

Full Length Research Paper

Chemical constituents and antioxidant properties of *Rosmarinus officinalis* L. essential oil cultivated from the South-Western of Tunisia

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Essential oil extracted from the aerial parts of plant by hydrodistillation from *Rosmarinus officinalis* was analyzed by gas chromatography/mass spectrometry and the components identified were 15. The major constituents of the essential oil were 1, 8-cineole (35.32%), trans-caryophyllene (14.47%), borneol (9.37%), camphor (8.97%), α -pinene (7.9%) and α -thujone (6.42%). The *R. officinalis* essential oil was screened for their *in vitro* antioxidant activities using three different and complementary assays: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), β -carotene bleaching test and reducing power. The results of the DPPH assay showed an IC_{50} inhibitory concentration of 110.20 μ g/ml. In the β -carotene bleaching test, the IC_{50} valued was 20.00 μ g/ml. At higher concentration (70 μ g/ml), this essential oil exhibited a lower reducing power with an absorbance value of 0.72 ± 0.02 . The corresponding value EC_{50} was evaluated as 38.68 μ g/ml. Comparison of the antioxidant properties of the investigated essential oil with those expressed by butylated hydroxytoluene (BHT), used as a positive control, showed that this oil exhibited a slightly weak antioxidant potential than BHT and therefore acts as natural antioxidant agent.

Key words: *Rosmarinus officinalis*, chemical composition, essential oil, antioxidant activity.

INTRODUCTION

Recently, the importance of aromatic plants is considerable due to their applications in folk medicine and their potential for commercial exploitations, which are used as aroma and flavour enhancers, cosmetics and in pharmaceuticals (Boussaada et al., 2008). Among them, Rosemary, *Rosmarinus officinalis* L. belongs to the member of Lamiaceae family, is widely cultivated all over

the world as an ornamental and aromatic plant. This herbal spice native to Mediterranean basin was used in cuisine as dried leaves not only to improve or modify the flavor of food, but also to avoid its deterioration because of its antimicrobial and antioxidant activities (Fernandez et al., 2005). It was used as a food flavoring, is also known medicinally for, its powerful antibacterial, antimutagenic properties, and as a chemopreventive agent (Oluwatuyi et al., 2004), treatment of a number of therapeutic applications in curing or managing of a wide range of diseases such as respiratory disorders, stomach problems and inflammatory diseases (Erenmemişoğlu et al., 1997; Al-Sereiti et al., 1999; Kültür, 2007). At present, demand for *R. officinalis* is increasing for its use in traditional medicine, pharmaceutical industries, cosmetic

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Abbreviations: BHT, butylated hydroxytoluene; DPPH, 1, 1-diphenyl-2-picrylhydrazyl; SOD, superoxide dismutase; CAT, catalase; GC, gas chromatography; RI, retention indices; GC-MS, gas chromatography/mass spectrometry.

fields and agribusiness and for the quality of their essential oil. Due to their biological activities, the essential oils have also been reported to be useful in aromatherapy (Buttner et al., 1996), food preservation (Faid et al., 1995), and fragrance industries (Van de Braak et al., 1999).

Today, the interest towards its cultivation is strongly arising, due to the well recognized antioxidant action exerted by the essential oil extracted from its leaves and flowers. Which compounds confer to Rosemary's essential oil, its antioxidant properties is still unclear, but it seems likely that these effects must be attributed to a mixture of different principles, acting contemporarily and synergically. The diverse composition of the essential oils of Rosemary's ecotypes coming from different geographical areas could be the reason of their various level of antioxidant activity. The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of the many human diseases, including cancer, aging and atherosclerosis (Perry et al., 2000). Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), or by chemicals such as α -tocopherol, ascorbic acid, carotenoids, polyphenols and glutathione (Gulcin et al., 2002). Thus, to increase the antioxidant intake in the human diet is one important way to minimize such oxidative damage. Therefore, research works concerning essential oils as alternative potential antioxidant for treatment of human diseases prevention and treatment of free radical-related disorders and for food preservation were important. Concomitantly, public attention to natural antioxidants has been increasing during the last years and the industry needs to find natural sources of antioxidants that could replace synthetic antioxidants or at least reduce their use as food additives (Shahidi, 2000). The aim of this work is to investigate the chemical composition of the essential oil from *R. officinalis* and to evaluate its antioxidant activity by using the DPPH assay, the β -carotene bleaching test and the reducing power assay.

MATERIALS AND METHODS

Chemicals, reagents and plant materials

Chemicals and reagents were supported by Prolabo (Paris, France) and Pharmacia (Uppsala, Sweden). Plant materials (aerial parts) of *R. officinalis* were collected from the local area of the mount of Sidi Aich, in the government of Gafsa (southwest of Tunisia), in February to March 2009.

Distillation method

The dried aerial parts were ground prior to the operation and then 300 g of ground rosemary were submitted to water distillation for 4 h using a Clevenger apparatus. The distilled essential oils were dried over anhydrous sodium sulfate, filtered and stored at +4°C.

GC/MS analysis conditions

The essential oil was analyzed using an Agilent-Technologies 6890 N Network gas chromatography (GC) system equipped with a flame ionization detector and HP-5MS capillary column (30 m \times 0.25 mm, film thickness 0.25 μ m; Agilent-Technologies, Little Falls, CA, USA). The injector and detector temperatures were set at 250 and 280°C, respectively. The column temperature was programmed from 35 to 250°C at a rate of 5°C/min, with the lower and upper temperatures being held for 3 and 10 min, respectively. The flow rate of the carrier gas (helium) was 1.0 ml/min. A sample of 1.0 μ l was injected, using split mode (split ratio, 1:100). All quantifications were carried out using a built-in data-handling programme provided by the manufacturer of the GC. The composition was reported as a relative percentage of the total peak area. The identification of the essential oil constituents was based on a comparison of their retention times to *n*-alkanes, compared to published data and spectra of authentic compounds. Compounds were further identified and authenticated using their mass spectra compared to the Wiley version 7.0 library.

Identification of essential oil compounds

The components of the essential oil were identified by comparing the mass spectra data with spectra available from the Wiley 275 mass spectra libraries (software, D.03.00) and those in the literature (Adams, 2001), as well as by comparing the retention indices with the literature data (Adams, 2001; Sibanda et al., 2004). Further identification confirmations were made referring to retention indices (RI) data generated from a series of known standards of *n*-alkanes mixture (C9-C28) (Kováts, 1958) on the HP5 and HP-20 M columns and to those previously reported in the literature (Adams, 2001; Vagionas et al., 2007; Lopez-Lutz et al., 2008).

Antioxidant activity

DPPH radical scavenging assay

The ability of oil to scavenge free radicals of *R. officinalis* essential oil was assayed with the use of a synthetic free radical compound DPPH, according to the method employed by Bersuder et al. (1998). Briefly, a volume of 500 μ l of each sample was mixed with 500 μ l ethanol and 125 μ l 0.02% DPPH in 99.5% ethanol. The mixture was shaken vigorously and incubated in the dark. After 60 min, the absorbance was measured at 517 nm using a spectrophotometer. The DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical-scavenging activity} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100,$$

Where A_{blank} and A_{sample} are the absorbance of the control (blank) and the sample, respectively. The IC₅₀ value was defined as the amount of antioxidant necessary to inhibit DPPH radical formation by 50%. The synthetic antioxidant reagent BHT was used as a positive control.

β -carotene bleaching assay

The antioxidant assay using the β -carotene bleaching was determined according to the protocol previously described (Koleva et al., 2002). β -Carotene (0.5 mg) was dissolved in 1 ml of chloroform and mixed with 25 μ l linoleic acid and 200 μ l tween 40. The chloroform was evaporated under vacuum at 40°C, then, 100 ml of distilled water was added and the resulting mixture was vigorously stirred. About 2.5 ml of the obtained emulsion was

Table 1. Chemical composition, retention indices and percentage composition of the *R. officinalis* essential oil.

N°	Compound	RI	%	Identification
1	α -pinene	930	7.90	MS, RI
2	Camphene	947	1.53	MS, RI
3	β -pinene	980	3.35	MS, RI
4	1,8-Cineole	1046	35.32	MS, RI
5	α -thujone	1135	6.42	MS, RI
6	β -thujone	1148	2.57	MS, RI
7	Chrysanthenone	1158	0.97	MS, RI
8	Camphor	1180	8.97	MS, RI
9	Borneol	1205	9.37	MS, RI
10	Bornyl acetate	1338	0.92	MS, RI
11	α -copaene	1433	1.61	MS, RI
12	trans-caryophyllene	1477	14.47	MS, RI
13	α -Humulene	1511	2.01	MS, RI
14	Germacrene-D	1569	1.68	MS, RI
15	δ -cadinene	1577	2.33	MS, RI

The components and their percentages are listed in order of their elution on apolar column (HP-5) RI: Retention indice; %: Percentage.

transferred into different tubes containing 500 μ l of essential oil dissolved in ethanol at different final concentrations. The tubes were immediately incubated at 50°C for 120 min and the absorbance was measured at 470 nm before and after heat treatment. A control containing 0.5 ml of ethanol instead of the sample solution was carried out in parallel. BHT was used as positive control.

Reducing power antioxidant

The ability of oil to reduce iron (III) was determined according to the Yildirim's method with some modifications (Yildirim et al., 2000). An aliquot of 500 μ l of each sample at different final concentrations was dissolved in ethanol and mixed with 1.25 ml of reagent of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide. The mixture was incubated 30 min at 50°C, followed by addition of 1.25 ml of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 1500 g for 10 min. Finally, 1.25 ml of the supernatant solution was mixed with 1.25 ml of distilled water and 250 μ l of 0.1% (w/v) ferric chloride. After 10 min, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

RESULTS AND DISCUSSION

Chemical composition

Essential oil obtained by hydrodistillation of the aerial parts of *R. officinalis* had a light yellow color and a high odor. The percentages of the identified components are listed in Table 1 in the order of their elution on the HP-5MS column. Fifteen components representing 99.42% of the total oil could be identified. The major compounds, which were identified by gas chromatography/mass

spectrometry (GC-MS), were 1, 8-cineole (35.32%), trans-caryophyllene (14.47%), α -pinene (7.90%), borneol (9.37%), camphor (8.97%) and α -thujene (6.42%). The oil contains a complex mixture of 77.32% of monoterpene and 22.10% of sesquiterpene. The essential oil composition was dominated by oxygenated monoterpenes (64.54%), followed by sesquiterpene hydrocarbons (22.10%) and monoterpene hydrocarbons (12.78%). The major constituents of oxygenated monoterpenes were 1,8-cineole (35.32%), borneol (9.37%), camphor (8.97%) and α -thujone (6.42%) however the principal component of monoterpene hydrocarbons were α -pinene (7.90%) and camphene (3.35%). The oil was poor in oxygenated sesquiterpene but those hydrocarbons were mainly composed of trans-caryophyllene (14.47%) with others minor constituent at less than 3% (α -humulene, germacrene-D and δ -cadinene).

The results obtained in this study differ from all those reported elsewhere in the literature for this species with the specificity the presence of trans-caryophyllene (14.47%), a sesquiterpene hydrocarbon in higher amount. Tomei et al. (1995) investigated the essential oil from flowers and leaves of *R. officinalis* (collected from the wild in southern Spain) and found the main components to be camphor (32.33%), 1, 8-cineole (14.41%) and α -pinene (11.56%). Angioni et al. (2004) reported that the major components were α -pinene, borneol, camphene, camphor, verbenone and bornyl-acetate, present in Sardinian *R. officinalis* L. oil. Santoyo et al. (2005) found that α -pinene, 1,8-cineole, camphor, verbenone and borneol, constituting represent about 80% of the total *R. officinalis* oil. Sacchetti et al. (2005) were also reported that the major compounds of *R. officinalis* essential oils were verbenone (21.76%), camphor (14.6%) and bornyl-acetate (12.3%). Gachkar et al. (2007) found that the chemical composition of *R. officinalis* essential oil from Iran was dominated by piperitone (23.7%), α -pinene (14.9%), linalool (14.9%) and 1, 8-cineole (7.43%). Bozin et al. (2007) found that the main compounds of *R. officinalis* oil were limonene (21.7%), camphor (21.6%) and α -pinene (13.5%). More recently, Martos et al. (2010) reported that the major constituent of *R. officinalis* were 1, 8-cineole (23.59%), camphor (20.7%) and α -pinene (18.21%). Zaouali et al. (2010) determined that the main constituents of *R. officinalis* var. *typicus* and var. *trogodytorum* were 1,8-cineole (47.2 to 27.5%) and camphor (12.9 to 27.9%). These differences in chemical compositions of oils could be attributed to the geographical location of where the plant grows, to the climatic effects on the plants and to the genotypical differences (Sagnard et al., 2002; Adams et al., 2006).

Antioxidant activity

The screening of the potential activity of essential oil may

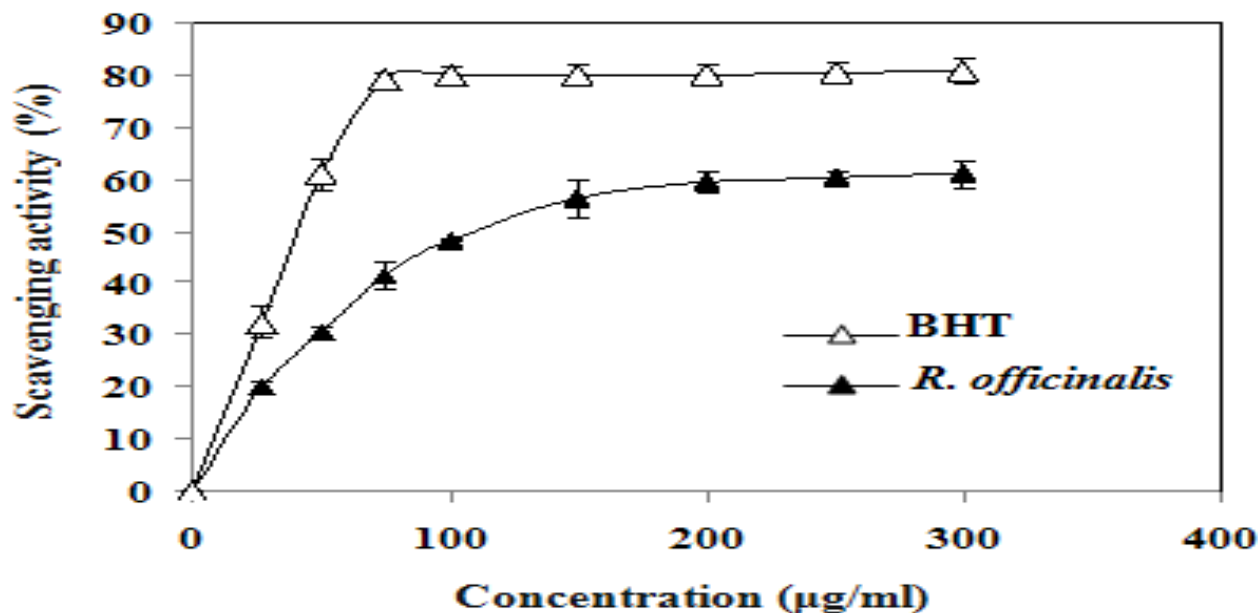


Figure 1. Free radical scavenging activity of *R. officinalis* essential oil and positive control (BHT). Scavenging activity was measured using the DPPH radical assay.

require a combination of different methods to describe the antioxidant properties of the sample in more detail. Therefore, the antioxidant activity of the tested essential oil of *R. officinalis* was determined by different *in vitro* methods such as, the DPPH free radical scavenging assay, β -carotene bleaching test and reducing power assay. The results were compared with the synthetic antioxidant BHT, which is an efficient synthetic antioxidant agent in food. All the assays were carried out in triplicate and average values were considered.

DPPH radical-scavenging activity

DPPH is a free-radical compound which has been widely used to test the free-radical scavenging ability of various samples. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Ebrahimzadeh et al., 2008). The antioxidant effect of essential oil on DPPH radical scavenging may be due to their hydrogen donating ability and it reduce the stable violet DPPH radical to the yellow DPPH-H. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Dehpour et al., 2009). Figure 1 depicts the effective concentrations of the essential oil required to scavenge DPPH radical and the scavenging values as inhibition percentage at various concentrations.

It can be seen that *R. officinalis* exhibited a dose-dependent increase with a radical scavenging effect of $61.00 \pm 2.30\%$ at 300 µg/ml, which is slightly lower than

the DPPH % inhibition of the positive control BHT ($80.70 \pm 2.40\%$) at the same concentration. DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Comparison of the DPPH scavenging activity of the *R. officinalis* essential oil (110.20 µg/ml) and those expressed by BHT (40.50 µg/ml) showed that the essential oil exhibited weakest antioxidant effects than BHT. Therefore, the antioxidant effect of the oil was about 3 times lower than that of the synthetic antioxidant BHT. As can be seen from the results summarized in Table 1, the DPPH scavenging ability of this oil can be attributed to the presence of some components that have antioxidant activity, for example, 1,8 cineole, α -pinene, β -pinene (Houghton, 2004) and camphor and α and β -thujone (Bozin et al., 2007).

β -Carotene bleaching method

The β -carotene bleaching method is based on the loss of the yellow color of β -carotene due to its reaction with radicals that are formed by linoleic acid oxidation in an emulsion. In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxide arising from linoleic acid oxidation. β -carotene undergoes rapid discoloration in the absence of an antioxidant, however the presence of antioxidant will be minimize its oxidation. This test measures the potential of the plant to inhibit conjugated diene hydroperoxide formation from linoleic acid oxidation. As

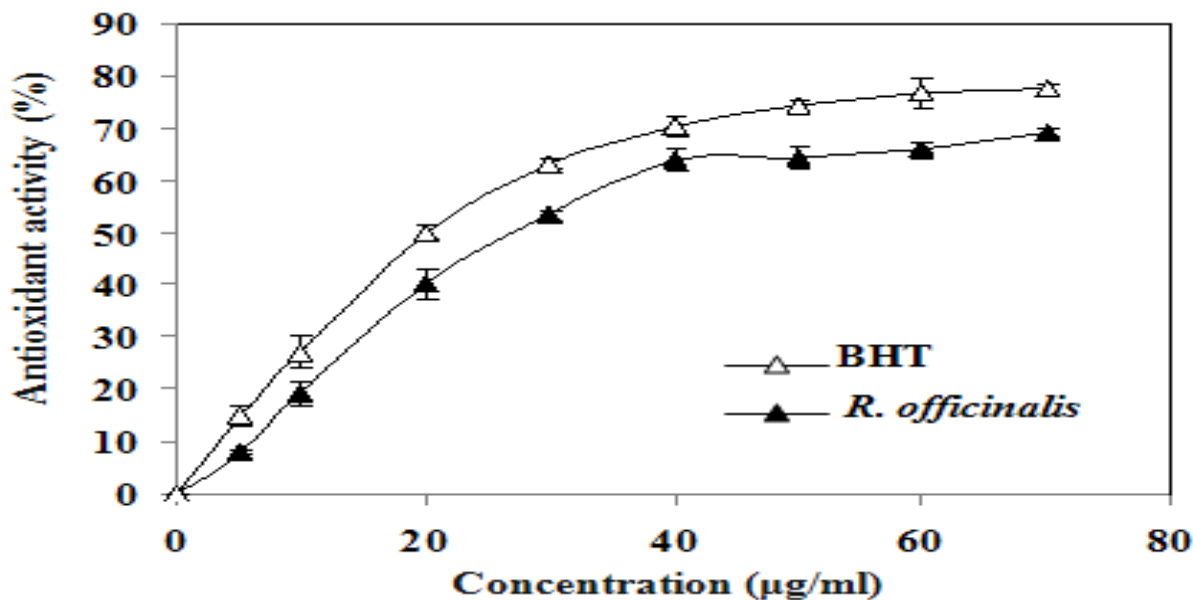


Figure 2. Antioxidant activity of *R. officinalis* essential oil and BHT determined by β -carotene bleaching test.

can be seen from Figure 2, the antioxidant activity was dose-dependent with a value of 69.09 ± 0.67 and 77.50 ± 1.00 at the final concentration of $70 \mu\text{g/ml}$ for the essential oil of *R. officinalis* and BHT, respectively. The potential of *R. officinalis* to inhibit lipid peroxidation was evaluated using the β -carotene/linoleic acid bleaching test by measuring the IC_{50} , with a value of 27.28 and $20.00 \mu\text{g/ml}$, obtained for the oil and the positive control BHT, respectively.

This activity was moderately lower than that of BHT and was attributed to the presence of 1,8 cineole, α -pinene and β -pinene (Wang et al., 2008) and to the presence of appreciable amount of antioxidant compounds such as 1,8-cineole, transcaryophyllene, borneol, camphor and α -pinene, and to the absence of phenolic compounds (Ebrahimabadi et al., 2010).

Reducing power antioxidant

Antioxidant activity was also determined by ferric reducing power using a spectrophotometer at 700 nm . In this assay, the presence of reducers causes the transformation of Fe^{3+} into Fe^{2+} by donating an electron. Then, the amount of complex can be monitored by measuring the formation of Perl's Prussian blue ($\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$) at 700 nm . Reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 3 illustrates dose-response curves for the reducing powers of the essential oil and the synthetic antioxidant, BHT, that is, there was an

increase in the values by increasing the concentration, which indicated an increase in the ferric reducing power. The *R. officinalis* essential oil exhibited a lower reducing power with an absorbance value of 0.72 ± 0.02 vs. 1.05 ± 0.01 for the synthetic antioxidant BHT obtained at $70 \mu\text{g/ml}$. The EC_{50} (concentration at which the absorbance is 0.5) value of *R. officinalis* essential oil was $38.68 \mu\text{g/ml}$ which was about three times lower than BHT ($13.80 \mu\text{g/ml}$). The reducing power behavior mainly due to the low phenolic content of this oil and related to the presence of reductant agent, which can reduce the oxidized intermediates of lipid peroxidation processes and consequently terminate radical chain reactions. By comparing the three assays, it was very difficult to attribute the antioxidant effect of a total essential oil to one or few active compounds. This can be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them since, since both minor and major compounds should make a significant contribution to the oil's activity (Wang et al., 2008). The antioxidant activity of this oil seems directly related to the presence of monoterpenes.

Conclusion

The present study is outlined to probe the chemical composition and *in vitro* antioxidant activity of essential oil of *R. officinalis* collected from the southwest of Tunisia. The aerial parts of plant was characterized by GC-MS and 15 volatile compounds were identified with a

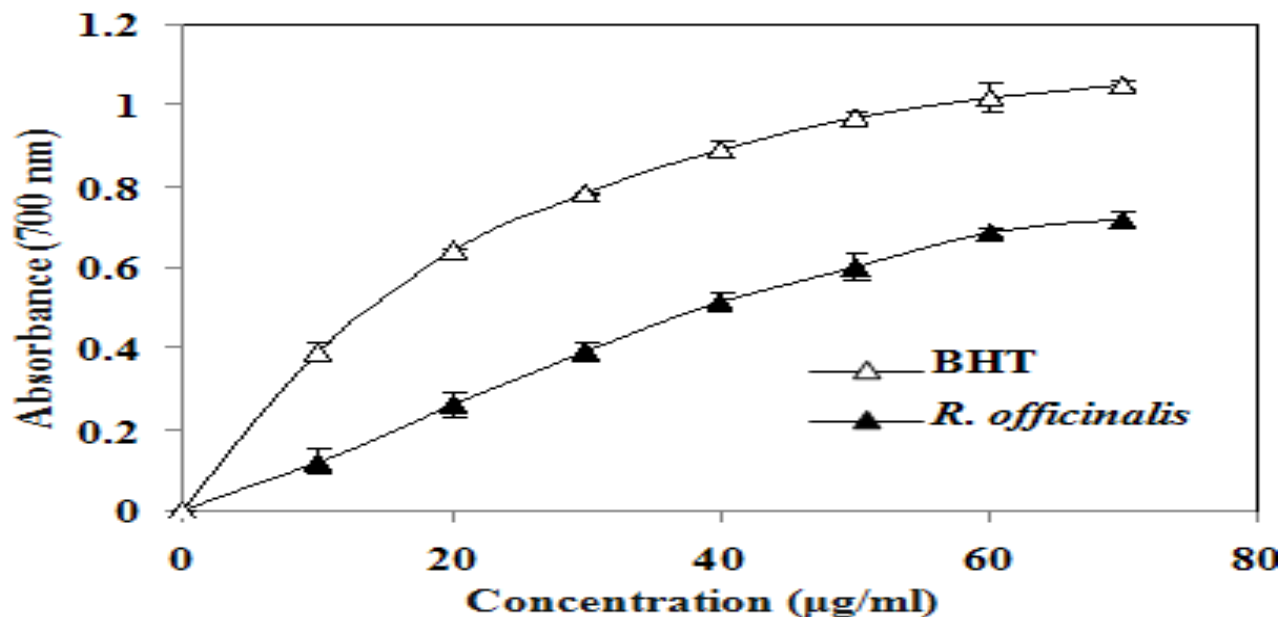


Figure 3. Reducing power of *R. officinalis* as compared to BHT.

new chemotype such as 1,8-cineole and trans-caryophyllene. A linear relationship between the results of the antioxidant effects of the investigated essential oil, obtained with different methods of assessment, which point out that essential oil could serve as antioxidant agent not only in food and cosmetics production but also as an important functional in the prevention and the treatment of various human diseases. Further isolation of bioactive compounds and determination of their biological activities *in vitro* and *in vivo* are needed to prevent the deterioration of foodstuffs, beverages and pharmaceuticals.

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