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GC/MS Analysis and *In vitro* Antibacterial Activity of the Essential Oil Isolated from Leaf of *Pistacia lentiscus* Growing in Morocoo

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Abstract: *Pistacia species* from the Anacardiaceae family are widely distributed in Morocco. In this work, the essential oils of *Pistacia lentiscus* collected in centre north from Morocco were obtained by hydro-distillation of the aerial parts and analysed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled to a mass spectrometry (GC/MS). The study was conducted to determine the phytochemistry and antibacterial activities of *Pistacia lentiscus* leaves oil against both bacteria using the disc diffusion method and minimum inhibitory concentration (MIC). For gram-negative: *Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia* and *salmonella typhi* and five gram-positive: *Staphylococcus aureus, Staphylococcus intermedius, Enterococcus faecalis, Bacillus sphericus* and *Enterobacter aerogens* were used as test bacterial strains. Twenty tree compounds were identified in leaves oil representing 77.22% of the total oil composition. The yield of essential oil of *Pistacia lentiscus* was 1.02% and the major compound in aerial parts were α-pinene (24.25%) followed by β-pinene (12.58%), limonene (7.56%), α-terpinen-4-ol (6.98%), α-terpineol (4.89%), β-Caryophyllene (3.15%), verbenol (3.05%), linalool (2.85%), camphene (2.32%) and myrcene (2.09%). The minimal inhibitory concentration (MIC) values against these bacteria ranged from.0.08 to 1.56 mg/mL.

Key words: *Pistacia lentiscus* · Essential oil · α-Pinene · Antibacterial activity

INTRODUCTION

Medicinal plants have been used by human being since ages in traditional medicine due to their therapeutic potential and the search on medicinal plants have led the discovery of novel drug candidates used against diverse diseases. According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [1]. Essential oils were used in ancient Rome, Greece and Egypt and throughout the Middle and Far East as perfumes, food flavours, deodorants and pharmaceuticals [2]. Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial, since ancient time. With the growing interest of the use of either essential oils or plant extracts in the food and pharmaceutical industries, screening of plant extracts for these properties has become of increasing importance [3]. Essential oils and their components are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances [4]. Essential oils have been traditionally used for the treatment of infections and diseases all over the world for centuries [5]. Multiple studies have been reported on the chemical composition of essential oil of *Pistacia lentiscus* belonging to different regions in the world [6-11]. The essential oil of leaves of *Pistacia* species has been the object of several studies of antibacterial [12-16], Antiproliferative [17] and antioxidant [18-20]. Antifungal activity of the essential oils and the leaf extracts of three *Pistacia species* including *Pistacea lentiscus* were also studied [21-23]. On the other hand, *Pistacia species* were reported to have various

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biological activities such as anti-atherogenic, hypoglycemic, anti-inflammatory, insectididal and antimutagenic activities [24-31]. The bark of *Pistacia lentiscus* used for the treatment of hypertension in some regions of Spain [32]. Furthermore, *Pistacia lentiscus* resin has been reported to possess anticancer activity [33], antiulcer activity [34]. Gum" mastic, oleoresin exudates from the stem of *Pistacia lentiscus* has a long history of use as a therapeutic agent with many reported medicinal properties [35]. It has been proven as a therapeutic agent against various gastric malfunctions, such as gastralgia, dyspepsia and gastric ulcer [34].

Morocco is blessed with a rich source of aromatic plants, many of which have not been previously investigated for their chemical constituents and biological potentials. *Pistacia lentiscus* is a plant belongs to the family Anacardiaceae, which grows in Morocco region and is a potential source of essential oils. Among them *Pistacia lentiscus* are the main oleoresin producing species and of immense economic and pharmaceutical importance [36]. The essential oil of *Pistacia lentiscus* is also used in cosmetics, perfumery and as a flavouring agent in food preparations [37]. The essential oil of this plant possesses anti-*Heliocobacter pylori* activity and can be beneficial in the treatment of peptic ulcer [34,38]. The aerial parts of *Pistacia. lentiscus* L. has traditionally been used in the treatment of hypertension and possesses stimulant and diuretic properties [39]. In this study we determinate the chemical constituents and evaluate the antibacterial activity of essential oils extracted from leaves of *Pistacia lentiscus* against for gram-negative: *Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumoniae* and *salmonella typhi* and five gram-positive*:* *Staphylococcus aureus, Staphylococcus intermedius, Enterococcus faecalis, Bacillus sphericus* and *Enterobacter aerogens* in vitro conditions.

MATERIALS AND METHODS

Plant Material: The aerial parts of *Pistacia lentiscus* was collected during May 2009 from remote areas in the suburbs of sekoura region 100 km from Fez city (Morocco) and identified by direct comparison with herbarium sample. The climate is semi-humid with strong continental influence with an annual average temperature of 20°C. The leaves were then isolated from the other specimen and conserved for extraction.

Extraction of Essential Oil: The leaves of *Pistacia lentiscus* were shade dried (25 days) at room temperature and immediately hydro-distilled for 3h according to the method recommended in the British Pharmacopoeia [40]. The oil was dried over anhydrous sodium sulfate and stored in the refrigerator (4°C).

Gas Chromatography Analysis (GC-FID): The isolated oil was diluted with hexane and 1µL was sampled for the gas chromatography with flame ionisation detector. Trace GC (ULTRA S/N 20062969, Thermo Fischer), gas chromatograph equipped with HP-5MS non polar fused silica capillary column (60 m x 0.32 mm, film thickness 0.25 µm) was used. Operating conditions: oven temperature program from 40°C (2 min) to 260°C at 3°C/min and the final temperature kept for 10 min; "split mode" ratio 1:20; carrier gas Azoth (N_2), flow rate 1 ml/min; temperature of injector and flame ionisation detector (FID) were fixed at 250°C and 270°C, respectively.

Gas Chromatography-mass Spectrometry Analysis (GC/MS): GC/MS analyses were performed on a Thermo Fischer capillary gas chromatograph directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; PolarisQ S/N 210729). HP-5MS non polar fused silica capillary column (60 m x 0.32 mm, 0.25 µm film thickness) was used under the following conditions: oven temperature program from 40°C (2 min) to 260°C at 2°C/min and the final temperature kept for 10 min; injector temperature, 250°C; carrier gas He, flow rate 1mL/min; the volume of injected specimen was 1µl of diluted oil in hexane; splitless injection technique; ionization energy 70eV, in the electronic ionization mode; ion source temperature 200°C; scan mass range of m/z 40-650 and interface line temperature 300°C.

The constituents of essential oils were identified in comparison with their Kovats Index, calculated in relation to the retention time of a series of alkanes (C_4- C_{28}) with those of reference products and in comparison with their Kovats index with those of the chemical components gathered by Adams table [41] and in comparison with their spectres of mass with those gathered in a library of (NIST-MS) type and with those reported in the literature [42-44].

Antibacterial Activity: In recent years, there has been target interest in biologically active constituents, isolated from plant species for the elimination of pathogenic micro-organisms, because of the resistance that micro-organisms have built against antibiotics [45] or because they are ecologically safe compounds [46].

The selected essential oil was screened against four: bacteria gram-negative (*Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia and salmonella typhi)* and five gram-positive *(Staphylococcus aureus, Staphylococcus intermedius, Enterococcus faecalis, Bacillus sphericus and Enterobacter aerogens)* bacteria. The minimal inhibition concentration (MIC) values were evaluated according to published procedures [47-48-35-42-49]. The minimal inhibitory concentration (MIC) was determined only with micro-organisms that displayed inhibitory zones. MIC was determined by dilution of the essential oils in dimethyl sulfoxide (DMSO) and pipetting 0.01 mL of each dilution into a filter paper disc. Dilutions of the oils within a concentration range of 0.08- 1.56mg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth [50]. The bacterial plates were incubated at 37°C and the zone of inhibition measured in mm after 24h, 48h and 72h of growth. A control experiment was set up by using an equal amount of sterile distilled water in place of different extract concentrations. Many screening reports, using disc diffusion and dilution techniques, have established an antimicrobial activity of *Pistacia* extracts from various species against a number of pathogens including [51] (*Staphylococcus aureus, Escherichia coli* and *Streptococcus pyogenes*), [52] (*Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus aureus*) and [53] *(Streptococcus mutans* and *Mutans streptococci*).

RESULTS AND DISCUSSION

Chemical Composition of the Leaves Essential Oils: The constituents of leaves essential oil of *Pistacia lentiscus* from Morocco are listed in order of their elution on the HP-5MS column, Figure (1).

In total twenty three volatile compounds, representing 77.22% of the total composition, were identified in the leaves oils (Table 1). Monoterpene hydrocarbons were found to be the major group of compounds, the main one being α pinene (24.25%) followed by β-pinene (12.58%). The most abundant components found in the leaf oil were α-pinene (24.25%) followed by β-pinene (12.58%), limonene (7.56%), α-terpinen-4-ol (6.98%), α-terpineol (4.89%), β-caryophyllene (3.15%), verbenol (3.05%), linalool (2.85%), camphene (2.32%) and myrcene (2.09%). The essential oil yield of *Pistacia lentiscus* collected from Fez city (Morocco) was 1.02%. It is relatively higher than other plants industrially exploited as a source of essential oils: *Achillea ligustica* (0.43-0.88%) [54], *Pistacia vera* (0.1%) [55], Thymus (1%) [56], lavender (0.8-2.8%), menthe (0.5-1%), néroli (0.5-1%) and Laurel (0.1-0.35%) [57], Artemisia (0.65%) [58] and *Teucrium polium* L (0.75%) [59].

The chemical compositions revealed that this leaves had compositions similar to those of other *Pistacia lentiscus* essential oils analyzed in Turkey [22] which the major constituent in lab sample have been

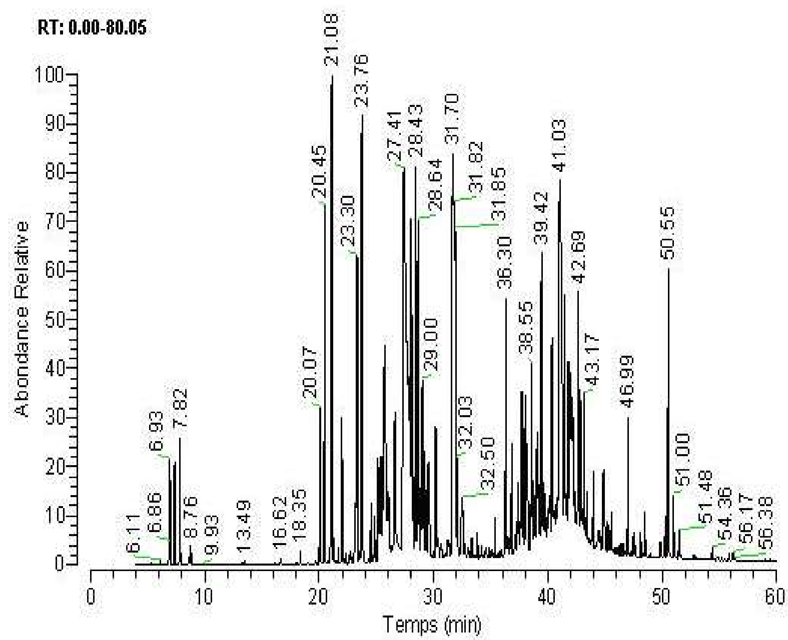

Fig. 1: Chromatogram of *Pistacia lentiscus*

Table 1: Chemical composition of essential oils of *Pistacia lentiscus* from Morocco

Peak	Constituents	Area (%)	Retention time (min)	Kovats index	Molecular ion (m/z)	Main fragment ions (m/z)
1	Isoledene	0.07	6.93	1419	204	161,105,119,41,91,204,133,55,93,81
2	3-carene	0.98	7.82	1005	136	93,91,79,77,92,121,80,136,94,105
3	Cymene	0.01	8.76	1042	134	119,134,91,120,117,41,77,39,65,115
4	α-phellandrene	1.15	20.07	964	136	93,77,91,136,79,94,41,80,92,39
5	Verbenol	3.05	20.45	1122	152	109,41,94,81,39,69,55,97,43,57
6	α-pinene	24.25	21.08	928	136	93,91,39,121,77,92,79,43,41,105
7	Linalool	2.85	23.30	1082	136	71,41,43,93,55,69,80,39,121,27
8	β-pinene	12.58	23.76	966	136	93,91,69,39,77,92,79,53,41,27
9	p-cymen-8-ol	0.08	24.50	1042	134	119,134,91,120,117,41,77,39,65,115
10	Terpinolene	0.02	26.45	1052	136	93,121,91,136,79,77,105,39,41,107
11	Limonene	7.56	27.41	1018	136	68,93,39,67,41,27,53,79,94,92
12	Terpinene-4-ol	6.98	28.43	1137	154	71,111,93,43,86,41,69,55,68,154
13	β-caryophyllene	3.15	28.64	1494	204	93,133,91,41;79,69,105,107,120,77
14	Cis-ocimene	0.35	29.00	976	136	93,41,79,39,91,77,92,27,80,53
15	α -farnesene	0.07	29.25	1458	204	41,93,69,107,55,79,91,119,77,123
16	α-terpineol	4.89	31.70	1174	154	59,93,121,136,81,43,68,95,67,41
17	y-terpinene	4.45	31.82	998	136	93,91,136,121,77,92,79,43,41,105
18	Camphor	0.12	32.03	943	152	95,41,81,39,55,69,108,67,83,27
19	Borneol	0.10	32.50	1138	154	95,41,110,93,55,67,139,121,96,69
20	Spathulenol	0.05	34.08	1536	220	43,41,205,119,91,93,159,105,162,107
21	Camphene	2.32	36.30	949	136	93,79,91,77,41,121,67,27,107,39
22	Myrcene	2.09	42.69	948	136	41,93,69,39,27,53,79,77,67,91
23	Globulol	0.05	44.11	1530	222	43,41,69,81,109,55,95,67,107,93
Total Identified Compounds		77.22				
Yields (%)		1.02				

Retention time obtained by chromatogram (Figure 1)

Kovats Index was determined by GC-FID on HP-5MS column

Molecular ion and Main fragment ions were determined by mass spectrometry (PlarisQ)

reported as *α*-Pinene, *β*-pinene, limonene, terpinen-4-ol and *α*-terpineol. In Tunisia [15], Twenty-seven compounds were identified, α-Pinene (17%), γ-terpinene (9%) and terpinen-4-ol (12%) were characterized as the main constituents and in United Kingdom [60], which the major compounds identified were α-Pinene, β-myrcene, β-pinene, limonene and β-caryophyllene. On the other hand, α- Pinene, γ-terpinene and terpinene-4-ol have been characterized as the main constituents of the essential oil of the *Pistacia lentiscus* leaves [22]. Chemical composition of the essential oil of *Pistacia lentiscus* L. from Morocco revealed that the major oil components from Oulmes were α-pinene (16.5-38.5%), *β*-myrcene (10.2-11.5%) and limonene (6.8-9.8%), while terpinen-4-ol (32.7-43.8%), α-pinene (7.1-13.5%) and bornyl acetate (6.8-10.3%) [61] and from Tafoghalt, 513 km northeast of Rabat (Morocco) the main constituents of the oil were: myrcene (39.2%), limonene (10,3), β-gurjunene (7.8), germacrene (4.3%), α-pinene (2.9%), muurolene (2.9), α-humulene (2.6), Epi- bicyclosesquiphellandrene (2.5), β-pinene (2.2) [62]. Intraspecific chemical variability of the essential oil of *Pistacia lentiscus* L. from Corsica studies has reported the occurrence of myrcene, limonene, terpinen-4-ol, α-pinene, β-pinene, α-phellandrene, sabinene, p-cymene and γ-terpinene [63]. Other studies [49] reported the major constituent in leaves of *Juniperus phoenicea* collected from Atlas median in the region of Boulmane (Morocco) as α-pinene (49.15%). Contrary this composition is different to the composition of essential oil of leaves of *Pistacia lentiscus* study in Greece which the major components were myrcene, α-pinene? trans-caryophyllene and germacrene D [64-65]. Furthermore, the essential oils obtained from leaves of *Pistacia lentiscus* in Turky contained: terpinene-4-ol (29.2%), β-caryophyllene (29.2%) and *p*-cymene (7.1%) [9]. In a previous study, [29], reported that myrcene (38%), limonene (15.5%), *p*-cymene (10.1%) and α-phellandrene (7.6%) were the major compounds. In others studies, the essential oils obtained from leaves of *Pistacia lentiscus* in Algeria contained approximately 95% of the components were identified among which terpinen-4-ol (17.3-34.7%), α-terpineol (10.4-11.0%) and germacrene D (8.4-15.8%) were the major constituents [66]. Intensive research on the chemical characteristics has been conducted on this species [67-69-63]. The essential oil of *Pistacia lentiscus* has been reported in varying detail

Table 2: Constituents of essential oil of other *Pistacia* species

Plant species	Major compounds	Essential Oil (%)	References
Pistacia atlantica	α -pinene	70	[70]
	bornyl acetate	21.50	[71]
Pistacia lentiscus	myrcene	68.20-71	[72]
	myrcene	39.20	[61]
	α -pinene	64.43-86.38	[63]
	d-3-carene	65	[7]
	β-caryophyllene	13.10	[62]
Pistacia khinjuk	α -pinene	60.13	[73]
Pistacia vera	α-pinene	60.13	[54]
Pistacia terebinthus	limonene	34.20	[74]
	α-cadinol	6.90	[75]
Pistacia palaestina	(*E*)-ocimene	41.30	[76]

Table 3: Antibacterial activity of leaves essential oils of *Pistacia lentiscus*

Bacteria	Micro-organisms	Disc diffusion assay (inhibition zone mm)	MIC (mg/mL)
Gram negative			
	Salmonella typhi	38	0.08
	Escherichia coli	34	0.12
	Klebsiella pneumonia	24	0.21
	Pseudomonas aeroginosa	10	1.01
Gram positive			
	Enterobacter aerogens	35	0.07
	Enterococcus faecalis	23	0.68
	Staphylococcus aureus	14	0.91
	Staphylococcus intermedius	11	1.02
	Bacillus sphericus	7	1.56

Disc diameter 6 mm average of three consecutive trials

MIC: Minimal Inhibitory Concentration, concentration range 0.08-1.56mg/mL

[62-61-15-70]. In this work the yield and total oil composition of essential oils of *Pistacia* collected from sekoura region (Morocco) where 1.02% and 77.22%. The total oil composition (77.22%) is heigher than other *Pistacia lentiscus* which in Greece witch is (13.50%) [64], in Tinisia which is recorded (58%) [15]. [21] studied the composition and antifungal and antioxidant activity of *Pistacia lentiscus* oil from Italy and they reported that the yields and the total oil obtained were 0.09-0.32% and 97.5-98.4% respectively and the composition is characterized by a high content α-pinene (14.8-22.6%), β-myrcene (1-19.4%), p-cymene (1.6-16.2%) and terpinen-4-ol (14.2-28.3%). Furthermore, in Greece [55] the yields were 0.5 and 0.1% respectively. Twenty one compounds were identified in the essential oil of the fruits and the major components were α-pinene (54.6 %) and terpinolene (31.2%). Thirty three compounds were identified in the essential oil of the leaves and the major components were found to be α-pinene (30.0 %), terpinolene (17.6 %) and bornyl acetate (11.3%). These differences in oil composition are correlated with different regions or countries where the plant is cultivated (Table 2).

The essential oil content shows variations in plants of different geographical origins, periods and also in different part of the tree: [78] reported the total composition of essential oil of *Artemisia herba-alba, Artemisia absinthium* and *Artemisia pontica* plant collected in three regions of Morocco as 83.10%, 80.72 and 43.95% respectively. In a previous study, on the chemistry of *Juniperus phoenicea* [79], considerable differences were observed in the essential oil composition between leaves and berries: α-pinene (38.22% and 39.30%), (α-cedrol (31.23%) and sabinene (24.29%) respectively. Furthermore, studies on the chemistry of *Pistacia lentiscus* [61], considerable differences were observed in the essential oil composition between 3 samples collected from three regions of Morocco (Mehdia, Oulmes and Chaouen). The main components of Mehdia oil were terpinen-4-ol (14.5-19.3%), caryophyllene oxide (6.5-10.3%) and limonene (6.7-8.1%) while α-pinene (16.5-38.5%), β-myrcene (10.2-11.5%) and limonene (6.8-9.8%) were the abundant constituents of Oulmes oil. From Chaouen oil, terpinen-4-ol (32.7-43.8%), α-pinene (7.1-13.5%) and bornyl acetate (6.8-10.3%) were the major compounds.

Antibacterial Activity: The results obtained from the antibacterial activity study of the leaves essential oils of *Pistacia letiscus* from Morocco are shown on Table 3. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the test samples where the absence of growth was recorded [80]. With the agar disc diffusion assay, oils were found to be active against *salmonella typhi*, *Escherichia coli, Enterobacter aerogens, Klebsiella pneumonia* and *Enterococcus faecalis* at a minimal inhibitory concentration (MIC) of 0.08, 0.12, 0.07, 0.21 mg/mL and 0.68 mg/mL.Against *Pseudomonas aeroginosa, Staphylococcus aureus, Staphylococcus intermedius* and *Bacillus sphericus* the oil from the leaves was found to be more active; the oils showed MIC values of 1.01, 0.91, 1.02 mg/mL and 1.56 mg/mL, respectively. The data indicated that *salmonella typhi*, *Escherichia coli* and *Enterobacter aerogens* were the most sensitive strain tested to the oil of *Pistacia lentiscus* with the strongest inhibition zone of 38, 34 and 35 mm, respectively. The *Klebsiella pneumonia* and *Enterococcus faecalis* were, found to be more sensitive among bacteria with inhibition zone of 24 and 23 mm, respectively. Modest activities were observed against *Pseudomonas aeroginosa, Staphylococcus aureus, Staphylococcus intermedius* and *Bacillus sphericus* with inhibition zones of 10, 14, 11 and 7 mm, respectively. The bactericidal activity revealed that this leaves had similar to those of other activity of *Pistacia lentiscus* essential oil study by [81] which the bacteria in lab sample have been reported as *Staphylococcus aureus, Lactobacillus plantarum, Pseudomonas fragi* and *Salmonella enteritidis.* In our study parallel to the study of [52], it was found that essential oil mastic obtained from *Pistacea. lentiscus* had serious antibacterial effect on *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus aureus) in vitro* conditions. Furthermore, *Pistacia lentiscus* resin has been reported to possess antibacterial activity on Salmonella and Staphylococcus [82-33]. Also, *Pistacea lentiscus* resin has been reported to have a fairly good antibacterial activity against *Helicobacter pylori* [83-85]. The antimicrobial activities revealed that this leaves had similar to those of *Juniperus* essential oils analyzed by [86] in which the major component was α-pinene.

Essential oils rich in α-pinene demonstrated potential antibacterial activity [87-88-49]. The major components of this oil, α-pinene, have been known to exhibit antimicrobial activity against the bacterial strains (*Escherichia coli, Staphylococcus aureus, Micrococcus luteus* and *Bacillus subtilis*) [59]. Monoterpenes hydrocarbons, terpinenes, have also shown antimicrobial properties that appear to have strong to moderate antibacterial activity against Gram positive bacteria [89]. The bridged bicyclic monoterpenes *α*-pinene and *β*-pinene showed considerable biological activity [90-91]. The antimicrobial activities have been mainly explained through C_{10} and C_{15} terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils [92]. On the other hand, enantiomers of α-pinene, β-pinene, limonene and linalool have a strong antibacterial activity [64]. Pinene-type monoterpene hydrocarbons (α-pinene and β-pinene) are wellknown chemicals having antimicrobial potentials [93]. It should be noted that the two major volatile constituents, α-pinene and terpinolene, are compounds with interesting antibacterial [94-95]. The antibacterial efficacy of essential oil of *pistacia lentiscus* is due to a number of its components working synergistically. *Pistacia lentiscus* proved to have strong antibacterial activity in almost all oil substrates examined.

CONCLUSION

The present study was conducted to investigate the essential oils of *Pistacia lentiscus* from Morocco and *in vitro* evaluation of its antibacterial activity. The leaves oil obtained from *Pistacia lentiscus* was characterized by GC-MS, GC-FID and Twenty three volatile constituents were identified which made up 77.22% of the total essential oil. The essential oil yield of the leaves was 1.02%. The major compounds were α-pinene (24.25%) followed by β-pinene (12.58%), limonene (7.56%), α-terpinen-4-ol (6.98%), α-terpineol (4.89%), β-Caryophyllene (3.15%), verbenol (3.05%), linalool (2.85%), camphene (2.32%) and myrcene (2.09%). Gram-negative: *Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia* and *salmonella typhi* and gram-positive*: Staphylococcus aureus, Staphylococcus intermedius, Enterococcus faecalis, Bacillus sphericus* and *Enterobacter aerogens* bacteria tested were found to be sensitive to essential oils studied and showed a very effective bactericidal activity with the strongest inhibition zone 7 at 38mm. It can be concluded that the essential oils of *Pistacia lentiscus* can be used antibacterial against.

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