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Original Article

CHEMICAL COMPOSITION AND ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *THYMUS SATUREIOIDES* COSS. ESSENTIAL OIL

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ABSTRACT

Objective: *Thymus satureioides* is a well-known aromatic perennial shrub widely used throughout the Mediterranean basin as a culinary herb, in traditional medicine for the treatment of a variety of diseases, and as a natural preservative ingredient in the food industry. The aim of this study was to analyze the chemical composition of *T. Satureioides* essential oil and to assess its antibacterial and antioxidant activities.

Methods: The chemical composition of the essential oil was investigated by Gas chromatography-mass spectrometry (GC-MS). The essential oil was evaluated for its antibacterial activity against Gram-positive and Gram-negative bacteria using agar diffusion method and macro-broth dilution. The antioxidant activity was tested by three different methods, namely DPPH free radical scavenging, β-carotene/linoleic acid and TBARS assays.

Results: Thirty compounds were identified, representing 85.52% of the total composition of this essential oil. Borneol (26.45%) and thymol (11.24%) were the major components. The oil had a bactericidal activity against all tested bacteria, with the exception of *Pseudomonas aeruginosa*. In addition, the *T. satureioides* essential oil revealed strong antioxidant activity in all conducted assays.

Conclusion: The findings suggest that essential oil of *T. satureioides* may be used as an alternative to synthetic antibiotics and antioxidants.

Keywords: *Thymus satureioides*, Chemical composition, Antibacterial activity, Antioxidant activity

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INTRODUCTION

Since their discovery, antibiotics have led to great advances in therapy and contributed to the development of modern medicine. The effectiveness of antibiotic therapy in controlling and limiting the spread of pathogens has raised hopes for eradicating all infectious diseases. Unfortunately, the emergence of antibiotic–resistant bacteria has decreased this wave of optimism. The rise of resistance is due to the immoderate and inappropriate use of antibiotics. Infections caused by resistant microorganisms often fail to respond to the conventional treatment, engendering prolonged illness, a rise in health care costs, and increased risk of death [1].

The oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Lipid peroxidation in fats and fatty foods deteriorates their quality, causes chemical spoilage, and generates free radicals and reactive oxygen species. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases [2]. Synthetic antioxidants, such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and tertbutyl hydroquinone (TBHQ), are suspected to cause negative health effects [3]. Thus, more attention has been focused recently on the development of natural, safe and efficient antioxidant compounds. The plant kingdom is a rich source of a wide range of natural products. About three-quarter of the world's population relies on plants and plant extracts for their healthcare [4].

Thymus species are aromatic plants of the Mediterranean flora with a wide range of biological properties that are commonly used as spices and as traditional medicine remedies [5-7]. Several studies have shown that they have strong antibacterial, antifungal, antiviral, and antioxidant activities [5, 8, 9]. Within the *Thymus* genus, *Thymus satureioides* is a North African species typical of arid habitats, which is used in Moroccan folk medicine to treat whooping cough, bronchitis, and rheumatism [10]. *T. satureioides* has been reported to possess several medicinal properties including analgesic [6], microbiocidal [11, 12], larvicidal [12], hypolipidemic and antioxidant activities [14]. These properties have been attributed to the essential oil (EO) contained in the species, as well as the presence of non-volatile compounds including polyphenols and flavonoids [15].

The aim of this study was to determine the chemical composition of the essential oil of *T. satureioides* and to investigate its antibacterial and antioxidant activities.

MATERIALS AND METHODS

Essential oil

The *Thymus satureioides* was collected from Taroudant (South of Morocco, its geographical coordinates are 30 °28'48" North, 8 °51'36" West) during April-July 2009. The essential oil was produced by *Santis* Company using steam distillation from the leaves and stems of *Thymus satureioides*.

Chemicals and reagents

Methanol, β-carotene, chloroform, linoleic acid, Tween 40, potassium chloride, Trichloroacétique acid (TCA), n-butanol, BHT (Butylated hydroxytoluene) and DPPH (2,2-diphenyl-1-picryl-hydrazyl) were purchased from Sigma-Aldrich, Germany. Ascorbic acid and thiobarbituric acid were obtained from Merck, Germany. All chemicals used were of analytical grade.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry analyses were performed with a gas chromatograph (Trace GC Ultra) coupled to a mass spectrometer (Polaris Q ion trap MS). The column used was a VB-5 capillary column (Methylpolysiloxane with 5% phenyl; 30 m x 0.25 mm x 0.25 µm film thickness).

The column temperature was initially kept at 40 °C for 2 min, then gradually increased to 180 °C at a rate of 4 °C/min and finally raised to 300 °C for 2 min at 20 °C/min. Fragmentation was performed by electron impact at 70 eV. The injection volume was 1 µl. Samples were injected in the split mode using helium as a carrier gas (1.4 ml/min).

Antibacterial activity

Bacterial strains

The essential oil was tested against eight bacteria. Three standard strains: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) (Microbiology Laboratory, Faculty of Pharmacy, University of Barcelona, Spain) and five clinically isolated strains: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Enterococcus faecium* (Microbiology Laboratory, CHU Ibn Rochd, Casablanca, Morocco).

Antibacterial screening

The agar diffusion method was used for the determination of antibacterial activity of essential oil [16]. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of essential oil and transferred into the Luria-Bertani Agar present in Petri dishes which had been previously seeded by spreading 1 ml of bacterial suspension adjusted to 10^6 CFU/ml. The Petri dishes were incubated at 37 °C for 24 h. The diameter of inhibition zones was measured in millimeters. Amoxicillin (25 µg/disc) was used as a standard antibacterial drug. All tests were performed in triplicate.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth macrodilution method was employed to determine the minimum inhibitory concentration (MIC) [17]. Serial dilutions of essential oil ranging from 20 µl/ml to 0.15 µl/ml were prepared in test tubes containing Luria-Bertani Broth with 0.15% agar. Each tube was inoculated with a bacterial suspension adjusted to 10^6 CFU/ml. Controls containing medium with either microorganisms or the essential oil alone were included. The tubes were then incubated at 37 °C for 24 h. MIC values were defined as the lowest concentrations of essential oil at which the absence of growth was recorded. To determine the minimum bactericidal concentration (MBC), 10 µl from tubes in which bacterial growth was not observed, was spread on Muller Hinton Agar and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of essential oil at which the incubated microorganism was completely killed. Each test was performed in triplicate.

Antioxidant activity

DPPH free radical scavenging activity

The hydrogen atom or electron donation abilities of the essential oil were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [18]. 100 µl of each concentration of essential oil was mixed with 1.3 ml of DPPH methanol solution (0.004%). After incubation in darkness at ambient temperature, the absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1800). Ascorbic acid was used as a positive control and methanol as a negative control. All analyses were carried out in triplicate. The percent inhibition of the DPPH radical was calculated according to the formula:

$$\% \text{ Inhibition} = \left(\frac{Ab - Aa}{Ab}\right) \times 100$$

Where Ab is the absorbance of the blank sample and Aa is the absorbance of the essential oil. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against the concentration of the sample.

β-carotene/linoleic acid assay

The β-carotene/linoleic acid test evaluates the ability of a product to inhibit the formation of conjugated diene hydroperoxides resulting from oxidation of linoleic acid [19]. A mixture of β-carotene and linoleic acid was prepared by adding 0.5 mg β-carotene to 1 ml chloroform, 25 µl linoleic acids, and 200 mg Tween 40. The chloroform was then completely evaporated, and 100 ml of oxygenated distilled water was added to the mixture with vigorous stirring. 350 µl of various concentrations of essential oil were added to 2.5 ml of the above emulsion in test tubes. BHT was used as a positive control and methanol as a negative control. The absorbance values were measured at 490 nm after 1 h, 2 h, 3 h, 4 h, 6 h, 24 h and 48 h of incubation at ambient temperature. Antioxidant activity (AA) was calculated using the formula:

$$AA\% = (Ae/Ac) \times 100$$

Where Ae is the absorbance in the presence of essential oil and Ac is the absorbance in the presence of BHT (positive control). All tests were carried out in triplicate.

TBARS assay

The TBARS assay quantifies oxidative stress by measuring the peroxidative damage to lipids that occurs with free radical generation. Free radical damage to lipids results in the production of malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) under conditions of high temperature and acidity generating a chromogen that can be used to quantify oxidative damage (lipid peroxidation) [20].

The assay was carried out according to the method reported by [21]. The egg yolk (10%, v/v) solution was prepared in KCl (1.15%, w/v). It was homogenized for 30 sec and ultrasonicated for 5 min. 500 µl of the homogenate was added to 100 µl of essential oil dissolved in methanol, and the volume was brought up to 1 ml with distilled water. 0.5 ml of this mixture was added to 0.5 ml of trichloroacetic acid (20%, v/v) and 1 ml of TBA (0.67%). Samples were vortexed and left in a 100 °C water bath for 15 min. When they had cooled, 4 ml of n-butanol was added. After centrifugation at 3000 rpm for 15 min, the absorbance was measured at 530 nm. Antioxidant Index percentage (AI %) was calculated using the following formula:

$$AI\% = (A0 - A1/A0) \times 100$$

Where A0 is the absorbance of the completely oxidized sample and A1 is the absorbance of the test sample.

Statistical analysis

The antioxidant and antibacterial results are stated in mean±standard deviation. Analysis of variance was performed by ANOVA procedures (SPSS 20.0 for Windows). Significant differences between means were determined by Tukey post hoc tests. Differences at $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Chemical composition

As shown in table 1, thirty compounds were identified, representing 85.52% of the total composition of Thymus satureioides essential oil. The predominant components were borneol (26.45%) and thymol (11.24%).

These results are in accordance with those reported by [22] for the *T. satureioides* growing in the Agoundis Valley (Morocco) in which the borneol content ranged between 22.67 and 37.47%. Other studies have shown the presence of different volatile constituents in some Moroccan *T. satureioides* populations. Lattaoui *et al.* [11] reported the major compounds to be borneol (31.2%), camphene (27.4%), α-pinene (17.5%) and linalool (6.3%). Jaafari *et al.* [23] and El Bouzidi *et al.* [24] have shown the codominance of borneol (21.1–30.03%) and carvacrol (26.5–35.9%)

The intraspecific chemical variability of Moroccan *T. satureioides* can be attributed to the environmental conditions [25]. In fact, Kasrati *et al.* [26] compared the oil composition obtained from the three *T. satureioides* populations from Morocco and showed that the oil obtained from Er-Rich populations was characterized by carvacrol (45.3%), p-cymene (8.9%), linalool (8.4%), borneol (7.5%), (E)-caryophyllene (6.4%) and γ-terpinene (6.3%) as the main compounds. The oil obtained from the Taws population was characterized by carvacrol (25.3%), borneol (19.7%), camphene (7.6%), p-cymene (6.6%), (E)-caryophyllene (5.4%) and γ-terpinene (5.0%) as the major constituents; whereas, the population from Ourika was composed of carvacrol (26.5%), borneol (20.1%),

camphene (8.0%), (E)-caryophyllene (5.7%), γ-terpinene (5.6%), p-cymene (5.4%) and linalool (3.8%).

Antibacterial activity

The antimicrobial activity of *T. satureioides* essential oil against eight species of microorganisms was assessed by evaluating the diameter of the inhibition zones and the determination of MIC and MBC values, as shown in table 2.

T. saturejoides essential oil inhibited growth of all the tested microorganisms, including the clinically–derived strains, except *Ps. aeruginosa.* This Gram-negative bacterium is notorious for its resistance to antibiotics and involvement in nosocomial infections.

This resistance appears to be related to the nature of the outer membrane, which is composed of lipopolysaccharides that form an impermeable barrier to hydrophobic compounds [27].

Table 1: Chemical composition of *Thymus satureioides* essential oil by GC-MS

	Component	Retention time	Area (%)
1	α-Pinene	7.39	0.35
2	α-Phellandrene	7.62	0.29
3	Camphene	8.25	7.16
4	3-Carene	9.20	0.68
5	α-Terpinene	10.68	0.39
6	β-Cymene	10.94	2.18
7	Alloocimene	11.11	1.56
8	Santolina triene	13.72	1.93
9	α-Campholene aldehyde	15.07	0.70
10	Borneol	15.97	26.45
11	4-Terpineol	16.35	1.35
12	α-Terpinyl acetate	16.86	10.99
13	Isobornyl formate	18.07	0.53
14	2-Isopropyl-4-methylanisole	18.72	1.51
15	Isobornyl acetate	20.08	1.87
16	5-Isopropyl-2-methylphenol	20.50	0.76
17	Thymol	20.75	11.24
18	α-Copaene	23.04	0.37
19	Hexahydroindan	23.31	0.11
20	Guaia-3,9-diene	24.10	0.24
21	β-Caryophyllene	24.39	8.27
22	Calarene	24.69	0.17
23	α-Guajene	25.42	0.37
24	α-Ferulene	25.65	0.32
25	Ledene	26.49	0.11
26	Valencene	26.72	0.16
27	α-Muurolene	26.89	0.25
28	γ-Cadinene	27.28	0.79
29	Caryophyllene oxide	29.23	0.50
30	Pentasiloxane, dodecamethyl-	43.62	3.92

Table 2: Antibacterial activity of *T. satureioides* essential oil

Tested microorganisms	Inhibition zone diameter (mm)		MIC (µl/ml)	MBC (µl/ml)
	Essential oil	Antibiotic control		
Gram-negative bacteria				
E. coli ATCC 25922	15±0	25±0	0.625	0.625
E. coli	21±0	0±0	1.25	1.25
Ps. aeruginosa ATCC 27853	0±0	0±0	>20	>20
Ps. aeruginosa	0±0	0±0	>20	>20
Enterobacter cloacae	15.5±0.7	12±0	0.625	0.625
Gram-positive bacteria				
Staph. aureus ATCC 29213	23±0	16±0	0.312	0.312
Staph. aureus	15±0	12±0	0.625	0.625
Enterococcus faecium	16±0	18±0	1.25	1.25

Values represent mean±standard deviation of three replicates.

According to the disc diffusion results, the inhibition zones of bacteria ranged from 0±0 to 23±0 mm (table 2). The MBC values were equivalent to the MIC values, confirming the bactericidal effect of the essential oil tested. *Staph. aureus* ATCC 29213 was the most sensitive strain with MIC = MBC = 0.312 µl/ml.

The bactericidal activity of *T. satureioides* essential oil may be attributed to the presence of high concentrations of borneol and thymol. Thymol is an oxygenated monoterpene well known with its antimicrobial potential [28, 29] which results from its ability to induce structural and functional alterations in the cytoplasmic membrane [30]. In fact, thymol affects membrane permeability and results in the release of K^+ ions and ATP [31-33]. It can also interact with membrane proteins and intracellular targets.

However, it is difficult to attribute the antimicrobial activity of essential oil, characterized by a complex mixture, to a single or particular constituent. In fact, some studies have shown that the antibacterial activity of whole essential oils is greater than that of the sum of their individual components [34]. Therefore, synergistic and antagonistic effects between several components in the oil are possible, and may contribute to the overall antibacterial activity of these oils.

Antioxidant activity

The antioxidant activity of essential oil of *T. satureioides* was determined by three different test systems–namely the DPPH, β-carotene/linoleic acid, and TBARS assays. The reduction ability of DPPH radicals formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The scavenging ability of *T. satureioides* essential oil increased with increasing oil concentrations. As demonstrated in table 3, the oil showed high scavenging value (IC_{50} = 0.25±0.03 mg/ml). There was no statistically significant difference (p>0.05) between EO and ascorbic acid (positive control).

Table 3: Antioxidant activity of *T. satureioides* essential oil, as measured by the DPPH, β-carotene/linoleic acid, and TBARS tests

Antioxidant tests	Essential oil	Ascorbic acid	BHT
DPPH assay (IC_{50} mg/ml)	0.25±0.03a	0.14±0.001a	-
β-carotene/linoleic acid assay (I%)	81.78±0.37a	-	98.13±0.94b
TBARS assay (I_{50} mg/ml)	300.32±1.50	-	-

Values represent mean±standard deviation of three replicates. For each test, values followed by the same letter are not significantly different (p>0.05).

In the β-carotene/linoleic acid test, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The EO exhibited high antioxidant activity, with I% = 81.78±0.37%, but this was still lower than that of the synthetic antioxidant BHT (I% = 98.13±0.94%) (table 3).

The thiobarbituric acid-reactive substances (TBARS) assay spectrophotometrically measures, at 532 nm, the pink pigment produced by the reaction of thiobarbituric acid (TBA) with malonaldehyde (MDA), a secondary lipid peroxidation product. The antioxidant activity of *T. satureioides* essential oil was dose–dependent. The highest activity (I_{50} value of 300.32±1.50 mg/ml) was observed at high EO concentrations. However, in other studies, Thyme EO exhibited stronger antioxidant activity in the TBARS assay, with an IC_{50} value of 0.004 µg/ml for *T. serphyllum* and 0.31 µg/ml for *T. algeriensis* [35].

EOs are complex mixtures of several classes of compounds, and this complexity often makes it difficult to explain the activity pattern. The antioxidant activity of *T. satureioides* EO could be attributed to the presence of thymol. In fact, the *in vitro* antioxidant activity of the essential oils of several *Thymus* species has been reported previously [36,37], and this activity has been attributed mainly to their content of phenolic components, especially thymol and carvacrol [38], which are capable of donating hydrogen atoms to inhibit lipid peroxidation [39]. The presence and the number of free hydroxyl groups are key determinants of the antioxidant activity of phenols [40]. Beside their antiradical activities [41], thymol and carvacrol express a strong antioxidant action, similar to that of α-tocopherol, which is used as a reference antioxidant in lipid systems [37]. Other constituents of the *T. satureioides* essential oil, such as borneol could also be taken into account for their possible synergistic or antagonistic effects.

CONCLUSION

The results obtained in this study confirm the antibacterial and antioxidant activities of *T. satureioides* essential oil. The essential oil tested represents a natural source of bioactive compounds. Thus, this study suggests that *T. satureioides* essential oil may be used to prevent the growth of bacteria and to replace synthetic preservatives. However, further research is needed to evaluate the safety and the effectiveness of this EO in food systems.

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CONFLICTS OF INTERESTS

Declared none

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