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EXPERIMENTAL PAPER

Antimicrobial activity of four essential oils extracted from plants commonly used in traditional medicine against some clinical strains

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Summary

Introduction: Recently, efforts regarding the discovery of the effectual components of plants possessing antimicrobial properties are advanced. Herbal essential oils are widely used for treatment of various diseases, and they play an important role in healthcare considerations.

Objective: This study aims to evaluate the effectiveness of *Cinnamomum verum*, *Eucalyptus globulus*, *Lavandula angustifolia* and *Mentha pulegium* essential oils against *Candida albicans* and some pathogenic bacteria.

Methods: The antibacterial activity of four essential oils (EOs) against different microbial strains was evaluated using the disk diffusion method as well as determination of the minimal inhibitory concentration

(MIC), and bactericidal concentration (MBC). For *Candida albicans*, the MFC of the plant oils was determined using a macro broth dilution assay. A range of concentrations (50 to 0.2 mg/ml) were prepared in Mueller Hinton Broth medium in flasks. Tween 80 (0.01% v/v) was included to enhance oil solubility. Each flask was inoculated with 10^8 CFU/ml of *C. albicans*. The flasks were incubated at 35°C for 48 hours. From each flask 13 μ l of culture was inoculated onto Mueller-Hinton Agar plates and incubated at 35°C for 48 h. The plates were observed and the MFC was determined as the lowest concentration of plant oil completely inhibiting the growth of *C. albicans*.

Results: The obtained results showed that all bacteria and yeasts tested were sensitive to cinnamon essential oil with an inhibition zone ranging from 22 to 39.33 mm and a MIC ranging from 0.20 mg/ml to 1.56 mg/ml. At low concentrations ranging from 0.2 to 3.13 mg/ml, this essential oil has shown the most important bactericidal effect. Eucalyptus essential oil showed the highest inhibitory effect on *Staphylococcus aureus* with a diameter of 21.33 ± 1.15 mm. The antibacterial effect of mint indicates that the most sensitive bacterium is *A. boumanningii*. However, *S. enteritidis*, *C. albicans*, *K. pneumoniae* and *P. aeruginosa* are resistant germs whose inhibition diameter varies from 7.33 ± 1.15 mm to 11.33 ± 1.15 mm. Lavender EO has an inhibitory effect against *S. aureus* (20.67 ± 1.15 mm) and an intermediate effect against *Streptococcus pyogenes*, *Serratia marcescens* and *Enterococcus faecalis*.

Conclusions: The antibacterial activity of essential oils, especially those of cinnamon against the strains studied, supports their potential use as a remedy against infectious microbial diseases.

Key words: *essential oils, antimicrobial activity, Candida albicans, pathogenic bacteria*

Słowa kluczowe: *olejki eteryczne, aktywność antybakteryjna, Candida albicans, bakterie patogeniczne*

INTRODUCTION

Infectious diseases, particularly those caused by bacterial microorganisms, are still among the top causes of mortality in the world [1]. During the last decades, rapid evolution and spread of resistance among clinically important bacterial species have been observed. Due to this growing increase of resistance, many antimicrobial agents are losing their efficacy [2-4]. The development of antibiotics bacterial resistance demonstrates the necessity of novel antibacterial agents to combat bacteria that have become resistant to currently used antibiotics. Thus, the quest for safer alternatives has led to studies on plant extracts, such as essential oils. Indeed, it has been known that aromatic plants and spices, as well as their essential oils, have varying degrees of antimicrobial activity, for this reason, extracts from these plants can be used to delay or inhibit the growth of pathogenic or spoilage microorganisms [5, 6]. EOs (essential oils) are a complex mixture of natural, volatile, and aromatic compounds synthesized by aromatic plants that have been often used in traditional medicine [7]. They are classified as monoterpenes and sesquiterpenes, according to the number of isoprene units, monoterpenes being the most abundant in EOs components.

Several researchers have also investigated the antimicrobial properties of plant extracts and many plants have been examined so far [8-10]. Various essential oils of different plants such as thyme,

oregano, mint, cinnamon, lavender, and clove, have been observed to possess strong antimicrobial properties [11]. Therefore, the present study was conducted to determine the antibacterial and antifungal activity of four essential oils: *Cinnamomum verum*, *Eucalyptus globulus*, *Lavandula angustifolia* and *Mentha pulegium*, against *Candida albicans* and some pathogenic bacteria isolated from Biomedical Laboratory of HSSAN II Hospital, Agadir, Morocco.

MATERIALS AND METHODS

Plant material

Cinnamomum verum, *Eucalyptus globulus*, *Lavandula angustifolia* and *Mentha pulegium* used in this work, for extraction of essential oils, were obtained from Herbalist in the Agadir province (tab. 1).

Extraction of essential oils

Fresh leaves of *E. globules*, *M. pulegium*, flowers of *L. angustifolia* and barks of *C. verum* were subjected to hydrodistillation (HD) for 3 h with 500 ml

Table 1

Main botanical characters, traditional use and therapeutic benefits of the four essential oils studied

Common name	Botanical name	Botanical characters	Traditional use	Therapeutic benefits
Cinnamon	<i>Cinnamomum verum</i>	Small tree 10–15 m tall with persistent foliage. The bark is gray outside and reddish inside. The large leathery leaves, 10 cm long in dark green glossy, are pointed oval shaped at the end. The yellowish white flowers are small and all hairy (covered with hair) in long, narrow clusters emerging at the axils of the leaves. The fruits are small red brown berries.	Can be taken as a decoction of dried bark, as an infusion or in the form of essential oil.	Has mainly antioxidant, antibacterial and anti-inflammatory properties. It is used mainly against colds, flu, digestive disorders, as it stimulates blood circulation and immune system.
Eucalyptus	<i>Eucalyptus globulus</i>	It is a tree native to Tasmania (Australia). Eucalyptus globulus is 30 to 60 meters tall, with a smooth trunk and white to gray color. Its bark is easily detached in long strips. The young leaves are waxy, oval, clear, opposite and sessile. But these are leaves growing on the old branches that are officinal because they are the only ones to have essence pockets on the underside. The flowers, visible in spring, are born in the axils of the leaves, the calyx has the shape of a bumpy top whose broad part is covered by a operculum which is detached at the moment of the flowering revealing numerous stamens. The fruit is the angular capsule of the chalice, it contains two types of seeds.	The usual forms of traditional use of eucalyptus are infusions, leaf decoctions and fumigations.	This plant is effective for treating colds, flus and sore throat. She acts as a potent expectorant used in the treatment of lung infections, including bronchitis and pneumonia.
Lavender	<i>Lavandula angustifolia</i>	Lavender prefers siliceous soils. It is a shrub with four-branched twigs. The discreet foliage is gray or gray green. The flowers are small, dark purple, united in short pedunculate, compact, quadrangular, topped with a bundle of large purple sterile bracts. The fruit is a tetra-alkene.	Can be taken as a decoction of leaves, or in the form of essential oil.	The flowers in decoction soothe hysteria, ingested as they are, they would also be effective to calm cough and asthma. The essential oil is a valuable remedy for first aid, it is antiseptic, accelerates the healing of burns and wounds and calms inflammations due to insect stings. They use it to treat scabies and lice. In massage, it is applied on the head to calm its evils, to relax and tone the nervous system
Pennyroyal	<i>Mentha pulegium</i>	It is a perennial by rhizomes, low, 10 to 55 cm high, frequent in humid environments, which exhale a strong aromatic odor. The leafy, quadrangular, spreading or lying stems emit adventitious roots very easily on the underside of the nodes. The flowering stems are more or less erect. Leaves, opposite, small, are oval almost entire (slightly serrated) and provided with a short petiole. The flowers, which appear from July to late September, are pink lilacs sometimes white and are grouped in the axils of leaves in glomeruli (false whorls) staggered along the stem. The fruits are achenes.	Can be taken as an infusion or in the form of essential oil.	Traditionally used in herbal medicine to help digestion and relieve flatulent dyspepsia and intestinal colic. In infusion, it has antispasmodic and stimulating properties. Applied on a tissue at the temples has relieves headaches. It is also used to relieve nervous disorders.

distilled water using a Clevenger-type apparatus according to the European Pharmacopoeia [12]. The extracted oil were collected and dried over anhydrous sodium sulfate, then stored in sealed glass vials in a refrigerator at 4°C prior to analysis.

Antimicrobial activities assays Microorganisms and media culture

The microorganisms used in this study were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Serratia marcescens*, *Salmonella enteritidis*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Staphylococcus aureus* and one strain of *Candida albicans*. They were provided by the laboratory of medical analyzes of Hassan II Hospital of Agadir-Morocco.

Media used for carrying out this study were Brain Heart Infusion Broth (Biokar diagnostics), Mueller Hinton Broth (MHB (Biokar diagnostics) and Mueller-Hinton Agar (MHA) ((Biokar diagnostics). Stock cultures were maintained at 4°C on nutrient agar slants for bacteria and Sabouraud agar for *Candida albicans*. Active cultures for experiments were prepared by transferring a loopful of culture to 5 ml of Brain Heart Infusion Broth and incubated at 37°C for 24 hours.

Essential oils activity assay Disk diffusion assay

Screening of essential oils for antimicrobial activity was done by the disk diffusion method. It was performed using an 18 h culture at 37°C for bacteria and 48 h culture at 35°C for *C. albicans* in 10 ml of Mueller Hinton Broth. Cultures were adjusted to approximately $1,5 \cdot 10^6$ CFU/ml, to turbidity comparable with that of McFarland 0.5 standard with sterile saline solution. Two ml of inoculum is deposited on each Petri dish for both control and test plates. After impregnation for 5 minutes, the excess inoculum is removed by aspiration. Under aseptic conditions, filter paper disks (Whatman 6 mm dia) were impregnated with 10 µl of different essential oils and placed on the agar surface. Antibiotics used as controls are those to which the bacteria studied showed resistance (Gentamicine (CN), Cefoxitin (FOX), Vancomycin (NA), Imipenem (IMP)). All Petri dishes were sealed with parafilm to avoid eventual evaporation of test samples. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then they

were incubated at 37°C. After the incubation period, the zone of inhibition was measured with a calliper. Studies were performed in triplicate, and statistical analysis was done with the aid of xlstat752. The results were expressed as mean ±SD. The results are expressed according to three levels of activity: resistant ($D < 12$ mm), intermediate ($12 \text{ mm} < D < 19$ mm) and sensitive ($D > 19$ mm) [13].

Minimum inhibitory concentration, minimum bactericidal and fungicidal concentration

The Minimum Inhibitory Concentration (MIC) values were evaluated by using the broth serial dilution method. The stock solution of each essential oil is prepared in the following manner: 100 µl of the HE is added to 900 µl of the Mueller Hinton Broth (MHB medium) supplemented with Tween 80. Serial dilutions of the high concentration were made in sterile test tubes contained (MHB + Tween 80 (0.01% v/v), to develop a concentration range from 50 to 0.2 mg/ml. After pre-culturing, microorganism suspensions (10^8 CFU/ml) were inoculated into fresh broth (MHB + Tween 80 (0.01% v/v) containing one EO. After the incubation at 37°C for bacteria and 35°C for *C. albicans* MIC values were determined as the lowest concentration of the essential oils at which the completely inhibited the visible growth of microorganisms.

After MIC determination, 13 µl from clear culture of the MICs test were placed on Mueller-Hinton Agar (MHA). MBC and MFC values were defined as the lowest concentration of sample which resulted in ≥99.9% kill of the initial inoculum.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

The antimicrobial activity of four essential oils against all microorganisms is summarized in table 2. The results revealed that the diameters of inhibition, generated by the essential oils, in comparison with those produced by the antibiotics are very variable, according to the oil used. Indeed, the essential oil of *C. verum* produces inhibition diameters much greater than those produced by Gentamicin, Vancomycin, Cefoxitin and Imipenem. In contrast, the antibacterial activity of essential oils of *E. globulus*, *L. angustifolia* and *M. pulegium* remains generally lower than those produced by the antibiotics tested.

Table 2

Antimicrobial activity of essential oils against studied strains by disc diffusion method. Each value shows the average from three independent experiments

Strains	Inhibition zone [mm]							
	Essential oils				Antibiotics controls			
	<i>C. verum</i>	<i>E. globulus</i>	<i>L. angustifolia</i>	<i>M. pulegium</i>	Gentamycin	Cefoxitin	Vancomycin	Imipenem
<i>E. coli</i>	26.67±1.15	8±0	12.34±0.58	20.33±1.53	18±1.15	12±0	19±1.15	12.33±0.5
<i>S. marcescens</i>	29.33±1.15	13.33±1.15	19.67±0.58	18.67±1.15	19.5± 1.15	13.5±0.58	20.6±0.58	15.33±1.13
<i>E. faecalis</i>	36±0	13±1.73	18±2	17.33±1.15	22± 1.15	17.44	21.7±1.15	9.33±0.5
<i>S. aureus</i>	37.67±1.53	21.33±1.15	20.67±1.15	15.67±1.53	23.6±0.58	16±0	19±0	28.67±1.12
<i>P. aeruginosa</i>	22.67±1.15	6±0	6±0	7.33±1.15	14.7±1.15	15± 0.58	15±0.58	9.67±0.52
<i>P. mirabilis</i>	34.67±2.31	14.67±0.58	11.33±0.58	12.33±0.58	19±0	11.55±1.15	22.67±1.15	11.33±1.17
<i>S. pyogenes</i>	39.33±1.15	16.67±2.31	19±1.73	15.33±1.15	21±0.58	13±0	24.67±2.31	17±0
<i>K. pneumoniae</i>	26.33±1.53	9±0	10±0	9.33±0.58	20±0	17.33± 1.15	19.33±1.15	15±0
<i>A. boumannii</i>	30±0	10±0	12.33±0.58	28.67±1.15	21±1.15	19.44±1.15	21±1.15	19.33±1.15
<i>C. albicans</i>	27.33±1.15	6±0	9.67±0.58	9.67±0.58	17±0	20±0.58	17±0	21±1.15
<i>S. enteritidis</i>	22±2	9.33±1.15	6±0	11.33±1.15	12.5±0.58	18.5±0.58	15±0	12.33±1.23

On the other hand, the *C. verum* oil showed significant inhibitory effect against all bacteria and yeast tested, with a zone of inhibition ranging from 22 to 39.33 mm. *Streptococcus pyogenes* is the most sensitive strain with a zone of inhibition (39.33±1.15 mm), followed by *Staphylococcus aureus* (37.67±1.53 mm), *Enterococcus faecalis* (36 mm), *P. mirabilis* (34.67±2.31 mm), *A. boumannii* (30 mm), *Serratia marcescens* (29.33±1.15 mm), *C. albicans* (27.33±1.15 mm), *E. coli* (26.67±1.15 mm), *K. pneumoniae* (26.33±1.53 mm), *P. aeruginosa* (22.67±1.15 mm) and *Salmonella enteritidis* (22±2 mm).

In general, *E. globulus* oil failed to inhibit all tested strains. Only *S. aureus* that is sensitive to this EO with an inhibition diameter of 21.33±1.15 mm. For *S. pyogenes*, *P. mirabilis*, *S. marcescens*, and *E. faecalis*, they showed an intermediate sensitivity with a zone of inhibition of 14.67±0.58 mm, 13.33±1.15 mm and 13±1.73 mm, respectively. However, *A. boumannii*, *Salmonella*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *C. albicans* show resistance to Eucalyptus EO with an inhibition diameter ranging from 6 to 10 mm.

On the other hand, *L. angustifolia* EO did not show an inhibitory effect against *P. aeruginosa*, *S. enteritidis*, *C. albicans*, *K. pneumoniae* and *P. mirabilis*, while lavender EO causes an intermediate effect against *E. faecalis*, *E. coli* and *A. boumannii*.

According to the *M. pulegium* essential oil, the most sensitive bacterium is *A. boumannii* with a diameter of 28.67±1.15 mm, followed by *E. coli* 20.33±1.53 mm. The intermediate strains

are *S. marcescens*, *E. faecalis*, *S. aureus*, *S. pyogenes* and *P. mirabilis* with a diameter ranging from 12.33±0.58 mm to 18.67±1.15 mm. On the other hand, *S. enteritidis*, *C. albicans*, *K. pneumoniae* and *P. aeruginosa* are resistant germs with a diameter of inhibition varying from 7.33±1.15 mm to 11.33±1.15 mm.

Minimal inhibitory, minimum bactericidal and minimum antifungal concentrations

Results according to the MICs and MBCs of each essential oil against microbial strains studied are showed in table 3. All microbial tested were sensitive to the essential oil of cinnamon with a MIC ranging from 0.20 mg/ml to 1.56 mg/ml. The highest concentration of cinnamon oil 1.56 mg/ml was effective against *E. coli*, *P. aeruginosa*, *S. enteritidis* and *S. pyogenes*. The *C. verum* value of MIC 0.20 mg/ml was observed in *E. faecalis*, *P. mirabilis* and *A. boumannii*. The MICs recorded for *E. globules* and *L. angustifolia* EOs range from 25 mg/ml as a minimum value to 50 mg/ml as the maximum value. The MIC obtained by the HE of *M. pulegium* varies between 3.13 mg/ml and 25 mg/ml, recorded respectively in *A. boumannii* and *S. pyogenes*. The CMBs values of essential oils vary from strain to strain. At low concentrations, ranging from 0.2 to 3.13 mg/ml the EO of *C. verum* showed the most important bactericidal effect. Thus, among all essential oils the *C. verum* exhibited a Fungicidal

Table 3

Minimal inhibitory, minimum bactericidal and minimum antifungal concentrations (mg/ml) of selected essential oils determined by broth dilution methods

Strains	Essential oils											
	<i>C. verum</i>			<i>E. globulus</i>			<i>L. angustifolia</i>			<i>M. pulegium</i>		
	MIC	MBC, MFC	MBC or MFC/MIC	MIC	MBC, MFC	MBC or MFC/MIC	MIC	MBC, MFC	MBC or MFC/MIC	MIC	MBC, MFC	MBC or MFC/MIC
<i>E. coli</i>	1.56	3.13	2	-	-	-	-	-	-	6.25	6.25	1
<i>S. marcescens</i>	0.39	0.78	2	-	-	-	25	25	1	6.25	6.25	1
<i>E. faecalis</i>	0.20	0.78	3.9	-	-	-	25	>50	1	6.25	12.5	2
<i>S. aureus</i>	0.78	3.13	4	50	>50	>1	25	50	2	12.50	50	>4
<i>P. aeruginosa</i>	1.56	1.56	1	-	-	-	-	-	-	-	-	-
<i>P. mirabilis</i>	0.20	0.20	1	25	25	1	-	-	-	-	-	-
<i>S. pyogenes</i>	1.56	3.13	0.32	50	50	1	50	50	1	25	50	2
<i>K. pneumoniae</i>	0.39	3.13	8	-	-	-	-	-	-	-	-	-
<i>A. boumanii</i>	0.20	0.39	1.95	-	-	-	-	-	-	3.13	3.13	1
<i>C. albicans</i>	0.39	0.39	1	-	-	-	-	-	-	-	-	-
<i>S. enteritidis</i>	1.56	3.13	2	-	-	-	-	-	-	-	-	-

Concentration (MFC) 0.39 mg/ml. For the *M. pulegium* EO the MBC varies from 3.13 to 50 mg/ml, then those of *L. angustifolia* and *E. globulus* it oscillates between 25 and 50 mg/ml.

In order to determine the bacteriostatic, bactericidal or effect of the HE, we calculated the MBC/MIC ratio (tab. 2). Indeed, if MBC/MIC is less than 4, the effect is bactericidal or and if MBC/MIC is greater than or equal to 4, the effect is bacteriostatic. In generally, results indicate that only *C. verum* EO that showed a bactericidal or bacteriostatic effect on all bacterial and *C. albicans* strains studied. The bactericidal and fungicidal effect of this oil is recorded in both gram negative and positive bacterial strains and *C. albicans*. While the bacteriostatic effect is recorded in *S. aureus*.

DISCUSSION

Plants produce an enormous array of functional relevant secondary metabolites such as essential oils (phytochemicals) that exhibit a diversity of medicinal properties [14]. In this study, the antimicrobial activity of four EOs was investigated against clinical bacteria and *Candida albicans*. The obtained results showed that *C. verum* essential oil exhibited a strong activity against all pathogens, with a zone of

inhibition ranging from 22 to 39.33 mm. Among all microorganisms tested *S. pyogenes* is the most sensitive strain with a zone of inhibition (39.33 ± 1.15 mm). According to MICs values, all bacteria and yeast tested were sensitive to cinnamon with a MIC ranging from 0.20 mg/ml to 1.56 mg/ml. At low concentrations, ranging from 0.2 to 3.13 mg/ml, the EO of *C. verum* showed the most important bactericidal and fungicidal effect. This result consistent with precious study, which showed that the cinnamon essential oil, extracted from the bark and leaves, had considerable ability to inhibit the growth of foodborne pathogens [15-17]. Other studies Matan *et al.* [18] have shown that cinnamon had strong and consistent inhibitory effects against various pathogens. In one study, Oulkheir *et al.* [19] reported that cinnamon possesses an important antimicrobial activity against Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 27853), *Salmonelle spp.* and Gram-positive bacteria; *Streptococcus* and *Staphylococcus aureus* (ATCC 25923) with an inhibition zones ranging from 26 to 32 mm. Other examples are described in the literature by different authors. Matan *et al.* [18], Mau *et al.* [20] Sathishkumar *et al.* [21] revealed that cinnamon oil have exhibited higher antimicrobial activity. According to Singh *et al.* [15] cinnamon is also used as a flavoring substance. These authors reported that in

addition to flavor, it could also be used as antioxidant agent to prevent food oxidation. Investigators have found that the cinnamon essential oil, extracted from the bark and leaves, had considerable ability to inhibit the growth of foodborne pathogens [15-17]. Even though earlier studies have reported better antimicrobial activity for *E. globulus* oil [22]. Our study showed least inhibitory activity of *E. globulus*. It was found that eucalyptus oil failed to inhibit all tested strains. Only *Staphylococcus aureus* that is sensitive to this EO with an inhibition diameter of 21.33 ± 1.15 mm. However, *Acinetobacter boumannii*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* show resistance.

Results obtained from this study showed that essential oil from *L. augustifolia* did not show antibacterial properties against *P. aeruginosa*, *Salmonella*, *C. albicans*, *K. pneumoniae* and *P. mirabilis*. In contrast, this EO causes an inhibiting effect against other strains. According to the *M. pulegium* essential oil, the most sensitive bacterium is *A. boumannii* with a diameter of 28.67 ± 1.15 mm, followed by *E. coli* 20.33 ± 1.53 mm. Finding of this study indicates that MICs of *M. pulegium* varies between 3.13 mg/ml and 25 mg/ml, recorded respectively in *A. boumannii* and *S. pyogenes* and MBCs varies from 3.13 to 50 mg/ml.

The regular use of antimicrobials for treatment therapy or prophylaxis promotes the development of antibiotics bacterial resistance. Results obtained from this study were particularly interesting. Antibacterial activity of essential oils especially those of cinnamon against studied strains, support their potential use as a remedy for bacterial infectious diseases.

Conflict of interest: Authors declare no conflict of interest.

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