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Research Article

Cytotoxicity and *In Vitro* Antioxidant Potential of *Quercus Brantii* Acorn Extract and the Corresponding Fractions

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ABSTRACT
The present study was mainly aimed to evaluate antioxidant activity and cytotoxicity of hydroalcoholic extract and three corresponding fractions of *Quercus brantii* acorn. A 70% ethyle alcohole extract of the plant were prepared and sequentially partitioned with n-hexane, chloroform, ethylacetate and n-butanol. The antioxidant potential of all these fractions was evaluated by the 2,2 diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity method. Cytotoxic activity was tested against two normal cell lines (African green monkey kidney [Vero] and human dermal fibroblasts [HDFs]) by MTT assay. The results revealed that the n-butanol fraction exhibited the lowest IC_{50} value (6.5±0.6 µg/ml) with the highest antioxidant activity as compared to the other fractions. The IC_{50} values of the chloroform fraction, the n-butanol fraction, the crude extract, and the n-hexane fraction were found to be significant ($p<0.05$) as compared with butylated hydroxytoluene (BHT). The results of cytotoxicity showed that the chloroform fraction exhibited the highest cytotoxicity toward Vero and HDFs cell lines at concentration of 60.6±23 and 287.8±38 µg/ml, respectively.
We conclude that at least, n-butanol fraction of this plant with high phytoconstituents and less toxicity could be a promising source of medicinally important natural compound. Our findings, therefore, suggest that overall the studied extract/fractions exhibit low cytotoxicity on normal cell lines.

Keywords: antioxidant, cytotoxicity, medicinal plants, *Quercus brantii*

INTRODUCTION

As an accessible source of innovative active biological compounds, medicinal plants are increasingly analyzed to determine the components on the structural level[1]. These new compounds are basic for the discovery of new herbal medicines. Some kinds of side effects such as carcinogenesis have been attributed to synthetic antioxidants and thus, there is increasing interest to replace them with naturally occurring antioxidants[2,3]. Antioxidant activity is one of the most important biological properties of these natural compounds. As natural antioxidants are capable of promoting food quality and stability and could act as nutraceuticals to end free radical chain reaction in biological systems, an increasing attention has been recently paid to directly evaluating the antioxidant properties of plant extracts as the source of natural antioxidants[4,5]. Many of naturally occurring antioxidants have been found to be free radical or active oxygen scavengers[6,7] and thus plants have been investigated for their biological activities and antioxidant properties[8-11]. Recent reports about antioxidant activity and polyphenol contents of herbals, used for fighting cancer, show that polyphenols, i.e. flavonoids, tannins, and phenolic acids, have antioxidant properties[12,13]. Belongs to the family of Fagaceae, Genus *Quercus* contains 500 species, some of which such as *Quercus brantii L.* or *Quercus* are predominant in central and northern regions of Iran[14]. The fruit of Oak tree is called acorn and is placed within a cup called Gland. Vitamines, nutrients, and Carbohydrates have been reported to comprise a large portion (48-85%) of acorn components. Acorn 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 include Iranian Oak with use of the acorn and bark in treatment of some diseases such as chronic dermatological diseases, eczema, and varis[2,8,15,18-20]. Additionally, some of the biologically active substances of acorn are utilized in preparing functional foods[21-23]. To the best of our knowledge, to date, there has been no reports on the phytochemical analysis and antioxidant potential of different fractions of *Quercus brantii* acorn. This study is mainly aimed to evaluate phytochemical screening, antioxidant activity and cytotoxicity in two normal cell lines (African green monkey kidney [Vero] and human dermal fibroblasts [HDFs]) of crude hydroalcoholic extract and three corresponding fractions of *Quercus brantii* acorn. The results of this study will help to further understand the antioxidant properties and cytotoxicity of the plant and may lead to the identification of corresponding fractions of plants with high antioxidant

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Table 1. DPPH radical-scavenging activity of the crude extract and various fractions of *Q. brantii* fruits.

Sample	Concentration (µg/ml)	Scavenging of DPPH radical activity inhibition (%)	IC50 / (µg/ml)
Crude extract	25	80.9±0.5	15±3[#a]
	20	66.4±1.1	
	15	49.7±0.9	
	10	34.5±0.3	
	5	19±1	
n-hexane fraction	150	55.3±1.5	144.5±5[#]
	125	47.5±2	
	100	40±2/1	
	75	37.7±.08	
	50	17.4±.3	
Chloroform fraction	25	91.5±1.5	12.7±1[#ab]
	20	80.1±1.4	
	15	62.5±0.8	
	10	40.2±1.3	
	5	22.9±2.4	
n-Butanol fraction	10	75±1.5	6.5±0.5[#b]
	7.5	58.2±1.2	
	5	47.3±0.3	
	2.5	25.8±1.3	
	1	8.6±1.2	
BHT	50	90.8±1.5	25.41±1.89
	40	78.3±1.2	
	30	55.5±0.7	
	20	40.09±1.7	
	10	22±1.06	

All results are presented as mean ± standard mean error of three assays.
Significant when compared with the reference standard (BHT); [a,b] values with the same letter in each column are not significantly different according to Tukey test ($p < 0.05$);
Abbreviations: DPPH, 1,1-Diphenyl-2-picrylhydrazyl; IC_{50}, the concentration of a sample at which 50% inhibition of free radical activity is observed; BHT; Butylated hydroxytoluene

Table 2. Fifty percent inhibitory concentration (IC_{50}) of hydroalcoholic extract and three correspond fractions of *Quercus brantii* acorn on two cell lines.

Cell line Extract/fraction	Vero (µg/ml)	HDFs (µg/ml)
Crude extract	333.7±45	411.1±21
n-Hexane fraction	1172.2±56	983.5±123
Chloroform fraction	60.6±23	287.8±38
n-Butanol fraction	155±35	205±6

activity for further investigation and development of value-added food products and nutraceuticals.

MATERIAL AND METHODS

Plant collection

The fruits of Oak were gathered from southwest region of Iran. Then, in Herbarium of Medical Plants Research Center of Shahrekord University of Medical Sciences (Iran), genus and species of the plant were identified and confirmed.

Extraction and fractionation of plant material

The fruits were harvested and powdered three times per maceration method. The plant material was dissolved in 70% ethyle alcohole (Ghadir, Iran) and kept at room temperature for 96 h. After that, the mixture was filtered and concentrated under nearly vacuum pressure and at 40°C using rotary evaporator. Having crude hydroalcoholic extract of the plant, three fractions of the crude extract, with different polarity through in-solution isolation and using the difference in various secondary metabolites' polarity, were prepared. To isolate hexane fraction, the extract concentrated, suspended in 70% ethyl alcohole, and mixed with equal volume of normal hexane with sufficient shaking. The remaining plant material, from which the methanol was removed, mixed with distilled water and with cholorphorm in equal volume, shaked, anhydrated using sodium sulphate and used as the cholophorm fraction. To prepare butanol fraction, equal volume of butanol was added to the remaining aqueous phase of the material, shaked and concentrated at 40°C and in vacuum condition. The extract/fractions were kept in sterile bottles, under refrigerated conditions, until further use.

Determination of the Free-Radical Scavenging Activity

The free-radical scavenging activity was measured by the 2,2 diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, with some modification[24]. Different amounts of each extract and methanol were added to a solution of 0.3 mg/mL methanolic solution of DPPH (Sigma, USA) to make up a total volume of 3.0 mL. After standing for 15 min at room temperature, the absorbance was measured at 517 nm using UV–Vis pectrophotometer (UNICO 2100: USA). High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxytoluene (BHT; sigma, USA) was used as positive control. Inhibition of free radical by DPPH was calculated as follows: Antiradical activity (%) = (Acontrol – Asample)/Acontrol×100. The IC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and was calculated based on linear regression of plots of the percentage antiradical activity against the concentration of the tested compounds[25]. The experiment was carried out in triplicate and the results are average values.

Determination of Cytotoxicity

Cells: Vero (African green monkey kidney) cell line was purchased from Pasteure Institute of Iran. Human dermal fibroblasts (HDFs) cell line was kindly provided by Cellular and Molecular Research Center of Shahrekord University of medical science, Iran. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco, USA), 100 µg/mL of streptomycin (Sigma, USA), 100 UI/ml of penicillin (Sigma, USA) and

0.25 µg/mL amphotericin B (Gibco, USA), at 37°C in a humidified air atmosphere containing 5% (v/v) CO2.

MTT assay for Cytotoxicity: Cells were seeded onto 96-well plates (SPL, Korea) at a concentration of 6000 cells per well to a final volume of 100 µl per well. After incubation at 37°C with 5% CO_2 for 24 h, overlay medium 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 extract and the fractions (in triplicates) and incubated at 37°C with 5% CO_2 for further 48 hour. The number of living cells in the culture medium was determined by the ability 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 and the procedure was followed as described previously[26]. Briefly, the supernatant was removed from the wells and 50 µL of an MTT (Sigma, USA) solution (1mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 100 µL of DMSO (Samchun korea) was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker (IKA, Germany) for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader (STATA FAX 2100, USA) at 492 nm. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC_{50}) of each extract/fractions as the percentage survival of the treated cancer and normal cultured cells was calculated according to the formula provided below:

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

The IC_{50} was defined by regression analysis and related models with probit regression model procedure using SPSS program.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

Our results showed that the n-butanol fraction exhibited the lowest IC50 value (6.5±0.6 µg ml) as compared to the other fractions under study. The crude extract, chloroform fraction, and n-hexane fraction had IC50 values of 15±3, 12.7±1, and 144.5±5µg/ml, respectively. The results are expressed relative to butylated hydroxytoluene (BHT), a reference standard having IC50 of 25.41±1.89 µg/ml. The IC50 values of the chloroform fraction, the n-butanol fraction, the crude extract, and the n-hexane fraction were found to be significant ($p<0.05$) as compared with BHT (Table 1).

Based on the scavenging capacity of the free radicals, the n-butanol exhibited the highest antioxidant activity with the highest percent inhibition of the DPPH radical, followed by the chloroform fraction with higher percent inhibition of the DPPH radical compared to that of BHT. Also, the n-hexane fraction contained lower percent inhibition of the DPPH radical compared to BHT. Phenolic compounds by donation of an H+ cause scav enging of free radicals and inhibition of macromolecules damage. In other words, it is possible to obtain compounds with more phenolic compounds by fractionation of plant extract. A study has reported that the total phenolic compounds of Salvia mirzayanii ethyl acetate fraction are high[27]. The antioxidant potential of our plant extracts is due to presence of phenolic components. Additionally, as tannin has been reported to be a significant component of phenolics in Q. brantii fruit[28], notable antioxidant potential of our fractions could be also due to their tannin content. Our results, based on the DPPH method, showed that with increasing the polarity of the extracts, their radical scavenging activity will increase. Similar findings were reported on the rate of antioxidant property of the bark of *Quercus robur*[15]. The presence of phenols and flavonoids in the plant, as its major constituents, plays a major role in controlling antioxidants. Phenolic compounds are very important plant constituents because their phenolic groups are responsible for high antioxidant activity and their hydroxyl groups confer scavenging ability.

Determination of Cytotoxicity

For evaluation of cytotoxicity activity of crude extract and three corresponding fractions of *Q. brantii* acorn on Vero and HDFs cell lines, the cells were treated with different concentrations of these compounds for 48h and cell viability was determined by MTT assay. The results showed that the IC50 was affected differently in each cell line. The chloroform fraction showed the highest cytotoxicity toward Vero and HDFs cell lines at concentration of 60.6±23 and 287.8±38 µg/ml, respectively. The n-Hexane fraction showed the lowest cytotoxicity toward Vero and HDFs cell lines at concentration of 1172.2±56 and 983.5±123 µg/ml, respectively. Comparison of the tested cell lines showed that HDFs exhibited the lowest cytotoxicity of all extract/fractions (Table 2). Over the last few decades, several in vitro methods using mammalian cell cultures have been developed, thereby avoiding the frequent use of laboratory animals which is costly, time consuming and often involves ethical problems. Cell culture systems can be more sensitive and more reproducible than animals[29]. All extract/fractions exhibited an extremely high value of IC50 (>100 µg/ml) against tested HDFs cell lines, indicating low cytotoxic to the cells[30]. Comparison of the tested cell lines indicated that HDFs had the lowest cytotoxicity of all extract/fractions, which might be due to the type of cell lines (connective tissue) and, among various fractions types, the n-Hexane fraction had the lowest cytotoxicity of all tested cell lines. Vero and HDFs (normal) cell lines, were treated with different concentrations of the compound for 48h and cell viability was determined using MTT assay. The IC_{50} was defined by regression analysis procedure using SPSS program; Vero: African green monkey kidney, HDFs: Human Dermal Fibroblasts.

CONCLUSION

The results of this study show that at least some fractions of *Q. brant*ii acorn can be used as an easily accessible source of natural antioxidants. However, since some of the fractions show lower activity in DPPH with the standard, further research on other extracts of this plant could be done to reveal and evaluate its antioxidant potential. Our

findings, therefore, suggest that all of these extract/fractions have low cytotoxicity for normal cell lines.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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