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AROMATHÉRAPIE

Chemical Composition, Antioxidant, and Antimicrobial Activities of *Rosmarinus officinalis* Essential Oil From Moroccan Middle Atlas

Composition chimique, activité antioxydante et antimicrobienne de l'huile essentielle de *Rosmarinus officinalis* cueilli dans le Moyen Atlas marocain

T. El Kamli · M. El Hamdani · N. Eloutassi · F. Errachidi · R. Chabir · A. Bour

Abstract This study was designed to evaluate antioxidant and antibacterial activities of essential oils from *Rosmarinus officinalis* obtained by three different extraction methods: DA: Artisanal distillation; the essential oils were obtained directly from small cooperatives using a very traditional (nonindustrial) method and based on the technique of Steam distillation; DI: Industrial distillation which is also based on steam distillation; and DC: hydrodistillation through Clevenger apparatus laboratory. The chemical analyses were carried out with gas chromatography/mass spectrometry (GC/MS); they identified 16 components representing more than 99.89% of the essential oil and indicate that the chemotype is 1,8-Cineole and varies according to the method used (DI: 49.09%, DA: 42.12%, and DC: 53.21%). The antioxidant activity was evaluated by the β-carotene bleaching test measuring percent inhibition of peroxidation in linoleic acid system. The disc diffusion and modified resazurin microtiter-plate assays were used, respectively, to evaluate the inhibition zones (IZ) and minimum inhibitory concentration (MIC) of *Rosmarinus officinalis* essential oil. In general, *Rosmarinus officinalis* L. essential oil showed a lower antioxidant and antimicrobial activity than 1,8-Cineole the major component of the essential oil.

Keywords *Rosmarinus officinalis* · Essential oil · Antioxidant · Resazurin assay

Résumé Cette étude a été conçue pour évaluer les activités antioxydantes et antibactériennes des huiles essentielles de *Rosmarinus officinalis* obtenues par trois méthodes d'extraction différentes : 1) DA : distillation artisanale ; les huiles essentielles ont été obtenues directement auprès de petites coopératives selon une méthode très traditionnelle (non industrielle) et fondée sur la technique de distillation à la vapeur ; 2) DI : distillation industrielle qui est également fondée sur la distillation à la vapeur ; 3) DC : hydrodistillation par le montage Clevenger dans le laboratoire. Les analyses chimiques ont été effectuées par chromatographie en phase gazeuse/spectrométrie de masse (CG/SM), elles ont identifié 16 composants représentant plus de 99,89 % de l'huile essentielle et indiquent que le chémotype est 1,8 cinéole et varie selon la méthode (DI : 49,09 % ; DA : 42,12 % et DC : 53,21 %). L'activité antioxydante a été évaluée par le test de blanchiment au β-carotène mesurant le pourcentage d'inhibition de la peroxydation dans le système d'acide linoléique. Les essais de diffusion sur disque et de microplaque de résazurine modifiée ont été utilisés respectivement pour évaluer les zones d'inhibition (IZ) et la concentration minimale inhibitrice (MIC) de l'huile essentielle de *Rosmarinus officinalis*. En général, l'huile essentielle de *Rosmarinus officinalis* L. a montré une activité antioxydante et antimicrobienne inférieure à celle de 1-8 cinéole, le principal composant de l'huile essentielle.

Mots clés *Rosmarinus officinalis* · Huile essentielle · Antioxidant · Resazurin

T. El Kamli (✉) · A. Bour
Laboratory of Biological Tests,
Food and Nutritional Transition Team (ETAN),
Ibn Tofaïl University Kenitra, Morocco
e-mail : elkamlit@yahoo.fr

M. El Hamdani
National Institute of Agronomic Research (INRA),
Rabat, Morocco

N. Eloutassi
Didactic Laboratory of Science and Educational Innovation,
Regional Center of the Trades of Education and Training,
Fes, Morocco

F. Errachidi
Laboratory of Physiology and Molecular Genetics,
Faculty of Sciences AïnChock, Casablanca, Morocco

R. Chabir
Laboratory of Physiology and Nutrition,
Faculty of Medicine and Pharmacy of Fez, Morocco

Introduction

In recent years, the demand for essential oils from medicinal plants has increased, rosemary (*Rosmarinus officinalis* L.) essential oil is one of the most requested and exploited oils. Recent studies have revealed that rosemary essential oil has antibacterial effects [1], antifungal effects [2], insecticidal [3], anticancer [4], antispasmolytic [5], anti-inflammatory, and antinociceptive effects [6], as well as antioxidant properties. All these activities show that rosemary is a source of active ingredients that can be exploited in the pharmaceutical and medical applications. The objective of this work is the qualitative and quantitative evaluation of the major compounds in the essential oils of rosemary extracted in the industrial and artisanal units. This study also aims to evaluate their antioxidant and antimicrobial activities.

Materials and Methods

Study Zone

The skoura m'daz site is located in the central part of Northern Morocco, between 33° 10′ and 33° 30′ north latitude and 4° 10′ and 4° 50′ east longitude.

Plant Materials

Plant materials (aerial parts of *Rosmarinus officinalis*) were collected in skoura m'daz site, in the former region of Fes-Boulemane (central part of Northern Morocco), in March 2014.

Hydrodistillation

The essential oils of rosemary were obtained by three different extraction methods: DA: artisanal distillation, the essential oils were obtained directly from small cooperatives using a very traditional (nonindustrial) method and based on the technique of steam distillation; DI: industrial distillation which is also based on steam distillation; and DC: hydrodistillation by Clevenger apparatus laboratory. The water content of the plant material was determined before distillation. The collected plant material was dried in a dark, shaded area until the weight stabilized (7 days). The yield of Essential oils is expressed relative to the dry matter. The distillation pressure used in the three techniques is carried out under normal atmospheric conditions.

GC/MS Analysis Conditions

The essential oil was analyzed using an Agilent Technologies 6890N Network GC system equipped with a flame ionization detector and HP-5MS capillary column (30 m × 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 program provided by the gas chromatograph manufacturer. The composition was reported as a relative percentage of the total peak area. The constituents of the volatile oils were also identified by comparing their GC retention indices. A mixture of aliphatic hydrocarbons (C8–C24) in hexane (Sigma–Aldrich—St. Louis, USA) was injected as under the abovementioned temperature program to calculate the retention indices [7].

β-carotene Bleaching Assay

This method evaluates the capacity of the oil to reduce the oxidative loss of β-carotene in a β-carotene linoleic acid emulsion [8]. Ten milligrams of β-carotene was dissolved in 10 ml of chloroform ($CHCl_3$), and an aliquot of 0.2 ml of this solution was added into a boiling flask containing 20 mg of linoleic acid and 200 mg of Tween 40. The chloroform was removed using a rotary evaporator at 40 °C for 5 min. Fifty milliliters of distilled water was slowly added to the residue with vigorous agitation, to form an emulsion. The emulsion was added to a tube containing 0.2 ml of essential oil. The absorbance was immediately measured at 470 nm, and the test emulsion was incubated in a water bath at 50 °C for 5 min; then, the absorbance was measured again. BHT was used as the positive control; for negative control, the essential oils were substituted with equal volume of ethanol. The antioxidant activity (%) of the oil was evaluated in terms of the bleaching of the β-carotene using the following formula:

$$\% \text{ inhibition} = [(at - ct) / (co - ct)] \times 100$$

where at and ct are the absorbencies of the essential oils and positive control, respectively, after incubation for 5 min, and co is the absorbance value of the positive control measured at zero time during the incubation. The essential oil concentration providing 50% antioxidant activity (IC_{50}) was calculated by plotting antioxidant percentage against oil concentration.

Antimicrobial Activity

Disc Diffusion Method

The antimicrobial studies were evaluated using the agar diffusion technique in petri dishes [9]; this activity was carried

out against five bacterial strains: *Staphylococcus aureus CIP 483*, *Bacillus subtilis CIP 5262, Escherichia coli CIP 53126, Pseudomonas aeruginosa CIP 82118*, and *Salmonella enteric* CIP 8039. Hundred microlitres of suspension contains approximatively 5.105 colony-forming units (CFU)/ml of bacteria cells on tryptic soy agar (TSA). The sterile filter discs of 6 mm diameter were separately impregnated with 15 µl of essential oils and placed on the agar which had previously been inoculated with the tested microorganism. These petri dishes were incubated overnight at 37 °C [10]. After incubation, all plates were checked for inhibition zones and the diameters were measured in millimeters; chloramphenicol antibiotics (50 mg/ml) and DMSO were used as positive and negative controls, respectively.

Resazurin Microtiter-Plate Assay

For the minimum inhibitory concentration (MIC) of the essential oils, a modified resazurin microtiter-plate assay was used [11]. Hundred microlitres of essential oil solution (2.5 mg/ml, w/v in DMSO 90%) was pipetted into the first row of the 96-well plates; then to all other wells of microtiter plates, we added 100 µl of tryptic soy broth. A serial dilution was achieved by transferring 100 µl test material from the first row to the subsequent wells in the next row of the same column, so that each well had 100 µl of test material in serially descending concentrations. Then 10 µl of bacterial suspension (108 cfu/ml) was added to each well. The microplates were placed in an incubator set at 37 °C for 24 h. Each plate had a set of controls: a column with an antibiotic as positive control (chloramphenicol in serial dilution—50 mg/ml), a column with all solutions except the test material, and a column with all solutions with the exception of the bacterial suspension. Finally, the resazurin solution was prepared by dissolving 270 mg of resazurin powder in 40 ml sterile, distilled water [12]. A volume of 10 µl of resazurin solution as indicator was added in each well after incubation. The microplates were placed again in an incubator at 37 °C for just 2 h. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value [13].

Results and Discussion

Composition of *Rosmarinus officinalis* Essential Oil

The chemical composition of *Rosmarinus officinalis* essential oil obtained by the three methods DI, DA, and DC is described in table 1.

The major compound in the three essential oils studied is 1,8-Cineole (DI: 49.09%, DA: 42.12%, and DC: 53.21%) followed by Camphor (DI: 17.93%, DA: 22.68%, and DC: 22.53%) and alpha-pinene (DI: 9.49%, DA: 10.11%, and DC: 8,15%). We found that the nature of the constituents is the same for the three essential types of oil, and that monoterpenes represent the totality of the compounds detected with the dominance of oxygenated monoterpenes (69% to 85%) against monoterpene hydrocarbons (13% to 24%); however, the content of the compounds differs according to the extraction method.

The essential oil of rosemary extracted in the laboratory by Clevenger apparatus gave an essential oil richer in 1,8-Cineole (53.21%), alpha-terpineol (5.04%), and borneol (2.80%). However, essential oil obtained by the artisanal method has a higher content of camphor (22.68%), alpha-pinene (10.11), beta-pinene (8.03%), and bornyl acetate (5.46%).

Antioxidant Activity

The antioxidant activities of the essential oil of *Rosmarinus officinalis* L. and its main components studied here were determined by β-carotene bleaching test.

The result of the lipid peroxidation inhibitory activity of the essentials oils assessed by β-carotene bleaching test is shown in figure 1. β-carotene usually undergoes rapid discoloration in the absence of an antioxidant; this is because the oxidation of β-carotene and linoleic acid generates free radicals [14]. The linoleic acid-free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecule; hence, β-carotene is oxidized, losing its orange color which is then monitored spectrophotometrically [15]. Our three essential oil samples from *Rosmarinus officinalis* DI, DA, and DC showed a high antioxidant capacity; in fact, they were able to protect 78%, 76%, and 82%, respectively, of β-carotene from oxidation against 84% with BHT. The 1,8-Cineole, a major component of the essential oil, has also demonstrated a strong antioxidant activity with 82% which is very similar to BHT. Table 2 shows the concentration inhibiting 50% (IC_{50}) of beta-carotene by *Rosmarinus officinalis* essential oils. There is a difference between the three extraction methods; the value of IC_{50} for DI method represented by 20.01 ± 0.09 is smaller than DA method with 24.23 ± 0.17; both values are higher than IC_{50} obtained by DC method which is 17.57 ± 0.14. IC_{50} of 1,8-Cineole and BHT equal, respectively, to 16.08 ± 1.3 and 11.51 ± 0.07 are lower than IC_{50} rosemary essential oils (Table 2, Fig. 1).

It is very difficult to attribute the antioxidant effect of a total essential oil to one or a few active principles because an essential oil always contains a mixture of different chemical compounds. In addition to the major compounds, minor molecules may make significant contributions to the oil activity. Therefore, the antioxidant property of rosemary

essential oils might be the combined activities of the various major and minor components of these oils.

In plant essential oils, oxygenated monoterpenes and monoterpene hydrocarbons are mainly responsible for the antioxidant potential [16] and were the main components of *Rosmarinus officinalis* essential oil [17].

Antibacterial Activity

Rosmarinus officinalis essential oil exhibited varying antibacterial activity as is shown by the inhibition zones (IZ) in figure 2. The results from the disc diffusion assay indicated that the tested essential oil showed higher antibacterial activity against gram-positive bacteria (IZ 17–27) than against gram-negative bacteria (IZ 15–23). It can be seen, also, that 1,8-Cineol, a principal component of *Rosmarinus officinalis*, had a better antibacterial activity than *Rosmarinus officinalis* essential oil (Table 3).

These results compared with other previous studies showed a high mean value (IZ 18.0–24.2) against gram-positive bacteria and (IZ 12.8–17.5) against gram-negative bacteria inhibition zones [13], a less mean value too with an average of 16–24 mm diameter of inhibition zone [18]. We can conclude that *Rosmarinus officinalis* essential oil collected from Moroccan middle atlas (Skoura M'daz) depicted better antibacterial activity as compared with these published papers. This might be due to a small variation in the chemical profile of *Rosmarinus officinalis* essential oil.

In the present study, we used the modified resazurin microtiter-plate assay to evaluate the antimicrobial activity

Table 1 Chemical composition, retention indices, and percentage composition of the *Rosmarinus officinalis* essential oils

Cmpds	Components[a]	RI[b]	Molecular formula	MW[c]	Relative percentage		
					DI	DA	DC
1	Alpha-pinene	939	C10H16	136	9.49	10.11	815
2	Camphene	954	C10H16	136	4.53	4.47	417
3	Beta-pinene	979	C10H16	136	3.72	8.03	0.19
4	Alpha-Terpinene	1017	C10H16	136	0.18	Tr	Tr
5	p-Cymene	1025	C10H14	134	2.35	2.19	0.74
6	Limonene	1028	C10H16	136	Tr	Tr	Tr
7	1,8-Cineole	1030	C10H18O	154	49.09	42.12	53.21
8	Beta-myrcene	1048	C10H18O	154	2.54	1.32	1.94
9	Linalool	1097	C10H18O	154	0.13	0.21	0.10
10	Camphor	1146	C10H16O	152	17.93	22.68	22.53
11	Borneol	1169	C10H18O	154	1.17	0.96	2.80
12	Alpha-Terpineol	1199	C10H18O	154	3.24	1.76	5.04
13	Verbenone	1205	C10H14O	150	0.61	0.39	0.11
14	Bornyl Acetate	1289	C12H20O2	196	4.89	5.46	1.02
15	Beta-Caryophyllene	1419	C15H24	204	Tr	0.11	Tr
16	Alpha-Caryophyllene	1423	C15H24	204	0.05	0.08	Tr
	Total				99.92	99.89	99.99

[a] Constituents listed in order of elution in DB-5 column

[b] RI: Retention index calculation using a temperature program according to *n*-alkanes; Tr: traces (% < 0.01)

[c] MW: molecular weight

Table 2 The IC50 values of DI, DA, DC *Rosmarinus officinalis* L. essential oil, 1,8-Cineole, and BHT by β-carotene bleaching test

	DI	DA	DC	Cineole	BHT
The IC50 values (mg/ml)	20.01 ± 0.09	24.23 ± 0.17	18.12 ± 0.14	16.10 ± 0.08	11.51 ± 0.07

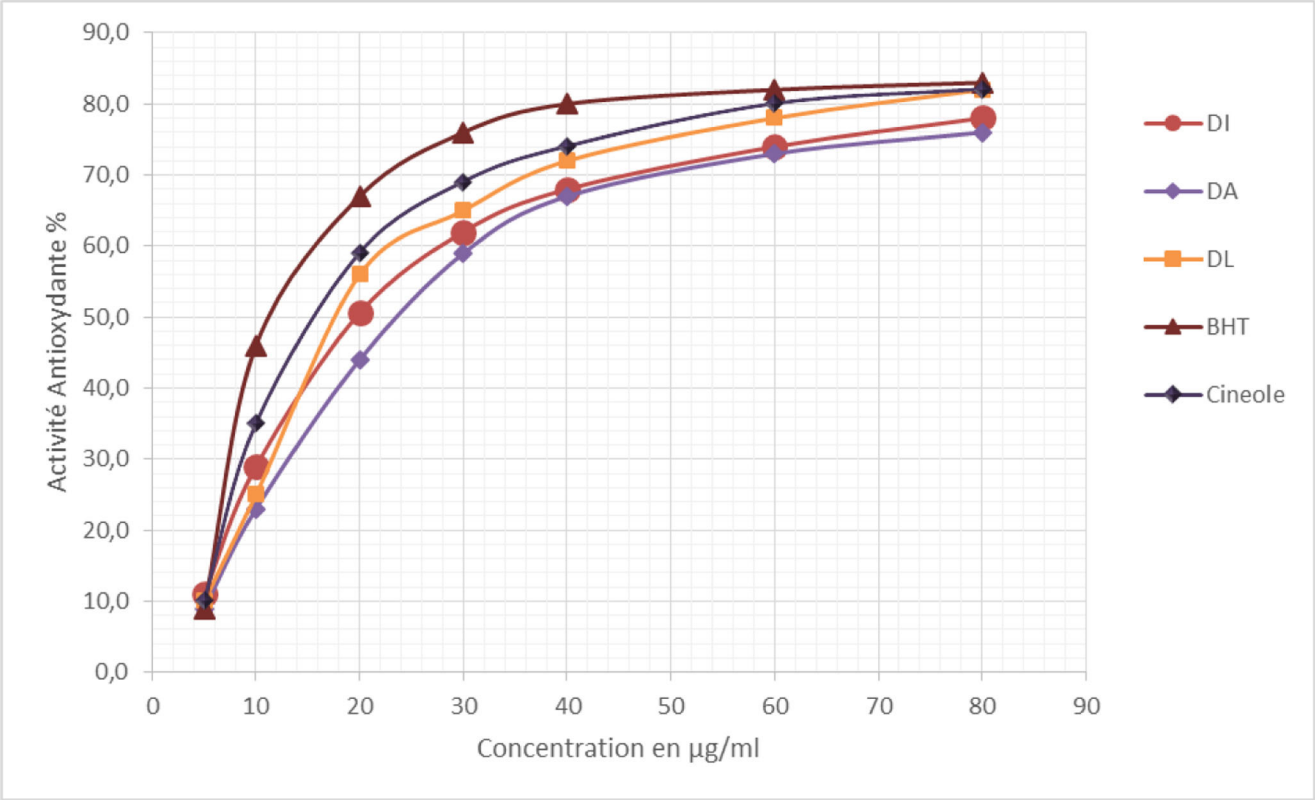

Fig. 1 Antioxidant activity percentage of *Rosmarinus officinalis* L. DI, DA, and DC essential oil and 1,8-Cineole by β-carotene bleaching test by comparison with the reference—butylated hydroxytoluene (BHT)

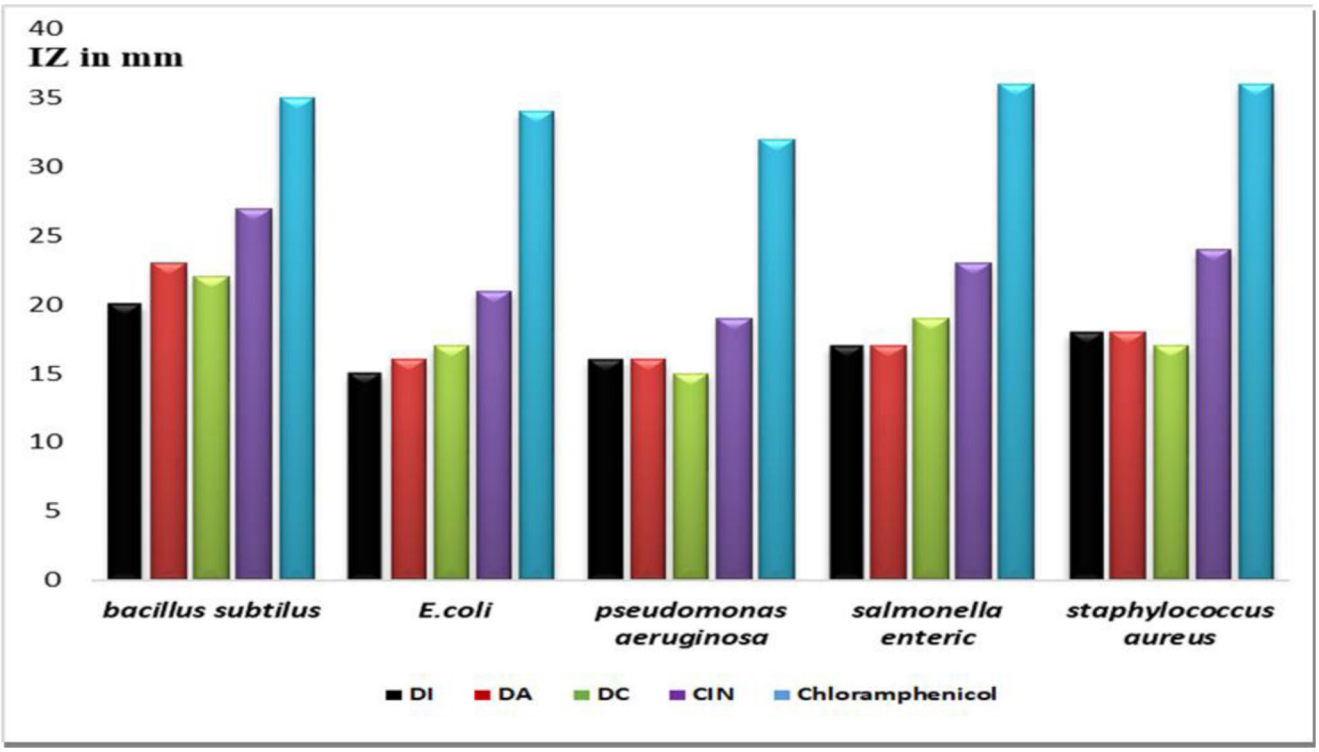

Fig. 2 Antibacterial activity [inhibition zone (IZ) measured in mm] of DA, DI, and DC essential oil of *Rosmarinus officinalis* against pathogenic bacteria

Table 3 Antibacterial activity minimum inhibitory concentration (CMI) of DA, DI, and DC *Rosmarinus officinalis* essential oil against pathogenic bacteria presented

	DA	DI	DC	1,8-Cineole	Chloramphenicol
Escherichia coli CIP 53126	0.54	0.54	0.55	0.68	0.050
Staphylococcus aureus CIP 483	0.34	0.33	0.34	0.41	0.095
Salmonella enterica CIP 8039	0.86	0.86	0.89	0.97	0.050
Bacillus subtilis CIP 5262	0.49	0.51	0.51	0.57	0.095
Pseudomonas aeruginosa CIP 82118	1.27	1.27	1.28	1.43	0.05

of essential oils. This method provided reproducible and accurate results and allowed direct comparison of the antibacterial activity of the tested essential oils.

The average of minimum inhibitory concentration of *Rosmarinus officinalis* essential oil varied between 0.34 mg/ml against *staphylococcus aureus* and 1.28 mg/ml against *pseudomonas aeruginosa*. Our results are in good agreement with the finding of [19] who reported that gram-positive bacteria (*staphylococcus aureus*) are more sensitive to plant essential oils than gram-negative bacteria (especially *Escherichia coli*). Furthermore, the antibacterial activity of essential oils may be due to the presence of alcohol and phenolic compounds that have an antibacterial power [20].

Chloramphenicol revealed stronger antibacterial activity with large IZ (36 mm) and small MIC values (0.05 mg/ml).

Conclusion

Consumer demand for healthy food products provides an opportunity to develop antioxidants as new functional foods. Following this idea, the results of the present study indicate that *Rosmarinus officinalis* essential oils collected from Morocco possessed very good antimicrobial potential as well as considerable antioxidant activity. The investigated essential oil may be used for the preservation of processed foods as well as pharmaceutical and natural therapies for the treatment of infectious diseases in humans and plants.

Conflicts of interests: the authors have no conflicts of interests to declare.

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