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Original article

Evaluation of antioxidant activity of *Ruta graveolens* L. extract on inhibition of lipid peroxidation and DPPH radicals and the effects of some external factors on plant extract's potency

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Abstract

The antioxidant properties of *Ruta graveolens* L. were evaluated by two different methods; free radical scavenging using DPPH and inhibition of lipid peroxidation by the ferric thiocyanate method. The IC_{50} value of the methanol extract in DPPH inhibition was 200.5 µg/mL which was acceptable in comparison with BHT (41.8 µg/mL). In thiocyanate method, the plant extract demonstrated activity as much as BHT in prevention of lipid peroxidation. Increasing the temperature during extraction, significantly decreased the extract power in inhibition of DPPH radicals. The storage time and temperature had no effect on lipid peroxidation inhibition.

Keywords: Antioxidant, extract, *Ruta graveolens* L., storage time, temperature

Introduction

In our body, free radicals and other reactive oxygen species (ROS) are produced by different natural mechanisms including phagocytosis [1], fatty acid beta oxidation [2] and arachidonate oxidation [3]. Excessive ROS, beyond the antioxidant defense capacity of the body can cause oxidative stress [4]. Superoxide anion radical ($O_2^{\circ-}$) is one of the strongest reactive oxygen species among the free radicals that is generated when oxygen is taken into living cells [5]. $O_2^{\circ-}$changes to other harmful reactive oxygen species and free radicals such as hydrogen peroxide and hydroxyl radical [4]. Hydroxyl radical can then attack macromolecules in the body, including lipids [6]. Lipid peroxidation can cause food rancidity [7] and some diseases such as cancer [6]. Therefore, antioxidants play an important role in prevention of such conditions. Toxicity and carcinogenicity of artificial antioxidants such as BHT and BHA [8] has made the consumption of natural antioxidants safer.

Rue (*Ruta graveolens* L.), which is native to Northern Iran, is used as a tonic and anti-bleeding agent in folklore Iranian medicine [9]. The anticancer activity of this plant [10,11] and the high content of rutin [12], drew our attention to select rue for evaluation of its antioxidant activity.

Since antioxidants act by different mechanisms, in this research we decided to

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evaluate the effect of the plant extract on radical scavenging and lipid peroxidation by two different methods; 2,2-diphenyl-1-picrylhydrazyl (DPPH) and thiocyanate, respectively. Moreover, the role of some external factors such as temperature and storage time on the quality of the extract has also been evaluated.

Experimental

Chemicals

Trichloroacetic acid (TCA) and iron (II) chloride were purchased from Aldrich (USA). 2-Thiobarbituric acid (TBA), linoleic acid, ammonium thiocyanate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and butylated hydroxytoluene (BHT) were purchased from Sigma (USA). Iron (III) chloride, absolute ethanol and methanol were purchased from Merck (Germany). All chemicals and reagents were of analytical grade.

Plant material

Plant sample was obtained from the herboratum of Faculty of Pharmacy, Tehran University of Medical Sciences and was identified by Dr Gholam Reza Amin.

Preparation of the plant extract

The specimen was dried at room temperature and powdered. A sample (10 g) of the dried powder was extracted thrice with 100 mL methanol for 24 h on each occasion through maceration. The extract was filtered and the combined filtrates were concentrated. For evaluation of the temperature effect during extraction on DPPH scavenging activity of the plant, extraction was carried out by soxhlet apparatus as well.

DPPH free radical scavenging activity

Experiments were carried out according to the method of Blois [13] with a slight modification. Briefly, a 0.1 mM DPPH radical solution in methanol was prepared and 1 mL of this solution was mixed with the sample solution (3 mL) in methanol. After 30 min, the absorbance was measured at 517 nm. Decreasing the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity. This activity is given as % DPPH radical inhibition calculated as:

$$\left(\frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\right) \times 100$$

The DPPH solution plus methanol was used as control. BHT was used as the positive control.

Inhibition of lipid peroxidation by ferric thiocyanate method

The antioxidant activity of the extracts was determined according to the ferric thiocyanate method as reported by Kikuzaki and Nakatani [14]. A mixture containing the extract (4 mL) in absolute ethanol, final concentration: 200 μg/mL, 2.51% linoleic acid in absolute ethanol (4.1 mL), 0.05 M phosphate buffer pH 7 (8 mL) and distilled water (3.9 mL), was placed in a vial with a screw cap, and then placed in an oven at 40 °C in the dark. 75% ethanol (9.7 mL) and 30% ammonium thiocyanate (0.1 mL) were added to 0.1 mL of the sample. Three minutes after adding of 20 mM ferrous chloride in 3.5% hydrochloric acid (0.1 mL) to the reaction mixture, the absorbance of the red color was measured at 500 nm each 24 h until one day after the absorbance of the control (without sample) reached maximum. BHT was used as the positive control.

%Inhibition of lipid peroxidation was calculated by the following equation:

$$\%\text{inhibition} = \frac{Ac - As}{Ac} \times 100$$

As is the absorbance of the sample on the day when the absorbance of the control is maximum, and Ac is the absorbance of the control on the day when the absorbance of the control is maximum.

The effect of the extract storage time on the antioxidant activity of the plant in prevention of lipid peroxidation was evaluated by the thiocyanate method every three months for six months; for understanding the effect of the storage temperature of the extract on the antioxidant activity of the plant in prevention of lipid peroxidation, the extract was kept in 4, 25 and 100 °C and the antioxidant activity was measured by thiocyanate method.

Results and Discussion

Free radical scavenging by using DPPH

Inhibition effect of different concentrations of the plant extract and BHT in scavenging of DPPH was evaluated. To determine the IC_{50} value of the extract and BHT, regression

equations of calibration curves were calculated using MS Excel software (y = 0.05x + 38.11, R^2 = 0.98 for the plant extract and y = 0.85x + 14.02, R^2 = 0.98 for BHT). From these equations the IC_{50} value for the extract and BHT were calculated as 200.5 and 41.8 µg/mL, respectively.

The IC_{50} value for the extract obtained by soxhlet apparatus was 378.3 µg/mL.

The inhibitory activity of the extract against linoleic acid peroxidation

The extract significantly decreased the absorbance and showed inhibitory activity against linoleic acid peroxidation. This inhibitory effect was as much as BHT (figure 1).

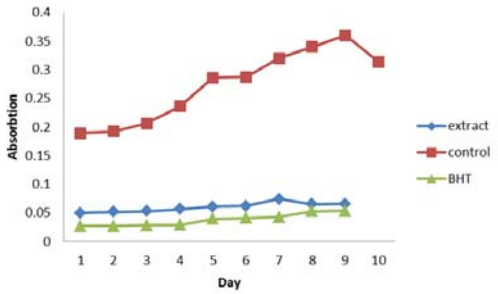

Figure 1. Total antioxidant activity of *R. graveolens* extract (final conc. 200 ug/mL), control and BHT, after the first month storage in refrigerator.

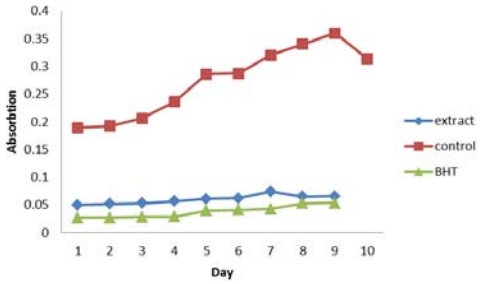

Figure 2. Total antioxidant activity of *R. graveolens* extract (final conc. 200 ug/mL), control and BHT, after 3 months storage in refrigerator

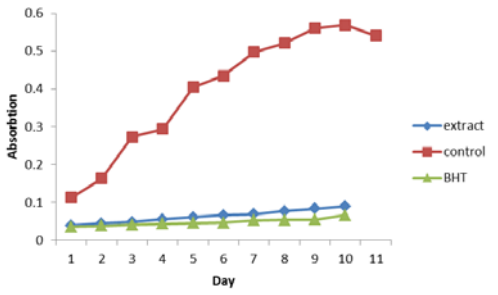

Figure 3. Total antioxidant activity of *R. graveolens* extract (final conc. 200 ug/mL), control and BHT, after 6 months storage in refrigerator.

For evaluating the effect of the storage time on inhibition of lipid peroxidation, the test was carried out 3 and 6 months after the first experiment. The results are presented in figures 2 and 3.

To evaluate the effect of the extract storage temperature on lipid peroxidation inhibition, instead of keeping the extract in refrigerator (4 °C), one part of the extract was kept in room temperature (25 °C) for 24 h and one part was placed in an oven (100 °C) for 60 min. the results of the effects of these extracts on lipid peroxidation have been shown in figures 4 and 5.

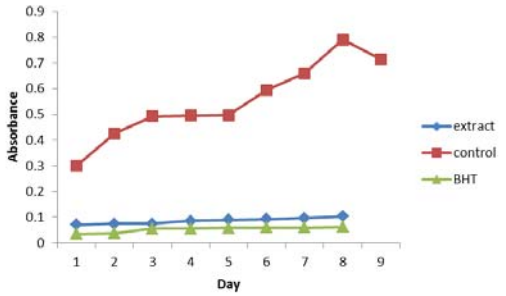

Figure 4. Total antioxidant activity of *R. graveolens* extract (final conc. 200 ug/mL), control and BHT, the first month kept for 24 h (25 °C).

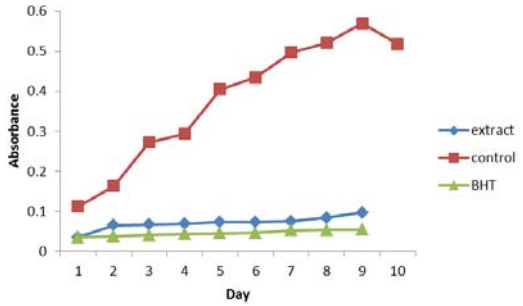

Figure 5. Total antioxidant activity of *R. graveolens* extract (final conc. 200 ug/mL), control and BHT, the first month kept for 60 min (100 °C).

Table 1. Inhibition of lipid peroxidation (%) of rue extracts on the day when the absorbance of the control was maximum

Sample	Inhibition of lipid peroxidation (%)
PE*, the first month, storage in refrigerator	81.66
PE, 3 months later, storage in refrigerator	87.22
PE, 6 months later, storage in refrigerator	84.18
PE, the first month, at room temperature (25°C) for 24 h	86.97
PE, the first month, oven (100°C) for 60 min	82.95
BHT	88.54

*plant extract

Inhibition of lipid peroxidation (%) of the extracts on the day when the absorbance of the control was maximum has been shown in table 1.

Free radicals can attack different macromolecules including lipids and therefore can cause many diseases, aging process and food rancidity [15]. Consumption of natural antioxidants such as plants [16] can prevent the occurrence of such events or postpone them, especially that synthetic antioxidants have shown toxic side effects [17].

Presence of three aromatic rings in DPPH structure has made this molecule very stable. This radical has the maximum absorbance in 517 nm. Every substance that can inhibit DPPH, decreases the absorbance in this wavelength, therefore, DPPH assay could be considered as an appropriate method for evaluation of samples in inhibition of free radicals [18].

Comparison of the result of rue (IC_{50} 200.5 µg/mL) in DPPH method with BHT (IC_{50} 41.8 µg/mL), demonstrated acceptable effect of the plant in free radical inhibitory activity.

Optimization of the extraction process could be a good way to keep the antioxidant capacity of the extract. Extraction temperature is an important factor which might be effective in chemical constituents and antioxidant capacity of an extract. In extraction by using soxhlet apparatus, the plant material is continuously flushed in with fresh solvent; this fresh solvent is formed by boiling the solvent, thus increasing the temperature in this method could decrease the time of extraction [19]. By increasing the temperature during plant extraction, the IC_{50} value increased significantly and reached 378.3 µg/mL. Such an increase was also observed for two other plants (*Artemisia absinthium* and *Myrtus communis*) which were investigated in our lab at the same time [20,21]. Therefore, the experimental results were in accordance with previous studies [22-24] and established that increasing heat could decrease radical scavenging activity of the *R. graveolens* extract.

Among the rue constituents [25], phenolics, flavonoids and cumarines are the most probable ones which could be able to have inhibitory activity on DPPH radicals. Flavonoids [26-28] and coumarines [29] are heat stable while phenolics are less stable [23-26] and may have structural changes and the decrease in inhibition of DPPH radicals might be due to the thermal deterioration of phenolics during the extraction.

Linoleic acid, an unsaturated fatty acid, can be easily peroxidased and produces various compounds such as aldehydes and epoxides [30]. By measuring the absorbance of these compounds, it is possible to find the level of peroxidation. If a compound inhibits lipid peroxidation, it decreases absorbance in specific wavelength [14]. Lipid peroxidation can be prevented by adding natural or synthetic antioxidant substances [31].

The results of the extract in lipid peroxidation inhibition method, demonstrated that the potency of the extract was nearly the same as BHT. Although phenolics can inhibit lipid peroxidation [6], it must be kept in mind that for the lipid peroxidation inhibitory effects, other factors such as the hydrophobicity of the compounds, besides the structural effects, should be taken into account [32]. It seems that in prevention of lipid peroxidation, the role of coumarins and flavonoids, which are less polar compared to phenolics, should be considered more important, especially that the increase of temperature did not have any effects on the lipid peroxidation inhibitory action, though further phytochemical studies are of necessity. Like temperature, the storage time of the extract also showed no effect on lipid peroxidation inhibition (table 1), it is concluded that *Ruta graveolens* can be considered as a potential factor for prevention of food rancidity and could be used in food industrials for prevention of food and fat rancidity especially due to the results of its antioxidant effects in high temperature and during time.

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