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In vitro antiproliferative and apoptosis-inducing activities of crude ethyle alcohole extract of *Quercus brantii* L. acorn and subsequent fractions

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[ABSTRACT] Cancer cell resistance to widely used chemotherapeutic agents is gradually developed. Natural products, mainly isolated from medicinal plants, have been considered as valuable sources for herbal anticancer drugs. The present study aimed to evaluate *in vitro* antiproliferative and apoptosis-inducing activities of crude ethyle alcohole extract and four fractions of *Q. brantii* acorn. Crude ethyle alcohole extract of *Q. brantii* acorn was prepared and subjected to fractionation with different polarity. Subsequently, the extract and the fractions wereevaluated for their *in vitro* antiproliferative activity in two cancerous (Hela and AGS) and one normal (HDFs) cell lines using MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumbromide] assay. To determine whether the cytotoxicity of these compounds involved the induction of apoptosis, Hela cells were treated with IC_{50} concentrations of test compounds, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. *In vitro* cytotoxicity assay showed that the cell viability was significantly reduced in a dose-dependent manner following treatment with crude ethyle alcohole extract and Cholophorm and *n*-Butanol fractions. Based on the probit regression model, antiproliferative activities of crude ethyle alcohole extract, Cholophorm fraction, and *n*-Butanol fraction on Hela and AGS cells and HDFs cells were significantly different ($P < 0.001$). The results of flow cytometric analysis showed that crude ethyle alcohole extract and two fractions of *Q. brantii* acorn induced early apoptotic cell death. These findings suggest that crude ethyle alcohole extract and Cholophorm and *n*-Butanol fractions of *Q. brantii* acorn suppress the proliferation of cancer cells through induction of early apoptosis.

[KEY WORDS] *Quercus brantii* Lindl; Cancer; Proliferation; Apoptosis

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Introduction

Chemotherapy is one of the most widely used approaches to the treatment of many cancers, but long-term use of these therapeutic agents may lead to drug resistance [1-3]. Therefore, developing medicinal plant products as new anticancer agents seems to be an urgent need in the field [4]. The World Health Organization (WHO) reports that traditional medications are used by about 80% of the population in developing countries for primary health care purposes [5]. Also, standardized herbal preparations are widely used as traditional medicine in some developed countries [5]. A broad spectrum of secondary metabolites present in herbs is effective in the treatment and/or prevention of various chronic diseases, including cancers [6-8]. These natural products have been considered as novel anticancer agents with several mechanisms, since 1981 [6-10].

A number of the mechanisms which are involved in cell death induced by plant products include programmed cell death (PCD)-type I (apoptosis), PCD-type II autophagic cell death, and necrosis [11]. Apoptosis is a mode of self-cannibalism of individual cells without any inflammation of the neighboring cells [12]. Some of the mechanisms of apoptosis include DNA fragmentation, nuclear condensation, and externalization of phosphatidylserine, membrane blebbing, and loss of mitochondrial membrane potential [13]. Understanding of these mechanisms has been interest of drug researches for many years.

A large number of medicinal plants are naturally grown

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in different regions of our country, Iran. Genus *Quercus* (Fagaceae) contains 500 species; *Q. brantii* L. is predominant in central and northern regions of this country [14]. Acorn, the fruit of Oak (Quercus tree), has been reported to contain Vitamins, nutrients, and carbohydrates [14]. It also contains considerable amounts of phenolic, tannin, catechin, epicathechin, and gallocatechin components [14-17].

There are some reports indicating different biological activities of some species of Genus Oak, including Iranian Oak with use of the acorn and bark in treatment of some diseases such as chronic dermatological diseases, eczema, and varis [15, 18-21]. Different species of *Quercus* have been reported to have antibacterial [15, 22-23], antiviral [24-25], and antioxidant activities [26-27] as well as gastroprotective effects [28].

To the best of our knowledge, to date, there has been no report on the antiproliferative activity of different fractions of *Q. brantii* acorn. The present research was aimed to prepare crude ethyle alcohole extract and four corresponding fractions of *Q. brantii* acorn and to evaluate the antiproliferative activity and apoptosis induction of these plant materials *in vitro*.

Materials and Methods

Plant collection

The acorns of *Q. brantii* were gathered from southwest region of Iran. The genus and species of the plant were identified and confirmed by Prof. M. Rafieian, in Herbarium of Medical Plants Research Center of Shahrekord University of Medical Sciences, Iran (Herbarium number: 325).

Extraction and fractionation of plant materials

The acorns were harvested and powdered three times using the maceration method. The plant materials were dissolved in 70% ethyle alcohol (Ghadir Industries, Iran) and kept at room temperature (RT) for 96 h. After that, the mixture was filtered and concentrated under nearly vacuum pressure and at 40 ºC using rotary evaporator. Four fractions of the crude extract were obtained with different polarity through in-solution isolation and using the difference in various secondary metabolites' polarity. To isolate the hexane fraction, the extract was concentrated, suspended in 70% ethyl alcohole, and mixed with equal volume of normal hexane (Merck, Germany) with shaking vigorously. The remaining solution from which the ethyle alcohole was removed was mixed with distilled water and with Cholorphorm (Merck, Germany) in equal volume, shaken, and hydrated using sodium sulphate to afford the Cholophorm fraction. To prepare *n*-Butanol fraction, equal volume of *n*-Butanol (Merck, Germany) was added to the remaining aqueous phase of the material, shaken, and concentrated at 40 ºC and in vacuum condition. The remaining aqueous phase was concentrated, under the similar condition as mentioned above to afford aqueous fraction. The crude extract and four fractions were kept in sterile bottles under refrigerated conditions until further use. The extract and fractions were dissolved at 37 °C in dimethylsulphoxide (DMSO 99.0%, Samchun Pure Chemical Co., LTD, Korea) to give a stock solution of 25 mg·mL^{-1}, dissolved in culture medium, and stored (−20 °C) until use. The low percentage of DMSO present in the wells (maximal 0.2%) in cytotoxicity assay described below was found not to affect cell growth under the experimental conditions [29].

Cells and cell culture

Hela (cervix adenocarcinoma) and AGS (human gastric carcinoma) cell lines were purchased from Pasteure Institute of Iran. Human dermal fibroblasts (HDFs) cell line was kindly provided by the Cellular and Molecular Research Center of Shahrekord University of Medical Science, Iran. All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen-Gibco, Carlsbad, California,) supplemented with 10% of fetal bovine serum (FBS; Gibco), 100 μg·mL^{-1} of streptomycin (Sigma-Aldrich Chemicals, St. Louis, MO, USA), 100 UI·mL^{-1} of penicillin (Sigma) and 0.25 μg·mL^{-1} amphotericin B (Gibco), at 37 °C in a humidified air atmosphere containing 5% (*V*/*V*) CO_2.

MTT assay

The cells were seeded onto 96-well plates (SPL Life Sceinces, Korea) at a density of 6 000 cells per well in a final volume of 100 μL per well. After incubation at 37 °C with 5% CO_2 for 24 h, overlay medium was aspirated to allow the cells attach to the bottom of each well. Subsequently, the cells were incubated with 100 μL/well of various concentrations of crude ethyle alcohole extract and the fractions in triplicate, at 37 °C with 5% CO_2 for additonal 72 h. The number of living cells was determined by the ability to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumbromide] by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product. The procedure was as described previously [30]. Briefly, the supernatant was removed from the wells and 50 μL of MTT solution (1 mg·mL^{-1} in PBS) was added to each well. The plates were incubated for 4 h at 37 °C, and 100 μL of DMSOwas added to each well to dissolve the MTT crystals. The plates were placed on a shaker (IKA company, Staufen, Germany) Germany) for 15 min and the absorbance at 492 nm of each well was read on an enzyme-linked immunosorbent assay (ELISA) reader (Stat Fax 2100, Awareness Technology, USA). Each experiment was carried out in triplicate and the percentage survival of the treated cancer and normal cultured cells was calculated according to the formula as follows:

Percentage of survival (%) = (Absorbance of treated cells/ Absorbance of control) × 100

The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis and related models with probit regression model procedure, using the SPSS software (version 16.0).

Flow cytometric analysis of cell apoptosis

To determine whether the cytotoxicity of the crude

extract and fractions involved the induction of apoptosis, Hela cells were treated with each of these preparations, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC). Subsequently, the apoptotic cells were detected by using an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer's instruction, and analyzed by flow cytometry. Briefly, Hela cells were seeded onto 6-well plates with a density of 10^5 cells/well with final volume of 2 mL per well. After incubation at 37 °C with 5% CO_2 for 24 h, the cell culture medium of cells was aspirated. The cells were incubated with 2 mL/well of the IC_{50} concentrations of crude ethyle alcohole extract and four fractions (crud extract = 343.9, *n*-Butanol fraction = 105.3, Chloroform fraction = 134.7, *n*-Hexane fraction = 1 463 and remaining aqueous fraction = 1 530.7 μg·mL^{-1}) and incubated at 37 °C with 5% CO_2 for additional 48 h. The cells were then collected, re-suspended in 100 μL of 1 × binding buffer containing 2 μL of FITC-conjugated annexin-V (BD Biosciences) and 1 μL (100 μg·mL^{-1}) of PI (BD Biosciences) and incubated for another 20 min in the dark. The results were then analyzed using flow cytometry (Partec GmbH, Munster, Germany).

Results

Antiproliferative activity

To evaluate the antiproliferative activity of crude ethyle alcohole extract and four fractions of *Q. brantii* acorn in Hela and AGS (cancerous) and HDFs (normal) cell lines, the cells were treated with different concentrations of these preparatins for 72 h and the cell viability was determined using MTT assay. The results showed that cell viability was significantly reduced in a dose-dependent manner following treatment with the crude ethyle alcohole extract (Fig. 1). Based on probit regression model, antiproliferative activity of the crude ethyle alcohole extract on the three cell lines studied was significantly different ($P < 0.001$). Also, IC_{50} values of this extract for Hela (343.9 μg·mL^{-1}) and AGS (255.9 μg·mL^{-1}) cells were lower than that of HDFs (411.1 μg·mL^{-1}) cell line (Table 1).

Following resolving of the crude ethyle alcohole extract in 70% (*V*/*V*) ethyle alcohole, sequential solvent partitioning with four different polarity solvents was used to further fractionate the *Q. brantii* acorn extracts. The cell viability of the three cell lines treated with *n*-Butanol and Chloroform fractions was significantly reduced in a dose-dependent manner. However, no antiproliferative activity was shown following treatment of these cell lines with *n*-Hexane and aqueous fractions (Fig. 2).

Based on the probit regression model, the antiproloferative activities of *n*-Butanol and Chloroform fractions in the three cell lines studied were significantly different ($P < 0.01$).

However, *n*-Hexane fraction and remaining aqueous fraction showed no significantly different antiproliferative activity on these cell lines (Fig. 2). The IC_{50} values of *n*-Butanol and Chloroform fractions for Hela and AGS cells were lower than that of HDFs cells (Table 1).

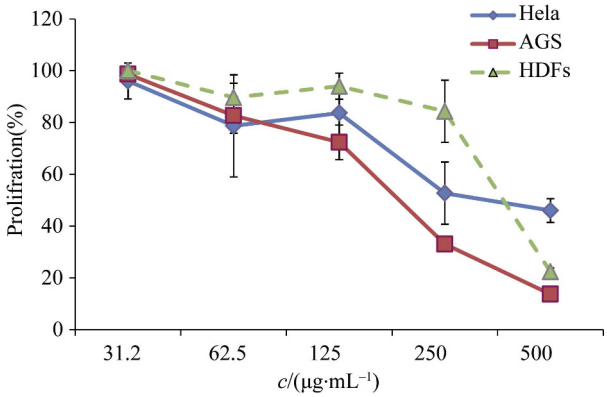

Fig. 1 Antiproliferative activity of crude ethyle alcohole extract of *Q. brantii* acorn on the three cell lines. Hela, AGS (cancerous) and HDFs (normal) cells, were treated with five different concentrations of the preparations for 72 h and the cell viability was determined using MTT assay. The data are shown as mean ± SD, *n* = 3. Using probit regression model, data (curves) show that antiproliferative activities of the crude extract between non cancerous (HDFs) and cancerous (Hela and AGS) cell lines were significantly different ($P < 0.001$)

Table 1 The IC_{50} values of crude ethyle alcohole extract and four subsquent fractions of *Q. brantii* acorn in three cell lines

Cell line / Extract/fraction	Hela (μg·mL^{-1})	AGS (μg·mL^{-1})	HDFs (μg·mL^{-1})
Crude extract	343.9 ± 29	255.9 ± 9	411.1 ± 21
n-Hexane fraction	1 530.7 ± 40	1 799.4 ± 56	983.5 ± 123
Chloroform fraction	134.7 ± 30	125.2 ± 7.5	287.8 ± 38
n-Butanol fraction	105.3 ± 16	144.6 ± 5.4	205 ± 6
Remaining aqueous fraction	1 463 ± 60	1 421.9 ± 40	1 619.2 ± 147

The IC_{50} values of ethyle alcohole extract, *n*-Butanol and Chloroform fractions in the three cell lines studied were significantly different ($P < 0.01$)

Apoptosis

To determine whether the cytotoxicity of these compounds involved the induction of apoptosis, Hela cells were treated with the corresponding IC_{50} concentrations of the extract and fractions, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. During the process of apoptosis, cell membrane asymmetry is lost, and phosphatidylserine becomes accessible on the cell surface, where it can bind to FITC-tagged anticoagulant Annexin V. PI stains late-apoptotic or necrotic cells whose membrane integrity has been compromised. The orthogonality of these dyes' binding and fluorescence allow for the simultaneous observation of early apoptosis and late apoptosis/necrosis [31].

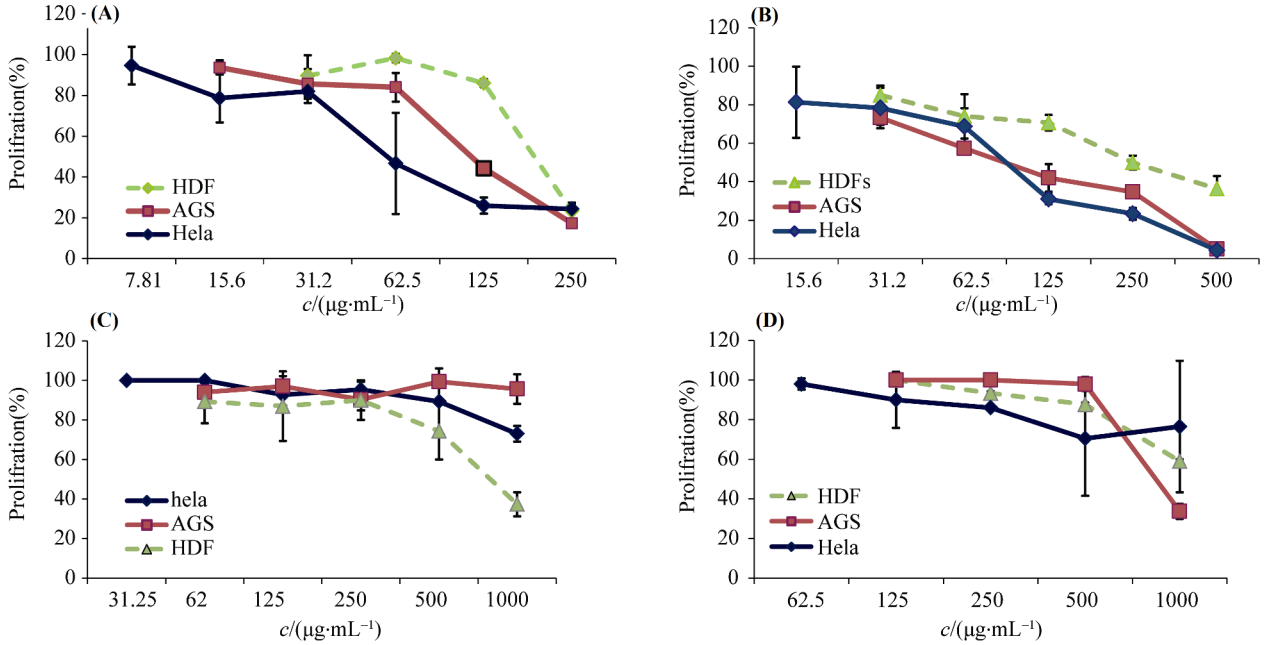

Fig. 2 Antiproliferative activities of four correspond fractions of *Q. brantii* acorn on three cell lines. Hela, AGS (cancerous) and HDFs (normal) cell lines, were treated with differents concentrations of the *n*-Butanol (A), Chloroform (b), *n*-Hexane (C), and Remaining aqueous (D) fractions for 72 h and cell viability was determined using MTT assay. The data indicate mean ± SD, *n* = 3. Using probit regression model, data (curves) show that antiproliferative activities of the Chloroform (panel A) and *n*-Butanol (panel B) fractions between non cancerous (HDFs) and cancerous (Hela and AGS) cell lines were significantly different ($P < 0.05$)

Flow cytometric analysis of apoptosis showed that the ethyle alcohole crude extract and four fractions of *Q. brantii* acorn induced cell death was though early apoptosis. The percentages of early apoptosis cells in the cells treated with the ethyle alcohole crude extract, *n*-Hexan, *n*-Butanol, Chloroform and aqueous fractions treatment were 13.8%, 18.11%, 36.5%, 31.1% and 6.5%, respectively. The cells treated with *n*-Butanol and Chloroform fractions, the most cytotoxic fractions in the set, exhibited more Annexin V-FITC staining than control cells (Fig. 3). The percentage of necrotic cells was low in each sample.

Discussion

Cancer remains one of the leading causes of death worldwide. Various cancer therapies have currently been tried, including the use of natural products from plants [32]. Therefore, the need to discover effective, novel and scientifically reliable natural compounds is urgent. Natural products provide a fertile ground for seeking out treatments with fewer side effects and equal or better efficacy. The beneficial effects of plant extracts are derived from their constituent phytochemicals that include polyphenols, carotenoids, alkaloids, and nitrogen and sulfur containing compounds [33]. Phytochemicals extracted from plants are excellent chemotherapeutic and chemopreventive agents, which are well tolerated, nontoxic, easily available, and inexpensive [34]. The present study investigated the antiproliferative activities of ethyle alcohole crude extract and four subsequent fractions of *Q. brantii* acorn.

Our results with *in vitro* cytotoxicity assay showed that cell viability of two human cancer cell lines, Hela and AGS, was significantly reduced in a dose-dependent manner, following treatment with crude ethyle alcohole extract of *Q. brantii* acorn. Based on probit regression model, antiproliferative activities of the crude ethyle alcohole extract on the three cell lines studied were significantly different ($P < 0.001$). Also, the IC_{50} values of this extract for Hela (343.9 μg·mL^{-1}) and AGS (255.9 μg·mL^{-1}) cell lines waeres lower than that of HDFs (411.1 μg·mL^{-1}) cells (Table 1). Some plant antioxidants have been suggested to contribute to their anti-carcinogenic effects and their flavanols have been reported to inhibit cancer cell proliferation *in vitro* [35]. Crude ethyle alcohole extract of *Quercus infectoria* galls has been shown to exhibit antiproliferative activity against cervical and ovarian cancer cells [36]. Chemical composition screening has shown that flavonoids, alkaloids, glycosides, tannins, phenolic compounds, resins, saponins, terpenes, and steroids are the main components of *Quercus* species acorn with no alkaloids and saponins compounds being detected [17, 37-38]. Therefore, in consistent with the above mentioned reports, our results may indicated that Tannin may be responsible for antiproliferative activity of crude ethyle alcohole extract of *Q. brantii* acorn.

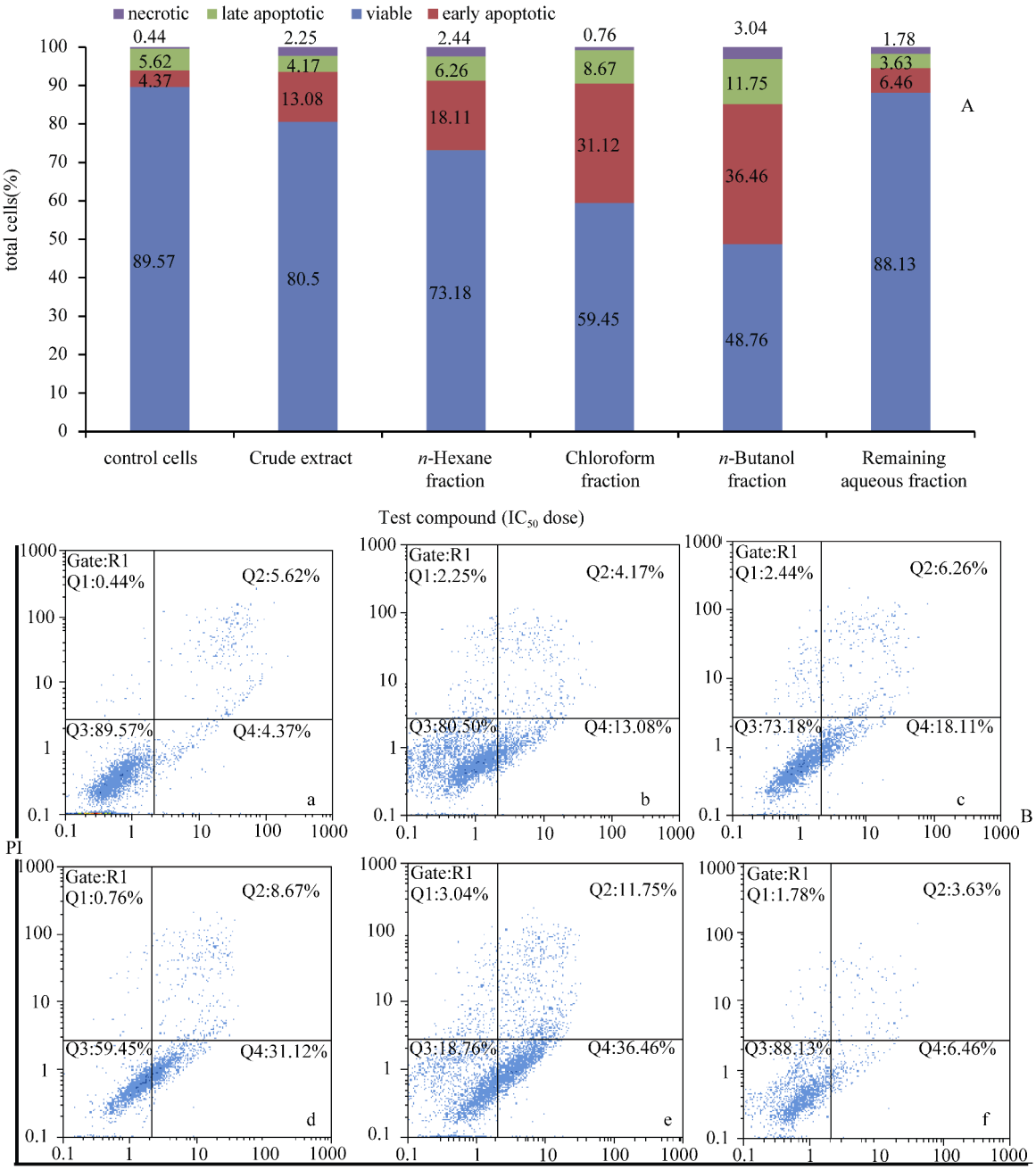

Fig. 3 Flow cytometric analysis of apoptosis in Hela cells. Hela cells were treated with the corresponding IC_{50} concentrations of crude ethyle alcohole extract and four fractions of *Q. brantii* acorn, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry; A: the apoptosis ratio of crude ethyle alcohole extract and four correspond fractions; B: Flow cytometry analysis of apoptosis induced activity of , a: control; b, c, d, e, and f: cells treated with Ethyle alcohole crude extract, *n*-Hexane fraction, Chloroform fraction, *n*-Butanol fraction and aqueous fractions, respectively.

In the present study, the crude ethyle alcohole extract of *Q. brantii* acorn was further fractionated using various solvents which possess different polarity index values: *n*-Hexane, Chloroform, and *n*-Butanol, We found that the *n*-Butanol and Chloroform fractions inhibited *in vitro* growth of Hela and AGS cells in a dose dependent manner, with IC_{50} values being less than that of the crude ethyle alcohole extract. Hasmah *et al.* have shown that methanolic fractionation of crude extract of *Hydnophytum formicarium* has promising IC_{50} values for HeLa and Caov-3 cell lines [39].

To determine whether the cytotoxicity of crude ethyle alcohole extract and four subsequent fractions of *Q. brantii* acorn involved the induction of apoptosis, Hela cells were treated with the corresponding IC_{50} concentrations of the

preparations, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. The results showed that crude ethyle alcohole extract and four subsequent fractions of *Q. brantii* acorn induced cell death through early apoptosis. The Hela and AGS lines treated with *n*-Butanol and Chloroform fractions, exhibited more Annexin V-FITC staining than that of the control cell line. The percentages of early apoptosis cells on the ethyle alcohole crude extract, *n*-Hexan, *n*-Butanol, Chloroform and remaining aqueous fractions treatment were 13.8%, 18.11%, 36.5%, 31.1% and 6.5%, respectively. The percentage of necrotic cells was low in each sample. The results from this experiment indicated that treatment with *n*-Butanol and Chloroform fractions led to an increased number of apoptotic cells compared to the untreated cells, without significantly increasing the percentage of necrotic cells. These results, coupled with those of the cytotoxicity assays, suggest that *n*-Butanol and Chloroform fractions may exert their antiproliferative activities through the induction of apoptosis in the cancer cells studied. Induction of apoptosis is one the most important marker of cytotoxicity of antitumor agents. Our results and other published results [1] indicated that some natural compounds may induce apoptosisin cancer cells. Therefore, at least some of these extracts and fractions could potentially be used to develop antitumor agents in the future. However, further investigations are needed to elucidate the active compound(s) of the extracts and their mechanisms of anticancer actions.

Conclusion

Based on the findings of the present study, crude ethyle alcohole extract and Cholophorm and *n*-Butanol fractions of *Q. brantii* acorn suppress the proliferation of cancer cells through induction of early apoptosis.

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