

Full Length Research Paper

Chemical composition and antioxidant activity of *Marrubium vulgare* L. essential oil from Tunisia

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In this study, the essential oil of the aerial parts of *Marrubium vulgare* L. obtained by hydrodistillation was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) in order to determine their chemical composition. Thirty-four (34) components in the oil of *M. vulgare* were identified. The results demonstrated that the major components of the essential oil were γ -eudesmol (11.93%), β -citronellol (9.90%), citronellyl formate (9.50%) and germacrene D (9.37%). Antioxidant effectiveness was examined by three different methods: The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, the β -carotene bleaching test and the reducing power assay. The results showed that this oil can be considered an effective source of antioxidants of natural origin. This is the first report on chemical composition of *M. vulgare* essential oil cultivated in Tunisia and the original study on the antioxidant activity of *M. vulgare* essential oil.

Key words: *Marrubium vulgare*, essential oil, chemical composition, antioxidant activity.

INTRODUCTION

In recent years, essential oils of plants and their other products from secondary metabolism have been in high demand from the manufacturers of foods flavoring, fragrance, cosmetics, and pharmaceutical industries due to the growing interest of consumers in ingredients from natural sources. Many plants have been used for different purposes, such as food, drugs and perfumery. They have been screened for their potential uses as alternative remedies for the treatment of many infections and preservation of foods from the toxic effects of oxidants (Barlow, 1990).

Marrubium vulgare L. is a perennial, herbaceous plant commonly known as "Horehound". As a medicinal plant, it was frequently employed in folk medicine to treat a variety

of ailments, exhibits antispasmodic and antinociceptive effects in different experimental models. It possesses tonic, aromatic, stimulant, expectorant, diaphoretic and diuretic properties. It is helpful for bronchial asthma and non-productive cough. It was formerly much esteemed in various uterine, visceral and hepatic affections and in phthisis (Chopra et al., 1956). The plant is reported to possess hypoglycemic (Roman et al., 1992), antihypertensive (El-Bardai et al., 2004), analgesic (DeSouza et al., 1998), anti-inflammatory (Sahpaz et al., 2002), and many other reported biological activities. In Tunisian folk medicine, it was used as hypotensive, hypoglycemic and cardiogenic. Essential oils extracted by distillation from aromatic plants are appreciated for their bioactive efficacy as fungicides, bacteriostatics, antioxidant, and other biological activities.

Synthetic antioxidants are widely used to retard undesirable changes as a result of oxidation in many foods. Excessively, oxidized fats and oils are not suitable for

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nutritive purposes. Because the oxidation products of oils have toxic effects, many synthetic substances such as propylgallate and citric acid are commonly used in lipids to prevent oxidation. Recently, these synthetic substances have been shown to cause effects, such as enlarging the liver size and increasing the microsomal enzyme activity. The use of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been restricted in food because of its carcinogenic effect. Therefore, the search for new natural antioxidant sources has been greatly intensified. In this field, plant originated antioxidants have been widely used in oils or lipid containing foods in order to prevent oxidative deterioration.

The main purpose of this study was to investigate the chemical composition of *M. vulgare* essential oil and to determine its antioxidant activity by means of three different antioxidant tests: Scavenging of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals, β -carotene bleaching test and reducing power assay.

MATERIALS AND METHODS

Chemicals, reagents and plant material

Chemicals and reagents were supplied by Prolabo (Paris, France) and Pharmacia (Uppsala, Sweden). Plant materials (aerial parts) of *M. vulgare* L. were grown in the vicinity of the village of Ouled Mnasser in Sidi Bouzid, Tunisia. The whole plants were collected during the period of June to July 2009.

Distillation of essential oil

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 (Gas chromatography/ mass spectrometry) 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 \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 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, 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.

Antioxidant activity

DPPH radical scavenging assay

The ability of *M. vulgare* oil to scavenge free radicals were 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 μ l of each sample was mixed with 500 μ l of ethanol and 125 μ l (0.02%, w/v) of 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 is 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. The values are presented as the means of triplicate analysis.

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 of linoleic acid and 200 μ l of 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 transferred into different tubes containing 500 μ l of essential oil dissolved in absolute ethanol at different final concentrations (5 to 70 μ g/ml). 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 blank containing 0.5 ml of ethanol instead of the sample test was carried out in parallel. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as positive control and all tests were carried out in triplicate.

Reducing power antioxidant

The ability of oil to reduce iron (III) was determined according to the Yildirim's method (Yildirim et al., 2000) with some modifications. 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 at 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 spectrophotometrically. Increased absorbance of the reaction mixture indicated increased reducing power. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as positive control and all tests were carried out in triplicate.

RESULTS AND DISCUSSION

Chemical composition

The percentages and the retention indices of the identified components are listed in Table 1 in the order of their elution on the HP-5MS column. GC-MS analysis of *M. vulgare* essential oil led to the identification of thirty four (34) compounds, accounting for 100% of the total oil. The

Table 1. Chemical composition, retention indices (RI) and percentage composition of the *M. vulgare* essential oil.

| N° | Compound | RI | % | Identification |
|----------------------------|---|------|-------------|----------------|
| 1 | N-trimethylsilyl trifluoroacetamide | 764 | 2.35 | MS, RI |
| 2 | N,N-bis trimethylsilyl trifluoroacetamide | 857 | 0.97 | MS, RI |
| 3 | α - Pinene | 932 | 1.16 | MS, RI |
| 4 | Camphene | 948 | 0.49 | MS, RI |
| 5 | 1,8-Cineole | 1044 | 3.72 | MS, RI |
| 6 | α -Thujone | 1131 | 2.29 | MS, RI |
| 7 | 1-Vinylcyclohexane | 1143 | 0.75 | MS, RI |
| 8 | Camphor | 1174 | 1.03 | MS, RI |
| 9 | Iso menthon | 1197 | 0.57 | MS, RI |
| 10 | Borneol | 1199 | 0.61 | MS, RI |
| 11 | β -Citronellol | 1266 | 9.90 | MS, RI |
| 12 | Geraniol | 1295 | 2.74 | MS, RI |
| 13 | Citronellyl formate | 1315 | 9.50 | MS, RI |
| 14 | Geranyl formate | 1344 | 6.25 | MS, RI |
| 15 | α -Copaene | 1419 | 1.37 | MS, RI |
| 16 | β -Bourbonene | 1429 | 1.96 | MS, RI |
| 17 | Trans-caryophyllene | 1462 | 2.15 | MS, RI |
| 18 | α -Muurolene | 1484 | 0.63 | MS, RI |
| 19 | α -Amorphene | 1490 | 0.81 | MS, RI |
| 20 | α -Humulene | 1495 | 0.68 | MS, RI |
| 21 | Neoalloocimene | 1502 | 0.91 | MS, RI |
| 22 | Neryl acetate | 1512 | 3.41 | MS, RI |
| 23 | Germacrene-D | 1521 | 9.37 | MS, RI |
| 24 | Ledene | 1534 | 5.35 | MS, RI |
| 25 | β -bisabolene | 1544 | 0.86 | MS, RI |
| 26 | δ -Cadinene | 1559 | 3.30 | MS, RI |
| 27 | α -Agarofuran | 1581 | 0.42 | MS, RI |
| 28 | Furan-2-one, 4-phenyltetrahydro | 1616 | 1.44 | MS, RI |
| 29 | γ -Eudesmol | 1647 | 11.93 | MS, RI |
| 30 | β -Cubebene | 1674 | 1.52 | MS, RI |
| 31 | Citronellyl butanoate | 1682 | 0.66 | MS, RI |
| 32 | Geranyl tiglate | 1712 | 5.53 | MS, RI |
| 33 | Cyclononasiloxane, octadecamethyl | 2198 | 3.08 | MS, RI |
| 34 | Eicosamethylcyclodecasiloxane | 2264 | 2.29 | MS, RI |
| Total identification | | | 100 | |
| Yield (g/100 g dry weight) | | | 00.34 | |
| Hydrocarbon monoterpenes | | | 01.65 | |
| Oxygenated monoterpenes | | | 40.02 | |
| Hydrocarbon sesquiterpenes | | | 42.70 | |
| Oxygenated sesquiterpene | | | 06.19 | |

yield of essential oil obtained by hydrodistillation from aerial part of plant was 0.34%.

The oil from the *M. vulgare* was found to be composed of approximately equal amounts of the oxygenated monoterpenes (40.02%) and sesquiterpenes hydrocarbons (42.7%). Indeed, the oil contains 41.67% monoterpenes, including 40.02% oxygenated monoterpenes, and 48.89% sesquiterpenes, including 42.7% sesquiterpenes hydrocarbons. In addition to γ -eudesmol (11.93%) that

was the major oxygenated sesquiterpenes, ledene (5.35%), δ -cadinene (3.30%), transcaryophyllene (2.15%), β -bourbonene (1.96%) and α -copaene (1.37%) were present in fairly good amounts. On the other hand, β -citronellol (9.90%) was the major oxygenated monoterpenes present in the oil. Other monoterpenes such as 1,8 cineole (3.72%), geraniol (2.74%) and α -thujone (2.29%) were also detected in appreciable amounts. Other compounds like ester were also found in good

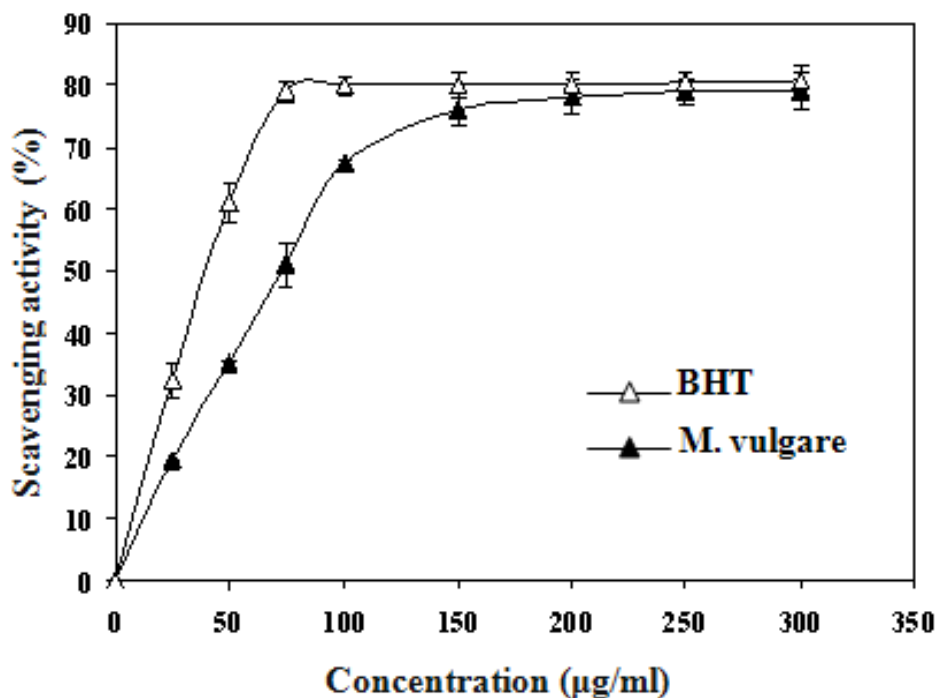


Figure 1. DPPH radical scavenging activity of *M. vulgare* essential oil and the synthetic antioxidant BHT, in different concentrations.

amounts such as citronellyl acetate (9.50%), geranyl tiglate (5.53%) and neryl acetate (3.41%). About the chemical composition of *M. vulgare* from different parts of the world, Saleh and Glombitza (1989) reported tricyclene, β -pinene, bisabolol, β -elemone and isomenthon-8-thiol as the main compounds of *M. vulgare*; Morteza and Saeedi, (2004) reported that the major constituents of the essential oil of *M. vulgare* from Iran were β -bisabolene (20.4%), 8-cadinene (19.1%) and isocaryophyllene (14.1%); Nagy and Svajdenka (1998) found that the main constituent of *M. vulgare* from Czech Republic were β -caryophyllene (45.8%) and germacrene D (14.4%); Weel et al. (1999) reported that (Z)- β -farnesene, β -caryophyllene, (E)-2-hexenal, α -humulene and germacrene D were the main components of *M. vulgare* growing in Lithuania; Khanavi et al. (2005) showed that the major component of *M. vulgare* from other region of Iran were β -bisabolene (25.4%), β -caryophyllene (11.6%), germacrene D (9.7%) and *E*- β -farnesene (8.3%), and Asadipour et al. (2005) found that caryophyllene oxide (18.7%), β -caryophyllene (12.8%) and germacrene D (10.0%) were the major compounds of *M. vulgare* collected from another region of Iran.

Interestingly, there were significant differences between the main components of this essential oil and the essential oil composition published in other studies. This oil possessed an original composition with the main component of γ -eudesmol (11.93%), which is not observed in the studies cited above. We note the presence of germacrene D as a common compound in all oils.

Antioxidant activity

The antioxidant potential of plant products can be evaluated using numerous assays. In this study, three methods were used to evaluate the antioxidant of *M. vulgare* essential oil activities and the results were compared with the synthetic antioxidant BHT which is an efficient synthetic antioxidant agent in food: Scavenging of DPPH free radicals, β -carotene bleaching test and reducing power assay.

DPPH radical-scavenging activity

Relatively stable organic radical DPPH has been widely used in the determination of the antioxidant activity of the essential oil. DPPH radical decreased in the presence of a hydrogen donor, that is, a free radical-scavenging antioxidant. In the DPPH-test, the ability of the essential oil to act as the donor of hydrogen atoms or electrons in the transformation of DPPH into its reduced form DPPH-H was measured spectrophotometrically. Assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H, reaching 50% of reduction with IC_{50} values. Lower IC_{50} value indicates higher antioxidant activity. The results represented in Figure 1 of the DPPH radical scavenging activities (% inhibition) of various concentrations of *M. vulgare* oil showed a concentration-dependent activity profile. As shown, it is clear that as the concentration increased, the scavenging effect also

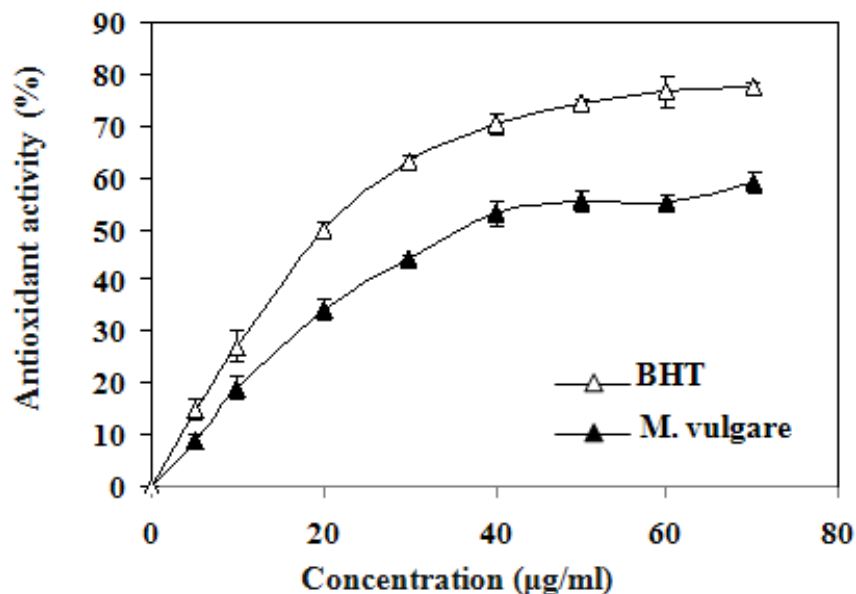


Figure 2. Antioxidant activity of *M. vulgare* essential oil and the synthetic antioxidant BHT, determined by β -carotene bleaching test, in different concentrations.

increased with inhibitory activity observed as was in the case of *M. vulgare* oil, reaching as high as $79.00 \pm 3.00\%$ at $300 \mu\text{g/ml}$. This value is too close to the activity potentials of synthetic antioxidants BHT ($80.70 \pm 2.40\%$) at the same concentration. The amount of the essential oil needed for 50% inhibition of free radical activity is expressed by IC_{50} (the concentration reducing 50% of DPPH). The lower the IC_{50} value is, the greater the free radical-scavenging activity. The results depicted in Figure 1 indicate that *M. vulgare* essential oil exhibited an IC_{50} value of $74 \mu\text{g/ml}$, which is about 2 times higher than the synthetic antioxidant (BHT). The efficiency of an antioxidant component to reduce DPPH essentially depends on its hydrogen donating ability, which is directly related to the less content of phenolic hydroxyl moieties.

β -Carotene bleaching method

The lipid peroxidation inhibitory activities of the essential oils were assessed by the β -carotene bleaching test which 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. The presence of different antioxidants can hinder the extent of β -carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. This method is widely used because β -carotene shows strong biological activity and is a physiologically important compound (Kumazawa et al., 2002). Furthermore, β -carotene is used as a coloring agent in beverages and its discoloration would markedly reduce the quality of those products (Sakanaka and Tachibana, 2006). This fact is

used in the evaluation of antioxidant activity of the *M. vulgare* oil. As shown in Figure 2, the ability of essential oil to inhibit the lipid peroxidation obviously increases with increasing essential oil concentration.

Although, at the same concentration ($70 \mu\text{g/ml}$), the antioxidant activities of the oil were somewhat lower than the BHT ($59.02 \pm 2.00\%$ vs. $77.50 \pm 1.00\%$). The concentrations providing 50% inhibition was expressed by IC_{50} . The IC_{50} of *M. vulgare* essential oil were estimated as $36.30 \mu\text{g/ml}$ compared to BHT ($20.30 \mu\text{g/ml}$). These values revealed that the antioxidant activity of *M. vulgare* oil was still less active than BHT and that *M. vulgare* essential oil inhibited the peroxidation of lipids.

Reducing power antioxidant

Determination of the ferric reducing/antioxidant power is a simple direct test of antioxidant capacity. In this study, assay of reducing activity was based on the reduction of Fe^{3+} /ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. As can be seen from the Figure 3, reducing power of the essential oil of *M. vulgare* increased as increasing essential oil concentration with a good potency to donate electron to reactive free radicals. The reducing power of *M. vulgare* essential oil at $70 \mu\text{g/ml}$ was 0.45 ± 0.032 , which remained significantly lower than that of BHT at the same concentration, used as positive control (1.05 ± 0.01). This

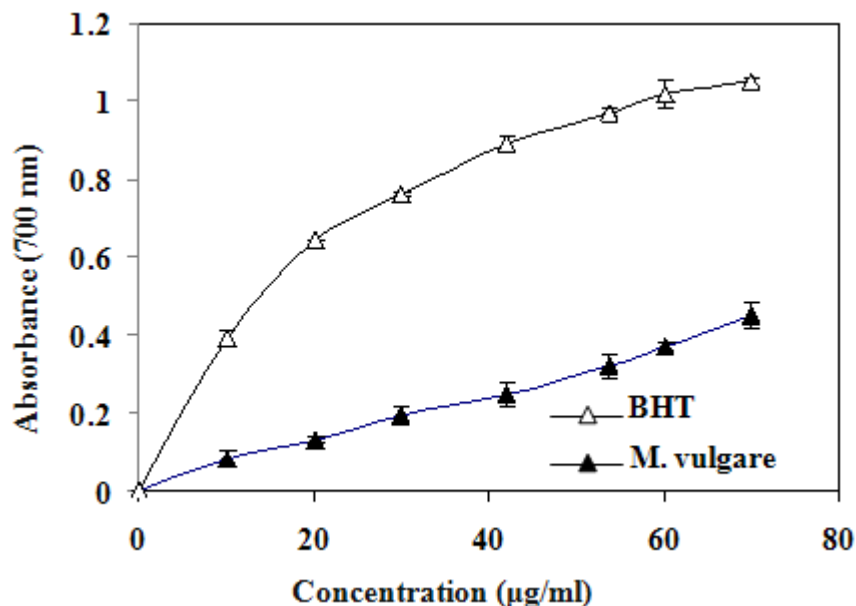


Figure 3. Reducing power of *M. vulgare* and the synthetic antioxidant BHT, in different concentrations.

trend may be attributed to the possibly effect of oil that contain lower amounts of reduction, which could react with radicals to stabilize and terminate radical chain reactions. Indeed, the reducing power property of a compound indicates that it is electron donor, and can reduce the oxidized intermediates of lipid peroxidation processes to convert them to more stable products and consequently terminate radical chain reactions.

From the results, we concluded that in the DPPH assay, the higher ability of essential oils of interest to act as donors of hydrogen atoms or electrons in transformation of DPPH• into its reduced form DPPH-H was attributed to the presence of higher amount of oxygenated monoterpene, the mixture of mono- and sesquiterpene hydrocarbons, and to the presence of biologically active compounds such as β -citronellol, thujones, 1,8-cineole and camphor. In the β -carotene bleaching test, the weaker activity of the *M. vulgare* as compared to BHT was associated with poor content of phenolic components. The lower reducing power of essential oil may be attributed to the less amount of 1,8-cineole, α -pinene and camphene.

Conclusion

Essential oil of *M. vulgare* from Tunisia had significant differences in the chemical composition as compared to the same essential oil from other country, which can be attributed to several factors. The results of the antioxidant activities evaluated by three different methods of assessment, point out strong protective activity against scavenging of DPPH free radicals and β -carotene bleaching test,

and a moderate reducing power effect. These activities found are probably in relation with the structure of the hydroxylated compounds, but a possible synergistic effect among oxygen containing compounds can be suggested too. These results indicated that the essential oil of *M. vulgare* could be considered as a natural food preservatives and enhance the human health as natural antioxidant. Complementary investigations of individual compounds are necessary to assess the effectiveness of this oil in food system, perfumes and pharmaceuticals fields.

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