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Antimicrobial and Insecticidal Activities of the Endemic *Thymus broussonetti* Boiss. and *Thymus maroccanus* Ball.

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Abstract: The objective of this study was to determine the antimicrobial and the insecticidal activities of essential oils (EOs) extracted from the leaves of *Thymus broussonetti and Thymus maroccanus*. These two endemic plants of Morocco, which are traditionally used in medicinal remedies, were collected from Marrakech-Tensift-Al Haouz region. The EOs were extracted by direct steam distillation and their chemical constituents were analyzed and quantified by gas GC-MS and GC. The dominant components identified were p-cymene (21.0%), borneol (16.5%), α-pinene (11.8%) and thymol (11.3%) for *T. broussonetti* and carvacrol (33.0%), p-cymene (25.3%) and α-pinene (11.6%) for *T. maroccanus*. The investigation by the agar-diffusion method of the antibacterial activity of EOs proved that they have antibacterial effects against *Staphylococcus aureus*, *Salmonella* sp., *Escherichia coli*, Non-O1 *Vibrio cholerae and Bacillus subtilis*. The obtained results showed that *T. maroccanus* EOs possessed higher antibacterial effects on some studied bacteria than *T. broussonetti* EOs. The EOs of *T. broussonetti and T. maroccanus* also presented insecticidal activity against the fourth instar larvae of *Culex pipiens*.

Keywords: Antibacterial activity; Essential oils; Insecticidal activity; *Thymus broussonetii*; *Thymus maroccanus*.

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1. Plant Source

Thyme species are among the medicinal plants largely used in the Mediterranean basin [1]. Many of them are used in popular medicine against a variety of diseases, aromatic, culinary as well as food preservative [2]. Several thyme species possesses numerous biological activities such as antispasmodic, antibacterial, antifungal activities and anti-inflammatory effects [3-7].

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 [8]. In Southwestern Morocco, particularly in Essaouira and Marrakech areas, *T. broussonetti* and *T. maroccanus* present a great demand [9]. 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, rheum and numerous infected areas of the body [10–11].

Aerial part of *T. broussonetti* and *T. maroccanus* were collected at flowering stage in July 2006 respectively in the South-western area, in the surroundings of "Essaouira" and in the High Atlas mountain, in "Ait Ourir". Voucher specimen was deposited in the Muséum d'Histoire Naturelle Marrakech - Cadi Ayyad University.

2. Previous Studies

According to our knowledge, little scientific informations are available on these two endemic species (*T. broussonetti* and *T. maroccanus)*. Some studies were carried out on the pharmacological properties of *T. broussonetti* which evoked the anti-inflammatory activity [12–13].

The most important compounds of EOs thyme are the phenols thymol and carvacrol, which constitute the major and more active constituents [14], as well as the monoterpene hydrocarbons p-cymene and γ-terpinene. *In vitro* studies showed that these compounds posses antimicrobial activity against gram negative or positive bacteria [15–16].

3. Present Study

With increasing interest in looking for antimicrobial and insecticidal properties of EOs extracted from aromatic plants [17–18], the present work investigates the antibacterial and the insecticidal activities of the EOs of the two endemic thyme species (*T. broussonetti* and *T. maroccanus)*. Six bacteria of sanitary interest were studied: *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* sp., *Escherichia coli* and Non-O1 *Vibrio cholerae*. The larvicidal activity of *T. maroccanus* EOs was evaluated using larvae of the insect *Culex pipiens*. Some researchers have demonstrated bactericidal and insecticidal activities of EOs thyme [4–19].

Furthermore, the main compounds of EOs of the two thymes species were determined. The leaves were separated by hand and dried at the shade for 16 days at room temperature (ca. 22 C°). EOs were obtained by the steam distillation for 4 hours. The obtained EOs were stored at -20°C until analysis by GC-MS and GC.

Each oil was analyzed by GC-MS using an Agilent 6890N/5973N system, with a HP-1 (polydimethylsiloxane, 50 m × 0.20 mm i.d. × film thickness 0.33 µm; Interchim, Montluçon, France); temperature program 60-250°C at a rate of 2°C/min and 250°C during 60 min under the following operation conditions: vector gas: Helium, constant flow 1 mL/min.; injector temperature: 250 °C, injected volume: 0.2 µL, split 1/100. Retention indices were determined with C_5 to C_{28} alkane standards as references. The mass spectra were performed at 70 eV of the mass range of 35-400.

GC analysis was carried out using an Agilent 5890 gas chromatograph, under the following operation conditions: vector gas: He, injector and detector temperatures: 250°C, injected volume: 0.2µl, split ration: 1/1000; HP1 column (J&W Scientific), polydimethylsiloxane (50 m × 0.20 mm i.d., film thickness 0.5 µm); temperature program :60-250°C at 2°C/min and 250°C for 60 min. Retention indices were determined with C_5 to C_{26} alkane standards as references. Relative amounts of individual components are based on peak areas obtained without FID response factor correction.

Three replicates were performed for each sample. The average of these three values and the standard deviation were determined for each component identified.

Identification of the constituents was based on computer matching against commercial (Wiley, Nist98, Mass Finder 2.1 Library) and our home made library of mass spectra built up from pure substances and MS literature data [20–24] and confirmed by comparison of retention indices with published index data [25–26].

Screening for antimicrobial activity: Tested bacterial strains were *Staphylococcus aureus* CCMMB3, *Salmonella* sp. CCMMB17, *Pseudomonas aeruginosa* CCMMB11, *Escherichia coli* CCMMB4, Non-O1 *Vibrio cholerae* isolated from wastewater and telluric *Bacillus subtilis* strain.
The studied species were known to be of sanitary interest and can cause diarrhoea, gastroenteritis, septicaemia, moreover; several works have shown that these species could be resistant to antibiotics [27–28].

Antimicrobial activity was assayed via agar diffusion method. The bacterial strains (10^6 colonies forming units/ mL) were grown on Mueller Hinton agar (Bio-Mérieux). A Whatman No. 1 sterile filter paper disc (6 mm diameter) was impregnated with 10 µL /disc of EOs and 30 µg /disc of the tested antibiotics (Gentamycin and Tetracycline). The plates were incubated at 37°C for 24h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone. Each assay was replicated three times.

Insecticidal activity assesment: C. pipiens (Diptera: Culicidae) is a mosquito which transmits many dreadful diseases like Western Equine, encephalitis [29] and many other diseases which can lead to disability and sometimes death. The most successful control method against *C. pipiens* is periodic treatment with chemical larvicides. However, this system of mosquito control has failed to show satisfactory results over the world because of resistance of mosquito to several classical larvicides. The aim of this part of the study is to determine the larvicidal activity of the EOs of *T. broussonetti* and *T. maroccanus.*

The insects were collected from a collection tank for irrigation water in a peri-urban area; the geographical coordinates given by GPS are N31.6375°, W008.1687° and 374m of altitude. The water conductivity of the aquatic tank is 558µs/cm, whereas the pH is 7.4.
The method of essays was inspired from the larvae test sensibility technique standardized by the World Health Organization [11].

Bioassays were made with the young fourth larval instars. Batches of twenty larvae were assayed in 99 mL of distilled water, adding one mL of insecticide or oil solution at the required concentration.
Five replicates per concentration, and four to six concentrations were used for each exposure. Temperature was maintained at 25 ±1°C during bioassays. Negative controls were made with 1mL of Ethanol. Positive controls were made with 1mL of 1ppm solution of Temephos.

For proper interpretation of results, control mortality should be below 5%. If the mortality is between 5% and 20%, Abbot Formula is used to correct mortality and exploitation of results [12]. If the control mortality is >20% the bioassay is to redone [23]. Doses of EOs were prepared by serial dilution with ethanol 95%. For each essential oil, 0.5ppm, 0.25ppm, 0.125ppm and 0.0625ppm concentrations were tested. Mortalities were recorded after 15, 60 min and 24 h of exposure, during which no food was offered to the larvae. Larvae were considered dead if they did not move when prodded with a wooden dowel. LC_{50} and LC_{90} values were determined using log. - Probit analysis.

The comparison of antibacterial and insecticidal effects of EOs, was carried out using one-way analysis of variance (ANOVA, $p<0.05$).

The chemical analysis of the two oils (Table 1) showed that the majority of substances are p-cymene (21.0%), borneol (16.5%), α-pinene (11.8%) and thymol (11.3%) for *T. broussonetti*, whereas carvacrol (33.0%), p-cymene (25.3%) and α-pinene (11.6%) dominated in the EOs of *T. maroccanus*.
Some studies have demonstrated that the two phenolic isomers; thymol and carvacrol led mortality of bacteria due to permeability of their membrane [27–28]. Cosentino et al. [30] have noted that more the phenolic contents are higher; more the EOs are effective. However, other compounds in EOs than phenols can present antibacterial activities. On the other hand, some studies have shown that the antimicrobial activities of EOs were greater than those noted with one of their components. Synergistic effects of the

chemotypes are important for the expression of a strong antimicrobial activity. The chemotype carvacrol of *T. maroccanus* appeared to present a great effect on some studied bacteria (Table 2). The EOs of *T. maroccanus* presented significant higher activities (ANOVA test, $p<0.05$) than the EOs of *T. broussonetti* against *B. Subtilis*, *S. aureus*, *Salmonella* sp. and *E. coli*. Other studies have showed that the carvacrol, dominant in *T. maroccanus* (Table 1), presented an important antimicrobial activity [15].
The antibacterial effects of *T. maroccanus* were significantly higher (ANOVA test, $p<0.05$) than the antibacterial effect of the antibiotic; the tetracycline against *B. subtilis*, *Salmonella* sp. and *E. coli*. Whereas, *T. maroccanus* presented higher significant activities than the gentamycin against *B. subtilis* (Table 2).

The EOs of both *Thymus* species showed larvicidal effectiveness (Table 3). From the dose of 0.125ppm, the EOs activities were significantly higher in comparison to the negative control (ANOVA test, $p<0.05$). The lethal concentrations for 50% of insect population (LC50), during exposure of the EOs at 24h, were 0.23 and 0.31 respectively for *T. broussonetti* and *T. maroccanus* (Table 4). Carvacrol and thymol compounds of the two thyme oils appeared to have effective toxicity on the larvae of *C. pipiens* according to the finding of Traboulsi et al. [29].

The present study provides evidence that the EOs extracted from *T. broussonetti* and *T. maroccanus* present potent antibacterial activities. Moreover the EOs could be used as substitute biopesticide in *C. pipens* control and would help to decrease the negative impact of chemical insecticides harmful to humans and the environment.

Table 1. Essential oil composition of *T. broussonetti* and *T. maroccanus* (%).

RI	Compounds	*T. broussonetii*	*T. maroccanus*
921	Tricyclene	0.3	t
924	α-thujene	1.3	0.8
934	α-pinene	**11.8**	**11.6**
937	Thuja-2,4(10)-diene	0.1	0.1
944	Camphene	**8.5**	**0.8**
964	Octen-3-ol	-	1.3
966	Sabinene	0.2	-
971	β-pinene	1.9	0.4
980	Octan-3-one	-	0.2
984	Myrcene	2.1	1.4
998	α-phellandrene	0.2	0.3
1006	3-δ-carene	0.1	-
1010	α-terpinene	1.0	1.1
1017	p-cymene	**21.0**	**25.3**
1024	Limonene	2.2	2.7
1052	γ-terpinene	**2.5**	**4.6**
1054	(E)-sabinene hydrate	0.3	0.2
1073	Dehydro-p-cymene	-	0.1
1079	Terpinolene	0.2	0.1
1085	Linalool	0.2	2.3
1122	Camphor	**0.1**	**0.2**
1140	Isoborneol	t	-
1148	Borneol	**16.5**	**0.8**
1161	Terpinen-4-ol	0.4	0.5

Table 1 continued

1171	Dihydrocarvone 1	0.4	0.3
1179	Dihydrocarvone 2	0.1	0.2
1214	Carvone	-	0.2
1233	Carvenone	t	-
1268	Thymol	**11.3**	**0.4**
1280	Carvacrol	**3.7**	**33.0**
1328	Eugenol	-	t
1345	Carvacryl acetate	-	t
1348	α-cubebene	t	t
1370	α-ylangene	-	t
1375	Copaene	-	0.1
1382	β-bourbonene	0.1	0.1
1386	β-patchoulene	-	t
1408	α-gurjunene	0.1	-
1416	β-caryophyllene	0.3	2.0
1425	β-cubebene	-	t
1437	Aromadendrene	2.1	1.4
1449	α-humulene	-	0.1
1456	allo-Aromadendrene	0.4	0.2
1469	γ-muurolene	0.2	-
1474	D-germacrene	-	t
1484	β-gurjunene	-	0.2
1491	Ledene	**3.2**	1.0
1504	γ-cadinene	-	1.3
1508	Calamenene	t	0.1
1516	Acetovanillone	-	0.9
1532	(Z)-α-bisabolene	-	t
1561	Spathulenol	0.2	0.2
1567	Caryophyllene oxide	-	0.1
1571	Globulol	-	t
1652	Guaiazulene	-	t
	Essential oil yield (%, w/w)	**1.6**	**1.0**
	Total	**93.1**	**96.7**

t= trace (<0.01 %)

Table 2. Antibacterial activity of the EOs of *T. broussonetti* (*T.b.)* and *T. maroccanus* (*T.m.*) antibiotics.

Bacterial strains		Inhibition Zone (mm)*			
		Essential oil		Standard antibiotics	
		T.b.	*T.m.*	Gentamycin	Tetracycline
Gram positive bacteria					
	Bacillus subtilis	33±0.4	43±0.1	37±0.4	22±0.3
	Staphylococcus aureus	19±0.8	23±0.1	22±0.2	20±0.3
Gram negative bacteria					
	Salmonella sp.	19±0.9	23±0.4	25±0.1	15±0.3
	Escherichia coli	21±0.1	23±0.1	29±0.1	11±0.1
	Vibrio cholerae	40±0.4	37±0.3	37±0.3	35±0.2
	Pseudomonas aeruginosa	9±0.1	11±0.1	23±0.1	9±0.1

*Values are reported as means ± standard deviations of three separate determinations.

Table 3. Mortality of young fourth instar larvae of *C. pipiens* by essential oils of *T. broussonetti* and *T. maroccanus*

		% Mortality (mean ± SD)		
	Doses (ppm)	Exposure time		
		15 min	60 min	24h
T. broussonetti	0.5	35±0.71	54±0.45	71±0.84
	0.25	15±0.71	34±0.84	56±0.84
	0.125	12±0.55	23±0.89	35±0.71
	0.0625	7±0.55	11±0.84	15±0.71
	Control (-)	8±0.55	10±0.71	10±0.71
	Control (+)	30±3.41	80±7.30	100±0.00
T. maroccanus	0.5	28±0.55	34±0.45	65±1.00
	0.25	12±0.55	24±0.84	48±0.89
	0.125	12±0.55	18±0.55	33±0.55
	0.0625	8±0.55	11±0.45	12±0.55
	Control (-)	5±0.71	6±0.45	7±0.55
	Control (+)	32±2.50	97±1.91	100±0.00

% Mortality (mean ± SD)
Control (-) : Ethanol.
Control (+) : 1ppm Temephos

Table 4. Lethal concentrations for 50 and 90 % of insect population

	T. broussonetti		*T. maroccanus*	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
15min	0.85	2.84	1.26	1.70
60 min	0.50	2.36	1.45	1.97
24h	0.23	0.76	0.31	1.53

Values are the mean (±S.D)
95% Confidence limits

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References

[1] H. Ismaili, L. Milella, S. Frih-Tetouani, A. Ilidrissi, A. Camporese, S. Sosa, G. Altinier, R. Della Loggia and R. Aquino (2004). *In vivo* topical anti-inflammatory and *in vitro* antioxidant activities of two extracts of *Thymus satureioides* leaves, *J. Ethnopharmacol*. **91**, 31-36.

[2] B. Dababneh (2007). Antimicrobial activity and genetic diversity of thymus species on pathogenic microorganisms, *J. Food Agr. Environ*. 5, 158-162.

[3] A. Meister, G. Bernhardt, V. Christoffel and A. Buschauer (1999). Antispasmodic activity of *Thymus vulgaris* extract on the isolated guinea-pig trachea: discrimination between drug and ethanol effects, *Planta Med*. ***65***, 512-516.

[4] T. Dob, D. Dahmane, T. Benabdelkader and C. Chelghoum (2006). Studies on the essential oil composition and antimicrobial activity of *Thymus algeriensis* Boiss et Reut, *Int. J. Aromatherapy*. **16**, 95-100.

[5] J. Bellakhdar (1997). La pharmacopée marocaine traditionnelle, *ed*: Ibiss Press, Paris, pp.358-359.

[6] H. Ismaili, S. Sosa, D. Brkic, S. Frih-Tetouani, A. Il Idrissi, D. Touati, R. Aquino and A. Tubaro (2002). Topical anti-inflammatory activity of extracts and compounds from *Thymus broussonetii, J. Pharm. Pharmacol.* **54**, 1137-1140.

[7] K. Elhabazi, A. Dicko, F. Desor, A. Dalal, C. Younos and R. Soulimane (2006). Preliminary study on immunological and behavioral effects of *Thymus broussonettii* Boiss. an endemic species in Morocco. *J. Ethnopharmacol*. **103**, 413-419.

[8] A. Benabid (2000*).* Flore et écosystèmes du Maroc. Evaluation et préservation de la biodiversité, *ed*: Ibis Press, Paris, pp.359-361.

[9] G. Milhau, A. Valentin, F. Benoit, M. Mallie, J. Bastide, Y. Pelissier and J. Bessiere (1997). *In vitro* antimicrobial activity of eight essential oils, *J.Ess.Oil Res*. **9**, 329-333.

[10] M. Mastouri, M. Nour , M. Ben Nejma, O. Bouallegue, M. Hammami and M. Khedher (2006). Antibiotics resistance of meticilline resistant *Staphylococcus aureus*: detection of the first glycopeptides low sensibility strains in Tunisia, *Pathologie Biologie*. **54**, 33-36.

[11] WHO (1996). Report of WHO in formal consultation on the evaluation and testing of insecticides CTD/WHO PES/IC/96.1, p. 69.

[12] W.S. Abbott (1925). A method of computing the effectiveness of an insecticide, *J. Eco. Entomol.*. **18**, 265-267.

[13] I. Rasooli and S.A. Mirmostafa (2002). Antibacterial properties of *Thymus pubescens* and *Thymus serpyllum* essential oils, *Fitoterapia*. **73**, 244-250.

[14] R. Di Pasqua, V. De Feo, F. Villani and G. Mauriello (2005). *In vitro* antimicrobial activity of essential oils from Mediterranean Apiaceae, Verbenaceae and Lamiaceae against foodborne pathogens and spoilage bacteria, *Ann. Microbiol.***55**, 139-143.

[15] S.A. Burt, R. Vlielader, H.P. Haagsman and E.J.A. Veldhuizen (2005). Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers, *J. Food Prot*. **68**, 919-926.

[16] G. Ozcan, O. Sagdic and M. Ozcan (2003). Inhibition of pathogenic bacteria by essential oils at different concentrations, *Int. J. Food Sci. Technol*. **9**, 85-88.

[17] Y.C. Yang, I.K. Park, E.H. Kim, H.S. Lee and Y.J. Ahn (2004). Larvicidal activity of medicinal plant extracts against *Aedes aegypti*, *Aedes togoi*, and *Culex pipiens pallens* (Diptera: Culicidae), *J. Asia-Pac. Entomol*. **7**, 227.

[18] K.M. Knio, J. Usta, S. Dagher, H. Zournajian and S. Kreydiyyeh (2008). Larvicidal activity of essential oils extracted from commonly used herbs in Lebanon against the seaside mosquito, *Ochlerotatus caspius, Biores. Technol*. **99**, 763–768.

[19] R. Pavela (2009). Larvicidal property of essential oils against *Culex quinquefasciatus* Say (Diptera: Culicidae), *Ind. Crop. Prod.***30**, 311–315.

[20] D. Joulain and W.A. König (2001). The Atlas of Spectra Data of Sesquiterpene Hydrocarbone. E.B.-Verlag, Hamburg, (1998). b) D. Joulain, W.A. König, D.H. Hochmuth, Terpenoids and Related Constituents of Essential Oils. Library of Mass Finder 2.1.

[21] F.W. Mc Lafferty and D.B. Stauffer (1989). The Wiley/NBS Registry of Mass Spectral Data, J. Wiley & Sons, New York.

[22] R.P. Adams (2001). Identification of essential oil components by Gas chromatography / quadrupole mass spectroscopy, Allured Publshing Co, Carol Stream, Illinois.

[23] H. Benhassaini, F.Z. Bendeddouche, Z. Mehdadi and A. Romane (2008). GC/MS analysis of the Essential Oil from the Oleoresin of *Pistacia atlantica* Desf. subsp. *atlantica* from Algeria, *Nat. Prod. Commun*. **3**, 929-932.

[24] N. Achak, A. Romane, M. Alifriqui and R.P. Adams (2008). Effect of the Leaf Drying and Geographic Sources on the Essential Oil Composition of *Juniperus Thurifera L.* var. *Africana* Maire from the Tensift-Al Haouz-Marrakech Region. *J.Ess. Oil Res.* **20**, 200-204.

[25] N.W. Davies (1990). Gas chromatographic retention indexes of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20M phases, *J. Chromatogr*. **503**, 1-24.

[26] ESO 2000 (1999). The Complete Database of Essential Oils, Boelens Aroma Chemical Information Service, Netherlands.

[27] I.M. Helander, H.L. Alakomi., K. Latva-Kala, T. Mattila-Sandh olm, I.E. Pol, E.J. Smid, L.G.M.Gorris, and A. Von Wright (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria, *J. Agric. Food Chem.* **46**, 3590–3595.

[28] R.J.W. Lambert, P.N. Skandamis, P.J. Coote and G.J.E. Nychas (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol, *J. Appl. Micro.* **91**, 453–462.

[29] A.F. Traboulsi, K. Taoubi, S. el-Haj, J.M. Bessiere and S. Rammal (2002). Insecticidal properties of essential oils against the mosquito *Culex pipiens* molestus (Diptera: Culicidae), *Pest Manag. Sci.***58**, 491-495.

[30] S. Cosentino, C.I.G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi and F. Palmas (1999). *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils, *Lett. Appl. Microbiol.* **29**,130-135.