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Antibacterial potential of essential oils of the needles of *Pinus halepensis* against *Staphylococcus aureus* and *Escherichia coli*

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the antimicrobial activity of essential oil of *P. halepensis* against *S. aureus* and *E. coli*. The activity was assessed *in vitro* based on two methods of antimicrobial evaluation (agar well diffusion and disc diffusion techniques). *P. halepensis* oil was found to be a promising antimicrobial agent in treatment of the infections caused by these two germs.

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ABSTRACT

Objective: To examine the *in vitro* antimicrobial activities of essential oil of the needles of *Pinus halepensis* (*P. halepensis*).

Methods: The antibacterial activity of essential oil of the needles of *P. halepensis* was determined using the agar well diffusion technique and disc diffusion method against *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922.

Results: The diameter of zones of inhibition exhibited by the essential oil was between 6 and 17 mm. The essential oils was compared favorably with gentamycin used as a standard control. The minimum inhibitory concentration determined by the agar well diffusion method was 0.52 mg/mL for *Staphylococcus aureus* and 2.15 mg/mL for *Escherichia coli*. The minimum bactericidal concentration of the oils against the two microorganisms was 4.17 mg/mL.

Conclusions: The results obtained from this study reveal that *P. halepensis* essential oils possess antibacterial activities and can be used as antimicrobial agents in the search for new drugs.

KEYWORDS

Pinus halepensis, Needles essential oil, Antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*

1. Introduction

The widespread and indiscriminate prescription of antibiotics has resulted in the emergence of a number of drug-resistant bacteria[1]. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are examples of multiresistant bacteria that are becoming an alarming problem within the healthcare system[2–6].

There is a strong necessity for the development of new drugs for the cure of infections provoked by these resistant and multi-resistant bacteria species[7]. Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries[8]. In recent years, there has been extensive research to explore and determine the antimicrobial activity of essential oils. It has been recognized that some of them have different antimicrobial

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activities against individual strains of microorganisms[7].

The Aleppo pine [*Pinus halepensis* (*P. halepensis*)], belonging to the family Pinaceae, is a tree species spread all around the Mediterranean basin including the islands. Its continental range extends from northern Africa (Morocco, Algeria, Tunisia and Libya) and Middle East (Syria, Lebanon, Jordan, Palestina and Turkey), up to Southern Mediterranean Europe (Eastern Greece, Croatia, Northern Italy, Eastern France and Eastern Spain)[9].

P. halepensis is used for timber and resin production and its wood is well known to be stable even at high humidity. It can be used for construction purposes, furniture making and to a lesser extent for the pulp and paper industry[10].

The medicinal and aromatic properties of the chemical compounds (*e.g.*, turpentine, resins and essential oil, *etc.*) of pine make it one of the most popular plants throughout all civilisation. Pine is also still widely used in traditional therapeutic practice in the world[11–13]. For example, in Italian popular medicine, decoctions of leaves, buds, resin and young female cones of *Pinus* spp. have been used against respiratory diseases, coughs, colds and rheumatic pains[14]. It is believed that part of these traditional and pharmaceutical uses of pines are due to their essential oils, which have been shown to possess good antimicrobial properties[15].

This study aimed to determine the antimicrobial activity of Algerian *P. halepensis* against *S. aureus* and *E. coli*. *P. halepensis* oils will be made from the needles by the hydrodistillation method and different concentrations of essential oils will be used to determine its antimicrobial activity, the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC) against *S. aureus* and *E. coli*.

2. Materials and methods

2.1. Plant material

The needles of *P. halepensis* were collected in April and May 2013 at the forest of Toghennif in Mascara region (Algeria). The specie was identified by Mr. Hafsi Mohamed in biology department, Mascara University (Algeria). The samples were dried in shade in ventilated place.

2.2. Isolation of the essential oils

The needles (40 g) were cut into small pieces and separately hydrodistilled for 2 h in a Clevenger–type apparatus with

water cooled receiver, in order to reduce hydrodistillation overheating artifacts. The essential oil was taken up in cyclohexane and dried over sodium sulphate and reduced at room temperature under vacuum on rotatory evaporator. The oil obtained was stored at 4 °C until analysis[11].

2.3. Microbial strains

Essential oils were tested against two microorganisms, *S. aureus* ATCC 25923 (Gram–positive), and *E. coli* ATCC 25922 (Gram–negative). The cultures were purchased from the Microbiology laboratory, Natural and Life Sciences Faculty of Oran University and were maintained on nutrient agar.

2.4. Disc diffusion method

Disc diffusion method was employed for the determination of antimicrobial activities of essential oil of *P. halepensis* with slight modification[16]. Briefly, a suspension of the tested microorganism prepared by diluting overnight (24 h at 37 °C) cultures in Muller Hinton broth medium to approximately 10⁶ CFU/mL was spread on the Mueller Hinton agar media plates. Whatman discs (6 mm in diameter) were impregnated with 5 µL of the essential oil and then placed on the inoculated plates. Gentamicine (10 µg/disc) (Pasteur institute, Algiers) was used as positive reference standard to determine the sensitivity of the microbial species tested. Diameters of growth inhibition zones were measured after incubation at 37 °C for 24 h.

2.5. Agar well technique

The tests cultures were spread evenly on the surface of a Petri dish containing solidified Mueller–Hinton agar. Five wells were made in the Muller Hinton agar plate using a sterile cork borer (0.5 cm).

Different doses of oil of *P. halepensis*, *i.e.*, 5, 10, 15, 20, and 25 µL were poured in the wells with the help of a sterile micropipette. The plates were incubated at 37 °C for 24 h and at the end of 24 h the diameter of the resulting zone of inhibition was measured and the average values were recorded[17].

2.6. Determination of MIC and MBC

Determination of MIC was carried out using the broth dilution method. About 50 µL of essential oil was added to test tube containing 2750 µL of sterile nutrient agar.

Dimethylsulfoxide was used as a solvent to prepare oil concentration of 8.32, 4.17, 2.15, 1.04, 0.52, 0.26, and 0.13 mg/mL. The tubes were then incubated with 30 μ L of the bacterial suspension and incubated at 37 °C for 24 h. The 8th test tube did not contain any essential oil, but a solution of pure solvent served as negative control. After incubation, the MIC of essential oil was determined by visual inspection of the tubes. The lowest concentration of the active ingredient that inhibited growth of the microorganism, as detected by lack of visual turbidity, was assigned to be the MIC. The MBC values were determined by making subcultures from the clear tubes which did not show any growth onto Mueller Hinton agar plates and incubated at 37 °C for 24 h. After incubation, the concentration at which no visible growth was seen was recorded as the MBC^[18,19].

3. Results

Hydrodistillation of dried leaves of *P. halepensis* yielded 0.34% (w/w) of a yellowish essential oil.

The results obtained in the disk diffusion assay regarding the growth inhibition zones of the tested microbes are shown in Table 1. The inhibition zones by *P. halepensis* essential oils were volume dependent for the two strains. The inhibition zones for tested bacteria ranged from 6 to 17 mm for *S. aureus*, and 6 to 14 mm for *E. coli*. The highest inhibition zone was observed against *S. aureus* (17 mm).

Table 1

Antibacterial activity of the essential oil from *P. halepensis* against *S. aureus* and *E. coli* by the agar well and disc diffusion assay.

		Zone of inhibition (mm)					
		Different amounts of oil dissolved					GN
		5 μ L	10 μ L	15 μ L	20 μ L	25 μ L	
<i>S. aureus</i>	Wells	7	9	10	13	15	–
	Discs	6	12	10	15	17	18
<i>E. coli</i>	Wells	6	9	9	13	15	–
	Discs	6	7	10	12	14	24

GN: gentamicin.

The data obtained from this test indicated that *S. aureus* was more sensitive (with the strongest inhibition zone 17 mm), than *E. coli* (with stronger inhibition zone 14 mm).

By agar well diffusion method, the essential oil from *P. halepensis* was effective against the two tested strains (Table 1). The least activity in terms of zones of growth inhibition was shown by 5 μ L of the essential oil against *E. coli* (6 mm), and *S. aureus* (7 mm) while the highest was demonstrated by 25 μ L, with a recorded zone diameter for *E. coli* and *S. aureus* (15 mm).

The samples of *P. halepensis* oils exhibited a great

inhibition effects on the growth of both germs but it stayed less important than that produced by the positive control, gentamicin, which induced a significant growth inhibition zone against *S. aureus* (18 mm) and *E. coli* (24 mm).

With an increasing dose of oil of *P. halepensis*, the resulting diameter of the zone of inhibition increased for the both bacteria.

P. halepensis essential oils exhibited significant antibacterial effects that expressed as MIC and MBC values against *E. coli*, and *S. aureus*. They showed very strong activity against *S. aureus* with the MIC 0.52 mg/mL. The results obtained from MBC experiment showed that MBC of the essential oils of *P. halepensis* for two types of microorganisms was 4.17 mg/mL.

4. Discussion

This yield was lower than the average oil yield reported by Hmamouchi (0.44%)^[15], Dob (0.52%)^[11], and Amri (0.85%)^[20], but similar with that reported by Saadou (0.3%)^[21], and Macchioni (0.33%)^[14]. Fekih reported that the yield of essential oils obtained from fresh aerial part in the ten locations in West and Northern Algeria of *P. halepensis* ranged from 0.13% to 0.63%^[13].

The zones of growth inhibition and MIC exhibited by the essential oil against the bacteria used in this study justify the use of *P. halepensis* in traditional medicine to treat respiratory diseases, coughs, colds and rheumatic pains.

The results of the study revealed that *P. halepensis* oil has antibacterial activity against Gram-positive as well as Gram-negative bacteria. Our results are in good agreement with the findings of Abi-Ayad and Saadou, who reported that Gram-positive bacteria are more sensitive to plant essential oils than Gram-negative bacteria, especially *E. coli*^[21,22]. In contrast, Ghanmi reported that *E. coli* is more sensitive to *P. halepensis* oil than *S. aureus*^[23]. Generally, the Gram-positive bacteria commonly seems to be more susceptible to the inhibitory effects of the plant extracts than the Gram-negative bacteria^[24–26].

The resistance of Gram-negative bacteria against essential oils has been attributed to the presence of a hydrophilic outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering^[26,27].

Results published in literature have shown variable response of the two microorganisms against *P. halepensis* oils. Recently, Fekih has found that essential oils of *P. halepensis* were ineffective against *S. aureus* and *E. coli*^[13].

On the contrary, some authors reported that *E. coli* and *S. aureus* were sensitive to essential oils extracted from *P. halepensis* which is in agreement with the results of the present study^[15,21–23].

Hmamouchi reported that the MIC of *P. halepensis* oils against *E. coli* and *S. aureus*, determined by agar dilution method, was superior to 100 µg/mL^[15].

Ghanmi has demonstrated the activity of *P. halepensis* essential oil against *E. coli* (MIC 1/500 v/v) and *S. aureus* (MIC 1/250 v/v)^[23].

Saadou reported that the tested essential oil of *P. halepensis* collected from two different regions located in the North East of Algeria (forest of Mellah Lake and the forest of Zaarouria in Souk Ahras) has a strong antimicrobial activity against *S. aureus* and *E. coli*^[21]. The zone of inhibition was found to be 10–11 mm for *E. coli* and 13–15 mm for *S. aureus*.

A similar study was conducted by Abi–Ayad who reported very low sensitivity of *S. aureus* (4.5 mm; 7.5 µL/mL ≤ MIC ≤ 10 µL/mL) and *E. coli* (0.5 mm; MIC ≥ 17.5 µL/mL) to the *P. halepensis* essential oil collected in Ghazaouet (Tlemcen), Northwest of Algeria^[22].

The antimicrobial properties of essential oils of *P. halepensis* are suspected to be associated with their high contents of oxygenated monoterpenes components^[13].

The so-called minor components present in the oils could play a more or less important role in the antimicrobial activity of the oil. Furthermore, it is possible that they may act together synergistically as has already been suggested^[15].

The present study has demonstrated that the essential oils of *P. halepensis* have significant antimicrobial potentials. Therefore, the *P. halepensis* essential oils may have a potential for further study and application for treatment of some bacterial infections. Further pharmacological and chemical composition studies will be needed and also the evaluation of the antimicrobial activities against a wide variety of pathogenic microorganisms.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

The Gram-positive bacterium *S. aureus* is mainly

responsible for post operative wound infection, toxic shock syndrome and food poisoning. *E. coli*, which is one of the Gram-negative bacteria, is present in human intestines and causes urinary tract infection, coleocystitis or septicemia. However, both germs exhibited multiple resistances to antimicrobial drugs and high prevalence of the antibiotics resistance.

Research frontiers

In the present study authors made an attempt to find a solution to antibiotic resistance. The essential oils are extracted from the needles of *P. halepensis* collected at the forest of Teggennif in Mascara region (Algeria). The activities of the essential oils were tested against two microorganisms, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922.

Related reports

Several studies have reported different essential oil yield of *P. halepensis*. The results are in agreement with the findings of Abi–Ayad (2013) and Saadou (2011), who reported that Gram-positive bacteria are more sensitive to plant essential oils than Gram-negative bacteria, especially *E. coli*.

Innovations & breakthroughs

Pine is widely used in traditional therapeutic practice in world, it have been used against respiratory diseases, coughs, colds and rheumatic pains. In the present study, authors have demonstrated the *P. halepensis* essential oils possess antibacterial activities against the two bacteria responsible for several infections.

Applications

From the literature survey it has been found that *P. halepensis* is safe to humans. This scientific study support and suggest the use of the essential oil of this plant as antimicrobial agents in the search for new drugs.

Peer review

This is a valuable research work in which authors have demonstrated the antimicrobial activity of essential oil of *P. halepensis* against *S. aureus* and *E. coli*. The activity was assessed *in vitro* based on two methods of antimicrobial evaluation (agar well diffusion and disc diffusion techniques). *P. halepensis* oil was found to be a promising antimicrobial agent in treatment of the infections caused by these two germs.

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