Apoptosis Induction by *Calystegia Sepium* HPLC Fraction can be *via* Dual Targeting of *MDM2* and *BCL-2* in Cancer Cells

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ABSTRACT

In this research the different extracts of plants were tested to find the special fraction of optimum extract that induce the apoptosis. Cytotoxicity of Methanolic leaf extracts of *Catharanthus roseus*, *Calystegia sepium*, Ethanolic leaf extract of *Tradescantia pallida* and *Nasturtium officinale* and the Ethanolic and Methanolic bulb extract of *Narcissus tazetta* treatment after 24 h exposure was investigated using MTT assay and flow cytometric cell cycle and apoptosis tests in HeLa and G292 cancer cells and HGF-1 normal cell. The most effective extract was separated into fractions by preparative HPLC. Fractions cytotoxicity and their effects on some genes expression involved in apoptosis were determined by Real-Time PCR. Results indicated that C5 fraction of *Calystegia sepium* leaf Methanolic extract created the strong cytotoxic effect on cancer cells when compared to doxorubicin as the standard drugs. In addition, C5 fraction increased the expression level of inflammatory transcription factor like nuclear-factor-KB1, cell cycle inhibitors like TP53 dependent G2 arrest mediator (*RPRM*) and Cyclin dependent kinase inhibitor 1A (*CDKN1A, p21*). In contrast, C5 fraction decreased the expression level of *MDM2* and *BCL-2* that dual targeting of both can consider as a therapeutic strategy. Thus, *Calystegia sepium* has a lot of potential to be introduced as a new anticancer plant.

Keywords: Apoptosis, Bioassay guided fractionation, Cell culture, HPLC, Natural products

INTRODUCTION

Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018 (WHO). Meanwhile, low- and middle-income countries are included approximately 70% of deaths from cancer. In addition, cancer causes the high pressure of economic and cost is increasing. The total annual economic cost of cancer in 2010 was estimated at approximately US$ 1.16 trillion [1]. Therefore, discovering of compounds and drugs as antitumor can provide the different ways of treatment. Recently, plant-based medicines could open new insights in cancer treatment and create a primary source for chemotherapeutic drugs in natural therapy. Some of the currently-used chemotherapeutic agents such as Vinblastine (VBL), vinorelbine (VRL), vincristine (VCR) and vindesine (VDS) are derived from *Catharanthus roseus*. In 2008,

another synthetic alkaloid from *Catharanthus roseus*, called vinflunine, was introduced and received permission for treatment in Europe [2-4]. Alkaloids of *Catharanthus roseus* also bind rapidly and reversibly to the binding sites of tubulin, which are different from those of taxanes, colchicine, podophyllotoxin, and guanosine-5'-triphosphate [5]. In addition, paclitaxel for breast cancer, vinca alkaloids for leukemia, and flavopiridol for colorectal cancer were initially derived from plants [6]. Accordingly, plants as herbal medicines possess the high potential to search for antitumor compounds. Known effects of *Catharanthus roseus* Methanolic extract could make that as a positive plant extract to compare with other plant extract.

There are many reports that have demonstrated that most phytochemicals act as anti-cancer through their apoptosis-inducing potential [7,8]. Apoptosis as a target for anticancer therapy plays a critical role in development as well as homeostasis and eliminate any unnecessary or unwanted cells through several cell-signaling pathways [7,8]. Since the suppression of apoptosis in carcinogenesis

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disturbs the balance between cell proliferation and program cell death, therefore, it results in the development and progression of cancer. The variety of molecular mechanisms in tumor cells also makes a complexity to suppress apoptosis [9]. Although the induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer chemotherapy.

Photochemistry studies of genus in *Convolvulaceae* family indicated that Convolamine and convolvine resins of *Convolvulus* genus, elymoclavine and pharbiticin in *Ipomea* genus, turpthein, convolvulin (jalapurgin) and jalapin are the main natural products found in this family [10]. In addition, previous researches have proven the anticancer effects of *Convolvulus arvensis* [11] and *Convolvulus galaticus* [12]. *Calystegia sepium* which is belonged to the family of *Convolvulaceae* was chosen to evaluate the anticancer effects on cancer cell lines. Furthermore, *Narcissus tazetta* is an herbaceous plant of the *Amaryllidaceae* family, which is one of the 20 most important alkaloid-containing plant families. So far, approximately 500 different alkaloids have been isolated from the *Amaryllidaceae* family, each with various interesting biological activities [13]. It has been reported that a glutamine-rich antifungal peptide from the *Amaryllidaceae* family possesses notable immunogenic and anti-reproductive activities [14]. Other alkaloids of *Narcissus* genus with anticarcinogenic properties are present in bulbs of the plants and include Lycorine, Galanthamine and Crinines [15]. Meanwhile*, Nasturtium officinale* is known as an important source of carotenoids [16]. Consumption of this plant in diet can repair DNA damages and increase the antioxidant capacity of the blood [17]. The extract of *Nasturtium* sp. indicated the inhibitory effects on various stages of cancer [18-20]. Previous studies have demonstrated that this plant contains flavonoids that are glycosylated with rhamnose at V position [21]. Among other plants, *Tradescantia pallida* was traditionally used to improve blood flow and it also acts as an anti-inflammatory and anti-toxic agent [22]. Moreover, two types of anti-cyanidinide, cyanidin-3,7,3'-triglucoside with three ferrolic acid molecules and one external glucose and the other one without exogenous glucose have been isolated from this plant [23].

The aim of this study was to investigate the cytotoxicity

effect of six plant extracts derived from five plants including *Catharanthus roseus*, *Calystegia sepium*, *Narcissus tazetta, Tradescantia pallida,* and *Nasturtium officinale* on cancer cells including HeLa and G292 cell lines and compared to HGF-1 as normal cells. In addition, the different phases of cell cycle and apoptosis in cancer cells and normal cells were evaluated by flow cytometry in response to extracts. The optimum extract that possesses the high efficiency of cell death was determined and the different fractions of optimum extract were investigated by cell death tests. Furthermore, molecular mechanism involved in apoptosis and cell death was determined by some genes that participate in cell cycle arrest and apoptosis.

MATERIALS AND METHODS

Plant Collection and Extraction

Five plants were collected including *Catharanthus roseus*, *Calystegia sepium*, *Narcissus tazetta, Tradescantia pallida* and *Nasturtium officinale* plants from Guilan province in northern of Iran. All voucher specimens (*Catharanthus roseus*: 4212, *Calystegia sepium*: 578, *Narcissus tazetta*: 2356, *Tradescantia pallida:* 3745 and *Nasturtium officinale:* 5748*)* were deposited at Guilan University herbal bank. Leaves of *Catharanthus roseus*, *Calystegia sepium*, *Tradescantia pallida, Nasturtium officinale* and the bulbs of *Narcissus tazetta* were separated, washed, and then dried. Next, 500 ml of pure Methanol or 80% Ethanol were added to 100 g of the separated tissues. The mixture was placed into a shaker incubator (50 rpm) in darkness for 24 h at 25 °C. The resulting extract was filtrated using a Whatman filter paper (No. 42) and subsequently concentrated by rotary evaporator to the final volume of 20 ml. Then, the concentrated extract was placed into the incubator and slowly dried at 37 °C. Eventually, 1 mg of dry matter was dissolved in 1 ml of 0.1% DMSO using RPMI medium and was subsequently added to the culture media.

Culture of Cancer Cells

The cell stocks (1 ml with the 10^6 cells ml^{-1}) from Hela and G-292 cancer cells and HGF-1 normal cells was added to 5 ml of RPMI-1640 culture medium supplemented with

10% FBS as well as 1% (w/v) streptomycin and 1% (w/v) penicillin antibiotics in 25 cm^2 flask and kept at 37 °C, 5% CO_2 for 24 h.

MTT Assay

After trypsinizing and counting the cells by Neubauer Chamber, The maximum number of 7×10^3 cells were cultured in 100 µl of RPMI-1640 culture medium accompanied by 10% FBS as well as streptomycin and penicillin antibiotics in 96-well microplates and subsequently incubated at 37 °C, 5% CO_2 for 24 h. In order to get the adequate number of cells per plate, the cells were allowed to stick to the bottom of the plate. The number and growth quality of the cells were continuously evaluated using inverted microscopy. Subsequently, supernatant was discarded. The concentration of 50 µg ml^{-1} of each extract was added to each well. After 24 h, the culture medium was discarded and 25 µl of PBS containing 0.25 mg ml^{-1} of MTT reagent was added to each well. Culture media containing MTT reagent were incubated at 37 °C, 5% CO_2 for 24 h. After removal of the medium containing MTT reagent, insoluble crystals of formazan were dissolved in DMSO and the absorbance of each well was determined at 570 nm using spectrophotometer.

Flow Cytometry

After 24 h, 1 ml of culture media containing at least 10^6 cells from treated and non-treated samples were dissolved in 0.5 ml of PBS and gently homogenized using vortex. Then, 1 ml of Ethanol was added to the cells, which were subsequently placed on ice for 2 h. The mixture was centrifuged at $300 \times g$ for 5 min and the supernatant was discarded. Next, 5 ml of PBS was added again and kept for 30 s. Then; the mixture was centrifuged at $300 \times g$ for 5 min. After removal of the supernatant, the residue was used for the calculation of cell cycle and apoptosis. According to the manufacturer's instructions from Sigma Kit, PI solution and the mixture of PI and Annexin IV were added to the cells for calculation of cell cycle and apoptosis, respectively. For measurement, the mixture was introduced to the flow cytometry instrument after 30 min.

HPLC Analysis

The extract of *Clystegia sepium* as a strong apoptotic inducer was selected for HPLC analysis and then the fractionations were performed by preparative HPLC. HPLC analyses were performed with Agilent liquid chromatograph (Santa Clara, United states) with two solvent delivery system pump (G1311A). Dried extracts were solved in water with 0.05% v/v TFA and subsequently filtered using 0.45 µm syringe filters and injected into 20 µl loop for analysis. The separation was achieved by a reversed-phase Acclaim C18 column (4.6 × 250 mm, 5 µm particle size, 100 Å pore size) with flow rate 1 ml min^{-1}. Agilent Chemstation is used as manager software. Oven temperature was 25 °C and UV detector was adjusted to 218 and 320 nm. The mobile phase A consisted of H_2O/0.1% TFA (v/v), and the mobile phase B was a mixture of acetonitrile/H_2O (90:10, v/v) with 0.1% TFA with a gradient elution of 5% (B) at 0-35 min; 100 % (B) at 35-45 min.

For preparative HPLC, the dried extract of *Calystegia sepium* (200 mg) were resolved in 10 ml of water with 0.05% v/v TFA and injected into loop. Preparative washing the C18 column (20 × 250 mm, 10-µm particle size, 100 Å pore size) was done according to obtained results from analytical HPLC with 15 ml min^{-1} flow rate. The mobile phase consisted of H_2O/0.1% TFA (v/v) (A) and acetonitrile/H_2O (90:10) with 0.1% TFA (v/v) (B) with a gradient elution of 5% (B) at 0-35 min; 100 % (B) at 35-45 min. The column temperature was maintained at 25 °C. The fractions were collected based on peak display. Fractions solvent was evaporated using freeze drier for 36 h. Dried fractions were weighted and drug stocks were prepared with 1 mg ml^{-1} concentration.

IC50 Calculation

For primary detection of effective extract, each extract with the concentration of 50 µg ml^{-1} was used for MTT and flow cytometry tests. According to obtained results from MTT and flow cytometry, IC50 amount were calculated using 25, 50, 75, 100, 125 and 150 µg ml^{-1} for each extract on each cell line during 24 h. After selection of effective fraction using primary MTT test, the IC50 for Active fractions was calculated in 24h.

RNA Extraction and Real-Time PCR

First, all cells were treated with the value of IC25 of C5 and Doxorubicin on each cell line (5.02, 5.68 and

8.59 μg ml^{-1} for doxorubicin on Hela, G292 and HGF-1; 1.09, 1.16 and 13.53 μg ml^{-1} for C5 fraction on Hela, G292 and HGF-1, respectively) to compare genes expression changes of live cells under drug treatments. After 24 h media containing dead cells was discarded and live cells were trypsinized. Total RNA was extracted from the cells using RNX-Plus solution (CinnaGen) according to the manufacturer's instructions. cDNAs were synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). A semi-quantitative PCR including six genes; Nuclear Factor Kappa B1 (*NFKβ1*; NM_001165412.2), TP53 dependent G2 arrest mediator (*RPRM;* NM_019845), B-cell lymphoma 2 (*BCL-2;* NM_000633.2), Apoptotic peptidase activating factor 1(*APAF-1;* NM_001160.3), Cyclin dependent kinase inhibitor 1A (*CDKN1A, p21;* NM_000389.5), and MDM2 proto-oncogene (*MDM2;* NM_001145337.3) were amplified with their pair primers (Supplementary file 1). The PCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific) with 40 cycles of annealing at 55-60 °C for 20 s and 20 s synthesis at 72 °C, using *GAPDH* as internal standard to quantify the steady-state transcript levels of genes. Quantitative Real-time PCR was performed using SYBRGreen Real Time PCR Kit according to the manufacturer's protocols. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ Method (Livak and Schmittgen, 2001).

Statistical Analysis

All experiments were carried out in triplicate in Completely Randomized Factorial Design and for each independent experiment; three replicates were considered. Doxorubicin as a standard drug was applied to comparison with active fraction. Also, *Catharanthus roseus* Methanolic extract was applied as a standard plant extract to improve comparison between plant extracts. Cell lines of HeLa, G292 and HGF-1 treated with only DMSO 0.1% (by RPMI medium) were used as negative controls. IC50s were obtained using GraphPad Prism 7. Data analyses were calculated using SPSS 20 (SPSS Inc., Chicago, IL, USA). Data are presented as mean values + standard error of the mean (SE). The differences were considered to be statistically significant at P < 0.05.

RESULTS AND DISCUSSION

Cytotoxicity of Various Extracts

Analysis of MTT using 50 μg ml^{-1} concentration revealed that all extracts caused the cell death in cancer cells with different percentages of toxicity (Fig. 1). The high-level cytotoxicity in cancer cells was observed by CS.m treatment that can be a candidate for the next analysis. Evaluation of cytotoxicity revealed that treatment with TP.e and NO.e extracts not only did not result in normal cell death, but also increased the number of live cells in comparison with the control. .

In order to understand the relationship between cytotoxicity and extract efficiency in cell death, the IC$_{50}$ value of extracts against HeLa, G292 and HGF-1 cell lines were measured with various concentrations of different extracts including 25, 50, 75, 100, 125 and 150 μg ml^{-1} for 24 h (Table 1; Supplementary file 2). In comparison, the IC$_{50}$ value of Methanolic extracts of *Calystegia sepium* leaves (CS.m) was minimal for cancer cell lines including HeLa (5.32 μg ml^{-1}) and G292 (11.81 μg ml^{-1}), although it was maximum in normal cells like HGF-1 (465.2 μg ml^{-1}).

Apoptosis Induction by Crude Extracts

According to Fig. 2, Among plant extracts with a concentration of 50 μg ml^{-1}, Methanolic extracts of *Calystegia sepium* leaves (CS.m) for 24 h demonstrated the highest rate of apoptosis in different stages including early and late apoptosis based on flowcytometric cell cycle (Supplementary file 3) and apoptosis assays (Supplementary file 4). Highest apoptosis induction has been observed in HeLa cells (92.79%) (Figs. 2D and E) under CS.m treatment when compare to HGF-1 as normal cell line that it was 26.29% (Figs. 2A and B). CS.m also induced cell death at rate of 31.75% and 29.62% for early (EA) and late (LA) stages of apoptosis in G292 cell line, respectively (Figs. 2G and H). After CS.m, NT.e induced more the early apoptosis (EA, 58.03%) than late apoptosis (LA, 9.75%) in HeLa cells (Supplementary file 5). In contrast, the results of MTT assay related to NT.e showed low levels of cytotoxicity on HeLa cells that it can be because of a significant increase of cells in early apoptosis than late apoptosis. Comparing the results of MTT assay, TP.e extract could induce more early (EA) stage of apoptosis in G-292 tumor cells, whereas NO.e
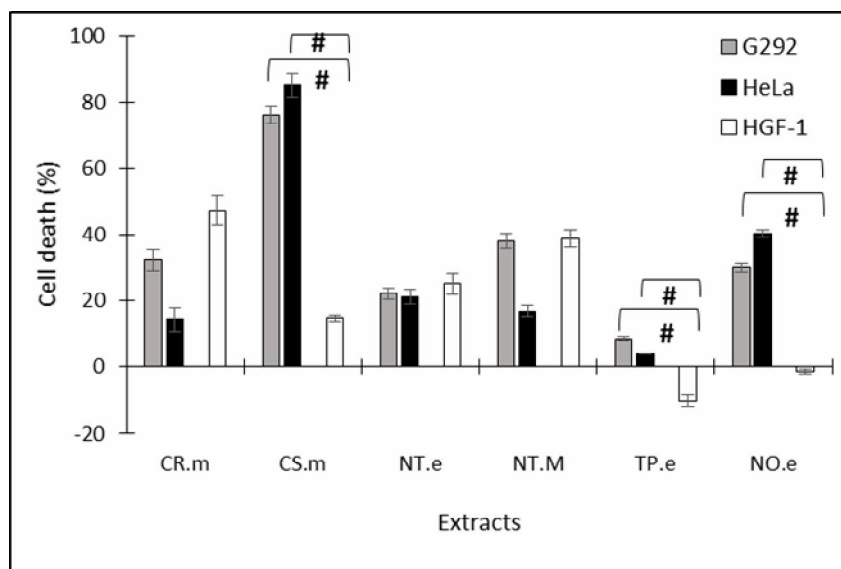
Fig. 1. The rate of cell death by MTT analysis in cancer (HeLa and G292) and normal (HGF-1) cells by the concentration of 50 µg ml^{-1} of all plant extracts after 24 h treatment. Negative score means that extracts cause an increase in cell division and growth than cell death in HGF-1 cells. CR.m; Methanolic extract of *Catharanthus roseus* leaves; CS.m; Methanolic extracts of *Calystegia sepium* leaves, NT.e: Ethanolic *Tradescantia pallida* leaves, NO.e; Ethanolic extracts of *Nasturtium officinale*. # means the significant increase of cell death by crude extracts in cancer cells in comparison with normal cells.

Table 1. The Value of IC$_{50}$ (µg ml^{-1}) for Different Extracts in Cancer Cell Lines Including HeLa and G292, and Normal Cell Line of HGF-1 after 24 h Treatment. CR.m; Methanolic Extract of *Catharanthus Roseus* Leaves; CS.m; Methanolic Extracts of *Calystegia Sepium* Leaves, NT.e: Ethanolic Extract of *Narcissus Tazetta* Bulbs, NT.m: Methanolic Extract of *Narcissus Tazetta* Bulbs, TP.e; Ethanolic Extract of *Tradescantia Pallida* Leaves, NO.e; Ethanolic Extracts of *Nasturtium Officinale*

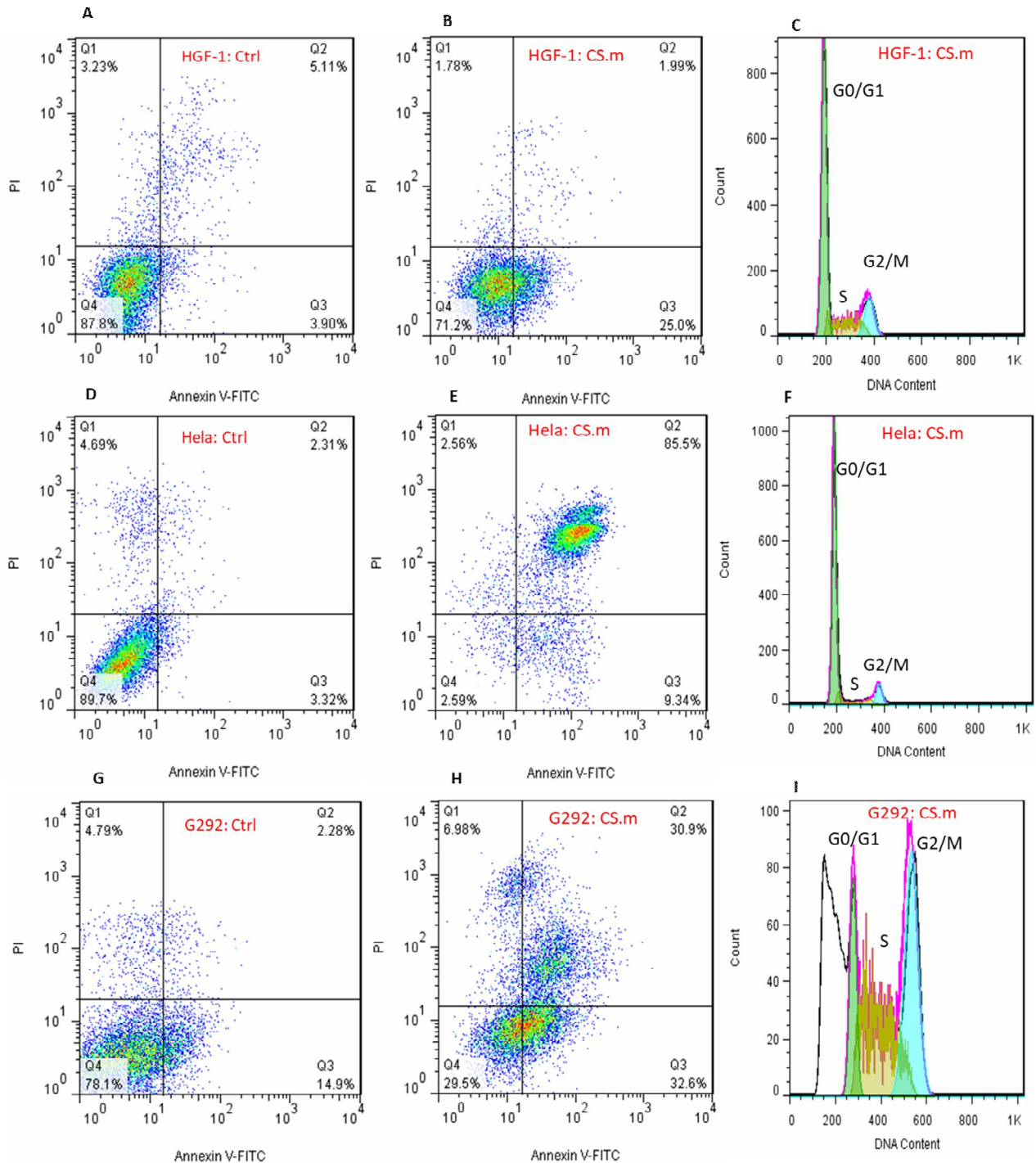| Extract	Cell line		
	HeLa (Mean ± SE)	G292 (Mean ± SE)	HGF1 (Mean ± SE)
CR.m	947.3 ± 19.67	179 ± 5.77	60.06 ± 3.85
CS.m	5.323 ± 0.52	11.81 ± 1.83	465.2 ± 8.69
NT.e	371.7 ± 16.82	278.7 ± 3.62	96.47 ± 4.94
NT.m	2272 ± 45.96	146.4 ± 4.28	90.45 ± 5.11
TP.e	48562 ± 101.9	14120 ± 29.95	-
NO.e	73.78 ± 4.18	74.73 ± 3.45	272.4 ± 9.48

Fig. 2. Analysis of apoptosis and cell cycle by flow cytometry in treated and non-treated cells with 50 μg ml^{-1} Methanolic extract of *Calystegia sepium* leaves (CS.m) including HGF-1 (A, B, C), Hela (D, E, F), and G292 (G, H, I). For apoptosis, the cells were stained with Annexin V PE and ReadiDrop Propidium Iodide after 24 h treatment. Apoptotic cells positive for Annexin V can be seen in the bottom right quadrant and dead cells positive for both Annexin and PI in the top right quadrant.

extract has a higher level of late (LA) induction of apoptosis (Supplementary file 5). In normal HGF-1 cells, the lowest number of live cells was observed under treatment with CR.m extract, which means the extract of *Catharanthus roseus* leaves had more significant lethal effect on normal cells compared to the G-292 and HeLa cancer cells, while CR.m extract indicated the safety effect on the live normal cells.

Induction of Cell Cycle Progression and Acceleration of Apoptosis

Different phases of cell cycle were considered and the sub-G1 peak resulting from DNA fragmentation as an indicative of apoptotic cells were detected in treated and control cells. Among the different extracts, treatment of G292 cell line with CS.m and CR.m indicated a significant increase in cells accumulation in the sub-G1 than control treatment (Fig. 3B). CR.m, NT.e, and NT.m indicated a sub-G1 peak and S-phase peak for HeLa cells when compared to control (Fig. 3C). These findings were not completely in accordance with the results of MTT assay, probably because apoptosis or necrosis occurred in a number of cells prior to the analysis of cell cycle, and hence they were not primarily calculated in cell cycle analysis. The results of cell cycle progression and apoptosis indicated that approximately 50 till 70% of cells were in G0/G1 in response to crude extracts for 24 h, except CS.m in G292, CR.m in HGF-1 and HeLA cell lines (Fig. 3). In consistent with MTT assay, treatment with TP.e and NO.e extracts led to the elimination of sub-G1 phase in HGF-1 normal cells, supporting the hypothesis that the extract would increase cell viability in HGF-1 normal cell line. In addition, all crude extracts were able to induce S phase and G2/M equally well in G292 and HGF-1 respectively (Figs. 3A and B). Meanwhile, the presence of large numbers of dying cells in proliferating cell populations has demonstrated, therefore the cell cycle study is not sufficient to prove apoptosis. Furthermore, cell death by apoptosis in various phases of cell cycle depends on the cell-type and inducer/stimulus.

Cytotoxicity of Various HPLC Fractions of *Calystegia Sepium*

To select the optimum extract with high level of lethal efficiency on cancer cells, several factors were considered

with together including IC50, cell number in sub-G1, and scatter gating of cell population in histograms of Annexin V and PI. Therefore, the Methanolic extract of *Calystegia sepium* leaves that was include a balance of all these factors and approximately indicated the high cytotoxicity level in cancer cell lines were selected as significant anticancer agent. In consistent with MTT assay, the lowest IC50 was also observed in treatment with CS.m on HeLa and G292 cancer cells. In next step, each fraction of CS.m was tested for having the ability of conduct cells to apoptosis in cancer cells. Crude extract was separated by HPLC into 6 fractions (C1-C6) based on peak and retention time in different time ranges including 0-7 (C1), 7-14 (C2), 14-20 (C3), 20 -26 (C4), 26-35 (C5) and 35-45 (C6) min (Figs. 4A and B). Cytotoxicity of CS.m different fractions were examined on HeLa and G292 cell lines. Cells were incubated with various fractions for 24 h in cancer cells and normal cells. The results demonstrated that 5th fraction had high significant of cell death on HeLa and G292 cancer cells and low toxicity on HGF-1 cells with 50 µg ml^{-1} (Table 2). In order to compare the cytotoxicity of obtained fractions of *C. sepium* (C5) with doxorubicin as a positive control, also, the rate of cytotoxicity on HeLa and G292 cancer cells and HGF-1 normal cells was tested (Supplementary file 6). Results showed that C5 fraction (Fig. 4B) could be toxic more in low concentrations on cancer cells when compared with doxorubicin. The IC50 value of C5 fraction against cancer cell lines including HeLa (5.1 µg ml^{-1}) and G292 (5.3 µg ml^{-1}) for 24 h was lower than normal cells (62.2 µg ml^{-1}), therefore, C5 fraction showed higher toxicity on cancer cells and lower toxicity on normal cells when compared to standard doxorubicin as a benchmark in cancer treatment (Fig. 5).

Regulatory Genes of Apoptosis in Response to C5 Fraction of *Calystegia Sepium*

Since the cell populations in different phases of cell cycle and apoptosis do not provide the data for cellular mechanisms involved in cancer cells, the molecular mechanism was investigated through the expression level of some genes that are indicative of cell death in response to optimum fraction (C5) of Methanolic extracts of *Calystegia sepium* leaves (CS.m). Also, doxorubicin was used as standard benchmark to compare its effects with C5 fraction
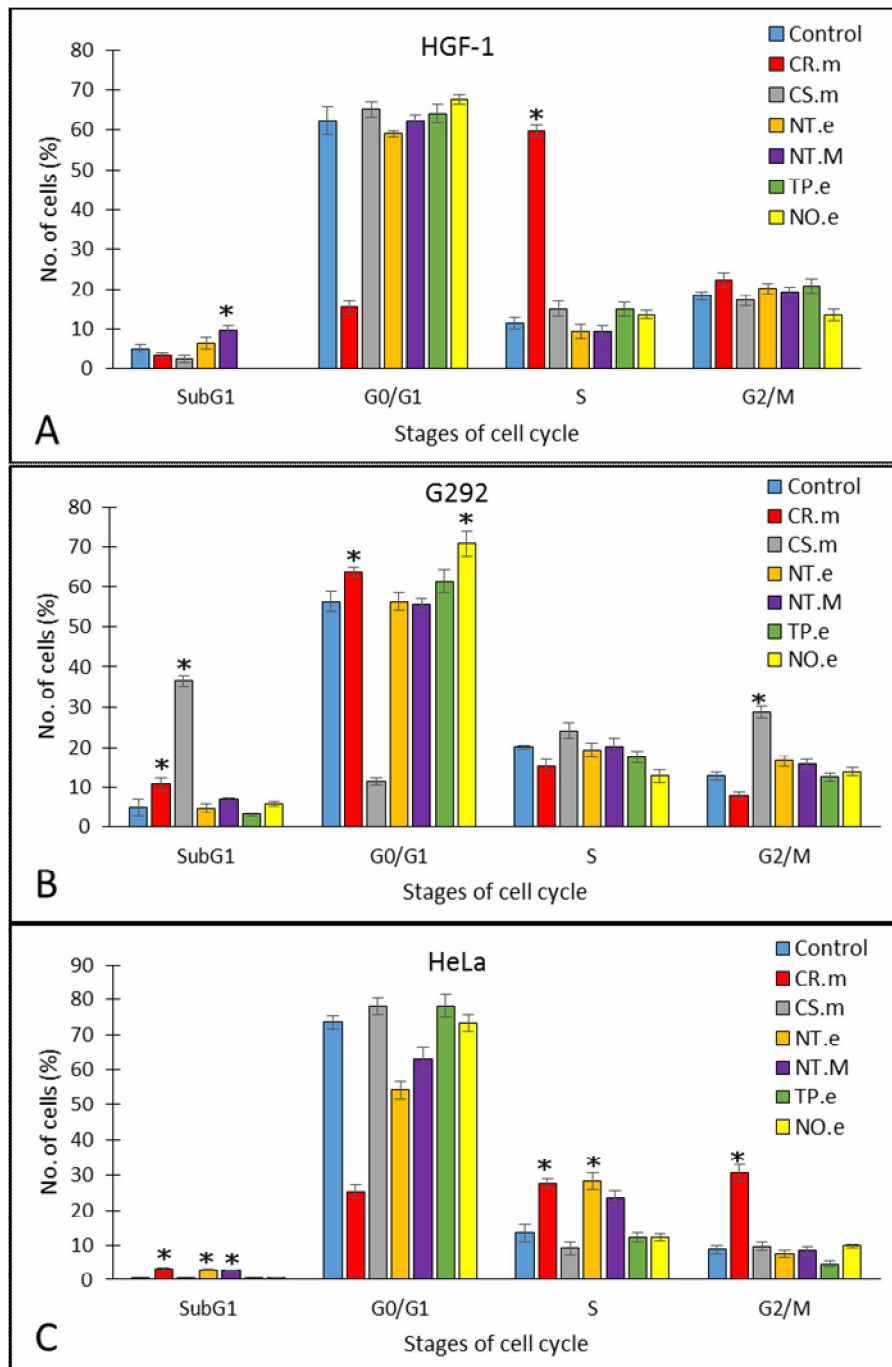
Fig. 3. Flow cytometry analysis of cell cycle stages in cancer (HeLa and G292) and normal (HGF-1) induced by crude extracts (50 μg ml⁻¹). CR.m; Methanolic extract of *Catharanthus roseus* leaves; CS.m; Methanolic extracts of *Calystegia sepium* leaves, NT.e: Ethanolic extract of *Narcissus tazetta* bulbs, NT.m: Methanolic extract of *Narcissus tazetta* bulbs, TP.e; Ethanolic extract of *Tradescantia pallida* leaves, NO.e; Ethanolic extracts of *Nasturtium officinale* after 24 h treatment.*means the significant increase of cell numbers in different phases of cell cycle than control.
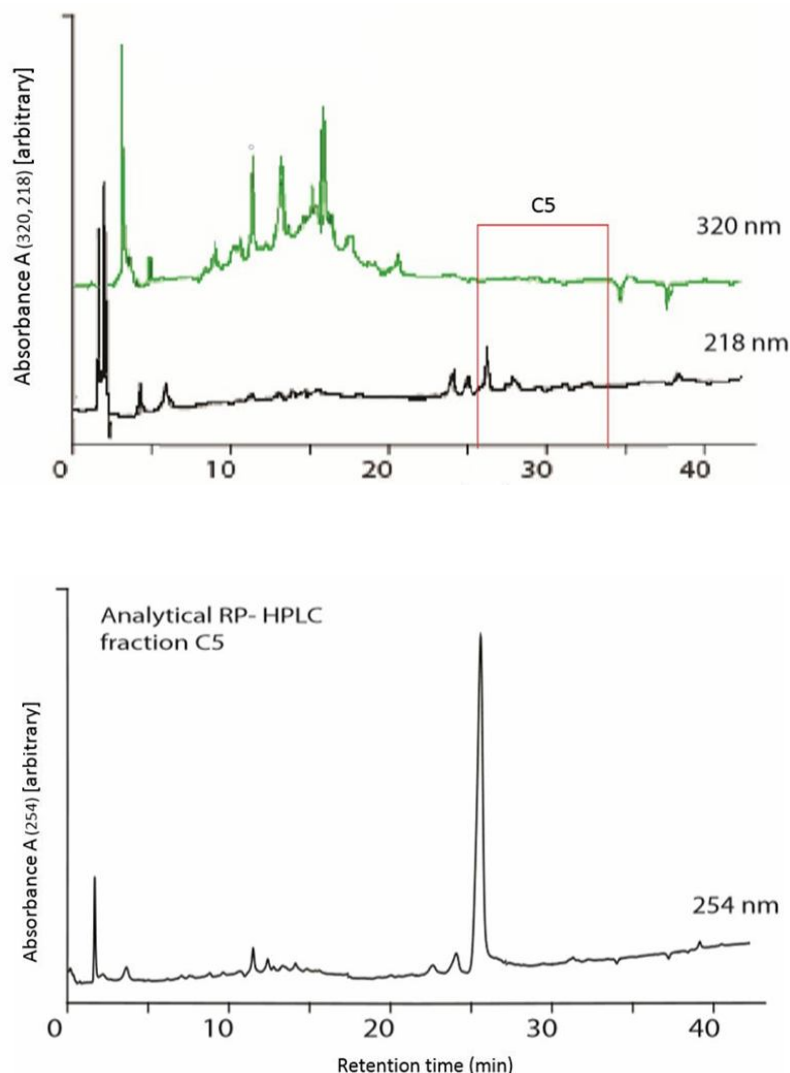
Fig. 4. HPLC chromatogram related to *Calystegia sepium* (a) Methanolic extract and (b) C5 active fraction.

in both cancer and normal cells. Results of genes expression indicated that behavior pattern for six genes is similar in cancer cells including G292 and HeLa. In some genes including *NFKβ1* and *APAF-1*, C5 fraction induced the expression levels more than doxorubicin. Compared to normal cells, the expression rate of *BCL-2* decreased more in cancer cells and possibly it led to a reduction of MDM-2 expression that resulted in the p53-induced apoptosis (Fig. 6). The expression changes of *p21* as a negative regulator of cell cycle were similar among cancer and normal cells and increased by both C5 fraction and doxorubicin treatments.

RPRM may be involved in the regulation of p53-dependent G2 arrest of the cell cycle that doxorubicin induced its expression more than C5 fraction.

Plant-derived drugs are widely applied for anticancer treatment as they are natural and readily available [24]. In addition, synthetic anticancer drugs have the adverse effects and drug interactions that are major restriction in modern medicine; therefore, plants are introduced as potential sources of anticancer agents in the world. Previous studies on *Catharanthus roseus* have showed the pharmacological effects of important alkaloids including vincristine,

Table 2. Percentages of Cancer (HeLa and G292) and Normal (HGF-1) Cell
Death (Cell Toxicity) Induced by Different Fractions of *Calystegia
Sepium* after 24 h Treatment with 50 μg ml^{-1} Concentration

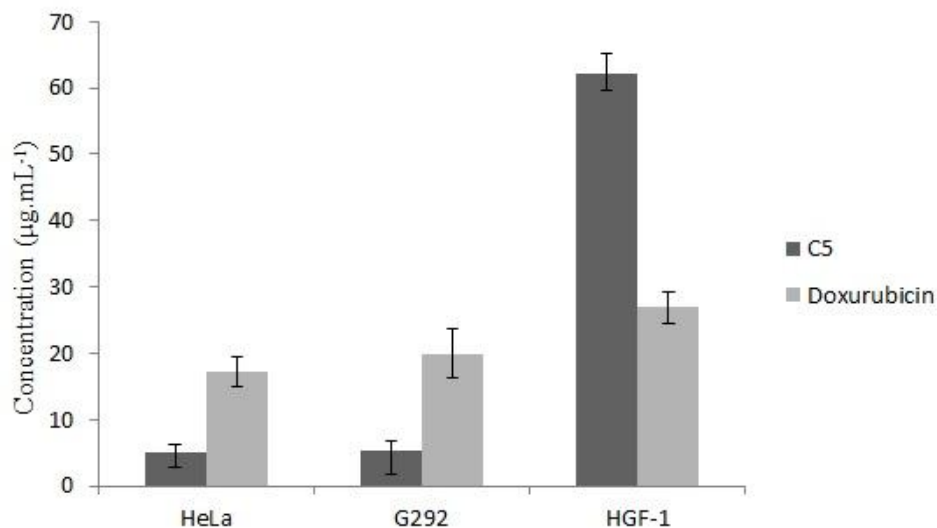| Fraction	Cell lines (Mean ± SE)		
	HeLa	G292	HGF-1
C1	12.43 ± 1.65	8.26 ± 1.07	7.57 ± 0.88
C2	11.31 ± 0.75	10.42 ± 1.69	8.86 ± 1.25
C3	15.95 ± 1.34	11.18 ± 0.76	7.48 ± 1.52
C4	34.86 ± 1.59	28.24 ± 1.06	19.26 ± 1.22
C5	91.41 ± 2.32	96.91 ± 2.57	22.83 ± 1.36
C6	50.17 ± 1.66	46.34 ± 1.90	14.39 ± 1.14

Fig. 5. The values of IC50s of C5 fraction of *Calystegia sepium* extracts (μg ml^{-1}) in comparison with Doxorubicin
on HeLa and G292 cancer cells and HGF-1 normal cells.

vinblastine and their derivatives that interact with growing ends of microtubules *via* end-capping process, disrupt their polymerization, and result in tubulin aggregation and production of tubulin paracrystals [25]. Today, vinblastine and vincristine are among the most widely-used drugs in chemotherapy. Vinblastine is applied for treatment of breast cancer, non-small cell lung cancer, head and neck cancers, as well as Hodgkin's lymphoma. Meanwhile, the effect of vincristine on Hodgkin's lymphoma, lymphoblastic leukemia and nephoroblastoma (Wilm's tumor) have been
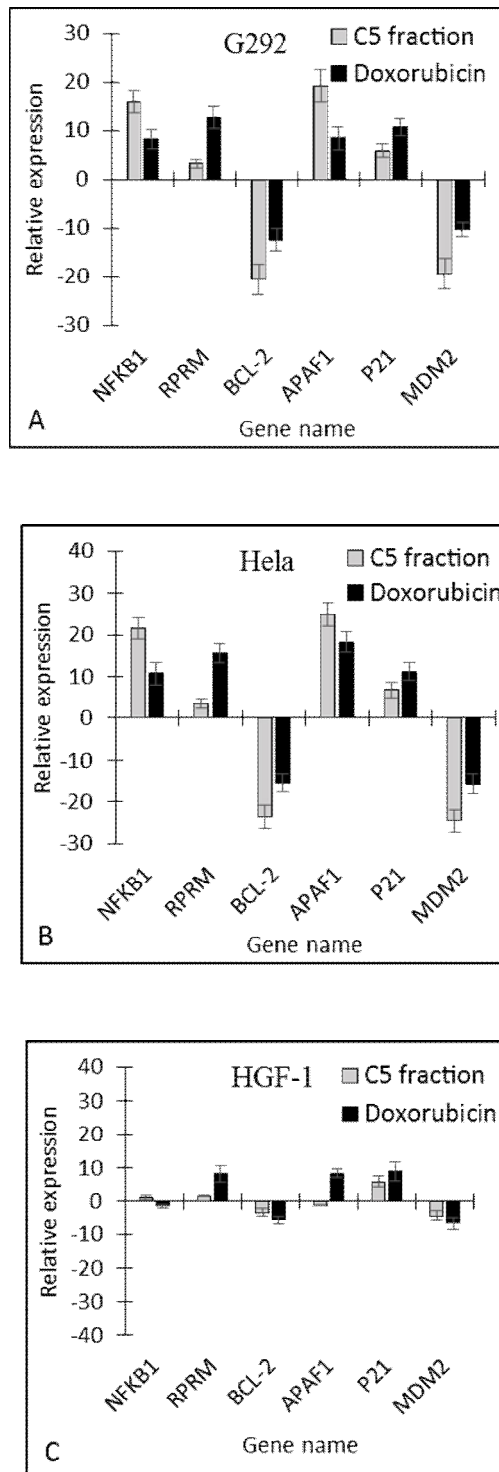
Fig. 6. Expression levels of six candidate's genes including *NFKβ1*, *RPRM*, *BCL-2*, *APAF-1*, *P21*, and *MDM2* after 24 h treatment in response to C5 fraction and doxorubicin in G292 (A) and HeLa (B) cancer cells and HGF-1 (C) normal cells.

frequently verified [26]. Among other extracts, Li *et al.,* (2017) indicated that MTT assay of aqueous extract of *Tradescantia pallida* leaves along with ZnO nanoparticles are able to kill 98.9% of cervical cancer cells (Li *et al.* 2017). In another study, cytotoxicity effect of Methanolic extract of *Tradescantia pallida* leaves showed the toxicity in brine shrimp lethality test with IC_{50} of 833.85 µg ml^{-1} [27].

Our results also indicated that Methanolic extracts of *Calystegia sepium* induced apoptosis and increased the cell accumulation in sub-G1 and cell cycle arrest in G0/G1. *Calystegia sepium* are known as a species of bindweed and contains lectin as carbohydrate-binding proteins [28]. Anticancer effects of lectinsvia ribosomal attachment, cell accumulation in either G0/G1 or G2/M phases, and apoptosis induction have been proven in several researches [29-31]. Although, among different fractions of Methanolic extract of *Calystegia sepium*, the optimum fraction that possesses the high efficiency of cytotoxicity was chosen. Tepfer *et al.* (1988) identified Calystegine as a alkaloid in different members of the family of *Convolvulaceae* that anticancer effects have been repeatedly studied [32]. In addition, results of MTT assay revealed that Methanolic extract of *Calystegia sepium* significantly increased the cell death in cancer cells than other extracts when compared to normal cells. Although, the minimum value of IC50 was an advantage for extract of *Calystegia* sepium to make it superior than other extracts as anticancer agent. In addition, with using PI/Annexin V analysis, the type of cell death that was responsible for Methanolic extract of *Calystegia sepium* cytotoxic property was determined.

Since genetic mutations and abnormal gene expression occur in cancer cells and act the extrinsic and intrinsic apoptotic pathways, therefore, signaling pathway-based cancer therapy might be an alternative way that can be indeed programmed by the activation of several key modulators. To find potential intracellular modulator(s) and molecular pathway (s) involved in apoptosis and cytotoxic property, effect of CS.m treatment on gene expression were investigated and compared to doxorubicin effect on HeLa and G292 cells. Since doxorubicin as an anti-tumor drug is applied for treatment of cancer diseases, it was used as standard benchmark to compare to Methanolic extract of *Calystegia sepium.* Activation of critical cellular

transcriptional factors such as nuclear fatoctor-κB (NF-κB) has been implicated in the control of proliferation and apoptosis in many types of cancer cells [33,34]. According to our results, C5 fraction from Methanolic extract of *Calystegia sepium* increased the NF-κB level that it was in consistent with doxorubicin effect. In addition, the increase of mRNA expression of *p21* are involved in the activation of caspases, which is associated with the activation of apoptosis. Gogada *et al.,* (2011) demonstrated that *p21*-deficiency resulted in reduced expression of *APAF-1*. *APAF-1* deficiency inhibited the activity of caspase-3, therefore, up-regulation of *APAF-1* can be a key factor in induction of apoptotic cell death. In our experiments, both doxorubicin and C5 fraction induced the expression level of *APAF-1* in cancer cells including G292 and HeLa [35].

Recently, many studies demonstrated that *BCL-2*/*MDM2* dual inhibition is a requirement for antitumor activity [36,37], that these results were in consistent to our experiments when C5 fraction decreased the expression levels of *MDM2* and *BCL-2*. As a novel strategy, C5 fraction from Methanolic extract of *Calystegia sepium* can provide new insights into the mechanisms of acquired resistance of *BCL-2* and *MDM2* inhibitors that led to as apoptosis-induction. Furthermore, activation of the p53 tumor suppressor indirectly downregulates many cell cycle genes and lead to cell cycle arrest [38]. In summary, CS.m controls a plethora of cell cycle genes through *p53*/*p21* and *BCL-2*/*MDM2* pathway and contributes to cell cycle arrest that is a target for cancer therapy.

According to all obtained results, *Calystegia sepium* extracts contains stable compound(s) with strong apoptotic effect on cancer cells while *Narcissus tazetta* bulbs extract has necrotic effect on all cells and *Nasturtium officinale* and *Tradescantia pallida* extracts help to survive normal cells with cytotoxicity on cancer cells.

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