

Total Phenols, Antioxidant Potential and Antimicrobial Activity of Methanol Extract of Rosemary (*Rosmarinus officinalis* L.)

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Abstract: In this work, we studied antioxidant activity and antimicrobial activity of rosemary leaves extract. Dry rosemary leaf powder was subjected to Soxhlet extraction with pure methanol. Yield extraction was 18.9% and the total phenolics were found to be 4.99 ± 0.054 g as gallic acid/100g dry leaves. The antioxidant activity of rosemary leaves extract and two synthetic antioxidant: BHA and TBHQ was assessed using the DPPH radical-scavenging method. IC_{50} values showed that rosemary extract (0.024 ± 0.005 mg/ml) has a higher antioxidant activity than BHA (0.034 ± 0.009 mg/ml). The antimicrobial activity of rosemary leaves extract against *Leuconostoc mesenteroides*, *Lactobacillus delbruekii*, *Saccharomyces cerevisia* and *Candida krusei* (*Issatchenikia orientalis*) were determined by minimum inhibitory concentration (MIC). The results indicated that among the tested microbes, rosemary extract were a stronger inhibitory effect on the bacteria. Minimum inhibitory concentration values for both bacteria *Leuconostoc mesenteroides* and *Lactobacillus delbruekii* ranged between 1.5 mg/ml and 1.75 mg/ml.

Key words: *Rosmarinus officinalis* L. • Total Phenolic Compound • Antioxidant Activity • Antimicrobial Activity

INTRODUCTION

Oxidative decline oils and fats in foods are accountable for rancid odors and flavors, with following diminished nutritional quality and safety made by formation of secondary, poisonous compounds. Addition of antioxidants is needed to conserve flavor and color and so keeps away from vitamin subversion. Between synthetic antioxidants, the most commonly used to conserve food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ). Researches exhibit that BHT and BHA could be toxic and the lower effectiveness and higher manufacturing of natural antioxidant such as tocopherols, with the increasing awareness of consumers with relation to food additives safety made a requirement for identification of substitute natural and safer sources of food antioxidants [1].

Herbs and spices have many photochemicals which are sources of natural antioxidants, such as flavonoids, phenolic diterpenes, tannins and phenolic acids. These compounds have antioxidant, anti-putrefaction and anticancer properties. From over 300,000 species of higher plants to occur in nature, only about 2 percent have been screened so far. Extracts of plants from 157 families have been reported to be active against microorganisms [2]. Among herbs and spices, intensified most attention is on rosemary as a source antioxidant [3]. Rosemary (*Rosmarinus officinalis* L.), belonging to the Lamiaceae family, is a pleasant-smelling perennial shrub that grow in several regions all over the world [4]. It is a well-known valuable medicinal herb that is widely used in pharmaceutical products and traditional medicine as a digestive, tonic, astringent, diuretic, diaphoretic and useful for urinary ailments [5-6].

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The antioxidant activity of plant extracts is primarily due to phenolic compounds. In rosemary extracts, these are categorized into three groups: phenolic diterpenes possessing abietic acid structure, flavonoids and phenolic acids. Carnosic acid (CA) and carnosol, abietane-type diterpenes and rosmarinic acid (RA), a hydroxycinnamic acid ester, are the major antioxidant compounds existing in rosemary. These compounds, collectively with other isoprenoids (sterols, isoprene, mono- and diterpenes, tocopherols or carotenoids) play a photo defensive function and are considered as bioactive constituents [7-8].

The aim of our work was to investigate antioxidant and antimicrobial activity of rosemary extract in concentrations that can be used as natural additives in lemon beverage.

MATERIALS AND METHODS

Materials: Solvents used in the experiments were HPLC grade and purchased from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and Folin-ciocalteu's reagent were obtained from Sigma.

Rosemary leaves were collected from Tehran university campus area and dried at 25-30°C for 4 days lacking applying any heat treatment to reduce the loss of active components. Dried leaves were separated from the branches, then blended in a blender and kept in refrigerator at 4°C until use.

Preparation of Rosemary Extract: Dried blend rosemary leaves were subjected to Soxhlet extraction using pure methanol as the solvent. Eight grams of the plant material and 160 ml of methanol were used in the extraction. Methanol containing the extract was then filtered through SandS(kind of paper)(no. 604) paper and the solvent was vacuum-distilled at 40 °C in a rotary evaporator [3] The remaining extract was finally dried in a vacuum oven at 30 °C for two hours to ensure the removal of any residual solvent. The extract was weighted to calculate the extraction yield (%). Final extract was kept in a dark bottle in refrigerator at 4 °C until use.

Determination of Total Phenols Content: Total phenols content in the obtained extracts were estimated by a colorimetric analysis based on procedures described by Singleton and Rossi [9] with some modifications. Briefly,

1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (CEILE CE 2502, 2000 spectrophotometer, England). Gallic acid was used for constructing the standard curve (0.01-0.4 mM). The results were uttered as gm of gallic acid equivalents/100 g of extract.

DPPH. Radical-Scavenging Assay: The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was carried out as described by Erkan *et al.* [3] with some modifications; 1.5 ml of various concentrations of the test materials (pure antioxidants or rosemary extracts) were mixed with 1.5 ml of methanolic solution containing DPPH radicals (2×10^{-4} mol/L). The mixture was shaken vigorously and after an incubation period of 30 min at 25 °C. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 515 nm. A blank test was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as a blank. The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\% \text{Inhibition} = 100(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}$$

Antioxidant activities of experiment materials or extracts were uttered as IC50, defined as the concentration of the test material necessary to cause a 50% reduction in initial DPPH? concentration.

Antimicrobial Activity

Microorganisms and Culture Conditions: The organisms used were *Saccharomyces cerevisia* PTCC 5269, *Candida krusei* (*I. orientalis*) PTCC 5295, *Leuconostoc mesenteroides* PTCC 1591 and *Lactobacillus delbruekii* PTCC 1333. Yeasts were maintained on Sabouraud Dextrose Agar (Merck, Germany) at 4 °C and bacteria were maintained on MRS (de Man, Rogosa and Sharpe) Agar (Merck, Germany) at 4°C.

Test Assays for Antibacterial Activity of Rosemary Extract: The amount of extract was estimated using the broth dilution susceptibility test [10]. The purpose of this method is testing decreeing accentuations of the antimicrobial agent(s), which usually are prepared in serial

two fold dilutions and placed in tubes of a broth medium that will support the growth of the test microorganism. MRS agar and MRS broth obtained from MST- England were used in this method. To perform the broth dilution susceptibility test, a standard inoculum of the microorganism (organisms 1×10^6 ml, a 1:500 dilution of a suspension of turbidity equal to a McFarland standard 1), was added to an equal volume of each concentration of antimicrobial agent and to a tube of the growth medium without antimicrobial agent,

Which has been served as a growth control adding a bacterial suspension dilutes both the suspension and the concentration of antimicrobial agent in the tube. An uninoculated tube of medium has been incubated to serve as a negative growth control. After overnight incubation, the tubes were cultured on the plates containing MRS agar and were incubated at 37°C (*Lactobacillus delbruekii*) and 26°C (*Leuconostoc mesenteroides*) for overnight [10].

Test Assays for Antiyeast Activity of Rosemary Extract:

The described method with some modification was also used for evaluating antiyeast activity. The amount of extract was estimated using the broth dilution susceptibility test [10]. The purpose of this method is testing decreasing accentuations of the antimicrobial agent(s), which usually are prepared in serial two fold dilutions and placed in tubes of a broth medium that will support the growth of the test microorganism. Sabouraud Dextrose Agar and Sabouraud Dextrose broth obtained from MST- England were used in this method. To perform the broth dilution susceptibility test, a standard inoculum of the microorganism (organisms 1×10^6 ml, a 1:500 dilution of a suspension of turbidity equal to a McFarland standard 1), was added to an equal volume of each concentration of antimicrobial agent and to a tube of the growth medium without antimicrobial agent, which has been served as a growth control adding a bacterial suspension dilutes both the suspension and the concentration of antimicrobial agent in the tube. An uninoculated tube of medium has been incubated to serve as a negative growth control. After overnight incubation, the tubes were cultured on the plates containing Sabouraud Dextrose Agar and were incubated at 35°C (*Saccharomyces cerevisia*) and 25°C (*Candida krusei*) for overnight [10].

RESULTS AND DISCUSSIONS

Total Phenolic Compounds: There are diverse reports on the amount of total compounds in rosemary. For methanol extracts of rosemary leaves and stems [11] it was reported

Table 1: Extraction yield and Total phenolic compounds (g/100 g dry leaves) of rosemary leaf extract

Parameters	Value ^a
Extraction yield	18.9±0.1
Total phenols ^b	4.99±0.054

^aMean value + (standard deviation), n=3.

^bCalculated as gallic acid equivalents.

Table 2: IC₅₀ value (mg/ml)^a

Sample	IC ₅₀ (mg/ml) ^a
Rosemary extract	0.024±0.005
BHA	0.034±0.009
TBHQ	0.014±0.008

^aResults are presented as mean ± SD (n=3)

Table 3: MIC and MBC values (mg ml⁻¹) of Rosemary extract against bacteria and yeasts

Species of bacteria or yeast	MIC	MBC
<i>Leuconostoc mesenteroides</i>	1.5	1.75
<i>Lactobacillus delbruekii</i>	1.75	2.0
<i>Saccharomyces cerevisia</i>	2.25	2.5
<i>Candida krusei</i> (<i>Ssatchenikia orientalis</i>)	2.5	3.0

a phenolic concentration of 5.07 gm gallic acid equivalents/100 gm of herb (dry weight). For dry rosemary leaves Dorman *et al.* [12] found the concentration in water extracts to be 185 mg gallic acid equivalents/gm of extract. Similar values were reported by Kosar *et al.* [13] for methanol extracts. In this work we found, for methanol extract of rosemary leaves, a phenolic concentration of 4.99 gm gallic acid equivalents/ 100 g of herb (dry weight) (Table 1). This result agrees with values reported by Shan *et al.* [11].

Antioxidant Activity: DPPH[•] is a stable radical showing a maximum absorbance at 515 nm. It can readily undergo decrease by an antioxidant (AH) which can be confirmed by the subsequent reaction.

DPPH[•]+AH→DPPH₂ H+A[•] ...Reac.1: Because of the relief and repose of this reaction, it now has common use in free radical-scavenging assays. Vanish of the DPPH radical absorption at 515 nm by the action of antioxidants is taken as a measure of antioxidant activity [3]. The antioxidant activities of rosemary extract and TBHQ, BHA were determined by DPPH assay. Results of the antioxidant activity can be seen in Table 2. The IC₅₀ of rosemary extract was lower than the synthetic antioxidant BHA and the IC₅₀ of synthetic antioxidant TBHQ was the lowest one. The variations in the antioxidant activities can be attributed to the higher phenolic content of the former

(162 mg GAE/g) than that of the latter (28.2 mg GAE/g). The proximate correlation between antioxidant activity and phenolic content of extract obtained from various natural sources has been demonstrated by many workers [14-15].

It was reported that the solvent used in extraction can also be important in the antioxidant activity of the extract, depending on the phenolic content. For example Liue *et al.* [14] found that phenolic and flavonoid contents of an endophytic *Xylaria* sp. were higher in methanol extracts than in hexane extracts. The antioxidant activity of phenolic compounds from plants is well known. This activity has been mainly attributed to flavonoids and ascorbic acid in citrus fruits (hesperidin, neohesperidin and eriocitrin) and to carnosol and rosmarinic acid in rosemary. All of these polyphenolic compounds have the ability to act as antioxidants by a free radical scavenging mechanism [16].

Antimicrobial Analysis: The study of antimicrobial power of plant phenolics is well known. [17-18]. In this work, we proposed the use of rosemary extract, as antimicrobials source. Some reports found that the mainly apolar phenolic compounds from rosemary extracts may be responsible for their antibacterial activity [18]. reported that rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative bacteria. High percent of the antimicrobial activity they attributed to carnosic acid and carnosol. It is clear that rosemary extracts have bioactive properties, but their antimicrobial activities have not been deeply characterized [19]. Rosemary extracts were screened against two (*L. mesenteroides* and *L. delbruekii*) bacterial strains and two (*S. cerevisia* and *C. krusei*) yeast strains. The results of the effect of the rosemary extract on the tested microbial strains are shown in Table 3. Rosemary extract inhibited the growth of both bacteria and yeast at MIC values ranging between 1.5 and 2.5 mg ml⁻¹. *L. mesenteroides* was more susceptible to rosemary extract (1.5 mg ml⁻¹) than the *L. delbruekii*. Generally, result MIC and MBC of tested bacteria and yeast showed that extract was strongly active against bacteria compared to yeast. Rosemary leaves extract can inhibit the growth of bacteria and yeasts and also its antioxidant activity is comparable with BHA and TBHQ. Therefore it can be used as a potential antimicrobial and antioxidant source of natural origin in foods.

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