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Bacteriostatic and Bactericidal Profile of Leaves and Twigs Essential oils of Moroccan *Pistacia lentiscus* L.

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ABSTRACT

In order to increase the Moroccan *Pistacia lentiscus* L. value, the antibacterial activity of its twig's and leave's essential oils was evaluated. The study of antibacterial activity was performed on Gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* by the microdilution method. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of leaves and twigs essential oils against the four studied strains were determined and showed that both essential oils tested have remarkable antibacterial activity. *Bacillus subtilis* was the most sensitive strain against the two essential oils while *Pseudomonas aeruginosa* was the most resistant one.

INTRODUCTION

For centuries, medicinal plants have been used as a remedy for various diseases. Over the past few years, the use of plant-based natural antimicrobials in the treatment of bacterial infections has gained much recognition (Kasrati *et al.,* 2014).The progressive interest of these plant's use has urged researchers to look for new methodologies (Hemaiswarya *et al.,* 2008), and for suitable design to develop new therapeutic value products with highly effective anti-infective agents in general and antibacterial agents in particular (Turgis *et al.,* 2012).

Pistacia lentiscus L. belong to the family of *Anacardiaceae*, also called pistachio mastic or mastic tree (Hmamouchi *et al.,* 1999). Shrub up to 5 m high, the mastic is one of the most characteristic trees of the Mediterranean region (Aafi et al, 2002), which grows on all kinds of soil (Bayer *et al.,* 2009) and it is a native species of Morocco (Aafi et al, 2002). The therapeutic properties of this species were known, long time ago, when the Egyptians used mastic for embalming (De Pooter *et al.,* 1991). The essential oil of mastic tree has been shown to have antibacterial (Derwich *et al.,* 2010), anti-fungal (Darua *et al.,* 2003), insecticides (Bachrouch *et al.,* 2010) and antioxidants effects (Barra *et al.,* 2007). Mastic tree is also used in cosmetics, perfumes and as a flavoring in food preparations (Daferera *et al.,* 2002). The aim of this study was to evaluate the antibacterial activity and to identify the chemical profiles of essential oils from twigs and leaves of *Pistacia lentiscus L.* collected in Taounate region (Morocco).

MATERIAL AND METHODS

Plant material

Leaves and twigs of *P. lentiscus* L. were randomly collected from natural populations at the flowering stage during May 2013 in the Ifrane's forest at Taounate region in Morocco (Altitude: 475 m, 34° 35'12.5" N 4° 38'31.1" W). *Pistacia lentiscus* leaves were separated from stem, and the twigs were cut into small pieces to facilitate the extraction of essential oils.

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Extraction of essential oil

The essential oils were extracted from *P. lentiscus* L. by hydrodistillation process in Clevenger-type apparatus (Clevenger, 1928). Plant material was dried at 105 °C for 4 hours to determine the moisture (Zrira *et al.*, 1995) and the yield was expressed relatively to the dry matter. Essential oils obtained were stored in opaque glass bottles at 4 °C.

Chemical analysis of essential oil

The essential oil was analyzed using Gas chromatography (GC) coupled to mass spectrometry GC / MS (Polaris Q ion trap MS). Hence, analyses were performed on a Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a 5% phenyl methyl silicone HP-5 capillary column (30m x 0.25 mm x film thickness 0.25 µm). The temperature was programmed from 50°C after 5 min initial hold to 200°C at 4°C/min. Chromatography carrier gas was N2 (1.8 ml/min), split mode was used (Flow: 72.1 ml/min, ratio: 1/50), temperature of injector and detector was 250 °C, final hold time was 48 min. The machine was led by a computer system type "HP Chem Station", managing its functioning and allowing to follow the evolution of chromatographic analyses. Diluted samples (1/20 in methanol) of 1µl were injected manually.

Antibacterial activity

Bacterial strains

The *in vitro* antibacterial effect of essential oils was tested against the following bacterial strains: *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 3366, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922. All of these strains were maintained in 20 % glycerol at -20 °C as stock.

Inoculum preparation

The direct colony suspension method was used for the inoculum preparation. Briefly, bacteria were sub-cultured in the Luria Bertani agar (LB). Plates were incubated at 37 °C for 24 h. A loop full of isolated colony was aseptically transferred into physiologic saline solution and the turbidity of the suspension was adjusted to 0.5 McFarland (Murray *et al.*, 2007).

Antibacterial screening

The screening of antibacterial activity was performed by the agar disc-diffusion method (Murray *et al.*, 2007). Petri dishes (90 mm in diameter) containing LB- agar were seeded using the previously prepared inoculum. The seeding was done so as to ensure a homogeneous distribution of bacteria, then excess liquid was eliminated with a Pasteur pipette and the Plates were dried for 20 minutes. The sterile filter paper discs (6 mm in diameter) were individually soaked with 10 µl of each essential oil then placed on the surface of plates seeded, which were placed at 4 °C for 2 h. After incubation for 24 h at 37 °C, inhibition diameters were measured. All the tests were performed in triplicate.

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

The broth microdilution method was used to evaluate the minimum inhibitory concentration (MIC) according to the CLSI guidelines M7-A7 (CLSI, 2007), with slight modifications. Briefly, agar at 0.15% (w⁄v) was used as emulsifier and resazurin was used as bacterial growth indicator (Mann and Markham, 1998). Firstly, 50 µl of Mueller Hinton Broth (MHB) supplemented with bacteriological agar (0.15% w/v) were distributed from the second to the 12th well of a 96-well polypropylene microtitre plate. Essential oils dilutions were prepared in MHB supplemented with agar (0.15% w/v), 100 µl of these suspensions were added to the first test well of each microtitre line, 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 was added to each well at a final concentration of approximately 10^6 CFU/ml. The final concentration of the essential oil was between 16 and 0.0015% (v⁄ v) for leaves and twigs. After incubation at 37°C for 20 h, 5 µl of resazurin was added to each well (Mann and Markham, 1998). After further incubation at 37°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in resazurin color (CLSI, 2007). Experiments were conducted in triplicate.

To determine the minimum bactericidal concentration (MBC), 2 µL of each negative well, in which microbial growth was not observed, were spotted on LB plates and incubated at 37 °C for 24 h. The MBC corresponded to the lowest concentration of the essential oil at which the incubated microorganism was completely killed (Bassole *et al.*, 2001). Each test was performed in triplicate.

RESULTS AND DISCUSSION

Yield and chemical composition of the leaves and twigs essential oils

The hydrodistillation of *Pistacia lentiscus* leaves and twigs gave essential oils with yields of 0.3% and 0.5% (v/w) respectively.

The results of chromatographic analysis of *Pistacia lentiscus* leaves and twigs essential oils are presented in Table 1. In order to simplify the analysis of the results, only compounds having abundance more than 0.5% were selected. Twenty nine compounds, which represented 77.90% of the total leaves essential oils, were identified. The major constituents of the *Pistacia lentiscus* leave's essential oil were Tricyclene (7.71%), terpinen-4-ol (7.44%), sabinene (6.96%), caryophyllene (6.62%), caryophyllene oxide (6.05 %), p-cymene (5.04 %), 3-carene (4.44%), α-terpineol (4.16%) and trans-β-Ocimene (3.89%).

Twenty six compounds, which represented 84.72% of the twigs essential oils, were identified. The major constituents of this essential oil were α-pinene (19.24%), tricyclene (8.16%), trans-β-Ocimene (6.9%), caryophyllene (6.18%), 3-carene (5.18%) and germacrene (5.17%). Similar compounds have been reported at

different percentages by other authors (Amhamdi *et al.,* 2009). However, the number of compounds identified in leaves and twigs essential oil (29 and 26) respectively, was inferior to the number of compounds identified in a previous study (Hafsé *et al.,* 2013). This could be explained by: environmental factors such as geography, temperature and collection period, etc., which were considered to play a key role in the chemical composition of essential oils (Derwich *et al.,* 2010).

Table 1: Main constituents (%) of *Pistacia lentiscus* leaves and twigs essential oil.

RI (min) [a]	Compound [b]	Leaves (%)	Twigs (%)
915	Tricyclene	7.71	8.16
939	α-Pinene	-	19.24
953	Camphene	1.65	1.21
974	Sabinene	6.96	-
1006	α- Phellandrene	2.61	3.36
1011	3-carene	4.44	5.18
1026	p-Cymene	5.04	3.52
1050	trans-β-Ocimene	3.89	6.90
1072	γ-Terpinene	0.63	-
1089	p-Cymenene	0.59	0.55
1180	Terpinen-4-ol	7.44	0.86
1192	α-Terpineol	4.16	0.65
1195	Myrtenal	-	0.50
1219	Trans-Carveol	0.62	0.56
1266	Geraniol	0.58	-
1288	Bornylacetate	3.32	1.96
1294	Undecanone	0.80	0.62
1348	α-Cubebene	1.06	1.21
1381	α-Copaene	0.83	-
1395	β-Elemen	0.94	1.74
1418	Caryophyllene	6.62	6.18
1454	α-Caryophyllene	1.65	1.75
1462	Benzoic acid, pentyl ester	0.57	-
1464	Aromadendrene	0.65	0.85
1480	Germacrene- D	-	5.17
1499	α-Muurolene	1.83	1.84
1513	γ-Cadinene	0.68	1.76
1523	Calamenene	0.80	-
1528	δ-Cadinene	1.97	3.98
1538	α-Cadinene	1.90	-
1576	Spathulenol	-	0.86
1581	Caryophylleneoxide	6.05	2.29
1648	Aromadendreneoxide	-	3.82
1652	α-Cadinol	2.72	-

a: retention indices on HP-5 capillary column.
b: Compounds present in trace amounts (<0.5%) were not registered.

Antibacterial activity

Results of both essential oil's antibacterial activity against *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa* are shown in table 2. As can be noted in this finding, both essential oils tested have shown a remarkable antibacterial effect. So, crude essential oils of the two *Pistacia lentiscus* parts were active against all strains examined. Indeed, the MIC values ranged from 4 to 0.015% (v/v) for leave's essential oil and from16 to 0.5 % (v/v) for twig'*s* essential oil. Hence, leaves essential oil exhibit a higher antibacterial effect with MIC values 0.015, 0.5, 1 and 4 % fold least compared to twigs essential oil with MIC values 0.5, 4, 4 and 16% against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* respectively. Also, it can be noted that *E. coli* and *P. aeruginosa* (Gram- negative) were more resistant to the leaves and twigs essential oil compared to *S. aureus* and *B. subtilis* (Gram-positive). Regarding the MBC values of both essential oils tested (Table 3), we found that MBC values could well be similar to their MIC values against *P. aeruginosa* and *B. subtilis* and two fold higher toward *S. aureus* and *E. coli* for twigs essential oil. In addition we noted that MBC values were 2, 4, 8 and 2 fold higher toward *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* respectively than MIC for leaves essential oil.

Table 2: Determination of minimum inhibitory concentration (MIC) values of *P. lentiscus* essential oils against bacteria tested.

Concentration	Leaves essential oil				Twigs essential oil			
%	B. s	S. a	E. c	P. a	B. s	S. a	E. c	P. a
16	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	+
4	-	-	-	-	-	-	-	+
2	-	-	-	+	-	+	+	+
1	-	-	-	+	-	+	+	+
0.5	-	-	+	+	-	+	+	+
0.25	-	+	+	+	+	+	+	+
0.125	-	+	+	+	+	+	+	+
0.062	-	+	+	+	+	+	+	+
0.031	-	+	+	+	+	+	+	+
0.015	-	+	+	+	+	+	+	+

B. s: *Bacillus subtilis*, E. c: *Escherichia coli*, S. a: *Staphylococcus aureus*, P. a: *Pseudomonas aeruginosa*

Table. 3: Determination of minimum bactericidal concentration (MBC)values of *P. lentiscus* essential oils against bacteria tested.

Concentration	Leaves essential oil				Twigs essential oil			
%	B. s	S. a	E. c	P. a	B. s	S. a	E c	P. a
16	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	+
4	-	-	+	+	-	+	+	+
2	-	-	+	+	-	+	+	+
1	-	+	+	+	-	+	+	+
0.5	-	+	+	+	-	+	+	+
0.25	-	+	+	+	+	+	+	+
0.125	-	+	+	+	+	+	+	+
0.062	-	+	+	+	+	+	+	+
0.031	-	+	+	+	+	+	+	+
0.015	+	+	+	+	+	+	+	+

B. s: *Bacillus subtilis*, E. c: *Escherichia coli*, S. a: *Staphylococcus aureus*, P. a: *Pseudomonas aeruginosa*

Antimicrobial activity of the essential oils of *P. lentiscus* against tested bacteria has shown that Gram-negative strains were more resistant compared to the Gram- positive ones. Similar findings have been reported by other authors (Hafsé *et al.,* 2013) who found that Gram negative strains were less sensitive to this essential oil than Gram- positive strains.

Pseudomonas aeruginosa has shown low sensitivity with MIC values of 16 and 8% for leaves and twigs essential oils respectively. Similarly, previous investigations showed that this bacterial strain was more resistant to leaves essential oils of *P. lentiscus* (Benhammou *et al.,* 2008). Typically, Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria, due to the differing structures of their cell wall. Outer membrane of the Gram-negative bacteria contains primarily lipopolysaccharides molecules and forms a hydrophilic barrier conferring protection against the effects of highly hydrophobic compounds (Trombetta *et al.,* 2005). The mechanism of action

assigned at both essential oils has not been studied in detail in the past. The results of this study confirm the findings in previous reports, which state that the strength and spectrum of activity varied between Gram type of target bacteria and the investigated parts of *P. lentiscus* (Djenane *et al.*, 2011).

The antibacterial activity of the essential oils of *P. lentiscus* could be attributed to their high content of different groups of chemical compounds known for their antibacterial effect. Nevertheless, the antibacterial activities of the essential oils are difficult to correlate to a specific compound due to their complexity (Mélanie *et al.*, 2012). The low antibacterial activity of twigs essential oil compared to the leaves one could be explained by their high content on terpene (α-pinene, tricyclene, 3-carene, trans-β-Ocimene and D-germacrene) known for their relatively low antibacterial activity (Inouye *et al.*, 2001). Moreover, many reports have shown that α-terpineol, present with high percentage in leaves essential oil, exhibit an inhibitory effect against *E. coli* (Alessandra *et al.*, 1999). Similarly geraniols, present only in the leaves oil, exhibit a higher antibacterial effect (Antonio *et al.*, 2007).

CONCLUSION

This work aims to evaluate the bacteriostatic and bactericidal profile of essential oils of leaves and twigs of *Pistacia lentiscus L.*against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*. Both studied essential oils presented remarkable antibacterial activity against tested strains. *B. subtilis* was the most sensitive strain in regard to the two essential oils while *P. aeruginosa* was the most resistant strain. The high antibacterial performance of *Pistacia lentiscus* essential oils from leaves and twigs should be studied in more details in order to make them a promising antibacterial agent for the control in food and pharmaceutical industries.

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