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Original Article

Antioxidant Activities and Phenolic Content of *Juniperus excelsa* Extract

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Abstract

Butyl-hydroxytoluene (BHT) is one of the major synthetic antioxidants with a wide range usage. Replacement of artificial antioxidants with natural antioxidants is highly considered. In this research, the antioxidant activity of *Juniperus excelsa* was investigated by using reducing power, DPPH radical scavenging and inhibition of lipid peroxidation. Also, the amounts of phenolic and flavonoid compounds were determined. Ethanol extract of *Juniperus excelsa* was fractionated based on increasing the polarity. The reducing power of ethyl acetate fraction was more than butanol fraction. The IC_{50} of ethyl acetate fraction (204.3±12.8 µg/ml) was less than that of butanolic (>400 µg/ml) fraction (p<0.0001). The IC50 of standard antioxidants such as BHT and gallic acid were 63.17±0.9 µg/ml and 2.49±0.06 µg/ml, respectively. In inhibition of β-carotene oxidation, ethyl acetate fraction had a remarkable effect (antioxidant activity coefficient, ACC= 960±20) which was more than the butanolic (ACC=550.7±15.3) fraction (p<0.01). The greastest amount of phenolic compounds (44.5±4.2 mg/g) and flavonoids (0.169±0.005 mg/g) were in ethyl acetate fraction. The results show that, the polar fractions of *Juniperus excelsa*, especially ethyl acetate, had more antioxidant activity.

Keywords: Antioxidant activities; β-Carotene bleaching; DPPH scavenging; *Juniperus excelsa*; Phenolics; Reducing power.
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1. Introduction

Fruits and vegetables in diet significantly reduce the mortality rates of cardiovascular diseases and cancers in human [1]. Many aromatic, spicy and medicinal plants contain antioxidant compounds. However, information about antioxidant properties of various plants that have been used in traditional medicine but are less used presently is not available. Therefore, the evaluation of such properties is attractive, particularly for finding new sources of natural antioxidants [2].

Antioxidant compounds such as polyphenols are common in plants, but high concentrations of these compounds are present particularly in fruits such as apples, grapes,

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Table 1. IC_{50} of *Juniperus excelsa* extract and its fractions compared with BHT and gallic acid.

Antioxidant compounds	IC50 µg/ml
Gallic acid	**2.49±0.6**
BHT	63.17±0.9
Juniperus excelsa crude extract	>400
Juniperus excelsa ethyl acetate fraction	204.3±12.8
Juniperus excelsa butanolic fraction	>400

blackberries and strawberries [3]. These compounds possess redical scavenging properties and exert protective effects against cardiovascular diseases [4].

Free radicals can be generated by metabolic pathways in the cell as well as by external sources such as food, drugs, and environmental pollution [2]. Free radicals can cause injury in different tissues and damage macromolecules such as proteins, DNA and lipids. Natural antioxidants such as food complements are used to scavenge or inactivate free radicals. Nowadays, natural antioxidants are attractive subjects, not only for their radical scavenging properties, but also because they have natural sources and are preferred by many people [2]. Thus, in this research, the antioxidant activity of *Juniperus excelsa* (JE) extract was investigated using different methods. *Juniperus excelsa* is a member of Conifers which is a small group of the Iranian flora (eight species of 8000 species). All aromatic Iranian conifers belong to the cupressaceae family. In Iran, this family contains *Platyclaudus, Cupressus* and *Juniperus* [5]. In other researches, the antioxidant activity of other species of *Juniperus*, *Juniperus virginiana* was reported [6].

2. Experimental

2.1. Materials

BHT (Butylated hydroxyl toluene), DPPH (2, 2- Diphenyl-1-picrylhydrazyl), gallic acid, were purchased from Sigma (ST. Louis, MO, USA). All other reagents were purchased from Merck Chem. Co.

2.2. Extraction and fractionation

Juniperus excelsa (JE) were collected from Genu mountain, north of Bandar Abbas, Hormozgan, Iran. The plant was authenticated by Mr. M. Soltanipour (Natural Resource

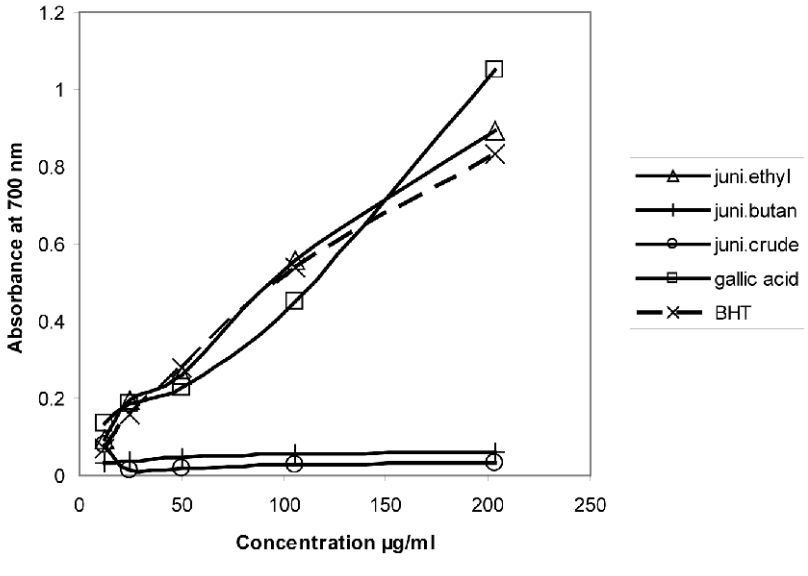

Figure 1. Reducing power of *Juniperus excelsa* extract and fractions compared to antioxidant standards (BHT and gallic acid).

Table 2. Antioxidant activity of *Juniperus excelsa* erxtract and fractions, BHT and gallic acid, measured using the BCBT[a].

Antioxidant compounds	ACC
Gallic acid	754.03±44.28
BHT	868.6±15.7
Juniperus excelsa crude extract	ND
Juniperus excelsa ethyl acetate fraction	960±20
Juniperus excelsa butanol fraction	550.7±15.3

[a]BCBT: β-Carotene Bleaching Test; [b]ACC: antioxidant activity coefficient by BCBT method given as ACC; ND: non detected

Research Center, Jahad-e-Keshavarzi, Hormozgan, Bandar Abbas, Iran). A voucher specimen (no. 323) was deposited at the Herbarium of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. Nine hundred g of the plant leaves were extracted with ethanol (4.9 L). After drying, the crude extract (110 g) was fractionated based on increasing polarity by n-hexane (3×300 ml), choloroform (3×280 ml), ethyl acetate (3×280 ml) and n-butanol (3×280 ml). The ethyl acetate and n-butanolic fractions which possessed more antioxidant activities were selected for further experiments.

2.3. Measurement of reducing power

The reducing power of the crude extract and fractions were determined by modification of the method described by Yen GC and Duh PD [7]. A series concentrations of JE extract (0.5 ml) was prepared (12.5- 200 µg/ml) in 1.25 ml of 0.2 M phosphate buffer (pH 6.6) containing 1% ferrocyanate (1.25 ml). The mixture was incubated at 50ºC for 20 min. To 2.5 ml of this mixture, 1.25 ml of 10% TCA was added and centrifuged at 3000 g for 10 min. The supernatant was separated and mixed with 1.25 ml of distilled water containing 0.025 ml of ferric chloride 1%. The absorbance of this mixture was measured at 700 nm. The intensity in absorbance could be the measurement of antioxidant activity of the extract [7]. Gallic acid and BHT were used as standards.

2.4. DPPH assay

The DPPH radical scavenging activity was performed according to the procedures described by Bruits *et al*. [8]. Samples for the

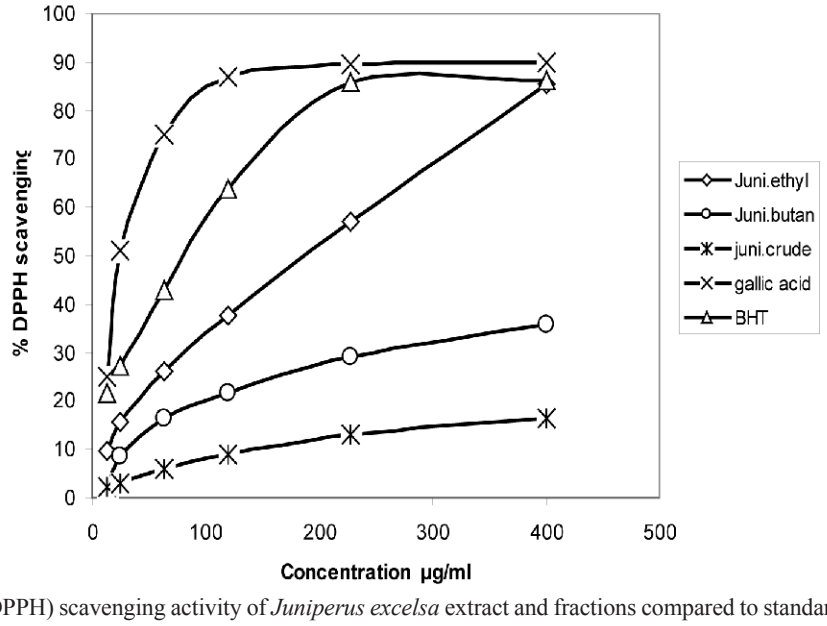

Figure 2. Radical (DPPH) scavenging activity of *Juniperus excelsa* extract and fractions compared to standards BHT and gallic acid (gallic acid is diluted 1:10).

Table 3. The amount of total phenolics and flavonoids in *Juniperus excelsa*

Samples	Total phenolic content mg/g	Total flavonoid content mg/g
Juniperus excelsa crude extract	1.7±0.045	ND
Juniperus excelsa ethyl acetate fraction	1.46±0.01	0.169±0.005
Juniperus excelsa butanolic fraction	0.732±0.04	0.146±0.001

ND: non detected

experiments (1 mg) were dissolved in 1 ml methanol and further diluted to appropriate concentrations (12.5-400 µg/ml) before being transferred to a 96-well microplate.

Controls were prepared with 20 µL methanol and 200 µL DPPH in triplicate. Each well contained 200 µL methanol solution of DPPH (100 mM) and 20 µL of samples. The microplates were incubated at 25°C for 30 min. and the absorbance was measured at 492 nm using a microplate reader (Panasonic KX-P108 athos 2020).

Gallic acid and BHT were used as antioxidant standards. The obtained data were used to determine the concentration of the sample required to scavenge 50% of the DPPH free radicals (IC_{50}). The percent inhibition was plotted against the concentrations of extract and the IC_{50} was obtained from the fitted linear curve. A lower IC_{50} denotes a more potent antioxidant. The results were expressed as the mean±SD of three replicates (Figure 1).

2.5. β-Carotene bleaching test

A modified method previously reported [9] was employed: 1 mg of β-carotene was dissolved in 5 ml of chloroform and 25 µl of linoleic acid. Two hundred mg of tween 40 was added to 1 ml of this solution. After chloroform was evaporated at 40°C under vacuum, 50 ml of oxygenated pure water was added (oxygenation was performed by bubbling air through water for 15 min.). This emulsion was freshly prepared before each experiment. Stock solution of the extract (0.1%) and antioxidant standards (0.01%) BHT and gallic acid were prepared in methanol. An aliquot of 250 µl of β-carotene /linoleic acid emulsion was distributed in each well of the 96 well microplate and 30 µl of methanol extract or fractions were added. An equal amount of this extract or fractions were used for blank sample. The microplate was incubated at 55°C and absorbencies were measured using a microplate reader model (Panasonic Kx-P108 athos 2020) at 492 nm. Reading of all the samples were performed immediately (t=0) and after 105 min of incubation [10]. After which no significant change in absorbance was detected. The antioxidant activity coefficient (ACC) was given by the equation:

$$ACC = [(A_{A105}-A_{B105})/(A_{B0}-A_{B105})]$$

A modified version of the formula of Chevolleau et al. was used [11] where A_{A105} and A_{B105} are the absorbencies of the test and blank sample at zero and 105 min., respectively, and A_{B0} is the absorbencies of blank sample at =0 min.

2.6. Content of phenolic compounds

The content of total phenolic compounds in JE methanolic extract was determined by modification of Folin Ciocalteu method [2]. For the preparation of calibration curve 0.5 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml methanol gallic acid solutions were mixed with 2.5 ml Folin Ciocalteu reagent (diluted ten –fold) and 2 ml (75 g/l) sodium carbonate. The absorption was read after 30 min at 20ºC, 765 nm and the calibration curve was drawn. Half of one ml methanol plant extract (10 g/L) was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of plant phenolics. All determinations were performed

in triplicate. Total content of phenolic compounds in the methanolic extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c.V/m$$

Where: "C" is total content of phenolic compounds, mg/g plant extract, in GAE; "c" is the concentration of gallic acid established from the calibration curve, mg/ml; "v" is the volume of extract, ml; "m" is the weight of pure plant methanolic extract [2].

2.8. Determination of total flavonoid content

The total flavonoid content of JE subfractions was determined by using colorimetric method. A 0.5 ml aliquot of appropriately diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15% $NaNO_2$ solution. After 6 min., 2 ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, then the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the mixture was determined at 510 nm versus a prepared water blank [12]. Quercetin was used as a standard compound for the quantification of total flavonoid. All values were expressed as mg of quercetin equiv per 1 g of extract. Data was recorded as mean±SD for three replications.

2.9. Statistical analysis

The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. *P* value less than 0.05 was considered significance. Correlation coefficients (R) to determine the relationship between variables were calculated using MS Excel software (CORREL statistical function).

3. Results

The reducing power of JE extract and its polar fractions as a function of their concentration are shown in Figure 1. The reducing power of the JE extract and polar fractions were increased with concentration. These assays were performed for 12.5 µg/ml to 200 µg/ml of the extract. The reducing power of JE extract and polar fractions were lower than BHT and gallic acid (antioxidant standards).

Figure 2 depicts the radical scavenging effects of JE extract butanolic and ethyl acetate fractions in equivalent concentration, and they are compared with BHT and gallic acid as standard radical scavengers. The concentrations in the study were between 12.5 µg/ml to 400 µg/ml. The IC_{50} of BHT and gallic acid were found to be 68 µg/ml and 2.49 µg/ml, respectively. The IC_{50} of ethyl acetate fraction is less than BHT and butanolic fraction (Table 1). The contents of total phenolic compounds in JE crude extract, ethyl acetate and butanolic fractlions were 24.8±0.6, 44.5±4.2 and 34.7±2.5 mg/g, respectively.

The amount of flavonoids of JE ethyl acetate and butanolic fractions were 0.169±0.005 mg/g and 0.146±0.001 mg/g, respectively.

4. Discussion

The boiled leaves or fruits of *Juniperus excelsa* M.-Bieb. subsp. *polycarpos* combined with animal fixed oil are used for treatment of ear ache in Hormozgan province, south of Iran [13].

In this research, the antioxidant activity of *Juniperus excelsa* is investigated. There are many methods to determine the antioxidant capacities which differ in terms of their determination principles and experimental conditions [14].

For measuring antioxidant activity, we determined reducing power capacity, free radical scavenging and inhibition of lipid peroxidation. In reducing power, the absorbance of samples at 700 nm will increase due to the conversion of oxidized Fe III to blue colored Fe II compound.

In radical scavenging activity, when DPPH expose to antioxidant compounds, its purple

color changes to yellow. The degree of discoloration of DPPH indicates the scavenging potential of the antioxidant extract [15-17]. In methodology, the DPPH method is easy and accurate in measuring the antioxidant activity of fruit and vegetable juices or extract. The results of DPPH scavenging are highly reproducible and similar to other free radical scavenging methods such as ABTS [18]. In bleaching of β-carotene (lipid peroxidation) the yellow color of β-carotene disappears due to the presence free radicals. In other words, in the presence of antioxidant compounds the yellowish color of β-carotene remains constant.

It was interesting that the reducing power of ethyl acetate fraction is equal to that of BHT (Figure 1) and more than that of the butanolic fraction (Figure 1).

In the present study, the radical scavenging activity of ethyl acetate fraction was more than that of the butanolic fraction and the crude extract $p<0.0001$, (Figure 2). It means that the IC_{50} of ethyl acetate fraction was less than those of others (Table 1). In other researches, it was also reported that the radical scavenging activity of ethyl acetate fraction was more than that of other fractions [19]. Also ethyl acetate fraction of JE displayed a strong effect in prevention of lipid peroxidation (AAC= 960±20). In other words, this effect of ethyl acetate fraction can be compared with the standard antioxidant compound (BHT), Table 2.

This result is similar to other results which showed that the ethyl acetate fraction of *Phlomis persica* had a remarkable effect in inhibition of lipid peroxidation [20].

Polyphenols are widely distributed in plants and often possess antioxidant activity [21]. Thus in this research, the total phenolic compounds of JE crude extract and fractions were determined (Table 3). The most important antioxidant compounds are phenolics specially flavonoids which are presented in different kinds of plants. These compounds show different chemical and biological properties [2]. It had been reported that the antioxidant activity of plant material is well correlated with the content of their phenolic compounds [22].

The content of phenolic compounds (mg/g) in the methanolic extract of JE and fractions was determined by using regression equation of caliberation curve. The amount of phenolic compounds was expressed in gallic acid equivalents (GAE). The greatest amount of phenolic compounds was found in crude extract of JE (1.7±0.045 mg/g) and the lowest was in the butanolic fraction (0.732±0.04 mg/g).

The greatest content of flavonoids is established in ethyl acetate fraction (0.169±0.005 mg/g; Table 3) and the lowest content of flavonoids is in the butanolic fraction (0.146±0.001 mg/g).

A low correlation was found between phenolic compounds and radical scavenging activity (R= 0.28). Other researches reported a moderate correlation (R = 0.65) between the DPPH radical scavenging and total phenolic compounds [22]. On the other hand, Gordana et al. [24] was reported a very close correlation between phenolic content and antioxidant activities. The differences of the results showed that the radical scavenging activity of a sample may not predict on the basis of its total phenolic content [22].

The correlation coefficient between flavonoids amounts and radical scavenging activity of JE extracts was 0.604. This result may show that the flavonoid compounds of JE participate in radical scavenging.

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