Essential oil extracted from the aerial parts of *Rosmarinus officinalis* by hydrodistillation 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%). *R. officinalis* essential oil was screened for its 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 IC₅₀ inhibitory concentration of 110.20 µg/ml. In the β-carotene bleaching test, the IC₅₀ value was 20.00 µg/ml. At a higher concentration (70 µg/ml), this essential oil exhibited a lower reducing power with an absorbance value of 0.72 ± 0.02. The corresponding value EC₅₀ was evaluated as 38.68 µg/ml. Comparison of the antioxidant properties of the investigated essential oil with those expressed by BHT (used as a positive control), showed that this oil exhibited a slightly weak antioxidant potential than BHT and therefore acts as a natural antioxidant agent.

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

**INTRODUCTION**

Recently, the importance of aromatic plants is being considered due to their applications in folk medicine and their potential for commercial exploitations as aroma and flavour enhancers, cosmetics and in pharmaceuticals (Boussaada et al., 2008). Among them, Rosemary (*Rosmarinus officinalis* L.), a member of the Lamiaceae family, is widely cultivated all over the world as an ornamental and aromatic plant. This herbal spice (native to the Mediterranean Basin) is used in cuisines as dried leaves not only to improve or modify the flavor of food, but also to prevent its deterioration because of its antimicrobial and antioxidant activities (Fernandez et al., 2005). It is used as food flavoring and is also known medically for its powerful antibacterial and antimutagenic properties, and as a chemopreventive agent (Oluwatuyi et al., 2004). It is used in a number of therapeutic applications as treatments in curing or managing a wide range of diseases, such as respiratory disorders, stomach problems and inflammatory diseases (Erenmemişoğlu et al., 1997; Al-Sereiti et al., 1999; Külțür, 2007).

At present, the demand for *R. officinalis* is increasing due to its use in traditional medicine, pharmaceutical industries, cosmetic 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. However, the compounds that confer on Rosemary’s essential oil its antioxidant properties are still unclear, but it seems likely that these effects must be attributed to a mixture of different components, acting contemporarily and synergically. The diverse composition of the essential oils of Rosemary’s ecotypes coming from different geographical areas could be the reason for their varying level of antioxidant activity.

The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of 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 a-tocopherol, ascorbic acid, carotenoids, polyphenols and glutathione (Gulcin et al., 2002). Thus, to increase antioxidant intake in human diet is an important way to minimize such oxidative damage. Therefore, research works concerning essential oils as alternative potential antioxidant for the treatment of human diseases, prevention and treatment of free radical-related disorders, and for food preservation are important. Concomitantly, public attention to natural antioxidants has been increasing during the last years, and there is need 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 DPPH assay, β-carotene bleaching test and reducing power assay.

MATERIALS AND METHODS

Chemicals, reagents and plant materials

Chemicals and reagents were supplied 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, during the Government of Gafsa (South-west of Tunisia), in February to March 2009.

Distillation method

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

GC/MS analysis conditions

The essential oil was analyzed using an Agilent-Technologies 6890 N Network GC system equipped with a flame ionization detector and HP-5MS capillary column (30 m x 0.25 mm, film thickness of 0.25 μm; 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 μl 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 gas chromatograph. 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, as compared to the published data and spectra of authentic compounds. Compounds were further identified and authenticated using their mass spectra when compared to the Wiley Version 7.0 library.

Identification of essential oil compounds

The components of essential oil were identified by comparing the mass spectra data with the 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-20M columns and to those previously reported in the literature (Adams, 2001; Vagionas et al., 2007; Lopes-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 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method employed by Bersuder et al. (1998). Briefly, a volume of 500 μl of each sample was mixed with 500 μl ethanol and 125 μl DPPH (0.02%) 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 is calculated as follows:

\[
\text{Radical-scavenging activity} = \left[ \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100
\]

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

β-carotene bleaching assay

The antioxidant assay using β-carotene bleaching was determined according to the protocol previously described by Koleva et al. (2002). β-Carotene (0.5 mg) was dissolved in 1 ml of chloroform and mixed with 25 μl linoleic acid and 200 μl tween 40. The chloroform was evaporated under vacuum at 40°C and then 100 ml of distilled water was added and the resulting mixture was vigorously stirred. About 2.5 ml of the obtained emulsion was transferred into different tubes containing 500 μl 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
Table 1. Chemical composition, retention indices and percentage composition of the *R. officinalis* essential oil.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Compound</th>
<th>Retention index</th>
<th>Percentage</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-pinene</td>
<td>930</td>
<td>7.90</td>
<td>MS, RI</td>
</tr>
<tr>
<td>2</td>
<td>Camphene</td>
<td>947</td>
<td>1.53</td>
<td>MS, RI</td>
</tr>
<tr>
<td>3</td>
<td>β-pinene</td>
<td>980</td>
<td>3.35</td>
<td>MS, RI</td>
</tr>
<tr>
<td>4</td>
<td>1,8-Cineole</td>
<td>1046</td>
<td>35.32</td>
<td>MS, RI</td>
</tr>
<tr>
<td>5</td>
<td>α-thujone</td>
<td>1135</td>
<td>6.42</td>
<td>MS, RI</td>
</tr>
<tr>
<td>6</td>
<td>β–thujone</td>
<td>1148</td>
<td>2.57</td>
<td>MS, RI</td>
</tr>
<tr>
<td>7</td>
<td>Chrysantheneone</td>
<td>1158</td>
<td>0.97</td>
<td>MS, RI</td>
</tr>
<tr>
<td>8</td>
<td>Camphor</td>
<td>1180</td>
<td>8.97</td>
<td>MS, RI</td>
</tr>
<tr>
<td>9</td>
<td>Borneol</td>
<td>1205</td>
<td>9.37</td>
<td>MS, RI</td>
</tr>
<tr>
<td>10</td>
<td>Bornyl acetate</td>
<td>1338</td>
<td>0.92</td>
<td>MS, RI</td>
</tr>
<tr>
<td>11</td>
<td>α-copaene</td>
<td>1433</td>
<td>1.61</td>
<td>MS, RI</td>
</tr>
<tr>
<td>12</td>
<td>trans-caryophyllene</td>
<td>1477</td>
<td>14.47</td>
<td>MS, RI</td>
</tr>
<tr>
<td>13</td>
<td>α-Humulene</td>
<td>1511</td>
<td>2.01</td>
<td>MS, RI</td>
</tr>
<tr>
<td>14</td>
<td>Germacrene-D</td>
<td>1569</td>
<td>1.68</td>
<td>MS, RI</td>
</tr>
<tr>
<td>15</td>
<td>δ-cadinene</td>
<td>1577</td>
<td>2.33</td>
<td>MS, RI</td>
</tr>
</tbody>
</table>

The components and their percentages are listed in order of their elution on apolar column (HP-5).

sample solution was carried out parallelly. However, BHT was used as a positive control.

**Reducing power antioxidant**

The ability of oil to reduce iron (III) was determined according to Yildirim et al. (2001) with some modifications. An aliquot of 500 µl of each sample at different final concentrations was dissolved in ethanol and mixed with 1.25 ml of 0.2 M phosphate buffer reagent (pH 6.6) and 1.25 ml of 1% potassium ferracyanide. The mixture was incubated for 30 min at 50°C followed by the 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 µl 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 strong 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 that 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 monoterpenes 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-thujene (6.42%). However, the principal components 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 other minor constituent at less than 3% (α-humulène, germacrene-D and δ-cadinene).

The results obtained in this study differed from those reported by other studies in the literature for this species with the specificity of the presence of trans-caryophyllene (14.47%) and 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, whereas Santoyo et al. (2005) found that α-pinene, 1,8-cineole, camphor, verbenone and borneol constituted and represented about 80% of the total *R. officinalis* oil. Also, Sacchetti et al. (2005) reported that the major compounds of *R. officinalis* essential oil 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), while Bozin
The antioxidant properties of the sample in more detail. The screening of the potential activity of essential oil may 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 the average values were considered.

**Beta-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 minimize its oxidation. This test measures the potential of the plant to inhibit conjugated diene hydroperoxide formation from linoleic acid oxidation. As 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 a final concentration of 70 µg/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 µg/ml, obtained 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 the essential oil on DPPH radical scavenging may be due to their hydrogen donating ability and it reduces 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 an 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 ± 2.30% at 300 µg/ml, which is slightly lower than the DPPH %inhibition of the positive control BHT (80.70 ± 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. Comparing the DPPH scavenging activity of *R. officinalis* essential oil (110.20 µg/ml) and those expressed by BHT (40.50 µg/ml), it was shown that the essential oil exhibited the 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), camphor, α-thujone and β-thujone (Bozin et al., 2007).

**Antioxidant activity**

The screening of the potential activity of essential oil may 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 the average values were considered.

**DPPH radical-scavenging activity**

DPPH is a free-radical compound which has been widely measured using the DPPH radical assay.

Figure 1. Free radical scavenging activity of *R. officinalis* essential oil and positive control (BHT). Scavenging activity was measured using the DPPH radical assay.
for the oil and 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). Also, it was attributed to the presence of an 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 (Fe$_3$[Fe(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 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 µg/ml.

The EC$_{50}$ (a concentration in which the absorbance is 0.5) value of *R. officinalis* essential oil was 38.68 µg/ml, about three times lower than BHT (13.80 µg/ml). The reducing power behavior may be mainly due to the low phenolic content of this oil related to the presence of a reductant agent, which can reduce the oxidized intermediates of lipid peroxidation processes and convert them to more stable products, thereby terminating radical chain reactions consequently.

By comparing the three assays, it is very difficult to attribute the antioxidant effect of the entire essential oil to one or few active compounds. This can be due to the high percentage of the main constituents, but may also be due to the presence of other constituents in small quantities or to synergy among them since both minor and major compounds make a significant contribution to the activity of the oil (Wang et al., 2008). However, the antioxidant activity of this oil seems to be directly related to the presence of monoterpenes.

Conclusion

This study is outlined to probe the chemical composition and in vitro antioxidant activity of the essential oil of *R. officinalis* collected from South-west Tunisia. The aerial parts of the plant was characterized by GC-MS and 15 volatile compounds were identified with new chemotypes, such as 1,8-cineole and transcaryophyllene. A linear relationship between the results of the antioxidant effects of the investigated essential oil, obtained with different methods of assessment, point out that the essential oil could serve as an antioxidant agent not only in food and cosmetics production, but also as an important function in the prevention and treatment of various human diseases. Further isolation of *R. officinalis*’ bioactive compounds and the determination of their biological activities (in vitro and in vivo) are needed to prevent the deterioration of foodstuffs, beverages and pharmaceuticals.

REFERENCES
