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Research Article

Antimicrobial activities of essential oil of five plant species from Morocco against some microbial strains

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ABSTRACT
The objective of this study is to assess the *in vitro* antimicrobial potential and also determine the minimum inhibitory concentration (MIC) of five selected species of medicinal plants from Lamiaceae family, namely *Satureja hochreutineri Briq, Teucrium polium, Thymus satureioides, Thymus broussonetti* Boiss. and *Thymus zygis* from wild and *in vitro* cultivated plants in Morocco with a view of searching a novel essential oil as a preservative in cosmetic products. The tests were carried out using disk-diffusion method, agar well diffusion method. The MICs of the essential oils were determined by broth microdilution method against five standardized microorganisms recommended by the The European Pharmacopoeia, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus brasiliensis*. Our results show that essential oils of genus Thymus, *Satureja hochreutineri* and *Teucrium polium* exhibited significant *in vitro* antimicrobial activity against all five tested bacterial and fungal strains except *P. aeruginosa* which resist to *T. polium* and *S. hochreutineri* essential oils with inhibition zone between 7.00–46.33 and MIC values ranged from 0.2 to 20 µl/ml. The results of this study revealed that these essential oils possesses antimicrobial properties as antibiotics, therefore, they can be used as a potential source of active ingredients for cosmetic preservatives.

Keywords: Antibacterial activity, antifungal activity, essential oils, Lamiaceae, Minimum inhibitory concentration

INTRODUCTION

Infectious diseases are a leading cause of morbidity and mortality worldwide, especially in developing countries[1]. The World Health Organization (WHO) has estimated that 55 million people died worldwide in 2011, and infectious diseases were responsible for one-third of all deaths. This situation is aggravated by the increasing number of disease causing microorganisms resistant to antibiotic therapy, which are able to recover and survive after antibiotic drug exposure through their ability to acquire and transmit resistance. Recently there has been a lot of attention focused on producing medicines and products that are natural. Thyme species are among the medicinal plants largely used in the Mediterranean basin[2]. Many of them are used in popular medicine against a variety of diseases, aromatic, culinary as well as food preservative[3]. Several thyme species possesses numerous biological activities such as antispasmodic, antibacterial, antifungal activities and anti-inflammatory effects[4-8]. In Morocco, the most known and studied species of thyme are *Thymus satureioides* Coss. and *Thymus willdenowii* Boiss. However, Morocco is rich by about twenty-one thyme species, twelve of them are endemic[9]. In Southwestern Morocco, particularly in Essaouira and Marrakech areas, *T. broussonetti* and *T. maroccanus* present a great demand[10]. Indeed, the leaves and stems barks of this two species were used as powder, decoction or infusion form to treat digestive disorder, diarrhea, fever, coughs, and numerous infected areas of the body[11,12]. Volatile oils are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). Other volatile compounds include phenylpropenes and specific sulphuror nitrogen-containing substances. Generally, the oil composition is a balance of various compounds, although in many species one constituent may prevail over all others[13]. Cultivation of genus Thymus plants such as *Thymus broussonetti* Boiss. is considered as an interesting possibility for providing additional agricultural revenue for farmers, due to their wide use in the food, cosmetic, and pharmaceutical industries. Genus Thymus plants are small aromatic perennial herbaceous plants that grow in well-drained calcareous soil and need full sun to develop to their full potential[14]. In order to develop a natural antimicrobial and preservative agents from plants, we selected from our ethnopharmacological study and literature some antiseptic species[4,15]. However, there are a few quantitative data related to the antimicrobial activity of essential oil obtained from different aromatic plants cultured in Morocco against skin infections.

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All of the essential oils are obtained from thymus genus domesticated and cultivated in the National Institute of Aromatic and Medicinal Plants (Morocco) and *T. polium*, *Satureja hochreutineri, Thymus zygis* harvested from Sefrou region. There are numerous studies on the biological activities of thym essential oil; however, *Satureja hochreutineri* Briq and *Thymus broussonetti* Boiss from *in vitro* culture are not studied to our knowledge. The present work highlighted the antimicrobial effect of essential oil of *Satureja hochreutineri* Briq*, Teucrium polium, Thymus saturoidess, Thymus broussonetti* Boiss., and *Thymus zygis* against a wide spectrum of Gram (+) and Gram (-) bacteria and fungi, five microrganisms recommended by the The European Pharmacopoeia in cosmetic products. Furthermore, by this preliminary study and other research in our laboratory (data not publuished), we attempt to contribute to the use of these medicinal plants as alternative preservatives agents in cosmetic products.

MATERIALS AND METHODS

Plant Material

*Thymus broussonotii, Thymus satureioidess and Thymus zygis (*Lamiaceae) were freshly harvested and collected on April-July in the garden of the National Agency of Medicinal and Aromatic Plants (ANPMA). The plants of *Teucrium polium, Satureja hochreutineri* Briq and *Thymus zygis (*Lamiaceae) were harvested in the region of Sefrou-Morocco. Plants were identified and deposited in the herbarium of the institute and center scientific of Rabat.

Extraction of the essential oils

The extraction of essential oils from the aerial part of plants was performed by a hydro-distillation method using Clevenger-type apparatus. The essential oils obtained were kept in dark at 4°C until further process.

Microbial strains

The following bacterial and fungal strains were used in the antimicrobial tests: *Staphylococcus aureus* ATCC 29213 (Gram-positive); *Escherichia coli* ATCC 25922 (Gram-negative); *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative); yeast *Candida albicans* ATCC 10231 and mould *Aspergillus brasiliensis* ATCC 16404 obtained from the American Type Culture Collection. The inocula of the bacterial and yeast strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity corresponding to 10^8 CFU/ml for bacteria, and fungal strains.

Disk diffusion method

The agar disk diffusion method was employed for the determination of antimicrobial activities of the essential oils in question with some modifications [16]. Briefly, Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates were inoculated with bacterial and fungal strain *candida albicans* respectively (0.5 McFarland size inoculum). Filter paper discs (6 mm in diameter) were impregnated with 10µl of the undiluted oil and were placed on the inoculated plates. These plates, after remaining at 4 °C for 2 h, were incubated at 37 °C for 24 h for bacterial strains, and at 30 °C for 24-48h for fungal strain. Streptomycin and Chloramphenicol were used as positive reference standard to determine the sensitivity of a strain of each tested bacterial species. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. All tests were performed in triplicate.

Antifungal activity

Antifungal activity of essential oils was demonstrated in a radial growth inhibition assay with some modification [17]. A fungal plug was placed in the center of the Potato Dextrose Agar plate. The essential oil 80µl of Thyme, *Origanum elongatum*, *Teucrium polium* and *Satureja hochreutineri* were screened for antifungal activity by Agar well plug method against *Aspergillus brasiliensis* ATCC 16404. The potential sensitivity of the essentials oils were obtained against this fungal tested. The fungicidal effect of the plant extracts can be assessed by the inhibition of mycelial growth of the fungus and is observed as a zone of inhibition near the wells.

Determination of Minimum Inhibitory Concentration (MIC)

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) as previously described with slight modifications [18], agar at 0.15% (w⁄v) was used as emulsifier and resazurin was used as bacterial growth indicator. Firstly, 50 µl of Mueller Hinton Broth supplemented with bacteriological agar (0.15% w/v) were distributed from the second to the 12^{th} well of a 96-well Microtiter plate. Essential oil dilution was prepared in Mueller Hinton Broth supplemented with bacteriological agar (0.15% w/v), 100 µl of these suspensions were added to the first test well of each Microtiter line, and then 50 µl of scalar dilution were transferred from the second to the 11th well. The 12^{th} well was considered as growth control. Then, 50 µl of a bacterial suspension were added to each well at a final concentration of approximately 10^6 CFU/ml. Plates were incubated at 37°C for 24 h and fungal plates at 25 °C for 48 -72 h. After incubation, 5 µl of resazurin were added to each well to assess bacterial growth. After further incubation at 37°C and 30°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in resazurin color. Bacterial growth is detected by reduction of blue dye resazurin to pink resorufin. Experiments were conducted in triplicate.

Minimum Bactericidal and fungicidal Concentration (MBC/MFC)

The minimum bactericidal and fungicidal concentration (MBC/MFC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at 37°C for 24 h and fungal plates at 25 °C for 72 h. It is determined by spotting 2 µl from negative wells on LB plates. Experiments were also conducted in triplicate.

Statistical analyses

All analyses were done at least in triplicate, and these values were then presented as average values along with their standard derivations.

RESULTS AND DISCUSSION

Table 1: Antimicrobial activities of the essential oils against different strains of bacteria and fungi by disk diffusion method.

	Zone of inhibition (mm)				
	Bacterial strains			*Fungal strains*	
	S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. brasiliensis*
Thymus satureioides[a]	34.67 ± 0.33	20.67 ± 0.27	7.00 ± 0.00	24.67 ± 0.67	NZ
Thymus broussonotii [c]	42.67 ± 1.45	29.33 ± 0.54	8.67 ± 1.20	35.67 ± 0.33	NZ
Thymus zygis [a]	37.33 ± 5.04	29.33 ± 1.44	10.00 ± 0.58	34.67 ± 0.33	NZ
Thymus zygis [b]	46.33 ± 0.33	28.67 ± 2.60	10.00 ± 0.58	36.00 ± 0.58	NZ
Satureja hochreutineri	12.67 ± 0.33	8.83 ± 0.14	-	11.67 ± 1.45	-
Teucrium polium	12.00 ± 0.00	-	-	10.33 ± 0.33	-
Chloramphenicol	19.00 ± 4.20	22.4 ± 9.10	10.00 ± 0.00	--	--
Streptomycin	12.67 ± 0.3	15.33 ± 0.3	21.66 ± 3.8	--	--

NZ: no measurable zone of inhibition ; - : no activity; positive control: bacterial suspensions and Mueller-Hinton Broth supplemented with agar (0.15 % w/v); a: cultivated plant; b: wild plant; c: from culture *in vitro* genotype 3; Standard: streptomycin: 10 µg/ml and chloramphenicol 30µ/disc

Table 2: Determination of MIC values of essential oils against the tested strains.

	MIC (µl/ml)				
	Bacterial strains			Fungal strains	
	S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. brasiliensis*
Thymus satureioides[a]	0.6	10	20	0.6	1.3
Thymus broussonotii [c]	0.2	1.3	20	ND	ND
Thymus zygis[a]	0.3	1.3	20	0.3	1.3
Thymus zygis[b]	0.6	0.6	20	0.2	0.6
Satureja hochreutineri	0.2	10	-	1.3	-
Teucrium polium	1.3	-	-	ND	-
Chloramphenicol[d]	4.0	6.25	-	--	--
Streptomycin[d]	4.0	8.00	32	--	--

-: no activity; positive control: bacterial suspensions and Mueller-Hinton Broth supplemented with agar (0.15 % w/v); a: cultivated plant; b: wild plant; c: from culture *in vitro* genotype 3; d: in µg/ml; ND: not determined

Table 3: Determination of MBC and MFC values of essential oils against the tested strains.

	MBC and MFC (µl/ml)				
	Bacterial strains			Fungal strains	
	S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. brasiliensis*
Thymus satureioide s[a]	0.6	10	>80	1.3	1.3
Thymus broussonotii [c]	0.6	1.3	>80	ND	ND
Thymus zygis[a]	2.5	2.5	80	1.3	1.3
Thymus zygis[b]	0.6	0.6	80	0.6	0.6
Satureja hochreutineri	2.5	>10	-	5.0	-
Teucrium polium	>4.0	-	-	ND	-
Chloramphenicol[d]	4.0	25	-	--	--
Streptomycin[d]	4.0	64	32	--	--

-: no activity; positive control: bacterial suspensions and Mueller-Hinton Broth supplemented with agar (0.15 % w/v); a: cultivated plant; b: wild plant; c: from culture *in vitro* genotype 3; d: in µg/ml; ND: not determined.

Antimicrobial Activity

As can be seen in Table 1 and Table 2, the essential oils were found to have good to moderate antimicrobial activities against all microorganisms tested. In the present study, thymus exhibit remarkable antibacterial activity against the Gram positive strains (*Staphylococcus aureus*), Gram negative strains (*Escherichia coli*) and moderate activity against *Pseudomonas aeruginosa*. However, Gram negative strains tested (*Escherichia coli* and *Pseudomonas aeruginosa*) were resistant to *Teucrieum polium* oil. Depending on of tested microorganism strain, the zone of inhibition varied between 7.00–46.33 mm and MIC values ranged from 0.2 to 20 µl/ml for essential oils. *Teucrieum polium* oil and *Satureja hochreutineri* oil did not affect the growth of *P. aeruginosa*.

Generally, all of tested essential oils reduced the growth of the bacterial and fungal strains used in these experiments and differed significantly in their activity against tested micro-organisms. As shown from data of microdilution method, the essential oils of thyme gave the highest antimicrobial effect on all strains while *Satureja hochreutineri* and *Teucrium polium* had a moderate effect. The lowest antimicrobial effect was recorded for *Teucrium polium*. These differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative cell wall is a multi- layered structure and quite complex[19,20]. In this context the work of Ghaly[21] found that thyme oil gave the maximum inhibitory action against *E. coli* and *S. aureus*, followed by *Origanum majorana* oil.

The antimicrobial activities of different Satureja species were shown in other previous studies[22-24]. In addition, Baser et al.[25] reported that antimicrobial activities result only from monoterpenes such as carvacrol and thymol and also *Saturja coerulea* essential oil rich in sesquiterpenes, which also displayed activity. Additionally, Purnavab et al., (2015) reported that α-Pinene and myrcene were of the highest percentage in *T. polium* essential oil. This result is in agreement with Purnavab et al., (2015), who concluded that *T. polium* essential oil did not show antibacterial activity against *P. aeruginosa*.

The previously published data indicate that the antifungal and antibacterial activity exhibited by Thymus genus essential oil has been demonstrated by several researchers[26-29]. The optimal effectiveness of medicinal plants may not be due to one main active constituent, but to the combined action of different compounds present in the plant[30]. These antimicrobial properties of thymus could be mainly attributed to its chemical composition, which are rich with monoterpenes hydrocarbon and oxygenated monoterpenes, and to their major compounds, including phenolic and alcoholic terpenes (thymol; linalool and casrvacrol) and γ-terpinene, p-cymene, α-pinene and 1.8-cineole[31,32]. Essential oils are known to confer antimicrobial activity by damaging the cell wall causing the permeabilization of membranes, loss of ions, leakage of macromolecules, and lysis[33]. The study of Esmaeili et al.[34] advanced a hypothesis regarding the potential mechanism of action of thyme essential oils against Helicobacter pylori and a gram-negative bacterium, and reported that the antimicrobial activity is mainly attributed to the presence of some lipophilic active constituents, whose hydrophobicity enables them for rupturing cell membranes and intra-structures. Thymol, carvacrol, p-cymene and γ -terpinene are the major compounds of genus Thymus plants which are responsible for its antibacterial effects[35,36]. It has been reported that thymol reduces bacterial resistance to some antibiotics such as penicillin[37]. Numerous reports have shown also that carvacrol has a potent protective role against bacteria, fungi, yeasts, mites and also insects[38-40]. Carvacrol is hydrophobic in nature and, due to the free hydroxyl group in the chemical skeleton as well as delocalized electron system, affects the cytoplasmic membrane of bacteria and in this way exerts its antibacterial effects[41]. It has also been reported that carvacrol possess antibacterial effects through inhibition of ATPase activity and induction of Hsp60[42]. Morever, p-Cymene is the biological precursor of carvacrol and is known as the most important hydrophobic antibacterial agent of thyme[43]. In addition, γ-terpinene is one of the major chemical components of thyme essential oils which possess potent biological activities, especially antioxidant and antibacterial properties[44].

CONCLUSION

It can be concluded that the essential oils obtained from genus Thymus plants, might have a beneficial role against different types of bacterial infections. The results of the study revealed that essential oils genus Thymus, *Satureja hochreutineri* and *Teucrium polium* Briq exhibited significant *in vitro* antimicrobial activity against all five tested bacterial and fungal strains except *P. aeruginosa* which resist to *Teucrium polium* and *Satureja hochreutineri* Briq essential oils. Despite the high antibacterial effects using *in vitro* studies, there is a lack of clinical trials on the antibacterial role of essential oils and their active constituents against bacterial infections in humans. In addition, this study provided an experimental basis of practical application of these essential oils as a natural antimicrobial and preservative agent.

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