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RESEARCH ARTICLE

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF A MOROCCAN HIGH ATLAS SPACY: *THYMUS SATUREIOIDES*

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ABSTRACT

The aim of our work is the characterization of the chemical composition and evaluation of the antibacterial activity of the essential oil and methanol extract of *Thymus satureioides* growing wild in the eastern High Atlas of Morocco. The extraction of the essential oil of the aerial parts of the plant was carried out by steam distillation. The yield of essential oil is 2,74%. The methanolic extract is obtained by maceration with methanol at 80%, and the yield is of the order of 11,66%.The results of the chromatographic analysis GC-MS of the *Thymus satureioides* Essential oils have allowed to identify around 35 constituents. The major ones are Carvacrol (30,47%); Borneol (18,15 %); β - cymene (12,69 %); Camphene (6,19%); α-pinene (3,77%) and γ-terpinene (3,66%). The antibacterial activity of the essential oils and that of the methanolic extract of *Thymus satureioides* has been studied with the help of the amatograms method through five pathogene bacterial strains for people: *Klebsiellapneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, wild Escherichia coli and acquired Escherichia coli penicillinase*. The obtained results showed that the essential oils of *Thymus satureioides* represent excellent activities vis-à-visall tested strains with MICs ranging from 0, 68mg / ml and 21,73mg/ml.Thisstrong antibacterial activity is attributed to the major constituents, especially 'carvacrol' known by these important antibacterial properties.However,this oil is more active than Imipenem and Amoxicillin used as a reference antibiotics. As for the methanol extract of this species, it represents a low activity vis-à-vis the same strains. The strong antibacterial activity of this species justifies its use in traditional medicine for the treatment of many infectious diseases.

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INTRODUCTION

Aromatic and medicinal plants are an important source of pharmacologically active substances. With the new techniques of extraction, identification and characterization of organic molecules, more than 25 to 50% of prescribed drugs nowadays for bioactive molecules active principles of medicinal plants (Calixto, 2005). Recent studies have shown that essential oils, in particular, their components have significant potential as antimicrobial agents applied in several medical and industrial fields (Baser *et al*., 2002; Dorman *et al*., 2000). Thyme is one of the most used as spices and extracts with strong antibacterial and anti-inflammatory in traditional medicine plants. Indeed, thyme is widely used in traditional medicine in several forms: the leaves are used in infusion against coughs, decoction to cure headache, hypertension, diarrhea and Fever, externally used as healing and antiseptic. Thyme leaves are rich in essential oil with properties put to use in herbal medicine. It is very antiseptic and used as such to treat lung infections. Its antiseptic effect is also active on the digestive system and in particular in cases of diarrhea and it is also a wormer (Bellakhdar, 1997; Ebrahimi, 2008). The pharmacological properties of the Moroccan thyme and essential oils have been studied by many researchers. These studies have shown interesting antimicrobial, antioxidant and antiviral proerties (Lattaoui, 1994; Ismaili, 2004; Jasna *et al*, 2012; El Bouzidi, 2013). In addition, previous work of Benjilali *et al*. (1984, 1986) allowed demonstrating the antimicrobial properties of several species of Thyme of Morocco against many strains of bacteria, yeasts and molds involved in food contamination. In Morocco, such kind of Thymus is represented by around thirty species and subspecies including some fifteen species are

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endemic (Fennane, 2007). These species are known locally as the common name "Zaetra" in Arabic and "Azoukeni" in Tamazight. Thymus satureioides, is a North African specy typical of arid habitats used in traditional Moroccan medicine as a decoction to treat whooping cough, Bronchitis and rheumatism and also for flavoring and preservation of several food products (Bellakhadar *et al*., 1991 and 2006). This is an endemic species of Morocco, it is widespread in forest clearings, scrubs and matorrals low and medium mountains to 2200m above sea level on siliceous limestone substrates and rocky soils more or less earthy but well drained (Chafai Elalaoui Ali *et al*., 2014).This work aims to characterize the chemical composition and evaluation of essential oils antibacte. This work aims to characterize the chemical composition and evaluation of the antibacterial activity of the essential oil and methanol extract of *Thymus satureioides* pushing growing wild in the eastern High Atlas of Morocco.

MATERIALS

Plant Material

Thymus satureioides was harvested in the rural commune of Mzyzle to15Km from the city of Rich to the High Atlas of eastern Morocco at the time of flowering (June 2014). The plant was dried in the shade for about ten days. The botanical species identification was conducted at the Floristic laboratory of the Scientific Institute in Rabat.

Biological material

We worked on five common bacterial strains in human pathologies, belonging to two different categories (Gram positive and Gram negative).The bacterial strains used are: *Klebsiella pneumonia* (Kp) (Gram negative) and *Staphylococcus aureus* (Sa) (Gram positive), *Pseudomonas aeruginosa* (Pa) (Gram negative) *Escherichia coliwild* (Ec1) (Gram negative), *Escherichia colipénicillinase acquired* (Ec2). They have all been provided by the Medical Analysis Laboratory Saiss Fez, Morocco. These bacterial species are responsible for skin infections (*Staphylococcus aureus*), urinary tract infections (*Escherichia coli*) and nosocomial infections (*Klebsiellapneumoniae* and *Pseudomonas aeruginosa*) that are major public health problems. The emergence of multidrug resistance in bacteria is a cause of major therapeutic failures.

METHODS

Extraction of essential oils

The extraction of essential oils from aerial parts was performed by steam distillation in a Clevenger type machine for three hours. The essential oils were dried over anhydrous sodium sulphate and stored in a refrigerator at 4 ° C until use. The essential oil yield is evaluated from three extractions of dry plant material.

Preparation of the methanol extract

The methanol extract, obtained by cold maceration, 30 g of plant powder (leaves and flowering tops) in 200 ml of methanol, 80% water for 48 hours. The obtained filtrate was subjected to evaporation in vacuo at 50 ° C, and then the residue is taken up with 5 ml DMSO.

Analysis and identification of essential oil constituents

Chromatographic analysis of the ET was performed on a gas chromatograph type Thermo Electron (Trace GC Ultra) coupled to a mass spectrometer type Thermo Electron Trace MS system (Thermo Electron: Trace GC Ultra, MS Polaris Q), fragmentation is done by electron impact with 70 eV intensity. The chromatograph is equipped with a DB-5 column (5% phenyl-methyl-siloxane) (30m x 0.25mm x 0,25µm film thickness), a flame ionization detector (FID) powered by a gas mixture of H2 / Air. The column temperature is programmed at a rate of a rose 4 ° C / min from 50 to 200 ° C for 5 min. The injection mode is split. The identification of the chemical composition of the essential oil *Thymus satureioides* was performed on the basis of comparing their indices of Kovats (IK) and Adams with those of well-known standard products in the literature (Kovats, 1965; Adams, 2007). It was supplemented by a comparison of indices and mass spectra with different references (Adams, 2007; National Institute of Standards and Technology, 2014). Kovats indices compare the retention time of any product with that of a linear alkane having the same number of atoms. They are determined by injecting a mixture of alkanes (standard C7-C40) in the same operating conditions.

Antibacterial activity

For the evaluation of the antibacterial activity of the essential oil and methanol extract of *T.satureioides* vis-à-vis the bacterial strains we used the technique of aromatogrammes (disk diffusion) (Girault, 1971; Belaiche, 1979), and in liquid area macrodilution for the determination of minimum inhibitory concentrations (MIC) and bactericidal (MBC) (Fauchère, 2002).

Evaluation of antibacterial activity by the disk diffusion method

A bacterial suspension of concentration 10^8 CFU / ml in sterile physiological saline was spread by flooding the surface of a Petri dish 90 mm in diameter; containing Mueller Hinton agar. After drying, the paper discs of Whatman sterile 6 mm diameter are deposited on the agar medium (a disk per box). They are then impregnated with 2 µl and 5 µl for essential oil to the concentration of methanol extract (100mg / ml), three repetitions were made. Reading the diameters of inhibition was done after 24 hours of incubation at 37˚C.

The negative controls used are physiological water and DMSO, and we used two antibiotics as positive controls: Imipenene (10 µg / disk) and Amoxicillin (25µg / disk). Susceptibility of the strains was classified, according to Ponce and al (2003), by the diameter of the inhibition zones: not sensitive (-) for diameters less than 8 mm; sensitive (+) for diameters from 8 to 14mm; very sensitive (++) for diameters from 15 to 19mm and extremely sensitive (+++) for the 20mm diameters.

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

This method consists of preparing a standardized bacterial inoculum in sterile saline; from a bacterial culture for 24 hours. An emulsification was carried out for the essential oil tested through a solutionde DMSO (dimethyl sulfoxide) at 30%. In test tubes containing broth Mueller Hinton culture and 40µl of the bacterial suspension (10^7UFC / ml), are added aseptically the different volumes of the essential oil emulated to achieve a growing range of final concentrations of essential oil from C_1 to $C_8$and whose values are respectively0,34; 0,68; 1,36; 2,72; 5,44; 10,87 ; 21,73 and 43,46 mg / ml.Three repetitions were made.The tubes that served as negative controls do not contain essential oil and those who have served as positive controls contain imipénène. After incubation for 24 hours at 37 ° C, observing the range allows access to the MIC, which corresponds to the lowest concentration of essential oil is capable of inhibiting bacterial growth. In addition, the MBC is assessed by subculturing 100 µl the tubes showing no turbidity on a Mueller Hinton agar culture medium. The petri dishes were incubated at 37 ° C for 24 hours.The report MBC / MIChas allowed us to determine the bactericidal and bacteriostatic power of the studied essential oil. When this ratio is greater than 4, the essential oil has a bacteriostatic power, and when it is less than or equal to 4, the power is bactericidal (Canillac, 2001).

RESULTS AND DISCUSSION

Yield of essential oils

Many factors influence the yield and chemical composition of essential oils such as the nature of the species, environmental conditions, the extraction technique, drying time, the period and the area of harvest, cultivation practices and age of plant material (Aberchane *et al*., 2001; Bourkhiss *et al*., 2011). The essential oil content, obtained from the aerial parts (flowers feuilleset) of our species is 2,74% .This result shows that our species is very rich in EO compared to other species like *Thymus zygis* collected in full bloom in the region of Khenifra (1,90%) and the species *Thymus algeriensis* collected Wiwan which is 1,40% (Belemalha, 2014**)**. A study on samples of *Thymus zygis* of Moroccan middle Atlas shows that the performance before during and after flowering was between 1,64 and 5.98% (Yacoubi, 2014). In general, average yields essential oils of different species of thyme were between 0,35% and 5,98% (Belemalha, 2014 and Yacoubi, 2014). The performance of *Thymus satureioides* in EO we got is still higher than the same species *Thymus satureioides* harvested in the Middle Atlas region of Morocco which is 1,1% (El oualilAlami, 2013).

Chemical composition of EO of Thymus satureioides

The critical analysis of the chemical composition of the oil of this plant has enabled us to identify 35 compounds in total, representing 99, 37% of the essential oil (Table 1).We also find that the fraction of oxygenated monoterpenes is the most abundant group of all compounds identified (62, 76%) followed by the fraction hydrogenated monoterpenes (30,13%) The majority compounds are: Carvacrol (30, 47%); Borneol (18,15%); β -cymène (12,69%); Camphene (6,19%); α-pinene (3,77%) and γ-terpinene (3,66%). These compound represent a percentage of 74,93% of the species. So our case is carvacrolchemotype. This result is in agreement with that found by ElbouzidiLaila and Al (2013) the majority of essential oil compounds of the same specy *Thymus satureioides* of Marrakech region are: Carvacrol (26,5%); Borneol (20,1%); Camphene (8%); γ-terpinene (5,6%); P-cymene (5,4%). Another study on a sample of EO*Thymus satureioides* Middle Atlas of Morocco shows that this oil contains as major compounds, p-cymene (27,59%); thymol (14,09%) and γ- terpinene (10,74%)(El Ouali Lalami Abdelhakim *et al*., 2013).

Works on *Thymus zygis* from three regions of the Middle Atlas of Morocco showed that three samples are mainly composed of carvacrol (16,07 to 74,33%), thymol (from 1,47 to 32,46%), p- cymene (6,97 to 40,26%) and γ -terpinene (2,68 to 22%) (Yacoubi *et al*., 2014).

Table 1. Chemical composition of the essential oil of *Thymus satureioides*

Constituents	Kovats Index	Formula	Pourcentage %
tricyclene	926	$C_{10}H_{16}$	0.35
α -Tujene	930	$C_{10}H_{16}$	0.85
α-pinène	939	$C_{10}H_{16}$	3.77
Camphène	954	$C_{10}H_{16}$	6.19
β-pinène	979	$C_{10}H_{16}$	0.70
Méta-mentha-7,8-diène	1000	$C_{10}H_{16}$	0.37
α -Phellandrene	1002	$C_{10}H_{16}$	0.06
DELTA-Carene	1011	$C_{10}H_{16}$	0.05
α -Terpinene	1017	$C_{10}H_{16}$	0.57
β -cymène	1024	$C_{10}H_{14}$	12.69
O-cymene	1026	$C_{10}H_{14}$	0.92
γ-Terpinene	1059	$C_{10}H_{16}$	3.66
Cis-Sabinene hydrate	1070	$C_{10}H_{18}O$	0.13
Mentha-2,4-diene(p)	1088	$C_{10}H_{16}$	0.09
Linalool	1096	$C_{10}H_{18}O$	4.94
1-terpinol	1132	$C_{10}H_{18}O$	0.06
Karahanaenone	1159	$C_{10}H_{16}O$	0.58
Bornéol	1169	$C_{10}H_{18}O$	18.15
Terpinen-4-ol	1177	$C_{10}H_{18}O$	1.32
α-terpineol	1188	$C_{10}H_{18}O$	6.40
Cis-Dihydrocarvone	1192	$C_{10}H_{16}O$	0.25
Cis-Sabinene hydrate acetate	1219	$C_{12}H_{20}O_2$	0.16
Carvacrol,methylether	1244	$C_{11}H_{16}O$	0.13
Isobornylacetate	1285	$C_{12}H_{20}O_2$	0.31
P-cymen-7-ol		$C_{10}H_{14}O$	0.46
Carvacrol	1299	$C_{10}H_{14}O$	30.47
E-caryophyllene	1419	$C_{15}H_{24}$	3.42
Cuaiene	1439	$C_{15}H_{24}$	0.10
γ-cadinene	1513	$C_{15}H_{24}$	0.09
δ -Cadinene	1523	$C_{15}H_{24}$	0.20
Caryophylleneoxide	1583	$C_{15}H_{24}O$	1.50
Caryophylle-4,8-diene-5β-ol	1640	$C_{15}H_{24}O$	0.10
α-Muurolol	1644	$C_{15}H_{26}O$	0.21
α- cadinol	1654	$C_{15}H_{26}O$	0.11
Guara-3,10-dien-11-ol	1677	$C_{15}H_{24}O$	0.06
Hydrogenated monoterpenes in%			30,13
Hydrogenated sesquiterpenes in%			3,9
Oxygenated monoterpenes in%			62,76
Oxygenated sesquiterpenes			1,98
Esters in%			0,6
Total			99,37

Furthermore *Thymus bronssonetii* of the Essaouira region is composed of majoriterement Carvacrol (43,4%); thymol

(12,3%); γ-terpinene (8, 9%); Borneol (8 ,5%); P-cymene (5,2%) and α-pinene (5%).*Thymus maroccanus* for the region Aitourir Marrakech majority compounds are: Carvacrol (71,6%), p-cymene (7,1%) and γ -terpinène (5 ,9%). A study conducted in 2014 in the region of Khenifra in Morocco on three species *T. zygis*; *T. munbyanus*; *T. algeriensis* showed that the major components are: Thymol (38.04%); p-cymene (18,94%); γ-terpinene (12,5%); carvacrol (11,6%) and borneol (6,63%) for T. zygis, and T. munbyanus: Thymol (24,02%); p-cymene (18,95%); γ-terpinene (23,35%); carvacrol (10,84%) and borneol (7,53%), while the compounds of *T. algeriensis* are: Thymol (37,07%); p-cymene (17,3%); γ-terpinene (16,3%); Borneol (8,43%) and carvacrol (0,09%) (Belemalha *et al*., 2014).Thymol and carvacrol are the two main components in most of the essential oils of thyme and non-aromatic terpenes may also be present as main constituents (Bellakhdar, 1997).

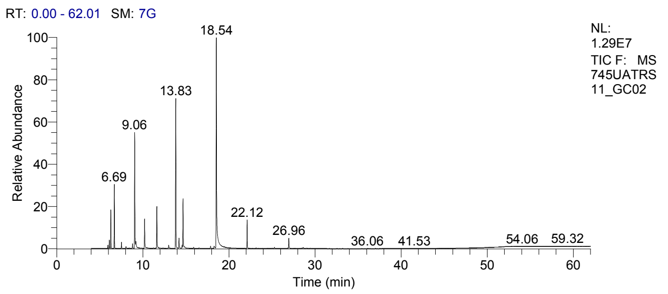

Figure 1. Chromatogram of the essential oil of *Thymus satureioides*

Antibacterial activitydisk diffusion method

The results on the effects of essential oil and methanol extract, from the *Thymus satureioides*, on the growth of bacterial strains performed by the disk diffusion method are shown in Table2. The results of the antibacterial activity "in vitro" obtained using the essential of aromatogrammes method show that the oil of *Thymus satureioides* showed an excellent activity vis-à-vis all the strains tested, with diameters of inhibitionqui zones vary between 21,33 mm and 47,33 mm (*Klebsiellapneumoniae*: 39 ± 1mm; *Staphylococcus aureus*: 21,33 ± 3,51mm*;* *Pseudomonas aeruginosa*: 34,33 ± 0,58mm; *Escherichia coli*wild: 47,33 ± 2,52mm; *Escherichia coli penicillinase acquired*: 29 ± 1mm) (Table 2). By against the methanol extract of *T.Satureioides* showed low activity against bacterial strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli wild*) and even showed no inhibition for some strains (*Klebsiellapneumoniae*, *Escherichia coli penicillinase acquired*) (Table 2).

Table 2. Comparison tests of the essential oil with the bacterial strains

	Diameter of the inhibition zone * (mm)				
	K.a	S.a	P.a	E.c1	E.c2
Essential oil	39±1,00	21,33±3,51	34,33±0,58	47,33±2,52	29±1
Methanol Extract	6 ± 0	8 ±1	8 ±1	7 ± 0	6± 0
Imipénène 10µg/disc	28±1	61±1	25±0	34,67±0,5	26±0
Amoxicillin 25µg/disc	6±0	19±0,5	6±0	20±0,5	6±0

*: The test results are expressed as mean ± standard deviation

We note that this result is in agreement with the results found by Ismaili *et al*. (2004) have confirmed that the *T.satureioides* methanol extract of the Marrakech region is inactive against the following strains: *Escherichia coli ATCC 25922*, *Pseudomonas aeruginosa ATCC 27853*, *Staphylococcus aureus ATCC 25923* and *Enterococcus faecalis ATCC 29212*. We conclude that the most sensitive strain against essential oil *T.satureioidesis Escherichia wild coli* (47,33 mm), followed *Klebsiellapneumoniae* (39 mm). On the other hand all the strains are resistant to the antibiotic amoxicillin except *Escherichia coliwild* (20mm) that showed asensitivity with respect to all antibiotics. But these strains are susceptible to imipenem and essential oil *T.satureioides*. Furthermore the essential oil antibacterial activity of *T.satureioisdes* is stronger than that of the antibiotic imipenem for all strains with the exception of Staphylococcus aureus. We also note that the essential oil of *T.satureioidesest* is active against *Escherichia coli strain acquired penicillinase* that is resistant to antibiotics of the penicillin family. This strong antibacterial activityof the essential oil of *T.satureioides* is attributed to major compounds, especially carvacrol known by its important antibacterial and antifungal properties (Castilho and Al, 2012; Kurdelas *et al*., 2012; Mothana *et al*., 2011). It has been reported that carvacrol causes disruption of the bacterial membrane and hence potentially may also exert an antibacterial activity on intracellular sites (Cristani *et al*., 2007). Chaima Alaoui Jamalia *et al*. (2013) have found that the essential oil of *Thymus maroccanus* present an antibacterial activity lower than that of our species with the following diameters (*Staphylococcus aureus*: 24 ± 2mm; *Klebsiellapneumoniae*: 22,3 ± 0.6 mm; *Escherichia coli*: 17 ± 2,6 mm and *Pseudomonas aeruginosa* 10 ± 0mm).

Determination of MIC and MBC

The results of MIC essential oil *T.satureioides* is endowed with a strong inhibitory activity vis-à-vis *Klebsiellapneumonia* and *Staphylococcus aureus* with MICof 0,68 mg / ml, followed by a MIC of 2,72 mg / ml vis-à-vis *Pseudomonas aeruginosa* and a MIC of 5,44 mg / ml vis-à-vis *Eschirichia coli* wild and a MIC 21,73mg / ml vis-à-vis *Escherichia coli penicillinase acquired* (Table 2).

Table 2. Results of the MIC and MBC of the essential oil of *Thymus satureioides*

Strain	K.a	S.a	P.a	E.c1	E.c2
MIC (mg/ml)	0,68	0,68	2,72	5,44	21,73
MBC(mg/ml)	1,36	10,87	>43,46	>43,46	>43,46

We note that the essential oil of *T.satureioides* has a bactericidal power vis-à-*vis Klebsiellapneumoniae* with a value of MBC is 1,36mg / ml, and a bacteriostatic power vis-à-vis *Staphylococcus aureus* with MBC (10,87mg / ml). Unlike the other strains which have MBC > 43,46mg / ml. We also note that in *Staphylococcus aureusa* there is not a correlation between the results obtained by the disc method (smaller diameter 21,33mm) and liquid-based method (low MIC value 0,68mg / ml) and that can be explained by the difference in operating conditions of the two techniques (Valgas *et al*., 2007). The results obtained in this study are comparable to those of literature within reach. Indeed, Laila El Bouzidi *et al*.

(2013) have found that the essential oil of *T.satureiodes* has presented significant activity against *Staphylococcus aureus* and *Escherichia coli* with MIC= 1, 78mg / ml. Moreover, the essential oil of the species *Thymus maroccanus* is very active against the same bacterial strains with MICs (*Staphylococcus aureus*: 0,3mg / ml; *Klebsiellapneumoniae*: 0,58mg / ml *Escherichia coli*: 0,58mg / ml and *Pseudomonas aeruginosa* >18,6mg / ml).

Conclusion

In this work, we have contributed to the enhancement of *Thymus satureioides* of the eastern Atlas of Morocco establishing a relationship between the chemical composition and antibacterial power. The essential qualitative and quantitative analysis of oil *Thymus satureioides* has identified 35 compound. Our species is chémotypecarvacrol. The results showed a strong antibacterial activity of the essential oil of this species against all bacterial strains with MICs ranging from 0,68mg / ml and 21,73mg / ml. Unlike the methanol extract of this species which has low activity. This study confirms the use of this species by local people to treat many bacterial infections.

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