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Original article

Novel anticancer activity and anticancer mechanisms of *Brassica oleracea* L. var. *capitata f. rubra*

R.R. Hafidh [a,b], A.S. Abdulamir [b,c,*], F. Abu Bakar [d,**], F.A. Jalilian [e], F. Jahanshiri [f], F. Abas [d], Z. Sekawi [f]

[a] *Department of Microbiology, College of Medicine, Baghdad University, P.O. Box 61023, Baghdad, Iraq*
[b] *Institute of Bioscience, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[c] *Department of Medical Microbiology, College of Medicine, Al-Nahrian University, P.O. Box 70046, Baghdad, Iraq*
[d] *Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[e] *Department of Medical Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, 69391 IUMS, Ilam, Iran*
[f] *Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

Abstract

Aim of the study: The anticancer activity of red cabbage (RC) and the underlying mechanisms against human cervical and hepatocarcinoma cancer cells were explored in this study.

Materials and methods: The cytotoxic activity of RC extract was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cytotoxicity assay. ELISA was used to detect the level of anticancer cytokines, TNFα and IFNβ, in the culture supernatants of cancer cells. Apoptotic cells were investigated by flow cytometry. The levels of apoptotic genes (Bax, BCL-2, Capsases 7–9), cell cycle regulatory genes (cyclin D, E, and A) and tumor suppressor proteins (p27, p21, and p53) were assessed by real time RT-PCR.

Results: The cytotoxic effect of the extract on normal human cells was significantly different from its effects on HeLa and HepG2 cells, 251.28 ± 4.3, 23.38 ± 1.87, and 28.66 ± 2.85 mg/ml, respectively. The selectivity index (SI) was 10.88 ± 0.82 for HeLa and 8.93 ± 0.81 for HepG2 cells. Increased levels of TNFα, but not IFNβ were observed in the treated HeLa and HepG2 culture supernatants when compared with untreated cells. RC extract induced G0/G1 phase arrest in the cancer cells. The extract induced apoptosis, in a dose and time dependent manner, in treated HeLa and HepG2 cells while no observed apoptosis was found in untreated cells. Moreover, RC IC50 showed remarkable influence on the expression of the apoptosis-related genes in a positive and negative manner on both HeLa and HepG2 cells.

Discussion/conclusion: RC extract could be considered as a novel anticancer agent providing new prospects for anticancer therapy using a natural product.

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Keywords: *Brassica oleracea*; Anticancer; Apoptosis; Cell cycle arrest; Anticancer cytokines

Aim of the study/Introduction

More effort is needed to control the increasing incidence of human malignancies worldwide. In an effort to win the combat against cancer, research across the globe has focused on identifying, characterizing, and providing the scientific basis to the efficacy of various plant-based drugs which could be used to control various human malignancies [1]. One of the reasons for focusing on natural products as potential anticancer agents is the growing resistance to chemotherapeutic drugs in cancer patients[2]. Moreover, numerous studies have shown that

* Corresponding author at: Department of Medical Microbiology, College of Medicine, Al-Nahrian University, P.O. Box 70046, Baghdad, Iraq.
Tel.: +964 770267 1741; fax: +964 77942 3552.
** Corresponding author at: Department of Food Science, Faculty of Food Science and Technology, University Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia. Tel.: +60 3 8946 8375.
E-mail addresses: ahmsah73@yahoo.com (A.S. Abdulamir), fatim@putra.upm.edu.my (F. Abu Bakar).

various natural products can interfere with different stages of cancer development, and may effectively block malignancy [1].

Recently, red cabbage (*Brassica oleracea* L. var. *capitata f. rubra* DC), which belongs to the family Brassicaceae, has attracted much attention because of its potential application on physiological functioning [3]. Red cabbage is a rich source of phenolic compounds, with the anthocyanins being the most abundant class; they have been found to be responsible for the main biological properties of RC [4,5]. Some studies have shown the ability of red cabbage extract to suppress oxidative stress in vivo [6,7]. Others have focused on its anticancer [8–10], anti-inflammatory [11], antibacterial [8], and anti-diabetic effects [12].

The current study explores possibly for the first time, the potential anticancer activity of red cabbage extract by using a sophisticated approach of molecular and cytotoxic methodologies. It also aimed to explore the possible mechanisms used by the active components of this extract, if any, to inhibit selectively the proliferation of human cancer cell lines. The current study was designed to determine whether RC extract had the potential to induce apoptosis in cancer cells and arrest the cell cycle using two techniques, namely flow cytometry and RT qPCR which together detect and measure apoptosis and apoptosis-inducing factors. Moreover, this study determined the level of anticancer cytokines and measured the expression level of genes with anticancer activity, namely genes involved in apoptosis, cell cycle regulation and tumor suppressor proteins, in human cancer cell lines after being treated with red cabbage methanol extract.

Materials and methods/Methodology

Preparation of the extract

Fresh red cabbage (RC) was purchased from local market grown in tropical area conditions (30 °C and 80+ humidity) in the state of Selangor, Malaysia. The dark colored medium sized leaves were collected in September. The cabbage leaves were cut into small pieces and left to dry in dark area for seven days at room temperature. Finally, leaves were ground to powder. The ground powder was extracted 1:10 wt/v with 2.4 mol/L HCl acidified methanol (Merck, Darmstadt, Germany) in order to extract all components of phenolic compounds, free and conjugated [13]; the ground powder was then soaked in a dark area for three days at room temperature. The solvent was removed by filtration and fresh solvent was added to the plant material. The extraction procedure was repeated twice and the extract was evaporated to dryness under vacuum at 40 °C. The pH of RC extract was neutral ranging from 6.8 to 7.2. The powder was stored at −18 °C in desiccant until further use. The stock extract was prepared by redissolving in 0.1% dimethyl sulfoxide (DMSO), (BIO BASIC INC., NY, USA) as this concentration was shown to be non toxic to cell culture [14]. The dissolved suspension was centrifuged at $134 \times g$ for 10 min and was filtrated by 0.22 μm Millipore filters (Nalgene, UK).

Cell culture

Two lines of human cancer cells along with normal human cells were used to evaluate the anticancer effects of RC extract. The cancer cell lines were cervix adenocarcinoma (HeLa; ATCC CCL-2) and hepatocellular carcinoma cells (HepG2; ATCC HB-8065), while the normal human cells were peripheral blood mononuclear cells (PBMC). Human PBMC were isolated by density gradient centrifugation technique from heparinized whole blood as described previously [15]. PBMC were propagated in humidified 5% CO_2 atmosphere at 37 °C using RPMI-1640 culture medium w/L-glutamine (biowest, FL, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany), 50 U/ml penicillin-streptomycin (biowest, Florida, USA), and 2.5 μg/ml amphotericin B (biowest, FL, USA). Trypan blue solution 0.4% (Sigma, Germany) was used to count the cells in order to adjust them to an appropriate concentration, and the viability of cells was checked to be within the required range of viability, which is 95–99%.

Cytotoxicity assay

MTS assay

In order to determine the cells viability, the MTS colorimetric method (the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA) was used. Briefly, purified mononuclear cells, 2×10^5 cell/well, were cultured, in quadruplicates, in 96-well U-bottom tissue culture plates (Orange Scientific, Europe) with 2-fold serial dilutions of RC extract in RPMI-1640 culture medium. The extract concentrations ranged from 317 to 9.9 mg/ml. The negative control wells, in quadruplicates, contained PBMC in RPMI-1640 culture medium while the positive control wells, in quadruplicates, contained PBMC with Concavalin A (Con A), (10 μg/ml), a T-cell specific mitogen (Sigma, Germany). The final volume in the wells was 200 μl/well. Cultures were incubated in humidified 5% CO_2 atmosphere at 37 °C for 72 h.

The cytotoxic effect of RC extract on the human cancer cell lines was performed according to the established method [16], where 1×10^5 cell/well viable HeLa and HepG2 cells were grown in RPMI-1640 culture medium in 96-well flat-bottom tissue culture plates (Orange Scientific, Europe). The plates were incubated in humidified 5% CO2 for 24 h at 37 °C. When cells reached >80% confluence, the medium was replaced with 200 μl/well of 2-fold serial dilutions of RC extracts from 250 to 15.62 mg/ml prepared in RPMI-1640 maintenance medium (with 2% FBS). The negative control wells contained DMSO (0.1%) in RPMI-1640 maintenance medium. The final volume in all of the wells was 200 μl/well, in quadruplicates. All the plates were incubated in humidified 5% CO_2 for 24 h at 37 °C. Later, the wells' contents were removed and replaced with 200 μl/well of RPMI-1640 maintenance medium. The plates were re-incubated for 48 h at the same conditions. Finally, MTS solution was added to each well. The plates were incubated for 4 h. The absorbance was measured at 490 nm (reference 690 nm) using a 96-well plate ELISA reader (Sunrise Basic

Tecan, Grödig, Austria). Each experiment was repeated for three times with four wells per dilution in each run.

The data of the extract cytotoxicity on PBMC and human cancer cell lines were calculated using the following formula:

$$\text{cytotoxicity\%} = \left[1 - \left(\frac{\text{ODt}}{\text{ODc}} \right) \right] \times 100$$

where (ODt) indicates the optical density of the tested extract and (ODc) indicates the optical density of the negative control. Accordingly, the concentrations of 50% inhibition (CC50 and IC50) were the concentrations that achieved 50% cytotoxicity against PBMC and the cancer cells, respectively. The selectivity index (SI) was the ratio of CC50 (cytotoxicity on PBMC) to the IC50 (cytotoxicity on human cancer cells) [17].

Cytokines production by human cancer cell lines

The level of anticancer cytokines, IFNβ and TNFα, was investigated in treated and untreated HeLa and HepG2 cells. HeLa and HepG2 cells at 1×10^5 cell/well were grown in RPMI-1640 culture medium in 96-well flat-bottom tissue culture plates in a humidified 5% CO2 atmosphere for 24 h at 37 °C. Later, serial 2-fold dilutions of RC extract, 20 to 0.62 mg/ml, prepared in RPMI-1640 maintenance medium were added to the cells. The negative control wells contained cells with RPMI-1640 maintenance medium only. The final volume of all of the wells was 200 μl/well, in triplicates. The plates were incubated for 48 h at the same conditions. Afterwards, the supernatants from all the wells were removed and centrifuged at $1000 \times g$ for 10 min to be ready for the ELISA.

The cytokine concentrations in the culture medium supernatant of cancer cells were determined by using sandwich ELISA kits. The manufacturers' instructions for each kit were followed to calculate IFNβ concentrations by human ELISA kit (abcam, USA) and TNFα concentrations by human enzyme immunometric assay (EIA) kit (Cayman, USA). Later, the absorbance was measured at 412 nm (reference 690 nm) and 450 nm (reference 620 nm) using a 96-well plate ELISA reader for TNFα and IFNβ plates, respectively. For each sample, the assay was repeated for three times. The concentrations of the anticacncer cytokines were determined by extrapolating their OD values with that of the generated standard curves.

Flow cytometry analysis

The cells (HeLa and HepG2) were seeded (1×10^5 cell/well) in 6-wells tissue culture plates and were incubated in humidified 5% CO2 atmosphere for 24 h at 37 °C. The medium was then replaced with RPMI-1640 maintenance medium with or without RC extract and was incubated for further 24 h at the same conditions. The cell treatment was divided into two groups. In the first group (dose-dependent group), the effect of three 2-fold serial dilutions of RC extract (concentration > IC50 > concentration) was investigated regarding the level of apoptosis, if any, after a fixed time (24 h). The concentrations of RC extract with HeLa were 46.76, 23.38, and 11.69 mg/ml while they were 57.32, 28.66, and 14.33 mg/ml for HepG2 cells. In the second group (time-dependent group), the IC50 of RC extract was used to investigate the level of cells' apoptosis as well as cell cycle arrest after different time intervals of incubation with the extract as indicated in the appropriate figure legend. For cell cycle analysis, the cells were fixed in 70% ethanol, stained with PI (MP Biomedicals, LLC, IIIKrick, France) and were analyzed using CyAn ADP apparatus (BECKMAN COULTER, USA). The data were analyzed by software Summit (V4.3). The assay was measured in duplicate for each sample in the specific time of treatment.

Analysis of apoptosis

RNA extraction

The cells (HeLa and HepG2) were seeded, 1×10^5 cell/well, in 6-wells tissue culture plates and were incubated in a humidified 5% CO2 atmosphere for 24 h at 37 °C. The medium was then replaced with RPMI-1640 maintenance medium containing RC extract or alone in duplicates and was incubated at the same conditions. The IC50 of RC extract for each type of cells was used. The IC50 of RC extract was 23.38 mg/ml for HeLa cells and 28.66 mg/ml for HepG2 cells. It was observed that incubation for 12, 16 and 20 h with RC extract was optimal for apoptosis and cell cycle arrest studying when using flow cytometry. At the end of time interval for each cell type, all of the cells were harvested and transferred to 15 ml tubes. The supernatants were discarded after being centrifuged at $134 \times g$ for 5 min. The pellets were resuspended in PBS and were washed four times. Total RNA was isolated according to the manufacturer's protocol using GF-1 kit (Vivantis Technologies, Malaysia). RNA quality and quantity were determined by Life Science UV/vis Spectrophotometer, DU Series 700 (BECKMAN COULTER, USA). The isolated RNA was stored at −80 °C and was ready for use.

Real time quantitative RT-PCR

One microgram of the isolated RNA from each sample was reverse transcribed according to the manufacturer's protocol iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, Canada). The complete reaction mix was incubated for (5 min 25 °C, 30 min 42 °C, then 5 min 85 °C) using Thermo Bath, ALB64 (FINEPCR, Seoul, Korea). Finally, cDNA was stored at −80 °C for the qRT-PCR reaction.

Real-time quantitative PCR reaction was conducted using SsoFast™ EvaGreen® Supermix (BIO-RAD, Hercules, Canada). According to the manufacturer's protocol, a final volume of 20 μl cDNA was run for 40 cycles by CFX96™ Real-Time System (BIO-RAD, Hercules, Canada) using the forward and reverse primers mentioned in Table 1. Cycling conditions were 3 min 95 °C, 10 s 95 °C, 30 s 55–61 °C, and 20 s 72 °C. PCR reaction for cDNA templates from untreated HeLa and HepG2 cells were used as negative controls. For each target gene, the PCR reaction was run in triplicates. PCR reaction mix without cDNA template was used to detect any contamination. Beta-actin was used as a housekeeping gene (reference gene) to normalize the mRNA expression of target genes. At the end of the amplification, Eva Green fluorescence was measured continuously for the conduction of the melting curve analysis by slow

Table 1
Primers used in Real-Time quantitative PCR analysis (Vivantis Technologies, Malaysia).

	Forward primer	Reverse primer
Bax	CAC CAG CTC TGA GCA GAT	GCG AGG CGG TGA GCA CTC
BCL-2	TAC CTG AAC CGG CAC CTG	GCC GTA CAG TTC CAC AAA GG
Caspase 7	GTC TCA CCT ATC CTG CCC TCA	TTC TTC TTC TGC CTC ACT GTC
Caspase 8	GAA AAG CAA ACC TCG GGG ATA C	CCA AGT GTG TTC CAT TCC TGT C
Caspase 9	CCA GAG ATT CGC AAA CCA GAG G	GAG CAC CGA CAT CAC CAA ATC C
Cyclin D	AGA CCT GCG CGC CCT CGG TG	GTA GTA GGA CAG GAA GTT GTT C
Cyclin E	CTC CAG GAA GAG GAA GGC AA	TCG ATT TTG GCC ATT TCT TCA
Cyclin A	GTC ACC ACA TAC TAT GGA CAT G	AAG TTT TCC TCT CAG CAC TGA C
p21	GTG ATT GCG ATG CGC TCA TG	TCT CTT GCA GAA GAC CAA TC
p27	GTC TAA CGG GAG CCC TAG CC	CTA ACC CCG TCT GGC TGT CC
p53	TGT GGA GTA TTT GGA TGA CA	GAA CAT GAG TTT TTT ATG GC
β-actin (reference gene)	TCA CCC TGA AGT ACC CCA TC	CCA TCT CTT GCT CGA AGT CC

Note: All primers are listed 5′–3′.

heating at 0.5 °Cs-1 increments from 70 to 95 °C. Accordingly, a melting curve was generated at the end of the PCR amplification for monitoring the specificity of PCR reaction. Melting curve analysis of the negative first derivative was pursued. The efficiency of PCR primers was done as described by [18]. The data were analaysed using software BIO-RAD CFX Manager (V 1.1.308).

It has been well known that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals [19]. Therefore, the Bax to Bcl-2 ratio was calculated using the following equation:

$$\frac{\text{Bax}}{\text{Bcl-2 ratio}} = (\text{mean PCR efficiency for Bax and Bcl-2})\,(\text{CtBcl2} - \text{CtBax})$$

Data analysis

Data are presented as mean ± 2SE. The data analysis was conducted by using SPSS software version (12.0.0.2). The effect of RC extract on the inhibition of cell growth was evaluated by using 95% confidence intervals. IC50 and CC50 values were calculated using linear regression index equations. The statistically significant effects of the extract on the ability of HeLa and HepG2 cells to synthesize selected cytokines were compared with the control groups using Student's *t*-test. For flow cytomteric analysis, R2 fraction represented sub-G apoptotic cells; moreover, the percentage of cells at different cell cycle phases was calculated from the total cells minus apoptotic cells. For quantitative real time PCR, the up- or down-regulation of mRNA expression of selected genes was measured as expression fold changes in term of mean ± 2SD. The significance of up- or down-regulation of the normalized mRNA expression of selected genes was determined by comparing the mean ± 2SD of any up- or down-regulation with the mean ± 2SD of control (untreated cells), which is of mean equal to 1 ± 2SD. *P* values less than 0.05 were considered significant.

Results

Cytotoxicity of RC extract on two human cancer cell lines

The results of thisexperiment revealed that the cytotoxic effects of RC extract on normal human cells (PBMC) were significantly different ($P < 0.05$) from that on human cancer cells. The cytotoxic effect of RC extract on PBMC (CC50 = 251.28 ± 4.3 mg/ml) was lower than on HeLa cells (IC50 = 23.38 ± 1.87 mg/ml) and HepG2 cells (IC50 = 28.66 ± 2.85 mg/ml). The results revealed that RC extract required high concentrations to be cytotoxic for the normal human cells (Fig. 1), while even low extract concentrations were enough to give the same effect on the human cancer cells (Fig. 2). These results reflected the good selectivity and safety of RC extract as a candidate anticancer agent.

The cytotoxicity of RC extract on PBMC, HeLa and HepG2 cells was dose dependent. In other words, the cytotoxicity of RC extract decreased with lower concentrations of the extract. The significant differences of RC cytotoxicity between normal and cancer cells were supported by the results of the
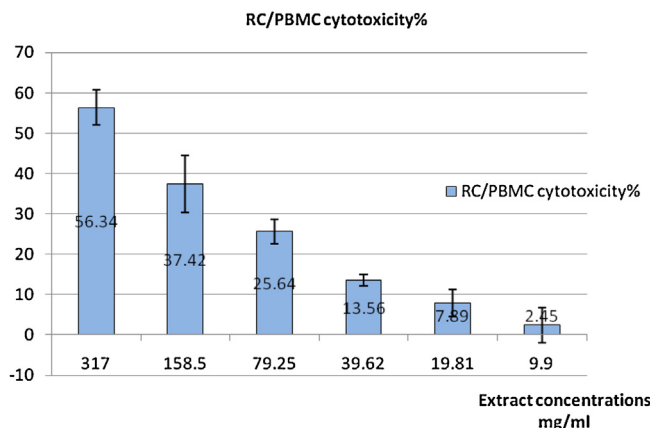

Fig. 1. The percentage of PBMC death after treatment with 2-fold serial dilutions of RC extract in term of mean ± 2SE (confidence interval CI 95%).
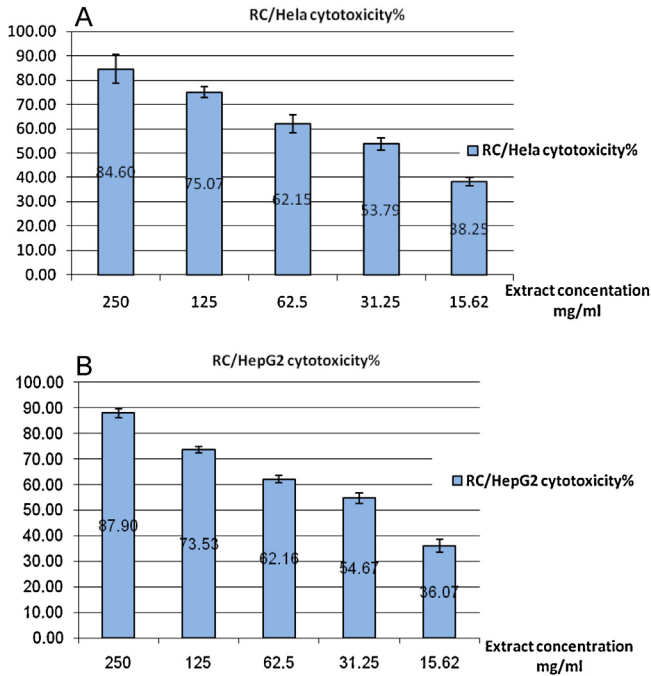
xxx.e5

Fig. 2. The percentage of (A) HeLa and (B) HepG2 cells death after treatment with 2-fold serial dilutions of RC extract in term of mean $\pm$ 2SE (confidence interval CI 95%).

selectivity index (SI) which represents the ratio of the highest concentration that causes 50% death to normal cells (CC50) to the lowest concentration that causes 50% death to cancer cells (IC50). The SI values of RC extract on HeLa and HepG2 cells were 10.88 ± 0.82 and 8.93 ± 0.81, respectively. There was no significant difference ($P > 0.05$) in the cytotoxic effect of RC extract on both cell lines.

Specific immune response by human cancer cells

The human cancer cell lines were treated with extract concentrations less than the IC50. These concentrations allowed the production of anticancer cytokines, if any, in the culture supernatants of HeLa and HepG2 cells (Figs. 3 and 4). The anticancer effect of RC extract on HeLa and HepG2 cells was partly investigated by detecting and measuring IFNβ and TNFα levels in the cell culture supernatants. No significant differences ($P > 0.05$) in IFNβ levels were found between the treated and untreated HeLa and HepG2 cells with RC extract while the levels of TNFα showed considerable increase ($P < 0.05$) in HeLa and HepG2 culture supernatants treated with RC extract in comparison with the control group (untreated cells). This increase was in a dose-dependent manner with RC extract concentrations.
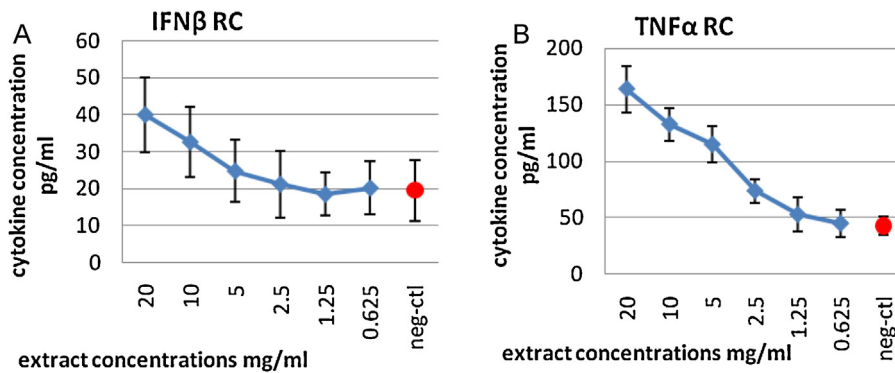

Fig. 3. Figures demonstrate the level of the produced cytokines, in comparison with negative control (neg-ctl), in culture supernatant of HeLa cell after treatment with red cabbage extract (RC): (a) IFNβ and (b) TNFα.
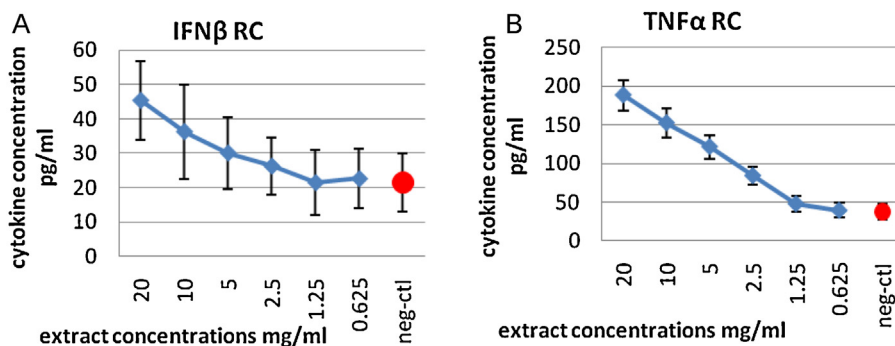

Fig. 4. Figures demonstrate the level of the produced cytokines, in comparison with negative control (neg-ctl), in culture supernatant of HepG2 cell after treatment with red cabbage extract (RC): (a) IFNβ and (b) TNFα.
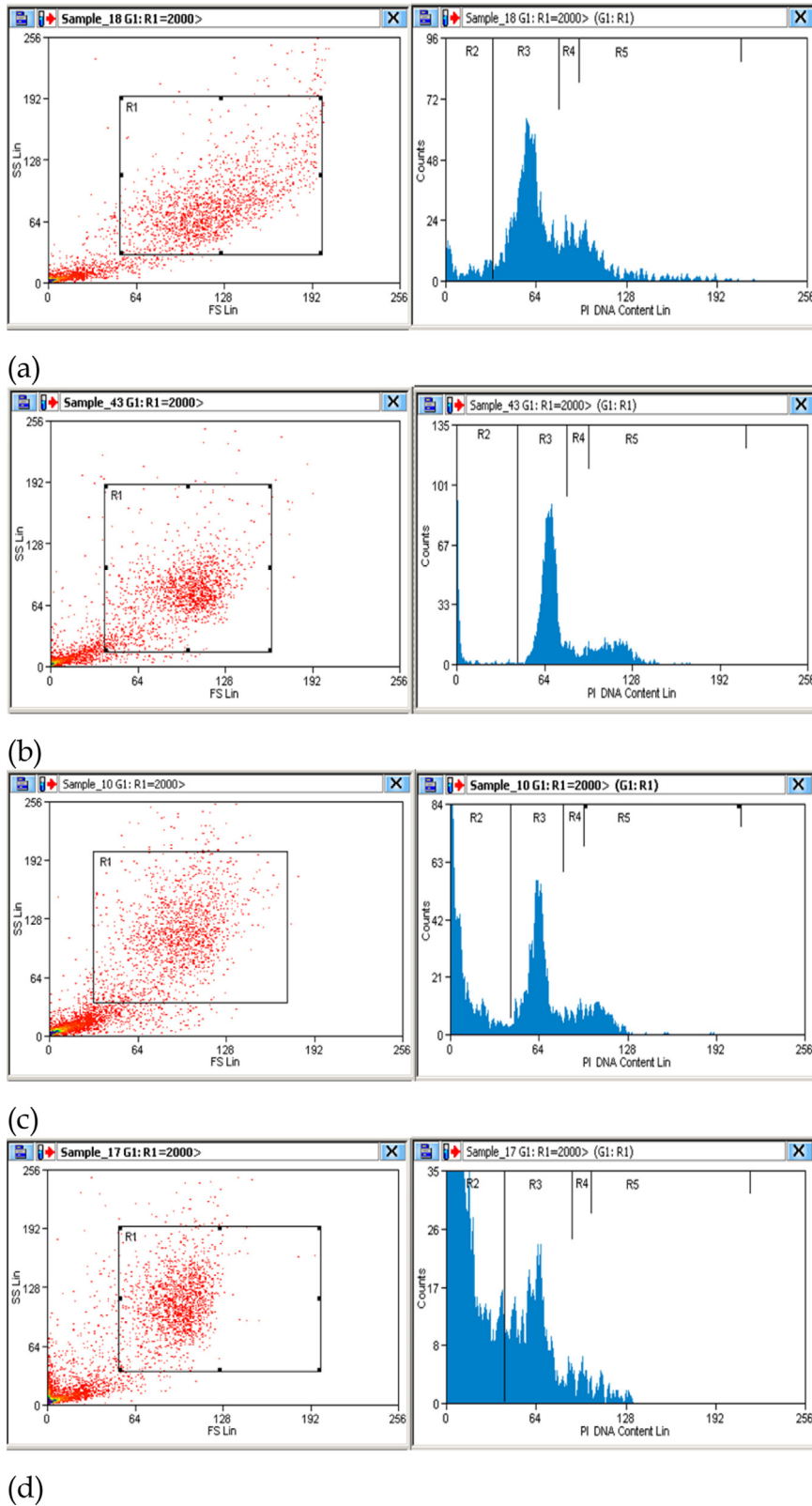
Fig. 5. DNA content frequency histograms representing HeLa cells after 24 h from (a) untreated cultures (b) cultures treated with RC extract concentration < IC50 (c) cultures treated with RC extract IC50 (d) cultures treated with RC extract concentration > IC50. The treatment affected the cell cycle distribution and induced apoptosis. The cells were stained with PI. Fluorescence of the PI-stained cells was measured using CyAn ADP appartus and Summit (V4.3) software. The software program provides the estimate of percentage of cells with fractional DNA content (apoptotic cells: R2) and cells in G0/G1 (R3), S (R4), and G2/M (R5) phases of the cycle. Total cell number (R1).
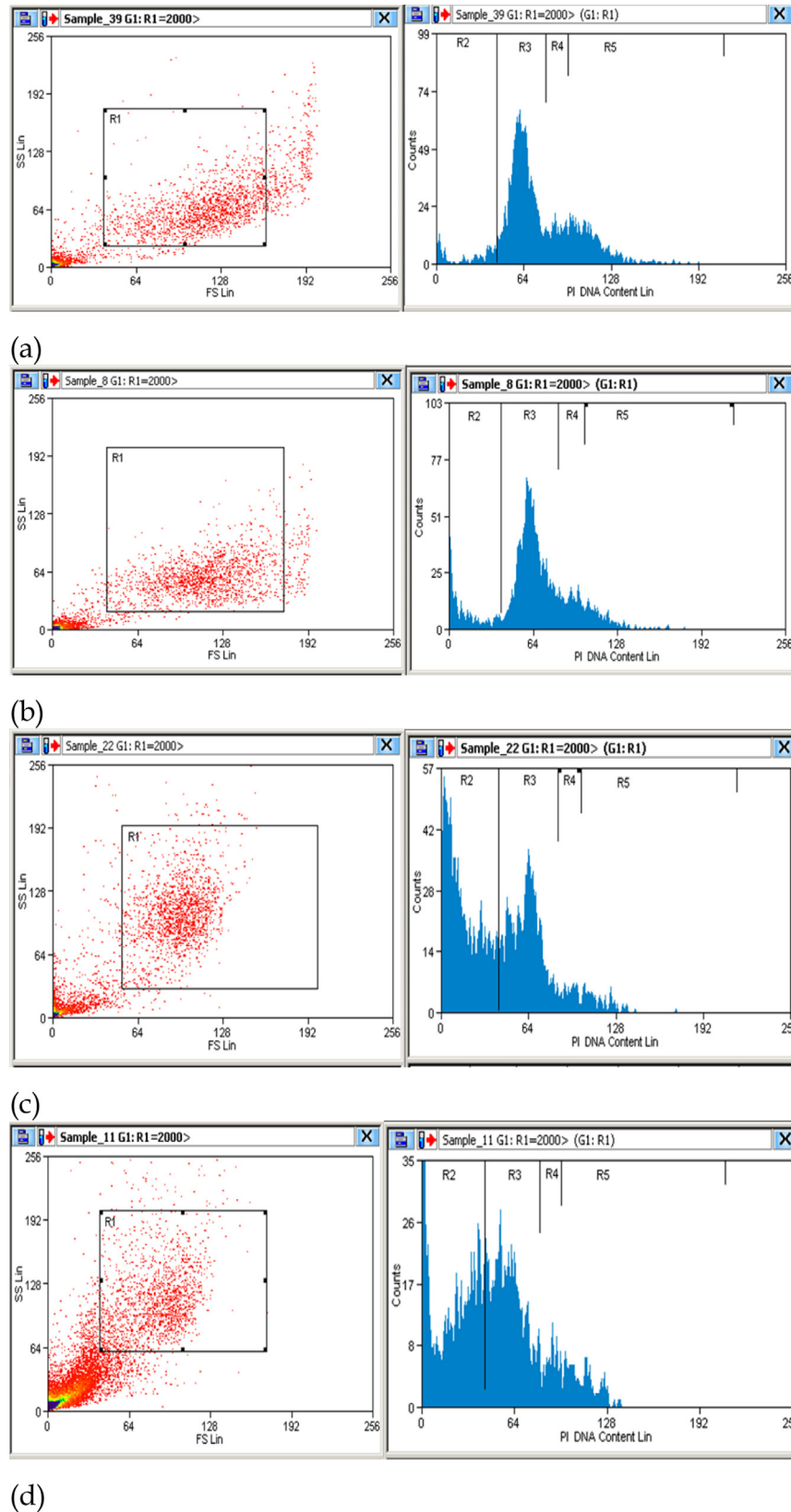
Fig. 6. DNA content frequency histograms representing HepG2 cells after 24 h from (a) untreated cultures (b) cultures treated with RC extract concentration < IC50 (c) cultures treated with RC extract IC50 (d) cultures treated with RC extract concentration > IC50. The treatment affected the cell cycle distribution and induced apoptosis. The cells were stained with PI. Fluorescence of the PI-stained cells was measured using CyAn ADP appartus and Summit (V4.3) software. The software program provides the estimate of percentage of cells with fractional DNA content (apoptotic cells: R2) and cells in G0/G1 (R3), S (R4), and G2/M (R5) phases of the cycle. Total cell number (R1).
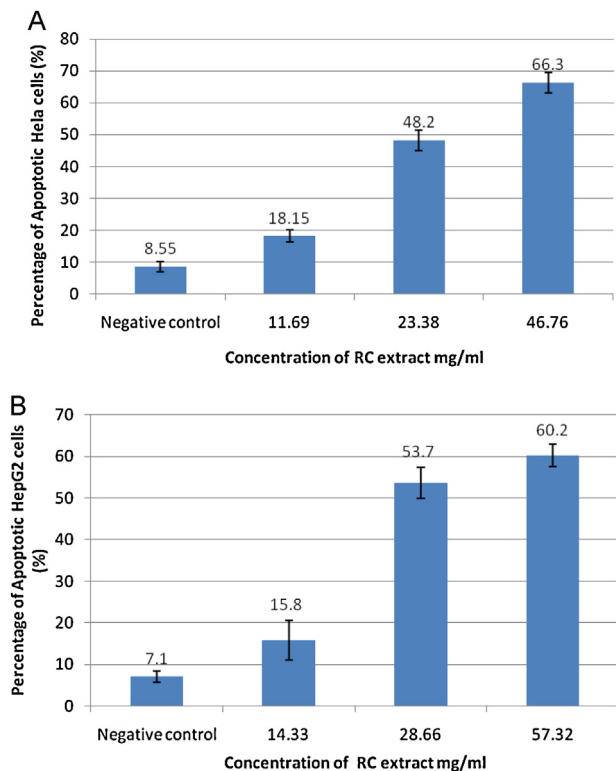
Fig. 7. The graph of flow cytometric analysis shows the percentage of apoptotic cells after treatment with RC extract for 24 h and in comparison with untreated cells (Negative control). The extract's concentrations represent 2-fold serial dilutions (concentration > IC50 > concentration). (A) Apoptotic % of HeLa cells. (B) Apoptotic % HepG2 cells. The increase in the percentage of the apoptotic cells was dose dependent.
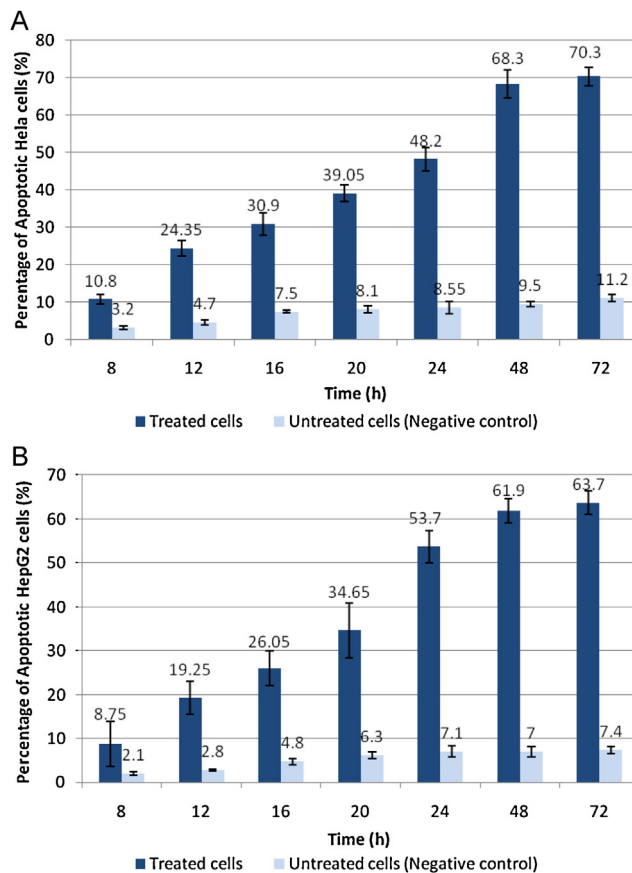

Fig. 8. The graph of flow cytometric analysis shows the percentage of apoptotic cells after treatment with IC50 of RC extract in comparison with untreated cells. The percentage of apoptotic cells increased with the time of treatment. (A) Apoptotic % of HeLa cells. (B) Apoptotic % of HepG2 cells. The increase in the percentage of the apoptotic cells was time dependent.

RC extract induced apoptosis and cell cycle arrest in human cancer cells

Apoptosis in the cells treated with RC extract was measured using flow cytometry. The cytotoxicity of RC extract was shown to be selective on cancer rather than normal cells. It is noteworthy to mention that physiological properties (pH, temperature and ionic strength) of the RC extract diluted in RPMI medium were within normal range; this minimizes the chance of extract-driven necrosis and suggests? That apoptosis was the type of cell death detected by flow cytometry. However, apoptosis was confirmed by integrating the results of both flow cytometry and RT-qPCR. The flow cytometric analysis showed remarkable potential of RC extract to induce apoptosis in the treated cells in comparison to untreated cells (Figs. 5 and 6). By testing the apoptosis for a fixed time interval, 24 h, and different doses of RC extract, the percentage of the apoptotic cells was directly correlated with the concentration of RC extract. The extract induced apoptosis, in a dose dependent manner, in treated HeLa and HepG2 cells while no observed apoptosis was found in untreated cells ($P < 0.05$). By using the IC50 of RC extract for 24 h treatment, 48.2 and 53.7% of HeLa and HepG2 cells, respectively, underwent apoptosis (Fig. 7).

The flow cytometric analysis of HeLa and HepG2 cells treated with the IC50 of RC extract revealed an increase in the percentage of the apoptotic cells in a time dependent manner. The

apoptotic cells increased significantly ($P < 0.05$) in the treated cell cultures when compared with untreated cell cultures, and this increase was directly proportional with duration of incubation with RC extract (Fig. 8). For the phases of cell cycle, except for 16 h time interval, there were no significant differences ($P > 0.05$) among the percentage of cells at different cell cycle phases, G0/G1, S, and G2/M, of HeLa and HepG2 cells treated with RC IC50. The replicates' mean percentage of cells at G0/G1 phase at all of the time intervals (8, 12, 16, 20, 24, 28, and 72 h) in RC extract-treated Hela and HepG2 cells, except for the 8 h interval in Hela cells, was significantly higher than the replicates' mean percentage of cells at G0/G1 phase for the corresponding time intervals in the untreated cells ($P < 0.05$). And the optimal time for maximal G0/G1 cell cycle arrest was found to be at 16 h (Figs. 9 and 10). In other words, RC extract significantly induced G0/G1 phase arrest in treated HeLa and HepG2 cells.

The apoptosis- and cell cycle-related genes in human cancer cells treated with RC extract

Cultivation of cells with RC extracts for 12, 16, and 24 h was optimal for measuring expression level of the apoptosis- and cell
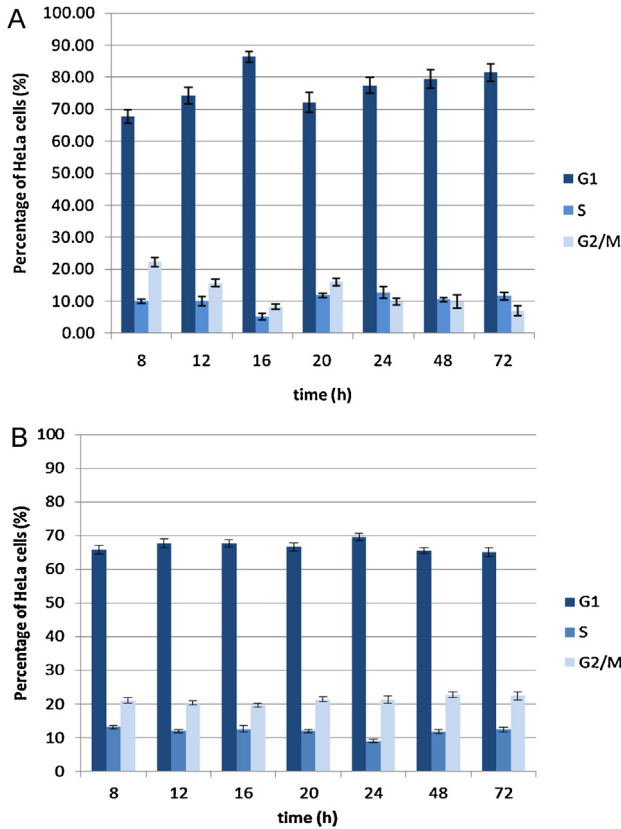
Fig. 9. The percentage of cells at G0/G1, S, G2/M phases of cell cycle as an indicator for cell cycle arrest in (A) HeLa cells treated by the IC50 of RC extract, and in (B) untreated HeLa cells at different time intervals at 8, 12, 16, 20, 24, 48, and 72 h.
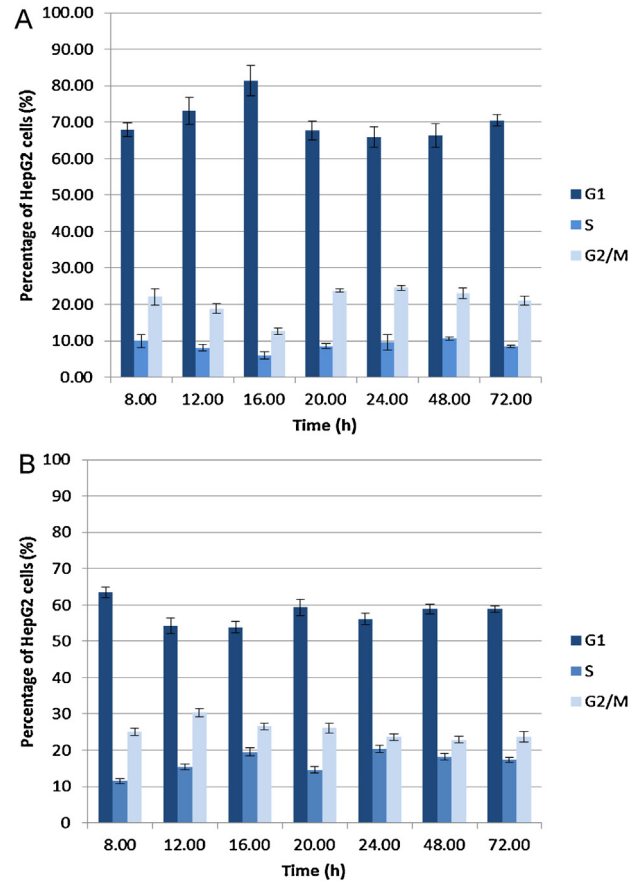

Fig. 10. The percentage of cells at G0/G1, S, and G2/M phases of cell cycle as an indicator for cell cycle arrest in (A) HepG2 cells treated by the IC50 of RC extract, and in (B) untreated HepG2 cells at different time intervals at 8, 12, 16, 20, 24, 48, and 72 h.

cycle-related genes using real-time quantitative PCR. The other treatments of 8, 24, 48, and 72 h were ignored because they either gave very low or very high percentage of apoptotic cells. Studying the apoptosis- and cell cycle-related genes cannot be covered well during very early phase of extract's treatment when some of the apoptosis genes are still not expressed; alike, during very late phase of apoptosis, most cells already died which renders measuring the expression of selected genes erroneous. The melting curve analysis showed single peak of melting temperature (Tm) 76–87 °C, while no significant premature peaks were found indicating that primer dimers were minimal and providing further evidence on the specific detection of the target mRNA genes (data not shown). The PCR efficiency of the primers used was greater than 90% and the correlation coefficients were greater than 0.99.

RC IC50 showed remarkable influence on the expression of the apoptosis-related genes in a positive and negative manner on both HeLa and HepG2 cells (Fig. 11). The expression of Bax gene in HeLa and HepG2 cells was upregulated ($P < 0.05$) after 12, 16, and 20 h treatments with RC IC50 ($P > 0.05$) while Bcl-2 gene expression was downregulated ($P < 0.05$) in HeLa cells after 12, 16, and 20 h, and in HepG2 cells after 12 h of treatment with RC IC50. Caspase 7 gene expression was found to be upregulated after 16 h of treatment of HeLa cells with RC IC50 ($P < 0.05$) while Caspase 7 gene expression in HepG2 cells was upregulated after 12 and 16 h of treatment with RC IC50

($P < 0.05$). Moreover, RC IC50 upregulated the expression of Caspase 8 gene in both HeLa and HepG2 cells after 12 and 16 h ($P < 0.05$). The current study also revealed that treating HeLa and HepG2 cells with RC IC50 upregulated Caspase 9 expression mainly after 12 and 16 h ($P < 0.05$).

The ratio of Bax to Bcl-2 proteins influences the apoptotic rate of cells; therefore, Bax/Bcl-2 ratio was calculated in treated and untreated HeLa and HepG2 cells. The Bax/Bcl-2 ratio in HeLa cells treated with RC IC50 for 12 h (7.26 ± 1.1) and 16 h (14.3 ± 3.5), but not at 20 h (3.71 ± 0.75), was higher ($P < 0.05$) than for untreated cells (0.22 ± 0.01). The Bax/Bcl-2 ratio in the treated HepG2 cells with RC IC50 after 12 h (11.15 ± 2.5), 16 h (12.49 ± 3.45), and 20 h (3.05 ± 0.67) was higher ($P < 0.05$) than in untreated cells (0.37 ± 0.04).

Flow cytometric analysis showed cell cycle arrest at G0/G1 phase of cancer cells treated with RC extract; therefore, the regulatory proteins of G0/G1 phase in the mammalian cell cycle, cyclin D, E, and A were studied. These cyclins are responsible for the activation of cyclin-dependent kinases (cdk) in G1 and S phases of the cell cycle in HeLa and HepG2 cells. Moreover, the mRNA expression of the proteins responsible for the inhibition of cyclin-cdk active complexes of G1 and S phases of HeLa and HepG2 cells treated with RC extract was studied as well, namely tumor suppressor proteins p27, p21, and p53 (Fig. 12).
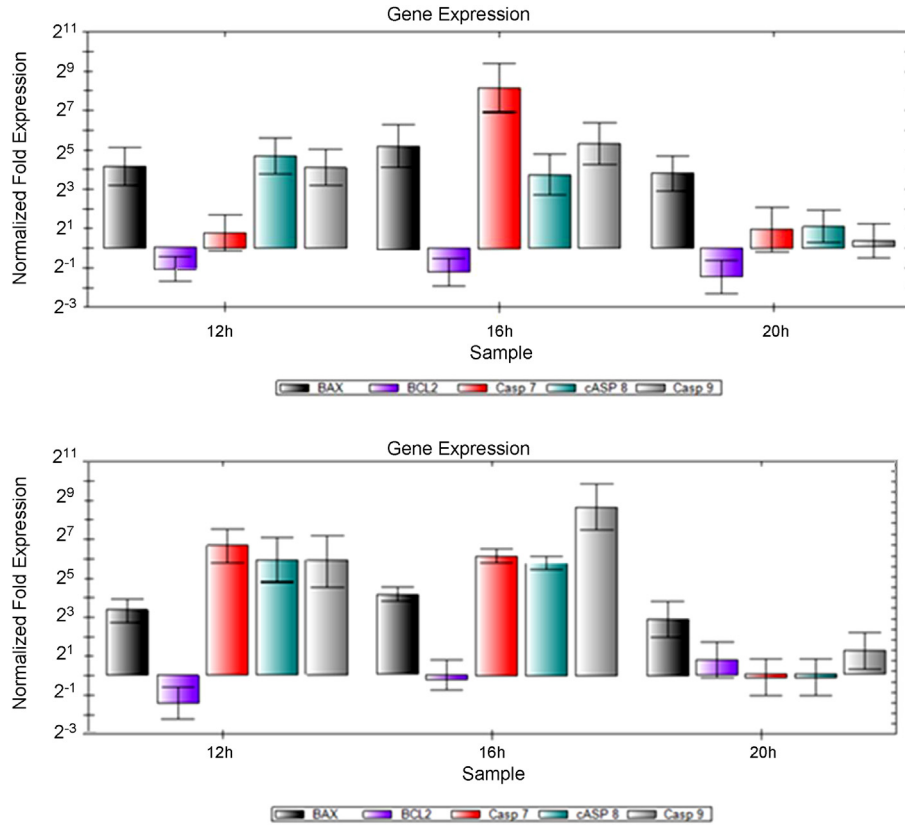
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Fig. 11. Real-time quantitative PCR analysis illustrates the gene expression in (A) HeLa cells and (B) HepG2 cells after 12, 16, and 20 h of treatment with the IC50 of RC extract. The gene expression was normalized with the reference gene (β-actin). The relative quantification of the target genes, Bax, Bcl-2, Caspase 7 (Casp 7), Caspase 8 (Casp 8), and Caspase 9 (Casp 9), by the delta–delta–Ct method was done using the software BIO-RAD CFX Manager (V 1.1.308).

Fig. 12. Real-time quantitative PCR analysis illustrates the gene expression in (A) HeLa cells and (B) HepG2 cells after 12, 16, and 20 h of treatment with the IC50 of RC extract. The gene expression was normalized with the reference gene (β-actin). The relative quantification of the target genes, Cyclin D (CYCD), Cyclin E (CYCE), Cyclin A (CYCA), p27, p21, and p53, by the delta–delta–Ct method was done using the software BIO-RAD CFX Manager (V 1.1.308).

The results of real time PCR granted valuable details on the underlying mechanisms of the potential of RC extract to induce cell cycle arrest. For HeLa cells, it was found that both cyclin D and E were not significantly affected by exposure to the RC extract after 12, 16, and 20 h ($P > 0.05$) except for cyclin E which was slightly downregulated after 16 h ($P < 0.05$). Surprisingly, cyclin A was upregulated significantly after 12 and 16 h ($P < 0.05$) but not after 20 h ($P > 0.05$); however, cyclin A upregulation was not prominent, up to 8–16 folds of expression only. On the other hand, p27 was significantly upregulated after 12 h, more than 8 folds, ($P < 0.05$) but not after 16 or 20 h ($P > 0.05$). This indicated that RC extract induced synthesis of p27 at early phases of exposure and its effect lasts for a short period of time. On the contrary, the upregulation of p21 and p53 was not significant ($P > 0.05$) after 12 and 20 h whereas p21 and p53 upregulation was significant after 16 h, up to 8 and 32 folds, respectively ($P < 0.05$). This finding provides evidence that the activity of p53 and p21 starts later than p27 for inhibiting cyclin-cdk complexes at G0/G1 phase of the cell cycle.

For the effect of RC extract on HepG2 cells, it was found that cyclin D and A expression was not affected by RC extract after 12, 16, and 20 h ($P > 0.05$) while cyclin E was found significantly, but slightly, upregulated after 12 and 20 h ($P < 0.05$) but not after 16 h ($P > 0.05$). These findings show the presence of more than one component in RC extract affecting, in different ways, the cell cycle of HepG2 cells. It appears that there is a component stimulates cyclin E rather than other cyclins, but this stimulation was counteracted by the stimulation of cdk inhibitors, p27, p21, and p53 especially after 16 h. The level of mRNA expression of p27 and p21 was largely upregulated after 12 and 16 h and of p53 after 16 h only ($P < 0.05$).

Discussion/conclusion

The anticancer activity of RC extract was investigated on two of the most important types of cancer in Asian countries. Depending on the recent studies, hepatocellular carcinoma is considered to be one of the most common cancers worldwide with an extremely poor prognosis [20,21]. On the other hand, cervical cancer continues to be the commonest cause of death among women in developing countries and is the second most frequent cancer among females worldwide [22,23]. This study focused on these two human cancers in an attempt to explore whether their anticancer therapy could be augmented with an under-investigated natural product, namely the red cabbage.

The findings in the current study revealed effective anticancer activities on HeLa and HepG2 cells by RC extract. Since the SI biological efficacy, or SI, ≥ 10 indicates specific cytotoxicity [24], the selectivity of RC extract on HeLa cells was significantly evident (SI = 10.88) while the selectivity of RC extract on HepG2 cells (SI = 8.93) was less remarkable. The noticeable anticancer activity of RC methanol extract on HeLa and HepG2 cells in this study is in agreement with the findings of a Korean study [8] which found that RC methanol fraction had an anticancer activity against HeLa and HepG2 cells. In addition, the chemopreventive effects of cruciferous vegetables, particularly RC, against different types of cancer have been demonstrated

by previous studies [25,26]. The chemopreventive effect of RC may be due to the high flavonoid contents, precisely, anthocyanins [27]. The critical anticancer activity of RC extract in this study is most probably due to the anticancer activity of flavonoids. It has been found that flavonoids act as antioxidants, essentially disabling the carcinogenic potential of specific compounds by having cytoprotective effects against ONOO- and HOCl mediated cytotoxicity [28,29]. Additionally, the ability of flavonoids to scavenge free-radicals and block lipid peroxidation raises the possibility that they may act as protective factors against carcinogenesis [30,31].

As mentioned earlier, anthocyanins are the major flavonoid components in RC. Thus, the results of the current study may be attributed in part to the anticancer activity of anthocyanins, as a subclass of the flavonoids. The anthocyanins anticancer activity was recorded by many studies [32,33]. It has been shown that anthocyanins demonstrate varied anticancer activities such as strong free radical scavenging and antioxidant activities [34], having the ability to inhibit the hormonal actions and metabolic pathways associated with cancer development, and inducing phase I or II detoxification enzymes [9,35,36]. Interestingly, different mechanisms, which may be used by anthocyanins as a subclass of flavonoids in RC, may be responsible for the promising anticancer activity of RC and may effectively reduce the chances for the development of anticancer resistance against this extract.

The current study also attempted to investigate the mechanism(s) of RC anticancer activities. Since the increased expression of anticancer cytokines, namely IFNβ and TNFα, explains the anticancer activity of any extract [37], we measured the expression levels of IFNβ and TNFα in the culture supernatants of HeLa and HepG2 cells treated with RC extract. The current study revealed an increased level of TNFα, but not IFNβ, in the culture supernatants of HeLa and HepG2 cells treated with RC extract, in a dose dependent manner, when comapred to untreated cells. The growth inhibition manifested in HeLa and HepG2 cells trated with RC extract may be, partly, due to the antitumor effect of TNFα. These findings could be supported by the fact that TNFα is a pleiotropic cytokine originally identified due to its antitumor activity [38]. Moreover, TNFα was one of the first cytokines used in treating cancer. It can induce cancer cell death via TNF-driven programmed cell death or apoptosis [39]. It was shown that anthocyanins can induce apoptosis by the extrinsic pathway [36]; alike, TNFα can induce apoptosis by the extrinsic pathway. So, the effect of anthocyanins in RC extract may induce the production of TNFα from human cancer cells which in turn induce cell death in these cancer cells.

Flow cytometry analysis was used to investigate the presence or absence of apoptotic cells in the treated HeLa and HepG2 cells. The results revealed that the RC extract induced apoptosis and cell cycle arrest in G0/G1 phase in the treated cells in a dose and time dependent manner. The cell cycle arrest and the induction of apoptosis in cancer cells have become the major indicators of anticancer effects [40]. The antitumor effects are attributed to altered biochemical mechanisms, including mainly inhibitions of proliferation, induction of cell cycle arrest at various cell cycle checkpoints, and enhanced apoptosis [41]. More to

the point, the current study is in agreement with previous studies which found that flavonoids, phenolic acids, and other antioxidants have the ability to inhibit the cancer cell cycle progression, cell proliferation and tumor growth by inducing cell-cycle arrest and apoptosis [42,43].

Subsequently, real-time quantitative PCR was used to confirm the RC-driven apopotisis detected by flow cytometry and to detect the mechanism(s) used by RC extract to induce apoptosis. Therefore, the expression of several apoptosis-related genes was investigated quantitively. Apoptosis is a broad network of signals that act through two major apoptotic pathways: the extrinsic death receptor pathway (via caspase 8), which triggers the activation of a caspase cascade, and the intrinsic mitochondrial pathway (via caspase 9), which shifts the balance in the Bcl-2 family toward the pro-apoptotic members and, consequently, toward caspase-mediated apoptosis [44]. Both caspase 8 and 9 are considered initiator caspases which in turn can activate the effector caspases, caspase 3 and caspase 7, leading to the dramatic morphologic changes of apoptosis [45]. In this study, RC extract upregulated the gene expression of caspase 8 in treated HeLa and HepG2 cells. This upregulation was manifested significantly after 12 h of treatment suggesting that this time was the beginning of the apoptosis induction via caspase 8 pathway. It has been shown that the activation of caspase 8 requires the involvement of apoptotic ligands such as TNFα and Fas ligand [44]. As mentioned earlier, RC extract stimulated TNFα production in the culture supernatants of the treated HeLa and HepG2 cells. Thus, we propose that TNFα which was produced by the treated cells might have an autocrine effect on the same producing cells. It might activate the extrinsic apoptosis pathway via caspase 8 by binding to its receptors on the surface of cancer cells.

The current results revealed that RC extract upregulated the expression of Bax gene after 12 h of treatment and this upregulation continued till 20 h. It has been stated that cytochrome *c* release from mitochondria could be controlled by Bax and the translocation of Bax can alter the outer mitochondrial membrane permeability, leading to cytochrome *c* release from the mitochondria to the cytosol then activation of the intrinsic apoptosis pathway [46,47]. Accordingly, RC extract promoted the intrinsic apoptosis pathway partly by its ability to upregulate Bax gene. Moreover, RC extract downregulated the expression of Bcl-2 gene after 12 h of treatment of both HeLa and HepG2 cells. These results revealed the predominance of Bax gene over Bcl-2 gene in all of the treated cells. It was proven that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals [19]. It was shown that changes in the Bax/Bcl-2 ratio could be caused by downregulation of Bcl-2 and upregulation of Bax [48]. For that reason, Bcl-2 proteins have emerged as an attractive target for the development of novel anticancer drugs [49] and this could be one of the targets hit by RC active compounds to induce apoptosis.

The upregulation of caspase 9 in the treated HeLa and HepG2 cells was evident after 12 h of treatment with RC extract. It is well known that caspase 9 can be activated by caspase 8 or can be activated independently on binding of cytochrome *c* release from the mitochondria [46]. Therefore, we assume that the intrinsic

apoptosis pathway induced by RC extract in the treated cancer cells might be provoked via direct upregulation of caspase 9 gene, via the activation of caspase 8 by the extrinsic pathway, or via the upregulation of Bax gene.

The upregulation of caspase 7 gene in the treated HeLa cells with RC extract started at 16 h while it started at 12 h in treated HepG2 cells. Moreover, caspase 7-dependent pathway without caspase-3 activation now is considered as caspase-independent apoptosis pathway. And caspase 7 can activate caspase 12 which results in the induction of apoptosis during endoplasmic reticulum stress [50]. Therefore, the results of this study disclosed a fact that RC extract might induce apoptosis by different pathways. The possible reason behind these findings might be dealing with a crude extract composed of different components that might trigger different pathways of apoptosis. Accordingly, using RC extract shows advantage of triggering two or three pathways of apoptosis simultaneously leading to vigorous induction of apoptosis. The induction of multi-pathway apoptosis usually leads to effective anticancer activity able to overcome any resistance that might be issued? By cancer cells against apoptotic signals. In addition, the current results highlight the ability to isolate more than one effective anticancer component from RC extract.

The regulatory proteins of the cell cycle studied in the current study were chosen carefully to give a clear image on the underlying mechanisms and pathways for the G0/G1 arrest observed in HeLa and HpeG2 cells treated by RC extract. The studied markers were cdk-activating proteins, namely cyclins and cdk-inhibitors, or CKI, namely tumor suppressor proteins such as p27, p21, and p53. Cdk activation requires cyclin binding and phosphorylation of conserved threonine residue by cdk-activating kinase. On the other hand, the activated cdk–cyclin complexes can be changed to an inactive state by phosphorylation of a conserved threonine–tyrosine pair or binding to cdk inhibitory subunits, or tumor suppressor proteins such as p53, p21, p27, p15, and p16 [51]. The mRNA expression of cyclins D, E, and A was assessed by real time PCR while other cyclins were not covered. Progression from G1 to S phase in mammalian cells is regulated by the accumulation of cyclins D, E and A, which bind to and activate different cdk catalytic subunits. The activation of cdk4–cyclin D and/or cdk6–cyclin D complexes is necessary for the transition from early to mid G1 phase. Transition through mid G1 to S phase is regulated by activation of the cdk2–cyclin E complex. And progression through late G1 to S phase also requires the presence of cdk2–cyclin A complex [52]. On the other hand, it was stated that cdk regulation involves a diverse family of proteins, termed the CKIs (cdk inhibitors), that bind and inactivate cdk–cyclin complexes. Of these proteins involved in G1 to S phases progression are p21, p53, and p27 [53].

The effect of RC extract on both HeLa and HepG2 cells was comparable but with minor differences. RC extract did not affect the expression of cyclin D in both HeLa and HepG2 cells indicating that cyclin D level is not the governing factor for the observed G0/G1 cell cycle arrest in flow cytometry. However, surprisingly, cyclin E in HepG2 cells and cyclin A in HeLa cells were both upregulated at some times in response to RC extract.

Actually this is a tumor progressing feature; however, the effect of upregulated cyclins A and E was counteracted strongly by extensive and synchronous upregulation of three cdk-inhibitors which are p27, p21, and p53 especially after 16 h of exposure to RC extract. Hence, any upregulation of the cyclins of G1 phase is counteracted and reversed by the activity of cdk inhibitor proteins. The current feature indicates the presence of more than one component of contradicting effects in RC extract but the cell cycle inhibitory effect is more prominent via the induction of p21, p53, and p27 proteins. Accordingly, it is highly suspect that RC extract harbors two classes of affecter agents, one cell growth stimulating and other agents with cell cycle inhibitory activities. Moreover, the differences found in the cyclins' activity in response to RC extract between HeLa and HepG2 cells provide evidence for the cell type- specific interaction of RC extract. However, the overall or net result of the interplaying factors in RC extract on both HeLa and HepG2 cells was clearly toward arresting the cell cycle at G1 phase via inducing tumor suppressor proteins.

Overall, the cell cycle inhibitory effects of RC extract showed a weak or absent influence on the expression level of cdk-activating cyclins. Instead, RC extract showed remarkable induction and upsurge of cdk-inhibitor proteins. Therefore, it is concluded that these cdk-inhibitor proteins are the main mechanism pursued by RC extract to exert G1 cell cycle arrest and ultimately final fate of cells, apoptosis. It is noteworthy to mention that the inhibitory effect on cell progression by p21 is largely exerted during G1 phase of the cell cycle, with preferential binding to cdk4- and cdk2-containing complexes [54]. In addition, the induction and regulation of expression of p21 is mainly dependent on the active form of p53, a transcriptional regulator that mediates cell cycle arrest following DNA damage [55]. Therefore, both p53 and p21 were expressed simultaneously acting together on slowing or arresting G1 phase. G1 phase, the first part of the cell cycle is a restriction point, since all other cell cycle compartments cannot be modified by interference with diverse proteins or enzymes. Therefore, stoppage of cells through transition from G1 to S is most commonly found among many cell cycle modifying chemicals. Another important cdk-inhibitor, p27, is mainly regulated by proteasomal degradation and its downregulation is often correlated with poor prognosis in several types of human cancers. The p27 protein is an unconventional tumor suppressor because mutation of its gene is extremely rare in tumors [56]; therefore, its upregulation by certain drugs or plants' extracts appear as fascinating goal for the anti-tumor therapy. P27 interacts with most actively engaged cdks of G1 phase, namely cdk 2, 4, and 6; moreover, it interacts with cyclins D, E, and A. Therefore, in the case of RC extract, the three cdk proteins, p53, p21, and p27, acted together powerfully in slowing cell cycle at G1 phase and inducing apoptosis.

The current study revealed an interesting finding; RC extract induced synthesis of TNFα from cancerous cells. TNFα is believed to be the central link between RC extract and its remarkable ability to induce cdk-inhibitors and/or downregulate cyclins. A previous report showed that TNFα induces p21 (waf1) protein in tumor cells and its binding to CDK2/4 and 6 proteins leading to inhibition of the activity of these complexes.

This inhibition leads the cells to G1 arrest; moreover, p21(waf1) was found to have an essential role in TNF-induced arrest and that the deregulation of cyclin D1 may be one of the mechanisms to escape physiological signals to restrict tumor growth [57]. In addition, Jaruga and Rayford revealed that p27Kip1 induces caspase -dependent and -independent stages of cell death that may involve TNF-signaling through TNF receptors 1 [58]. Besides, it was shown that TNFα plays a central role in downregulating cyclins A, E and D [59].

The anticancer activities of RC extract were investigated thoroughly in the current study. RC extract showed significant anticancer activities against human cervical cancer cells and human hepatocarcinoma cells. RC extract revealed selective anticancer action against HeLa cells while less selectivity of RC extract was shown with HepG2 cells. RC extract was found to be a potent inducer for apoptosis in the treated human cancer cells via caspase-dependent, both extrinsic and intrinsic, and caspase-independent pathways. RC extract also induced apoptosis and cell cycle arrest in human cancer cells via cell-type specific interactions. Cdk-inhibitor proteins (p21, p27, and p53) were the main mechanisms used by RC extract to exert G1 cell cycle arrest and ultimately the final fate of cells, apoptosis. In addition, RC extract induced synthesis of TNFα from cancerous cells and TNFα was most probably linked to the apoptotic and cell cycle slowing/arresting potential of this extract. Taken together, the findings of the current study indicated that RC extract is most likely a promising anticancer agent by affecting either known or new targets in the anticancer chemotherapy. And, because of the wide range of anticancer activity of RC extract, its anticancer activity is thought to be driven by more than one component giving the chance for RC extract to exert strong, multi-mechanism, and synergistic anticancer effect.

Conflict of interest

Authors declare that there are no competing interests of any kind associated with the current study.

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