African Journal of Biotechnology Vol. 7 (10), pp. 1563-1570, 16 May, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Antibacterial and antioxidant activities of *Origanum compactum* essential oil

Bouhdid, S.[1]*, Skali, S. N.[1], Idaomar, M.[1], Zhiri, A.[2], Baudoux, D.[2], Amensour, M.[1] and Abrini, J.[1]

[1]Laboratoire de Biologie et Santé, Equipe de Biotechnologies et Microbiologie Appliquée, Département de Biologie, Faculté des Sciences, Université Abdelmalek Essaâdi, BP 2121 93002, Tétouan. Maroc.
[2]PRANAROM International S. A. 37, Avenue Des Artisans, B-7822, Ghislenghien, Belgique.

Accepted 13 March, 2008

In the present study, essential oil of *Origanum compactum* was analysed and its chemical composition was identified by gas chromatography coupled to mass spectrometry (GC-MS). Among thirty two assayed constituents, carvacrol (30.53%), thymol (27.50%) and its precursor γ-terpinene (18.20%) were found to be the major components. The oil was investigated for its i*n vitro* antibacterial activity against a panel of standard reference strains using well diffusion and broth dilution methods. In solid medium, the oil was found to be remarkably active against all tested strains except *Pseudomona*s which showed resistance. In liquid medium the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBCs) ranged from 0.0078 to 0.25% (v/v). The antioxidant activity was investigated by three different methods; 1,1-diphenyl-2-picryl-hydrasyl (DPPH) radical scavenging assay, β-carotene bleaching test and reducing power. The results of this study revealed evidence that the essential oil of *O. compactum* possesses a good antioxidant effect with all assays; the antioxidant activity is dependent on the oil concentration and can be attributed to the phenolic compounds present in the oil.

Key words: Essential oil, *Origanum compactum*, chemical analysis, antibacterial activity, antioxidant activity.

INTRODUCTION

The reactive oxygen species (ROS) are a group of highly reactive molecules including the free radicals such as superoxyde ion (O_2^-) and hydroxyl radical (OH) as well as the no free radicals such as the hydrogen peroxide (H_2O_2). In human body, ROS are produced through normal aerobic respiration and during inflammatory process. Furthermore, aggressions especially such as radiations, stress, pollution, alcoholism and nicotinism increase the production of ROS (Yildirm et al., 2000). Natural protection against ROS is provided by enzymatic system (superoxyde dismutase, catalase and the selenium glutathion peroxidase) or by chemical molecules (scavengers and antioxidants) (Berger, 2006). The imbalance between ROS production and the defence mechanisms leads to oxidative modification in the intracellular molecules or the cellular membrane. Such alterations can be involved in high number of diseases including diabetes, cancer and cardiovascular diseases. (Kaplan et al., 2007). Moreover, ROS can cause lipids peroxidation in food during manufacturing process and storage which consequently leads to the loss of the food quality and safety (Mau et al., 2004).

Antioxidants are capable to prevent or delay oxidative processes by inhibiting the initiation or propagation of an oxidative chain reaction. They are important in the prevention of many oxidative-stress related diseases (Gerber et al., 2002) and are used to maintain the nutritional quality and to increase the shelf life of food. However, the most frequently used synthetic antioxidants in food industry such as 2,3-tert-butyl-4-methoxy phenol (BHA) and 2,6-di-tert-butyl-4-methyl phenol (BHT) have been suspected to cause undesired health effects (Namiki, 1990). Consequently, a large body of research is now focused on the antioxidant action of natural substances, especially those derived from plants.

On the other hand, there is an impetus for researches for natural antimicrobial agents to use as alternative for food preservation and human remedies. In fact, the excessive and inappropriate use of antibiotics in agriculture or in human's medication to treat infectious

*Corresponding author. E-mail: s_bouhdid@yahoo.fr.

diseases is responsible for the emergence of resistant organisms (Bronzwaer et al., 2002; Wegener, 2003; Väänänen et al., 2006).

Essential oils obtained by steam distillation from aromatic plants have recently gained popularity and scientific interest. Thanks to their flavour and fragrance properties, they are widely used in perfumery, cosmetic, pharmaceutical and food industries. Essential oils are a potentially useful source of molecules with many biological activities. Several of them are qualified as antioxidant and are proposed to replace synthetic antioxidants used in food industry where they do not affect the organoleptic characteristics of the final product. Also, numerous scientific reports have highlighted an important antimicrobial activity of essential oils (Delamare et al., 2007; Oussalah et al., 2007). These biological activities depend on the chemical composition (Chun et al., 2005) which vary according to the geographical origin, the environmental and agronomic conditions, the stage of development of the plant material and the extraction method (Goodner et al., 2006). Therefore, the evaluation of the biological activity of an essential oil should be supplemented with the determination of its chemical composition.

In Morocco, the Lamiaceae family (more than 30 genera and 225 species of which more than 90 are endemics) represents a great ecological and economic interest, as it includes many medicinal, aromatic and honey-plant species (Benabid, 2000). Within this family, the genus *Origanum* is represented by five species of which three are endemic, including *Origanum compactum*.

O. compactum, locally known as za'tar, is mainly used as a culinary condiment and largely employed in popular medicine for the treatment of ailments such as digestive and pulmonary disorders (Belakhdar, 1997; Benabid, 2000; Ennabili et al., 2000). *O. compactum* is also used as preservative for the melted butter (*smen*).

Till date, different extracts of *O. compactum* plant have been tested for their biological activities. The essential oil of *O. compactum* exhibited a significant antifungal action (Bouchra et al., 2003) and the ethyl-acetate extract showed a molluscicidal activity (Hmamouchi et al., 2000).

In the present study, we characterized the chemical constitution of essential oil of *O. compactum* and evaluated its antibacterial and antioxidant activities.

MATERIAL AND METHODS

Essential oil

The essential oil tested in the present study was kindly provided by Pranarom International (Ghislenghien, Belgique). It was extracted by hydrodistillation at a low pressure without chemical descalers and stored at 4°C before use.

Chemical analysis

The chemical composition of essential oil was analyzed by CG-MS using a Hewlett Packard GCD system equipped with a capillary column (HP-Innowax 60 m x 0.5 mm with 0.25µm film thickness). Helium was used as carrier gas (22 psi). GC oven temperature was kept at 50°C for 6 min. Then the temperature was programmed to increase at a rate of 2°C/min, then kept constant at 250°C for 10 min. The injector and detector temperatures were 250 and 280°C, respectively, injection in split mode, volume injected 1 µl of a solution 5/100 in hexane of the oil. Automatic calibration of the masses by autotuning was used in MS. The relative amount of individual components of the total oil is expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NKS library with 75 000 spectra and a personnel aromatic library.

Bacterial strains

Fourteen reference bacterial strains were used: *Escherichia coli* K12 and *Staphylococcus aureus* (Laboratory of Food Microbiology, UCL, Belgium: MBLA), *Pseudomonas aeruginosa* and *Proteus mirabilis* (Institute of hygiene, Rabat, Morocco: IH), *E. coli serovar* O157:H7 CECT 4076, *P.* aeruginosa CECT 110T, *P. aeruginosa* CECT 118, *Pseudomonas fluorescens* CECT 378, *S. aureus* CECT 976, *S. aureus* CECT 794, *Listeria innocua* CECT 4030, *Listeria monocytogenes serovar* 4b CECT 4032 and *Enterococcus faecium* CECT 410 (Spanish Type Culture Collection: CECT), *Bacillus subtilis* DCM 6633 (German Collection of Micro-organisms: DCM).

Bacterial strains were grown in LB broth and incubated at 37°C, and 25°C for *P. fluorescens* CECT 378.

Agar-well diffusion assay

A basal layer was prepared by Muller-Hinton agar. After the agar plates were solidified, sterile 8 mm diameter cylinders were deposited. Six ml of LB medium in superfusion containing 0.8% agar were inoculated by a fresh culture of indicator bacterial strain (a final concentration was 10^6 CFU/ml). After solidification, the wells were filled with 50 µl of pure essential oil. After incubation at appropriate temperature for 24 h, all plates were examined for any zone of growth inhibition, and the diameter of these zones was measured in millimetres. All the tests were performed in duplicate.

Minimum inhibitory and minimum bactericidal concentrations

All tests were performed in LB broth supplemented with bacteriological agar (0.15% (w/v)). Serial twofold dilutions, ranging from 2 to 0.0019% (v/v) of the essential oil, were prepared in a 96-well microtitre plate, volume being 50 µl. Then 50 of LB (0.15%, w/v) inoculated with tested bacteria were added onto microplates. Final cellular concentration was 10^6 cfu/ml. Plates were incubated at 37°C for 18 h. At that time, 5 µl of resazurin were added to the wells. After incubation at appropriate temperature for 2 h, the MIC was then determined as the lowest essential oil concentration prevented change of colouring of resazurin.

The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h. It is determined in broth dilution tests by subculturing 10 µl from negative wells on PCA medium.

Reducing power

The reducing power was determined according to the method of Oyaizu (1986). 1 ml of essential oil dilution was mixed with 2.5 ml of the phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferri-

Table 1. Quantitative and qualitative composition of *Origanum compactum* essential oil.

Compound	RT (min)	PA (%)
α-Pinene	15.4	0.71
α-Thuyene	15.7	0.98
β-Myrcene	25.4	1.87
α-Phellandrene	25.7	0.25
α-Terpinene	26.8	2.59
Limonene	28.3	0.37
1,8-Cineole	29.0	0.33
β-Phellandrene	29.1	0.33
γ-Terpinene	32.1	18.20
3-Octanone	32.0	0.15
P-Cymene	34.0	7.89
Terpinolene	34.9	0.12
1-Octen-3-ol	46.6	0.36
Trans-thuyanol	47.8	0.12
Camphre	52.6	0.08
Linalol	53.4	1.73
Cis-thuyanol	53.9	0.08
Terpinene-4-ol	57.9	0.65
β-Caryophyllene	58.0	2.85
Pulegone	61.5	0.36
α-Humulene	63.0	0.18
Neral	63.3	0.15
α-Terpineol	63.9	0.60
Borneol	64.4	0.25
β-Bisabolene	66.0	0.05
δ-Cadinene	68.2	0.18
γ-Cadinene	68.5	0.12
P-Cymene-8-ol	73.1	0.14
Piperitenone	78.6	0.10
Caryophyllene oxide	82.2	0.10
Thymol	91.5	27.50
Carvacrol	93.1	30.53
Total		99.92

RT: Retention time in minutes, PA: peak area.

cyanide (10 g/l). The mixture was incubated at 50°C for 20 min. 2.5 ml of the trichloroacetic acid (100 g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1 g/l) and the absorbance was measured at 700 nm. Ascorbic acid was used as positive control. Increased absorbance indicated increased reducing power.

DPPH free radical scavenging assay

The DPPH radical-scavenging activity was assessed using the method described by Blois, 1958. Briefly, 1 ml of a 1 mM solution of DPPH radical in methanol was mixed with 3 ml of essential oil solution. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. All tests were carried out in triplicate. Ascorbic acid and BHT were used as positive control. Inhibition of free radical DPPH in percent was calculated as follow:

$$\% \text{ DPPH radical scavenging} = [(OD_{Control} – OD_{Oil})/OD_{Control}] \times 100$$

β-Carotene-linoleic acid assay

This test was carried out according to the spectrophotometric method developed by Miller, 1971, based on the aptitude of various extracts to decrease the oxidative discolouration of β-carotene in an emulsion. 2 mg of β-carotene was dissolved in 10 ml of chloroform. 1 ml of this solution was pipetted into a round-bottom flask containing 20 mg of linoleic acid and 200 mg of Tween 40. Chloroform was completely evaporated using a vacuum evaporator. Then, 50 ml of distilled water was added with vigorous shaking. 5 ml of the obtained emulsion were transferred into different test tubes containing 0.2 ml of the oil dilution. The mixture was then gently mixed and placed in a water bath at 50°C for 120 min. Absorbance at 470 nm was measured every 30 min for 120 min. Blank solution was prepared in a similar way except that addition of β-carotene was omitted. All assays were performed in triplicates. BHT was used as positive control and the antioxidant activity was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(AA_{(120)} - AC_{(120)})/(AC_{(0)} - AC_{(120)})] \times 100$$

$AA_{(120)}$: the absorbance with antioxidant at t = 120 min
$AC_{(120)}$): the absorbance of the control at t = 120 min
$AC_{(0)}$: the absorbance of the control at t = 0 min

Statistical analysis

Results of antioxidant activity are presented as means ± SD of three measurements. Analysis of variance (one way ANOVA), followed by LSD post hoc determinations, were performed using the statistical software STATISTICA 6.0 (1997 edition). Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition

The analysis of O. *compactum* essential oil by gas Chromatography coupled to mass spectrometry revealed thirty-two constituents representing 99.92% of the total oil. Carvacrol (30.53%), thymol (27.50%) and its precursor γ-terpinene (18.20%) were found to be the major components (Table 1). These values are in accordance with those reported in previous studies. Indeed, the analysis of essential oils of *O. compactum* from various Moroccan regions revealed the presence of three main components with variable rates; thymol (0 - 43.4%), carvacrol (3.8 - 71%) and p-cymene (0 - 25.4%) (Van Den Broucke and Lemli, 1980). Similar results were obtained by other authors (Bouchra et al., 2003; Lahlou and Berrada, 2001).

Antibacterial activity

Table 2 summarizes the qualitative (diameters of inhibition zones) and quantitative results (minimal inhibitory

Table 2. Antibacterial activity of the essential oil from *Origanum compactum* in solid and liquid media.

Bacteria[a]	Source	Inhibition zone diameters[b]	MIC[c]	MBC[d]
Staphylococcus aureus	MBLA	27	0.0078	0.0078
Staphylococcus aureus	CECT 976	12	0.0312	0.1250
Staphylococcus aureus	CECT 794	10	0.0312	0.0625
Bacillus subtilis	DCM 6633	25	0.0312	0.0312
Enterococcus faecium	CECT 410	22	0.0312	0.0312
Escherichia coli K12	MBLA	20	0.0625	0.0625
Escherichia coli serovar O157:H7	CECT 4076	20	0.1250	0.2500
Proteus mirabilis	IH	32	0.0625	0.0625
Listeria innocua	CECT 4030	32	0.0312	0.0312
Listeria monocytogenes serovar 4b	CECT 4032	15.5	0.0625	0.1250
Pseudomonas fluorescens	CECT 378	5	0.2500	ND
Pseudomonas aeruginosa	IH	0	>1	>1
Pseudomonas aeruginosa	CECT 110T	2	>1	>1
Pseudomonas aeruginosa	CECT 118	1	>1	>1

[a]Final bacterial density was around 10^6 cfu/ml.
[b]Inhibition zone diameters (mm) produced around the wells by adding 50 µl of essential oil. Values are means of two measurements.
[c]MIC: minimum inhibitory concentration (as % v/v).
[d]MBC: minimum bactericidal concentration(as % v/v).

and bactericidal concentrations) of the antibacterial effect of the essential oil from *O. compactum* on the fourteen reference strains assayed. From the recorded diameters of inhibitory zone, we observed that except for *P. aeruginosa*, which showed obvious resistance, the oil was active against all other bacterial strains. However this activity varies between the test bacteria.

The minimum inhibition concentration (MIC) of the oil ranged from 0.0078 to 0.125% (v/v). In most cases the MIC was equivalent to the MBC indicating a bactericidal action of the oil. *S. aureus* MBLA remains most sensitive of all the bacteria studied with MIC=MBC=0.0078%, v/v.

Interestingly, *L. monocytogenes* serovar 4b, highly pathogenic bacteria associated with case of meningitis as well as *S. aureus* CECT 976, which produce enterotoxin A and beta-haemolysin involved in food poisoning incident were inactivated by tested essential oil with MBC=0.125% v/v. Moreover, *E. coli* serovar H7 O157 which is a cause of serious food poisoning and occasionally death was inhibited by oregano oil at 0.125% (v/v).

It is interesting to point out that the bacteria demonstrating the biggest inhibition zones by diffusion method are not always the ones that present the lowest MIC and MBC values. In fact, the diameter of the growth inhibition zone is affected by the oil solubility and volatility (Hernandez et al., 2005).

Interestingly, the recorded MICs showed that the Gram +ve strains of bacteria are more sensitive to *O. compactum* oil than Gram -ve strains with the MICs less than 0.0312% (v/v). Indeed, the majority of the essential oils assayed for their antibacterial properties showed a more pronounced effect against the Gram +ve bacteria (Wan et al., 1998). The resistance of Gram -ve bacteria to essential oils has been ascribed to their hydrophilic outer membrane which can block the penetration of hydrophobic compounds into target cell membrane (Inouye et al., 2001).

This greater antibacterial activity correlates with the strong content of carvacrol and thymol (30.53 and 27.5% respectively) of *O. compactum* oil analyzed in this study. These phenolic compounds are among the most efficient plant antibacterial agents known to date (Nazer et al., 2005). Several studies demonstrated that Carvacrol and thymol are capable to inactivate bacterial strains in synthetic media as well as in food system (Lambert et al., 2001; Knowles et al., 2005; Valero and Frances, 2006).

Carvacrol can destabilizes the cytoplasmic membrane and acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool eventually lead to cell death (Ultee et al., 2002). Furthermore, it has been reported that essential oils rich on phenolic components possess high levels of antimicrobial activity. However, the compounds present in the greatest proportions are not necessarily responsible for the total activity; the involvement of less abundant constituents should also be considered (Cimanga et al., 2002). Therefore, the activity could be ascribed to the presence of other components such as *p*-cymene, linalool and β-pinene also known to possess an antibacterial activity (Mourey and Canillac, 2002; Rasooli and Mirmostafa, 2002; Bagamboula et al., 2004; Sonbolia et al., 2006).

Moreover, the antimicrobial activity of essential oils may be due to the presence of synergy between the ma-

Table 3. Reducing power of essential oil from *Origanum compactum* and ascorbic acid used as positive control.

Sample	Concentration (mg/l)	Absorbance (700 nm)*
Control	0	0.046 ± 0.002
Origanum compactum	100	0.105 ± 0.012
	250	0.182 ± 0.008
	500	0.271 ± 0.001
	1000	0.484 ± 0.021
Ascorbic acid	10	0.166 ± 0.010
	50	0.714 ± 0.020
	100	1.277 ± 0.049

*Results are means ± SD of triplicate measurements.

jor components and other constituents of the oils leading to various degrees of antimicrobial activity. Accordingly, a synergistic effect against *B. cereus* vegetative cells has been observed between carvacrol and p-cymene at low concentrations. P-Cymene, which possesses relatively weak antibacterial activity, was responsible for the expansion of the bacterial cell membranes to a greater extent compared to carvacrol. By this mechanism p-cymene acts synergistically with carvacrol probably by enabling it to be more easily transported into the cell (Ultee et al., 2002).

In addition, it has been reported that the strains of *E. coli* that are not susceptible to the mixture of linalool-1,8-cineole are likely to be affected by linalool alone (Faleiro et al., 2003), which suggests that possible antagonistic and synergistic effects may occur according to the tested micro-organism.

Antioxidant activity

Due to the chemical complexity of plant extracts, different methods are required to assess their antioxidant activity. In this study, two complementary tests were selected to evaluate the primary and the secondary step of oxidation; the ability to scavenge free radicals and the capacity to inhibit lipid oxidation. The reductive potential was also measured.

Reducing power is an indicator of the antioxidant activity of a given reagent/product. This method evaluates the aptitude of plant extracts to reduce a potassium ferricyanide solution which leads to the increase of absorbance at 700 nm. As shown in Table 3, the reductive potential of all oil concentrations tested was higher when compared to the negative control ($p<0.01$). The reducing power of essential oil from *O. compactum* increased with increasing oil concentrations. However, it remained significantly lower than that of ascorbic acid (100 mg/l) used as positive control ($p<0.01$). Nevertheless, at 100 mg/l ascorbic acid gave an absorbance value 12-fold higher than that expressed by oregano oil (1.277 ± 0.049 and 0.105 ± 0.012, respectively). At 250 mg/l, the essential oil showed a reductive potential comparable to that presented by ascorbic acid at 10 mg/l ($p>0.05$).

The reducing power is associated with antioxidant activity. Indeed, the reducing power property of a compound indicates that it is electron donor, and can reduce the oxidized intermediates of lipid peroxidation processes and convert them to more stable products and consequently terminate radical chain reactions (Yen and Chen, 1995).

DPPH is a stable free radical generally used to determine the ability of compounds to scavenge free radicals. The method is based on the reduction of methanolic solution of DPPH in presence of hydrogen donating molecules. The reduction of DPPH solution is monitored by measurement of absorption at 517 nm. Colour changes from purple to yellow and scavenging activity correspond inversely to the absorption value measured after incubation time (Blois, 1958). Figure 1 illustrates a highly significant decrease of the DPPH radical concentration ($p<0.001$) due to the scavenging activity of each oil concentration and standards. Ascorbic acid and BHT at 100 mg/l showed a high radical scavenging activity (96.15 and 78.04%, respectively) while the oregano oil activity was significantly lower than ascorbic acid ($p<0.001$). 52.30% DPPH radical was scavenged in presence of 1000 mg/l essential oil. We have found that the activity to scavenge DPPH radical increases significantly with increasing oil concentration ($p<0.001$).

This activity is higher when compared to that of *O. syriacum* essential oil (Alma et al., 2003). The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Baumann et al., 1979).

β-Carotene-linoleic acid assay

β-carotene bleaching test is carried out in emulsion and based on the loss of the yellow colour of β-carotene due to its reaction with radicals which are formed due to linoleic acid oxidation. Lipid peroxidation inhibitory activity of oregano oil and standard (Figure 2) showed that BHT (100 mg/l) was highly active ($p<0.001$) and the inhibition of essential oil was concentration dependant ($p<0.001$). These data are consistent with the results obtained using DPPH assay (R^2= 0.9971).

Ascorbic acid, a well known polar antioxidant, showed a high antioxidant capacity by DPPH assay (96.15%), but it is ineffective when tested by β-carotene bleaching test (data not shown). Indeed, the polar molecules remain in aqueous phase and are consequently less efficient in protecting linoleic acid. This fact has been previously reported by other investigators (Fukumoto and Mazza, 2000). The DPPH assay allows the test of both lipophilic and hydrophilic substances while β-carotene bleaching

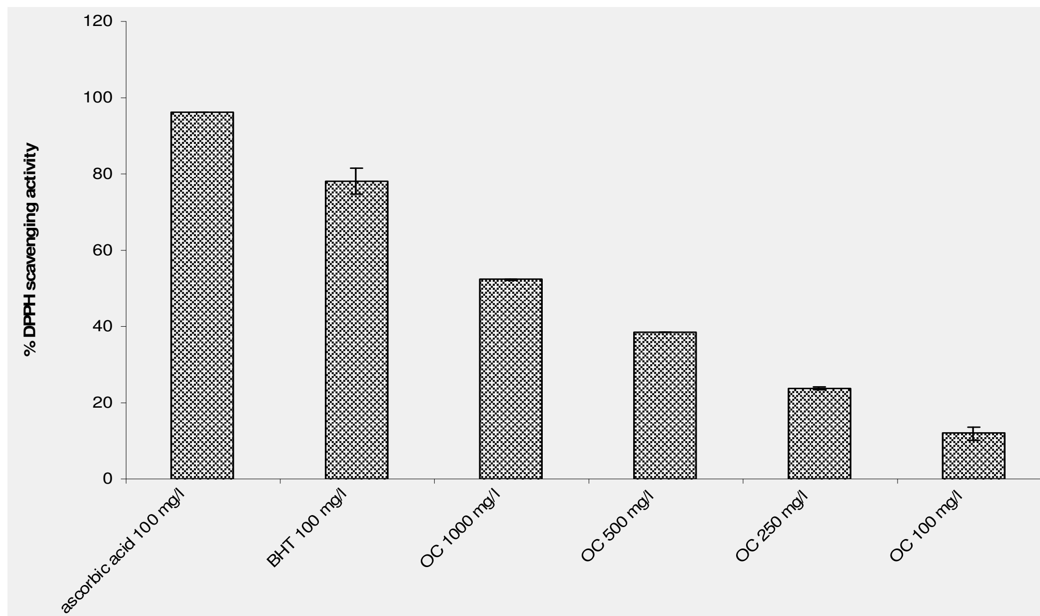

Figure 1. Free radical scavenging activity of *Origanum compactum* (OC) essential oil and positive controls (ascorbic acid and BHT). Scavenging activity was measured using the DPPH radical assay. Data are means of three measurements ± S.D.

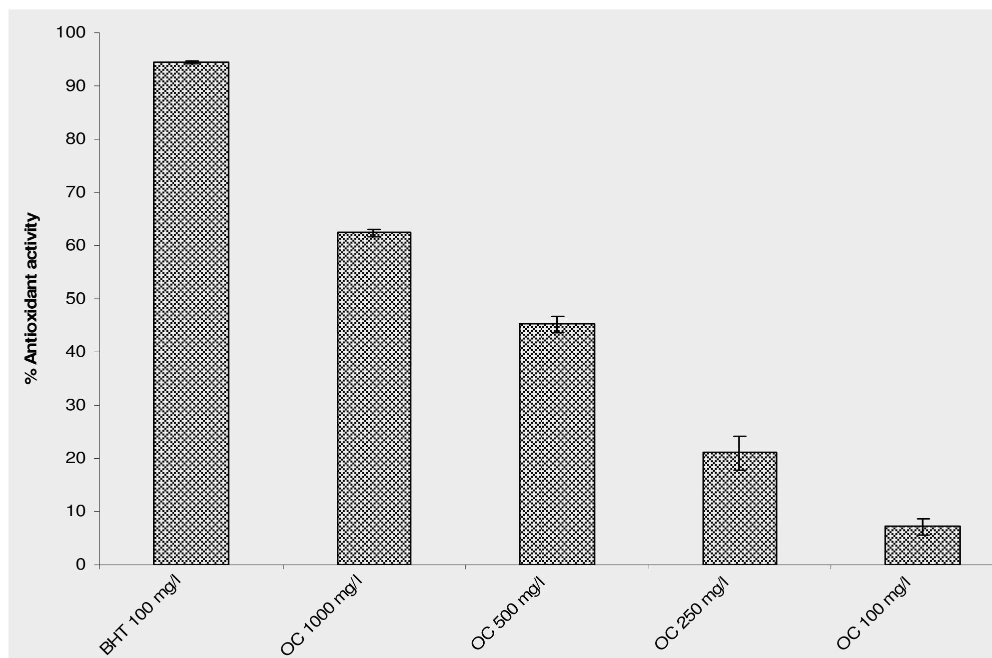

Figure 2. Antioxidant activity of *Origanum compactum* (OC) essential oil and BHT determined by β-carotene bleaching test. Each value is the mean of three measurements ± S.D.

test is dependent on substrate polarity. The high activity revealed by the β-carotene test indicates a good ability to function at the lipid water interface.

Our results revealed that oregano oil possesses a good capacity to scavenge free radicals and to prevent lipid peroxidation, which can be ascribed to the high content of the phenolic compounds in this oil. In fact, various studies highlighted the correlation between the phenolic content and the antioxidant capacity of plant extracts (Gulçin et al., 2004; Chun et al., 2005). Thymol and carvacrol, the main components of this oil, are two phenols known as antioxidant molecules (Juliani and Simon, 2002). This

activity is due to the presence of hydroxyl groups in their chemical structure. P-cymene and linalool have also been demonstrated to possess *in vitro* antioxidant properties (Dorman et al., 1995). A possible synergy between the various components of oil must also be taken into account (Tiwari, 2001).

Conclusion

In conclusion, *O. compactum* essential oil showed a good *in vitro* antioxidant and antibacterial effect. This activity could be attributed to the phenolic compounds present in this oil. The result presented here may explain the traditional use of this plant. Complementary investi-gations are necessary to assess the effectiveness of this oil in food system.

ACKNOWLEDGEMENTS

This work was supported by PRANAROM International Company and the Minister of Wallone region, Belgium. Acknowledgements to Dr. Bouchra Edderkaoui for valuable comments and corrections of the manuscript.

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