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Chemical composition and antibacterial activity of three essential oils from south of Morocco. (*Thymus satureoides*, *Thymus vulgaris* and *Chamaelum nobilis*).

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Abstract
The chemical composition of the essential oils from three Moroccan medicinal plants; *Thymus satureoides*, *Thymus vulgaris* and *Chamaelum nobilis* was determined, as well as the determination of its antibacterial activity. The essential oils were identified by (GC and GC/MS). In essential oils of *Thymus vulgaris* and *Thymus satureoides*, Carvacrol is preponderant (78.4% and 49.3% respectively) but in *Chamaelum nobilis*, 2-Undecanone is dominated by 24.7%. Results demonstrated that the essential oils of the two Thymus species (*Thymus satureoides* and *Thymus vulgaris*) showed an excellent inhibitory effect against the bacteria tested. In addition, Gram+ bacteria (MIC: 2.5-5 µl/mL) are more sensitive than Gram- bacteria (MIC: 5-20 µl/mL). These data would indicate the potential usefulness of the two Thymus species as antibacterial activity. The present study confirmed the antibacterial activity against four bacteria tested whose the problem relates to the emergence of strains that possess multiple resistances to a range of antibiotics. Thus, these results indicate that the essential oils represent a potential source of natural antibacterial substances that may be used against pathogenic systems.

Keywords: Essential oils, Chemical composition, Carvacrol, 2-Undecanone, Antibacterial activity.

1. Introduction
Aromatic and medicinal plants had acquired particular attention in the field of intensive research on the natural antimicrobial compounds. They constitute a constant source of active reagents against pathogen germs [1]. Among these products, Essential Oils (EO) produced by aromatic plants as secondary metabolites, gain a net interest by many investigators [2]. EO is volatile, natural, complex compounds and characterized by strong odor [3]. It has been recognized that some EO have antimicrobial, antifungal and antioxidants properties [4 - 8].
Morocco has an enormous unexplored potential of medicinal plants that are used in traditional medicine. The heterogeneous ecologic conditions have favored the proliferation of more than 42. 000 plant species [9 - 10]. In this article, attention was focused on Thymus L. species (Lamiaceae) and *Chamaelum nobilis* (Asteraceae). The genus Thymus are economically important due to their use in folk medicine[11], for flavor and

organoleptic enhancement, as well as food preservatives. Their antioxidant and antimicrobial properties provide the basis for many applications in raw and processed food preservation, pharmaceutical products, alternative medicine and natural therapies [6]. The genus *Chamaelum nobilis* has a long tradition in herbal medicine. The flowers were used in many cures including an herbal tea to cure insomnia. During the Second World War chamomile was also used as a disinfectant. The essential oil is useful in the treatment of aches and pains in muscles and joints [12]. Treatment of symptoms of PMS with Chamomile is also beneficial especially when the symptoms are related to stress. In this context, We were interested analyzing in the present work the chemical composition of essential oils obtained from three (3) endemic plants of Morocco (i.e. *Thymus satureoides*, *Thymus vulgaris*, *Chamaelum nobilis*) and antibacterial activity against four (4) pathogenic bacterial strains including, Gram+ bacteria: *Staphylococcus aureus*, *Streptococcus fasciens* and Gram- bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, in order to develop new type of disease control alternatives. The selection of medicinal plants is based on their traditional uses in Morocco.

2. **Experimental**

2.1. *Plant materials*

Plants of *Thymus* were collected in June 2014 from two region of Morocco: Tafraout and Errachidia. *Chamaemulum nobile* were collected from Errachidia, (South east of Morocco),
The plants were deposited at the Laboratory of Plants in the Scientific Institute in Rabat, Morocco.

2.2 *Oil isolation and analysis.*

2.2.1 Essential oils extraction

The entire Plants are dried in the shade and stored in the laboratory at room temperature (25°C). The extraction of essential oils of *Thymus vulgaris*, *Thymus satureoides* and *Chamaelum nobilis* was conducted by hydrodistillation using a Clevenger type apparatus [13]. These essential oils obtained was dried under anhydrous sodium sulfate and stored at -5°C in the dark before analysis.

2.2.2 *Gas chromatography analysis (GC-FID)*

GC analysis was carried out using a Perkin-Elmer Autosystem XL GC apparatus (Waltham, MA, USA) equipped with a dual flame ionization detection (FID) system and the fused-silica capillary columns (60m *0.22mm I.D., film thickness 0.25µm) Rtx-1 (polydimethylsiloxane) and Rtx-wax (polyethyleneglycol). The oven temperature was programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C for 35 min. Injector and detector temperatures were maintained at 280 °C. Samples were injected in the split mode (1/50) using helium as a carrier gas (1 mL/min) and a 0.2 µL injection volume of pure oil. Retention indices (RI) of compounds were determined relative to the retention times of a series of n-alkanes (C5–C30) (Restek, Lisses, France) with linear interpolation using the Van den Dool and Kratz equation and software from Perkin-Elmer.

2.2.3 *Gas chromatography mass spectrometry (GC-MS)*

Samples were analyzed with a Perkin-Elmer turbo mass detector (quadrupole) coupled to a Perkin-Elmer Autosystem XL equipped with the fused-silica capillary columns Rtx-1 and Rtx-wax. Carrier gas: helium (1 mL/min), ion source temperature: 150 °C, oven temperature programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C (35 min), injector temperature: 280 °C, energy ionization: 70 eV, electron ionization mass spectra were acquired over the mass range 35– 350 Da, split: 1/80, injection volume: 0.2 µL of pure oil.

2.2.4 *Components identification*

The identification of the essential oil constituents was based on: (i) comparison with the mass spectra of authentic reference compounds where possible and by reference to WILEY275,NIST 02 and Adams mass spectral libraries [14] (ii) comparison of their retention index (RI), calculated relative to the retention times of a series of C-5 to C-30 n-alkanes, with linear interpolation, with those of our own library of authentic compounds or literature data [14 - 15].

2.3 *Tests for antibacterial activity*

2.3.1 *Preparation of bacterial strains*

The tested microorganisms included the following bacteria: Gram+ bacteria: Staphylococcus aureus (ATCC 25923), Streptococcus fasciens (ATCC 29212) and Gram- bacteria: Escherichia coli (ATCC 4157), Pseudomonas aeruginosa (ATCC 27853). All microorganisms were derived from the culture collection of the Biology Department (Microthec unity) at the Faculty of Sciences (Rabat, Morocco). Prior the experiment working, cultures were prepared by culturing 1mL of each culture stock in 9 ml of BHI (Brain Heart Infusion) in order to obtain inoculate containing cultures in an exponential growth phase.

2.3.2 *Disc diffusion method*

The agar disc diffusion (ADD) method was employed for the determination of antimicrobial activities of the tested EO as described previously [16]. Briefly, the test was performed in sterile Petri plates containing BHI agar. Sterile filter paper discs (6 mm in diameter) were impregnated with 6 µl of oil and were placed on the Petri plates previously inoculated with a sterile microbial suspension, the suspension of bacteria was obtained from 18h cultures (one microorganism per Petri Plates). All Petri plates were sealed with sterile laboratory films to avoid eventual evaporation of the test samples, and then incubated at 37°C for 24 h. The diameters of inhibition (D) zones were measured in millimeters.

2.3.3 *Determination of Minimum Inhibitory Concentration*

For determining the minimum inhibitory concentration (MIC) values, of the three essential oils against four selected microorganisms. We tested seven serial concentrations of each EO (40 µl/mL, 20 µl/mL, 10 µl/mL, 5 µl/mL, 2.5 µl/mL, 1.25% µl/mL , 0.625 µl/mL) diluted in BHI broth with 0.15% agar and strongly mixed for 2 min using a vortex. For MIC determination, we adopted the technique of sterile microtitermicroplates[16 - 18], using the tetrazolium (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as an indicator of sustainability. In each well, poured 100 µl of liquid culture medium BHI more 100 µl of the test product. Serial dilutions were then performed. Each well is then inoculated with 10 µl of the bacterial suspension. At the end of the incubation period (24 h) at the appropriate temperature, 10 µL of MTT (0.4 mg/mL) is added to each well. The plates were reincubated for 30 minutes at 37 ° C. After this incubation time, Wells, where microbial growth occurs, show a blue-violet color. The MIC was then determined and corresponds to the lowest concentration substance which produces no bacterial growth.

3. **Results and discussion**

3.1. *Essential oils yields and chemical composition:*

Hydrodistillation of *Thymus vulgaris and saterioides* harvested in two distinct areas in Morocco (Tafrout and Errachidia), gave yellow essential oils characterized by a typical odor, the yield of EO (% v/w) was 1.42% for *Thymus vulgaris* and 1.78% for *Thymus saterioides*. For *Chamaelum nobilis* the EO obtained was yellow and characterized by a typical odor. The yield of this EO was 0.85%.

Table 1: Yields of essential oil in the 3 plants studied.

Plants	*Chamaelum nobilis*	*Thymus Vulgaris*	*Thymus Saterioides*
Yields (%)	0.85	1.42	1.78

T. Satureioides provided highest rate of yield with 1.78%, followed by *T. Vulgaris* a yield of 1.42%. However, the lowest content is obtained with *Chamaelum nobilis* 0.85%. The essential oils were identified by chromatographic analysis (GC and GC/MS). The results obtained by GC-MS analysis of the EO are presented in Table 2. The analysis of essential oil the *T. vulgaris* allowed the identification of 29 compounds that show a total of about 95.7% of compounds identified. 22 for *T. satureioides* essential oil (96%) and 27 for *Chamaelum nobilis* essential oil (88.6%) (Table 2). Comparing the two thyme species (*T.Vulgaris* and *T.Satureioides*) shows a polymorphism in the chemical composition with a common major compound is carvacrol. It represents 78.4% and 49.3% respectively. Essential oil of *Chamaelum nobilis* is dominated by 2-Undecanone 24.7%.

Table 2: Chemical composition (%) of Three Essential oils from *Thymus vulgaris*, *Thymus satureoides* and *Chamaelum nobilis* (C)

Component	Ir apol	Ir pol	*Thymus vulgaris*	*Thymus satureoides*	*Chamaelum nobilis*
Tricyclene	921	1012	-	0.2	0.2
α –Thujene	923	1023	0,1	0.9	-
α-Pinene	931	1023	0,4	2.4	0.8
Camphene	944	1068	0,1	3.5	0.3
1-Octen-3-ol	961	1441	0,2	-	-
Octan-3-one	964	1248	0,2	-	-
β-Pinene	971	1111	0,1	0.5	-
Octan-3-ol	979	1384	0,3	0.5	-
Myrcene	981	1159	0,2	1.3	0.1
α-Terpinene	1010	1179	0,3	1.0	-
p-Cymene	1014	1268	4,6	6.0	0.4
1,8-Cineole	1022	1209	0,2	0.3	1.3
Limonene		1200	0,4	0.5	0.4
ϒ-Terpinene	1049	1243	0,5	5.0	-
trans-Hydratesabinene	1053	1455	0,1	0.2	-
Terpinolene	1080	1281	-	0,1	-
Linalol	1084	1538	0,6	5.7	-
Camphre	1122	1506	0,2	0.1	9.2
transPinocarveol	1124	1639	0,3	-	-
Pinocarvone	1134	1558	-	-	0,3
Borneol	1150	1688	0,8	10.2	4.0
Terpinen-4-ol	1162	1595	0,7	1.2	0.2
α –Terpineol	-	-	-	1,5	-
Verbenone	1182	1681	-	-	0,8
Bornylacetate	1272	1576	-	-	13,5
2-Undecanone	1279	1594	-	-	24,7
Carvacrylmethylether	1226	1597	0,3	-	-
Carvacrol	1286	2193	78,4	49.3	4.1
2-Undecanol	1289	1711	-	-	1,8
E-Caryophyllene	1418	1592	3,1	4.6	4.8
Caryophyllene oxyde	1570	1967	-	1,2	-
2-Undecanol acetate	1419	1658	-	-	2,2
Aromadendrene	1437	1602	0,9	-	-
Alloaromadendrene	1457	1643	0,2	-	-
Ledene	1491	1684	0,6	-	-
Spathulenol	1563	2107	0,7	-	-
Caryophyllene oxyde	1569	1970	0,6	-	-
m-Camphorene	1964	2524	0,3	-	-
p-Camphorene	1999	2549	0,3	-	-
α-Humulene	1450	1650	-	-	0,2
4-Tridecanone	1458	1733	-	-	0,4
Z-E-a-Farnesene	1483	1720	-	-	0,7
E-E-a-Farnesene	1496	1742	-	-	0,3
NI	1511	1894	-	-	5,9
Tridecane 2,4-dione	1565	2008	-	-	9,6
2-Tetradecanone	1591	2224	-	-	0,8
NI	1742	2257	-	-	1,4
Total amount of compound			95,7%	96%	88.6%

Ir apol = retention indices on the apolar column (Rtx-1)
Ir pol = retention indices on the polar column (Rtx-Wax)

In recent paper [19], Carvacrol (26.5%), followed of borneol (20.1%) was reported as the most abundant compounds in *T. satureioides*. However, in our study, Carvacrol was present at higher percentage of 49.3 %, whereas borneol only reached 10.2 % of the total essential oil. The major constituents of *T. Vulgaris* essential oil was Carvacrol (78.4%), p-cymene (4.6%) and E-Caryophyllene (3.1%). These results are in accord with that found by M.Boukhatem et al, [20] who's the majority compounds are carvacrol (83.8%) and p-cymene (8.15%). Moreover, other results are in total contradiction with ours [21 - 22]. For example Shazia Shabnum[23] indicate that the essential oil of *T. Vulgaris* has a high rate in thymol (46.2%), γ terpinene (14.1%), P-cymene (9.9%), linalool (4.0%), myrcene (93.5%), α-Pinene (3%) and α -thujene (2.8%). Zambonelli et al., [24] found thymol (22-38%), γ-terpinene and p-cymene. Oil obtained from *C.nobilis* was characterized by 2-Undecanone (24.7%) as a major compound followed by Bornyl acetate (13.5%), Tridecane 2,4-dione (9.6%), camphor (9.2%), E-Caryophyllene (4.8%) and Carvacrol (4.1%), these results are different to that found by N. Dezfooli[25] and R. Omidbaigi[26]. These results show that each plant Species has a specific quantitative and qualitative composition. The reasons of this variability can be due to different geographical sources, the genotype and the climate; all of this variability influences the chemical composition and the relative concentration of each constituent [27 - 30].

3.2. *Antibacterial activity of the essential oils and MIC*

The results of the disk diffusion test indicate that each EO showed different degree of growth inhibition (Figure 1).

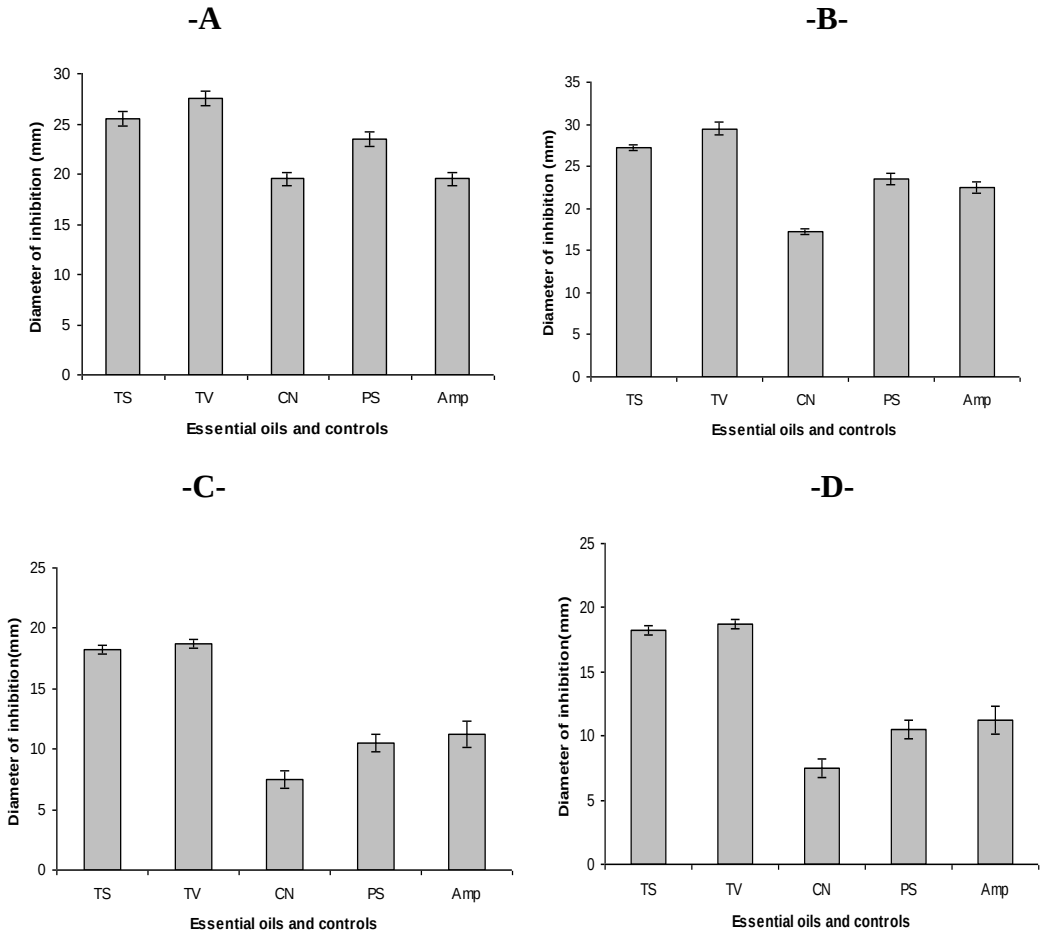

Figure 1: Antibacterial activity* of EO from *Thymus satureoides* (TS),*Thymus vulgaris*(TV) and *Chamaelum nobilis* (CN) against *Staphylococcus aureus* (A), *Streptococcus fasciens* (B), *E.coli* (C) and *Pseudomonas aeruginosa* (D). *: Mean zone of inhibition (Ø mm) and standard deviation. Control (PS: *Penicillin-streptomycin*, Amp: *Ampicillin*)

These EO were found to have antimicrobial activities against all microorganisms tested. The screening for antibacterial activity indicates that oils from *Thymus vulgaris* and *Thymus satureoides* present high antibacterial activity against all strains of tested bacteria (18mm≤D≤30mm, 2.5 µl/mL ≤MIC≤10 µl/mL) Figure 1, Table 2. The maximum inhibition was recorded against *Streptococcus fasciens* with the EO of *Thymus vulgaris* (30 mm) Figure 1B. The essential oil of *C. nobilis* (7mm≤D≤19mm) reveals a low level compared to *T. vulgaris* and *T. satureioides*. All these EO were found to be more active at lower dilution against the chosen pathogenic bacterial strains Gram+ than Gram- Table 2. In addition, *Pseudomonas aeruginosa* was more resistant to the oils studied (10 µl/mL ≤MIC≤20 µl/mL). In the case of *Chamaelum nobilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were most resistant (D≤ 9 mm) Figure 1A and MIC was of about 20 µl/mL of oil dilution Table 2.

Table 3: Minimal inhibitory concentrations (MIC) (µl/mL) of selected essential oils from *Thymus satureoides (T.S), Thymus vulgaris (T.V)* and *Chamaelum nobilis (C.N)*against four pathogenic bacteria.

	***Test organism* (MIC en µl/mL)**			
Plants species	***S. aureus***	***S. fasciens***	***E.coli***	***P.aeruginosa***
TS	2.5	2.5	5	10
TV	2.5	2.5	5	10
CN	5	5	20	20

Results obtained could be due to differences in chemical composition of the oils. In addition, the chemical composition of EO of the two Thymus species (*Thymus satureoides and vulgaris*) is characterized by some differences in the concentrations of individual components. The main volatile components of the two EO species were Carvacrol. It represents 78.4% and 49.3% respectively. For *Chamaelum nobilis* EO we found that 2-Undecanone 24.7% was the main components. Indeed, it has been reported that Carvacrol plays an important role in antibacterial activity [34]. Thus, one may take into consideration that the inherent activity of an oil can be expected to the chemical configuration of the components, the proportions in which they are present and to interactions between them [31]. The antibacterial activity can be correlated to a number of terpenoids and phenolic compounds presents such as carvacrol. Carvacrol is a monoterpene known for its antimicrobial activity against a wide range of bacteria in particularly, is also considered as a biocide, with its precursor, p-cymene, a low antibacterial, but probably acts synergistically with it by the expansion of the membrane, resulting in the destabilization of the membrane [32 - 34]. It's also one of the most documented active components of EO [35]. It has been reported that the presence of carvacrol and thymol in Oregan EO, contribute to a synergistic antibacterial activity against many bacteria [36 - 39]. In the case of *C.nobilis* low antibacterial activity may be due to the low concentration of carvacrol (4.1%) compared to the two other essential oils of thymus. The antibacterial activity varies from oil to another and from one strain to another, also essential oils act on both Gram positive bacteria than Gram negative bacteria [40]. However, gram negative bacteria appear less sensitive to their action [35, 41] and this would be directly related to the structure of their cell wall [35]. Nevertheless the bacterium *P. aeruginosa* (gram-negative) remains the least sensitive to the effect of essential oils [31, 42-43].

Conclusions

The results obtained in this study support the notion that higher antimicrobial of Moroccan Thymus oils is conferred by high Carvacrol content. In spite of some chemical variability, our findings may suggest the potential use of the two species of Thymus oils in treatment of infections caused by those pathogenic germs. these oils could potentially be used as natural preservatives in food against the well-known causal agents of foodborne diseases such as *S. aureus* and *E. coli*. The essential oils produced in Morocco offer a promising way for research

for phytochemical active principle in therapeutic indications. However, studies should be completed to determine the mechanism of action of each EO and compared to the most potent antibiotics used in therapeutics.

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