*, *R emodi* and *G gummifera* on induction of apoptosis in human prostate (PC-3) and colorectal (HCT116) carcinoma cells. PHF (0 - 2 µg/ml) exerted strong antiproliferative effect on both PC-3 and HCT116 cells as compare to its individual herbs (0-64 µg/ml). The PHF IC₅₀ values of 0.365 µg/ml and 0.374 µg/mL on PC-3 and HCT116 cells respectively (Fig. 1C) compared to the IC₅₀ values

of *R succedanea*, *R emodi* and *G gummifera* are 11.69, 11.09 and 23.25 $\mu\text{g/ml}$ on PC-3 cells (Fig. 1A); also to the IC_{50} values of *R succedanea*, *R emodi* and *G gummifera* are 8.72, 19.89 and 11.51 $\mu\text{g/ml}$ on HCT116 cells (Fig. 1B).

Many medicinal herbs have been widely used for cancer adjuvant therapies and are considered to be potential chemopreventive and chemotherapeutic agents. Because advanced malignancies require potent therapies targeting multiple cellular pathways, properly formulated herbal cocktails have the advantages of synergism and improved therapeutic efficacy compared with individual herbs with no or less side effects compared to other anticancer therapeutics including chemical compounds and targeting antibodies^{19, 20}. Apoptosis is the major mechanism of the cell in which the cancer cells have evolved multiple actions to resist against them. The modulation of apoptosis signaling pathways by natural compounds have been demonstrated to constitute a key event in anticancer activities²¹. In recent years, it was identified that better biological activity had a mixture of plant compounds in the formulation than a single plant compound. The effect of chemopreventive agent is mainly designed by the extent of inducing the apoptosis by the control cells when treated with anticancer drugs. In order to evaluate the apoptotic effect of the polyherbal extract, clonogenic assay was carried out and found that PHF significantly inhibited the number of survival of colony formation (99 to 100 %) in the treated (0.5, 1 and 2 $\mu\text{g/ml}$) PC-3 and HCT116 cells as compared to untreated control cells (100 %) as shown in Fig 2 A-C. Clonogenic assay is a time consuming assay and it refers to the ability of the cell to proliferate and retains its normal morphological characteristic when the drug was added to the cancer cells. The loss of reproducible ability and inability to proliferate is the common cause of the cell death, which was evident following a period of drug exposure. In the present study, it was established that the PHF has the antiproliferative activity when compared to the control PC-3 and HCT116 cells.

Apoptosis by DNA fragmentation assay has also been evaluated. The DNA fragments could appear in two patterns on agarose gel electrophoresis: as a “smear” of randomly degraded DNA and/or as “DNA ladder” because of endonucleases^{22, 23, 24}. As a characteristic of apoptotic cell death, DNA fragmentation assay was performed to assess the antiproliferative and cytotoxic effects of PHF were indeed due to apoptosis. The PHF induced PC-3 and HCT116 cells revealed DNA ladder like patterns after 24 h of treatment (Fig. 3). Breakdown of cellular DNA molecule and the release of enzymes from the cytoplasmic membrane loss is one of the signs of inhibition of DNA replication. This damage may be due to the inhibition of topoisomerase II, a key enzyme in DNA replication²⁵.

Additionally, the onset of apoptosis was also investigated by measuring the alterations in the cell cycle of PHF induced HCT116 and PC-3 cells. Flow cytometric analysis of apoptosis using PI revealed apoptotic inducing potential of PHF with the appearance of the G2/M peak (Fig. 4). The results of cell cycle analysis indicated that HCT116 and PC-3 cells treated with PHF for 24 h could induce cell cycle arrest in G2/M phase in a dose-dependent manner compared with control. Surprisingly PC-3 cells treated with 2 mg/ml of PHF induce cell cycle arrest in both subG0/G1 and G2/M phase. The dysregulation of the cancer cell cycle is one of the therapeutic targets for development of new anticancer agents/herbal formulation and several studies have indicated that cell cycle arrest might result in apoptosis due to the existence of cell cycle checkpoint and feedback control^{6, 11, 26}.

Some active chemical constituents were isolated from herbs of *R succedanea*, *R emodi* and *G gummifera* and their pharmacological effects and action mechanism were reported by previous studies^{27, 28, 29, 30, 31}. Especially, in traditional Chinese medicine (TCM), the combination of *S. baicalensis* and *C. japonica* has been used clinically in the treatment of various diseases including inflammation of the eyes and gingival bleeding¹¹. KIOM-C administration dramatically inhibits tumorigenic growth of HT1080 cells *in vivo* without adverse effects¹⁹. PC-SPESII inhibits human breast cancer MDA-MB-231 cell migration, invasion, and metastasis through *in vitro* and *in vivo* studies². Ellagic Acid is a polyphenolic compounds from pomegranate fruit extracts (PFEs) have been reported to initiate cell cycle arrest, apoptosis and also inhibit the invasion of human androgen-independent prostate cancer, PC3 cells, *in vitro*, markedly reduced the motility and the invasion of the cells³². Curcumin (diferuloylmethane), one of the major components of turmeric show an antiproliferative effect with a telomerase inhibitory potency on the T47D breast cancer cell line. This finding is important because toxicity and drug resistance problem of cancer chemotherapeutic agents, has led to challenge in the field of cancer research³³. From these points, it is possible that the anticancer effect of PHT on human prostate and colorectal cancer cells may come from the synergistic action of its individual herbs or active components. In addition active components of PHF may be overcome the problem of drug or chemotherapeutic agents' resistance cancer cells towards the apoptosis.

CONCLUSION

Our studies demonstrated here that a poly herbal formulation (PHF) significantly inhibits the cell viability in prostate and colorectal cancer cells and its antiproliferative effect is likely to be mediated by synergistic effects of individual herbal constituents. PHF effectively induces apoptosis through

inhibition of survival colonies, DNA fragmentation and the induction of subG0/G1 and G2/M cell cycle arrest at in human prostate and colorectal cancer cells resulting in the inhibition of cancer cell proliferation. Taken together, these results suggest that PHF has a potential to be developed as a potential therapeutic agent against prostate and colon cancer cells after *in-vitro* study of signaling pathway, check points of cell cycle molecules and *in-vivo* study using xenografts animal model.

CONFLICT OF INTERESTS

The authors declare that we have no conflict of interests.

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