ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS FROM TWO SPECIES OF GENUS *Thymus* GROWING IN DIFFERENT SITES OF NORTH EASTERN ALGERIAN

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Abstract. The flora of Algeria enjoys a considerable biodiversity; it includes aromatic and medicinal plants endowed with many therapeutic activities. To this end, and as part of the enhancement of this flora, we were interested in studying the antibacterial activity of the essential oils from two species of genus *Thymus* (*Thymus capitatus* and *Thymus hirtus* ssp. *algeriensis*) growing wild in the Souk Ahras region (northeast Algeria). The antibacterial activity of essential oils was evaluated by the agar diffusion test and we determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against six pathogenic bacteria: *Pseudomonas aeruginosa*, *Acinetobacter* spp, *Escherichia coli*, *Salmonella* spp, *Staphylococcus aureus* and *Enterocus faecalis*. The strains tested were highly sensitive to essential oils, except *Pseudomonas aeruginosa*, which showed some resistance. The MIC values found ranging between 0.05 % and 0.8 %. The MBC values were equivalent or equal to double the MIC, indicating a strong bactericidal effect of various *Thymus* essential oils tested.

Keywords: *Thymus*; essential oils; antibacterial activity; MIC; MBC.

INTRODUCTION

The *Thymus* commonly called "Zaâitra" by local people is known to have medicinal properties that make it among the most used plant in traditional pharmacopoeia. Belonging to the Lamiaceae family, the genus *Thymus*, widely distributed in the Mediterranean region [14], is represented by 220 species [13]. In Algeria, 12 species of *Thymus* colonize the country's territory [11]. The essential oils of these species have found their place in aromatherapy, pharmacy, perfumery, cosmetics and food processing as a potential source of natural bioactive molecules [1].

The alarming increase of multidrug-resistant bacteria, over recent decades, due to the excessive use of broad-spectrum antibiotics is causing serious health problems. It therefore seems important to find an alternative to the use of these agents [25].

In the context of research into new natural antibacterial products derived from medicinal plants, the aim of this work interested in studying the antibacterial activity of essential oils of two species of genus *Thymus* (*Thymus capitatus* and *Thymus hirtus* ssp. *algeriensis*), growing spontaneously in four regions of Souk Ahras (North Eastern Algerian).

MATERIAL AND METHODS

Plant material

The aerial parts (leaves and flowers) of the *Thymus* plants (*Thymus capitatus* and *Thymus hirtus* ssp. *algeriensis*) were collected, during the full flowering stage, from four sites (Table 1) in Souk Ahras region (north-east Algeria). The harvested plants were identified by Professor Azzedine CHEFROUR.

Table 1. Geographic location of collected species

Region	Species	Code	Geographical coordinates		
			Latitude (N)	Longitude (E)	Altitude (m)
Sedrata	*Thymus capitatus*	R1TC1	36° 10’ 33.7’’	007° 30’ 11.9’’	1277
Zouabi	*Thymus capitatus*	R2TC2	36° 05’ 32.8’’	007° 26’ 31.3’’	882
Dekma	*Thymus hirtus ssp. algeriensis* Boiss. et Reut.	R3TA1	36° 14’ 43.8’’	007° 54’ 26.5’’	695
Ain Seynour	*Thymus hirtus ssp. algeriensis* Boiss. et Reut.	R4TA2	36° 18’ 24.0’’	007° 50’ 43.0’’	1151

Essential oils extraction

The essential oil of each plant was extracted by hydrodistillation using a Clevenger-type apparatus, 100 g of air-dried samples was treated for 3 hours. Afterwards, all oils obtained were stored in tightly sealed brown glass vials at 4°C until analysis [7].

Study of the Antibacterial Activity

Bacterial strains

To evaluate the antibacterial capacity of our essential oils, six bacterial strains were used: four strains belonging to the ATCC batch: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 43300), *Enterococcus faecalis* (ATCC 29212) and two of clinical origin: *Acinetobacter* spp., *Salmonella* spp. These strains were selected for their high frequencies to contaminate foodstuffs and their pathogenicity. They were provided to us by the microbiology laboratory, Faculty of Medicine, University Annaba, Algeria).

They are maintained by transplanting them onto nutrient agar for 24 h at 37°C.

Qualitative evaluation of the antibacterial activity

The disc diffusion method was employed for the determination of qualitative antibacterial activities in vitro of essential oils according to Kherraz et *al*. (2019) [16]. The antibacterial activity was classified as follows: very strong response, zone diameter ≥30 mm; strong response, zone diameter 21–29 mm; moderate response, zone diameter 16–20 mm; weak response, zone diameter 11–15 mm; and little or no response, zone diameter ≤10 mm [20].

Fresh bacterial cultures were suspended in physiological solution to obtain a final density equivalent to 0.5 Mac Farland standard (10^8 CFU(Colony Forming Unit)/mL) [6]. Petri plate containing Mueller Hinton agar were inoculated using swabs soaked by standardized inoculum. Subsequently, sterile cellulose disc of 6 mm diameter impregnated with each dilution of essential oils (1/1, ½, ¼) were placed onto the inoculated agar surface (the dilutions are prepared with Di Methyl Sulf Oxide (DMSO). After the plates were incubated for 24 h at 37°C. The results were obtained by measuring the inhibition zone diameter [12]. All results expressed are mean of three individual replicates (n= 3±SD).

Quantitative evaluation of antibacterial activity

A. Search for minimum inhibitory concentrations MIC

The minimum inhibitory concentrations (MIC) were determined by the agar incorporation method as cited by Chouikh et *al*. (2015) [8]. A range of dilution of essential oil was carried in dimethylsulfoxide (DMSO). The dilutions thus obtained were incorporated into 19 ml of supercooling Mueller Hinton agar (45°C), so as to obtain the final concentrations: 1%, 0.8%, 0.4%, 0.2%, 0.1%, %, 0.05%, 0.025% and 0.0125% of essential oil per milliliter of culture medium [2].

Immediately, the mixture was distributed in Petri plates. After solidification of the medium, spots of 2 µl from standardized inoculum to 0.5 McFarland concentrations are placed on the agar plates using a micropipette. After an incubation period of 24 h at 37°C, we read the results; the distinction between susceptible and resistant strains depends on whether or not strains have grown at their location [4]. The MIC is defined as the lowest concentration in the presence of which no bacterial growth similar to the growth of the same strain on the control plate.

B. Search for minimum bactericidal concentrations MBC

MBC were defined as the lowest concentration that could kill 99.9% of the bacteria. The determination of MBC was performed by sampling bacterial strains from each of the plates that showed no growth in the MIC test. These samples were then transplanted onto Mueller Hinton agar and incubated at 37°C for 24 hours [8]. The MBC (%, v/v) of the essential oil is deduced from the first plate devoid of bacteria.

Statistical analysis

The results were expressed in means ± standard. Statistical analyses (ANOVA) were performed with Excel 2010. Two-way analysis of variance was conducted to determine the significance of differences between analytical results at $p<0.05$ significance level.

RESULTS

Antibacterial activity determination

Qualitative evaluation of the antibacterial activity

The inhibitory activity of four *Thymus* essential oils against four Gram-negative and two Gram-positive bacteria are presented in (Table 2). All the results obtained show that the essential oils studied have a wide spectrum of action; acting on both Gram positive (Fig. 1) as well as Gram negative bacteria (Fig. 2 and 3), although of differing degrees.

We note that this action is proportional to the concentration. The more concentrated the essential oil, the larger the muting range.

The antibacterial activity of each strain significantly different ($p < 0.05$) depending on the concentration of oils essential and location of collection of the two species.

Referring to the scale cited by [20], the maximum inhibitory activity was observed against *Staphylococcus aureus* with inhibition areas ranging from 19.46 to 52 mm (Table 2). The best result obtained with this strain is that recorded by R1TC1 essential oil which showed a very important activity up to ¼ concentration. Mean inhibitory activity was noted against *Enterococcus faecalis*, *Escherichia coli* and

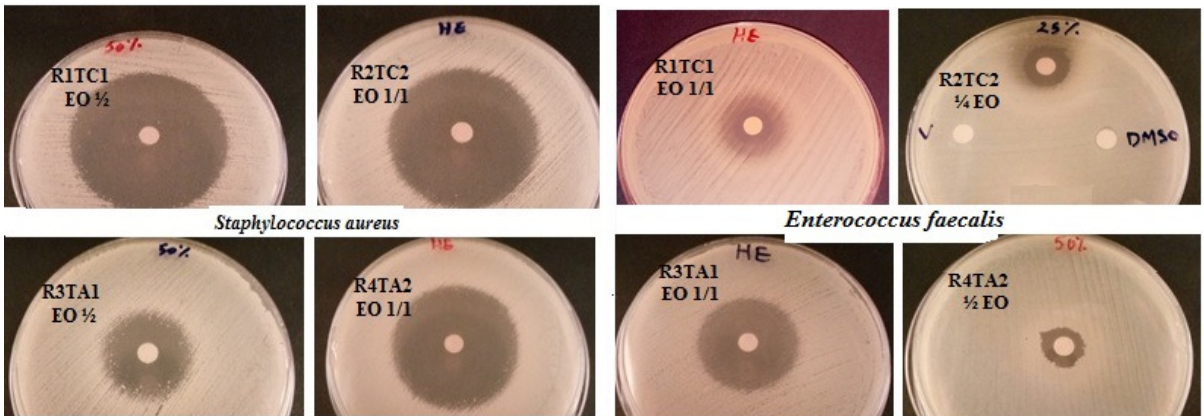

Figure 1. Antibacterial activity of essential oils of *Thymus* against strains pathogenic bacteria Gram positive

Table 2. Inhibition Diameters (mm) of *Thymus* essential oils (Eos) tested (n = 3)

Strains Bacteria	Concentrations Eos	R1TC1	R2TC2	R3TA1	R4TA2
Staphylococcus aureus.	**1/1**	52 ± 4.39	44.08 ± 2.78	36.33 ± 2.25	43.34 ± 1.44
	1/2	49.59 ± 0.26	38.48 ± 2.84	30.30 ± 0.64	34.18 ± 1.79
	1/4	42.45 ± 4.62	31.09 ±3.80	19.46 ± 3.22	33.28 ± 0.74
Escherichia coli	**1/1**	23.7 ± 2.14	23.19 ± 0.87	26.91 ± 0.64	28.62 ± 1.35
	1/2	18.49 ± 0.85	20.42 ± 1.84	21.85 ± 3.18	15.30 ± 0.68
	1/4	10.05 ± 0.70	15.26 ± 3.36	15.14 ± 3.25	12.72 ± 0.59
Enterococcus faecalis	**1/1**	12.15 ± 0.50	14.15 ± 0.15	13.95 ± 0.96	13.50 ± 0.31
	1/2	10.88 ± 0.37	13.41 ± 0.10	12.05 ± 1.00	12.50 ± 0.40
	1/4	9.98 ± 0.44	13.11 ± 0.08	9.48 ± 0.81	12.24 ± 0.20
Salmonella spp	**1/1**	16.72 ± 0.28	23.17 ± 1.59	21.41 ± 0.48	26.69 ± 1.42
	1/2	13.46 ± 0.99	16.16 ± 0.46	18.65 ± 0.85	15.06 ± 1.10
	1/4	11.12 ± 0.48	12.33 ± 1.26	10.29 ± 0.46	12.31 ± 1.20
Pseudomonas aeruginosa	**1/1**	8.85 ± 1.01	9.06 ± 0.78	10.44 ± 1.48	7.74 ± 0.20
	1/2	6.93 ± 0.76	8.10 ± 1.37	7.46 ± 0.27	6.82 ± 0.09
	1/4	6 ± 0.00	7.35 ± 0.74	6 ± 0.00	6.77 ± 0.25
Acinetobacter spp	**1/1**	14.28 ± 0.43	14.93 ± 0.17	14.67 ± 0.98	16.79 ± 0.28
	1/2	13.46 ± 0.21	13.69 ± 0.12	13.60 ± 0.54	13.13 ± 0.31
	1/4	10.96 ± 0.40	11.58 ± 0.84	10.12 ±0.11	12.41 ± 0.08

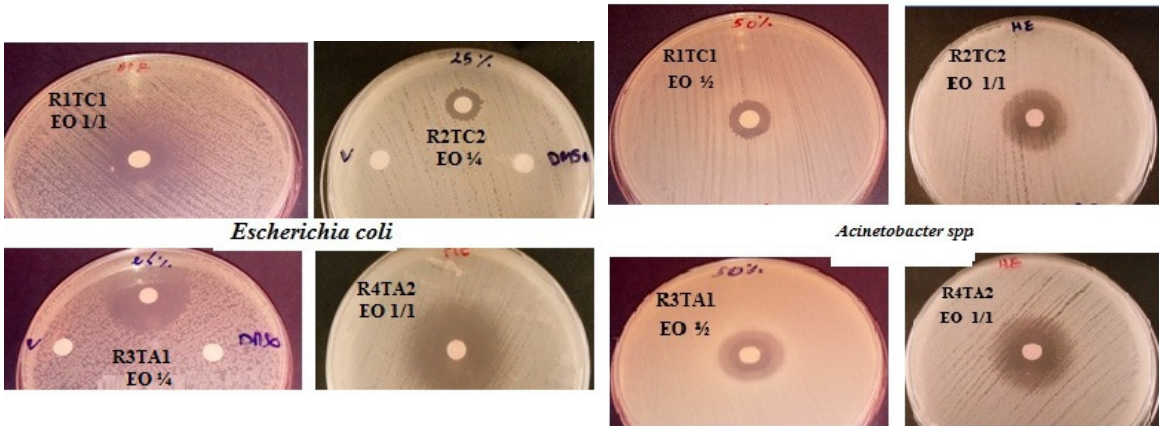

Figure 2. Antibacterial activity of essential oils of *Thymus* against strains pathogenic bacteria Gram negative (*Escherichia coli* and *Acinetobacter spp*)

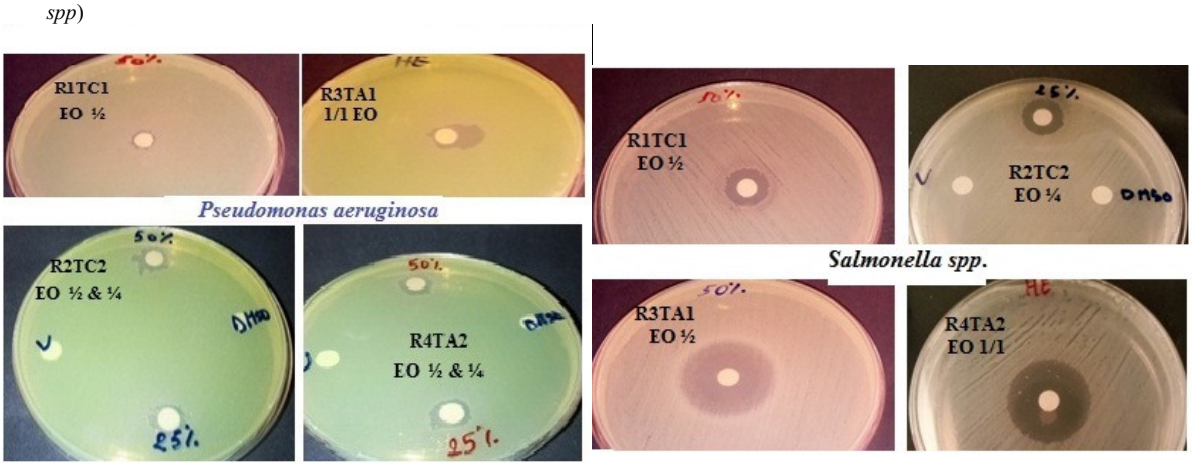

Figure 3. Antibacterial activity of essential oils of *Thymus* against strains pathogenic bacteria gram negative (*Pseudomonas aeruginosa* and *Salmonella spp*)

Salmonella spp., with inhibition zones ranging from 9.48 to 28.62 mm. According to the scale mentioned above, the essential oils studied showed a low inhibitory activity on *Acinetobacte*r spp. inhibition diameters ranging from 10.12 to 16.79 mm. However, with the exception of R3TA1 essence, which showed slightly inhibitory activity against *P. aeruginosa* (D>10mm), the EOs of R1TC1, R2TC2 and R4TA2 were found to be inactive on this strain (D<10mm).

Quantitative evaluation of antibacterial activity

The results of the MIC and MBC of various *Thymus* essential oils tested confirmed those found by

Table 3. MIC and MBC values of *Thymus* EOs tested

Strains	R1TC1		R2TC2		R3TA1		R4TA2	
	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)
***Staphylococcus aureus* (ATCC 43300)**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1
***Escherichia coli* (ATCC 25922)**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
***Enterococcus faecalis* (ATCC 29212)**	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.1
***Salmonella* spp.**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
***Pseudomonas aeruginosa* (ATCC 27853)**	0.4	0.8	0.8	0.8	0.8	1	0.4	0.8
***Acinetobacter* spp.**	0.05	0.1	0.05	0.1	0.05	0.05	0.1	0.1

the disk diffusion test (Table 3). *Thymus* essential oils from four regions of Souk Ahras exhibited important inhibitory activity against all bacteria tested, except for the *Pseudomonas aeruginosa* strain, which proved to be somewhat resistant.An essential oil concentration of 0.05% to 0.1% (v/v) was sufficient to stop the growth of all bacterial strains studied, except *P. aeruginosa* which resisted up to 0.4% (v/v) concentration against R1TC1 and R4TA2 EOs; and up to 0.8% (v/v) concentration against R2TC2 and R3TA1 essential oils (Table 3).

On all bacteria tested, the MBC values found are equivalent or equal to twice the MIC, indicating a strong bactericidal action of our essential oils.

According to the results given in (Table 2 and 3), the essential oils tested have effective against almost all bacteria studied.

DISCUSSION

The comparison of the sensitivity of the different strains to the *Thymus* essential oils tested, we can see that the efficiency of these oils differs from one bacterium to another. Unlike *Staphylococcus aureus*, which showed the highest sensitivity to the essential oils. The strain of *P. aeruginosa*, on the other hand, exhibited the highest resistance towards all essential oils applied. Our results are in agreement with the literature according to which Gram+ bacteria are more susceptible to essential oils than Gram- bacteria [23, 24]. It is likely that this result will be due to a difference in the penetration capacity of the active compounds present in the EOs. In Gram negative bacteria, the presence of a second membrane which has hydrophilic polysaccharide chains acting as a barrier to hydrophobic essential oils [5], and also to the presence of highly sophisticated bacterial efflux pumps in their cytoplasmic membrane; canals that transport any unknown substances to outside of the bacterial cells [22]. Gram positive bacteria with a simple membrane structure are less protected against the diffusion of fine essential oils particles [15].

The MBC/MIC ratio is less than or equal to 4, the EO is called bactericidal. When this ratio is greater than 4, the EO is bacteriostatic [21].

The results of this study are consistent with those found by Mebarki (2010) [19] in testing the antimicrobial essential oil effect of *Thymus fontanesii* from the Bouira region on several microbial strains. She reported that the EO tested had bactericidal and/or fungicidal power on all strains used. Extremely inhibitory essential oil activity of *Thymus numidicus* against several bacterial strains has been reported by Kouch (2015) [17], with MIC values ranging from 0.025 to 0.13%.

The important bioactivity of essential oils is related to their chemical composition. Indeed, several authors [1, 9] have reported that the antibacterial effect of EOs is attributed to the presence of phenolic derivatives. These compounds cause damage to the outer membrane of bacteria, resulting in increased membrane permeability to protons and potassium ions, reduced intracellular ATP reserves, disruption of motor proton force and denaturation of intracellular proteins [3, 5]. In addition to these majority compounds, minor compounds can significantly contribute to the activity of essential oils [18]. It is also likely that this antibacterial activity is not due to the presence of particular substances alone, but is the result of the interaction between various aromatic structures [10]. According to [21], these molecules would most often act synergistically.

In conclusion we can said the strains tested were highly sensitive to essential oils, except *Pseudomonas aeruginosa*, which showed some resistance. The MBC values were equivalent or equal to double the MIC, indicating a strong bactericidal effect of various essential oils extrcated from two especes of genus *Thymus*.

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