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Chemical composition and antimicrobial activity of the essential oils of *Pinus pinaster*

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PEER REVIEW

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Comments

This a good study in which the authors evaluated antimicrobial activity of essential oil from *Pinus pinaster*. The research could be useful in pharmaceutical and food industries.
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ABSTRACT

Objective: To investigate the antimicrobial activity and chemical composition of essential oils of *Pinus pinaster*.
Methods: Essential oils were extracted from the needles by hydrodistillation. The chemical composition of the obtained essential oils was analyzed using GC-MS technique. The antimicrobial potential has been tested against six microorganisms performing the disc diffusion assay.
Results: Twenty-three components have been identified. β-caryophyllene (30.9%) and β-selinene (13.45%) were predominant compounds. The essential oil exhibited a moderate activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, but did not affect the growth of *Erwinia amylovora*. *Aspergillus flavus* and *Aspergillus niger* were not inhibited by maritime pine essential oils.
Conclusions: The essential oils from *Pinus pinaster* can be used as an antibacterial agent.

KEYWORDS
Pinus pinaster, Essential oil, GC-MS, Antimicrobial activity

1. Introduction

Aromatic plants have been used around the world since ancient times for their preservative and medicinal attributes. Recently, there has been considerable interest in essential oils and extracts of medicinal plants.

Pinus pinaster Aiton (cluster, maritime pine) (*P. pinaster*) is a western Mediterranean species reaching the High Atlas and Tunisia in North Africa. This species occurs on a variety of substrates and under a range of Mediterranean climate regimes (semi-arid to humid)[1]. Maritime pine (*P. pinaster* Ait.) is one of the most important species in the Mediterranean region for its ecology and wood productiveness. As other conifers, this long lived species dominates different landscapes and can withstand severe environmental conditions. *P. pinaster* is a major resin producing species. It has been traditionally used for timber and as a source of turpentine[2]. However, previous studies on *Pinus* species reported the essential oil[3], flavonoid[4], and alkaloid content[5] are limited.

Essential oils are volatile, natural compounds obtained by steam or hydro-distillation, characterized by a strong odour. They are formed by aromatic plants as secondary metabolites and well known for their antimicrobial properties that could be used to control food spoilage and pathogenic bacteria[6,7]. In folk medicine, pine needle essential oils are mainly used for the treatment of respiratory infections. Generally, monoterpenes and sesquiterpenes are dominant components of pine needle essential oils. Because of their characteristic odor, pine

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essential oils are also appreciated in the cosmetic and perfume manufacture[8-10]. In the last decade, there has been an increased interest in essential oils and their antimicrobial activity due to the spread of antibiotic resistance. Some studies revealed that several essential oils possess strong antimicrobial activity against various microorganisms suggesting the possibility of using these oils as replacements of synthetic drugs.

The chemistry and the biological effects of essential oils of different pine species have been previously studied. In contrast, there are only a few studies on maritime pine essential oils. This work aims to evaluate the chemical composition of needle essential oil of *P. pinaster* and its antimicrobial effects.

2. Materials and methods

2.1. Plant material

The fresh needles of *P. pinaster* were collected during March 2013 in North Algeria from the Garden of SAFEX "Société Algérienne des Foires et Exportations", Algiers. The plant was identified in the Department of Botany, National Institute of Agronomy- El-Harrach, Algiers. Before extraction, the needles were washed with running tab water and once with sterile distilled water, in order to eliminate dust particles. Material was then air-dried under shade.

2.2. Extraction of the essential oils

The dried needles were finely chopped and hydrodistilled for 4 h using a Clevenger apparatus. A total of 100 g of plant materials were boiled with 500 mL of distilled water. After distillation, the essential oil was isolated and kept in sealed glass tube at 4 °C until analysis. The yield of the essential oil was calculated.

2.3. Identification of chemical components of the essential oil

The composition of the essential oils was carried out using GC-MS analysis method. The essential oils were analyzed on a DB-5MS capillary column (30 m×0.25 mm×0.2 mm). Mass spectra were recorded with an ionization energy of 70 eV. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The oven temperature was held at 60 °C for 5 min, and then programmed to reach 280 °C at a rate of 15 °C/min. The sample was diluted (1/100, v/v, in Hexane) and a volume of 1 μL was injected in the split mode. Injector temperature was 200 °C. The components present in essential oil were identified by comparing literature and estimated Kovats retention indices that were determined using mixture of homologous series *n*-alkanes (C_8-C_{24}) in hexane, under the same above mentioned conditions. The oil components were identified as the relative amount (%) of individual components of the oil and expressed as percent peak area relative to total peak area from MS analyses of the whole extracts.

2.4. Inoculum preparation

The microorganisms tested in this study included four bacterial strains [*Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli*, *Erwinia amylovora*], and two fungal strains responsible for food spoilage (*Aspergillus flavus*, *Aspergillus niger*). These microorganisms were obtained from the Culture Collection of the Department of Microbiology of Pasteur institute, Algeria. For preparation of inoculums, the Gram-positive and Gram-negative bacteria were subcultured on Muller Hinton medium at 37 °C for 24 h, while the fungal strains were inoculated on potato dextrose agar and incubated for 5 d at 25 °C, to allow the growing of the microorganisms.

After incubation time, colonies were collected from each plate and diluted in sterile saline water. The optical density was adjusted to a turbidity of 0.5 on the Mcfarland scale to prepare standardized inoculums of 10^6 CFU/mL.

2.5. Antibacterial assay

Disc diffusion assay method was carried out for the screening of essential oils. Bacterial and fungal suspensions were spread over the plates containing Muller Hinton medium for bacteria and potato dextrose agar for fungal strains, using a sterile cotton swab in order to get a uniform microbial growth.

Sterile filter Whatman paper discs (6 mm diameter) were impregnated with 20 and 40 μL of essential oils under aseptic conditions and placed on inoculated agar surface. The control was cultured without essential oil. The inoculated Petri dishes were left for 2 h at 4 °C for better oil diffusion then incubated at 37 °C for 24 h for bacteria and at 28 °C during 48 h for fungal strains. Each experience was carried out in duplicate. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

2.6. Statistical analysis

The results of antimicrobial activity were analyzed using analysis of variance (ANOVA). Experiments were replicated twice under the same conditions.

3. Results

3.1. The yield of essential oils

The oil yield was obtained by hydrodistillation of 100 g of the dried needles. Before distillation, humidity of the dried material was measured. The essential oil obtained by distillation of needles was characterized by a transparent color and fresh pine odor.

The yield was calculated from the relation between the essential oil mass obtained and the raw material used in the extraction. The yield of essential oil was 0.61%.

3.2. Identification of chemical composition of essential oils

The chemical composition of the essential oil of *P. pinaster* needles collected in Algeria was investigated using GC-MS technique.

The chromatogram showed that the essential oil was a mixture of numerous compounds; some of them were present in trace amounts. Twenty-three components of the aromatic oil were identified representing 88.6% of the total essential oil composition. The principal components of maritime pine essential oil were β-caryophyllene (30.9%) and β-selinene (13.45%). Other important compounds were identified included α-pinene (1.29%), myrcene (2.07%), methyl acetate (1.62%), α-terpenyl acetate (1.36%), α-copaene (5.05%), α-humulene (6.86%), germacrene-D (3.75%), α-murolene (2.59%), γ-cadinene (3.93%), δ-cadinene (7.81%), humulene epoxide II (2.33%), phenyl ethyl anthranilate (2.01%). The main constituents, Kovats retention indices and their relative percentage are summarized in Table 1.

Table 1
Major compounds of essential oils with their Kovats retention indices and relative percentage.

KI	Compounds	Composition (%)
927	α-Thujene	0.64
933	α-Pinene	1.29
991	Myrcene	2.07
1012	Δ3-Carene	0.38
1209	Verbenone	0.25
1296	Methyl acetate	1.62
1349	α-Terpenyl acetate	1.36
1379	α- Copaene	5.05
1394	β-Elemene	0.45
1426	β-Caryophyllene	30.90
1463	α-Humulene	6.86
1484	Germacrene-D	3.75
1488	β-Selinene	13.45
1501	α-Murolene	2.59
1508	γ-Cadinene	3.93
1525	δ-Cadinene	7.81
1595	β-Copaene	0.64
1608	Humulene epoxide II	2.33
1670	β-Bisabolol	0.64
1842	(E,E)-Farnesyl acetate	Tr
1968	Isophyllocladene	Tr
2120	Phenyl ethyl Anthranilate	2.01
2365	Cis-ferruginol	0.58
	Monoterpene hydrocarbons	4.63
	Oxygenated monoterpenes	2.98
	Sesquiterpene hydrocarbons	75.43
	Oxygenated sesquiterpenes	2.97
	Oxygenated diterpenes	0.58
	Others	2.01
	Total	88.60

KI: Kovats retention index estimated. Tr: traces.

3.3. Antibacterial assay

Results obtained in this study relieved that the essential oils of *P. pinaster* posses a weak antibacterial effect against the microorganisms tested, but did not present any activity against the fungal strains. At the concentration of 20 μL, the essential oils from *P. pinaster* did not affect the growth of any strain. The highest activities were observed for *S. aureus*, *B. subtilis* and *Escherichia coli* at a concentration of 40 μL, while the rest of the strains were not affected by the presence of the essential oil. Inhibition zones values are shown in Table 2.

Table 2
Antimicrobial activity (growth inhibition zones in mm) of *P. pinaster* needle essential oil tested by disc diffusion assay.

Microorganism		Zone of inhibition (40 μL)
Gram-positive bacteria	*S. aureus*	13.0±1.0
	B. subtilis	10.0±0.5
Gram-negative bacteria	*Escherichia coli*	8.0±0.6
	Erwinia amylovora	-
Filamentous fungi	*Aspergillus niger*	-
	Aspergillus flavus	-

-: No activity found.

4. Discussion

The major purpose of this study was to evaluate the antimicrobial properties of maritime pine essential oil against several microorganisms. The volatile oil was extracted by hydro- distillation. The yield of essential oil was 0.61% by mass (g of isolated oil/100 g). This value is in agreement with previous studies reported by Dob *et al.* and Kelkar *et al*[3,11]. Several factors can affect the amounts of active principles of plant material. The yield of essential oil is influenced by cultivation conditions, pretreatment of the leaves, ratio of water and leaves, extraction time, and collection season[12]. The oil yield of *P. pinaster* on this study might be affected by the age of plant used and the mode of extraction.

It can be seen from the chemical composition that this oil is characterized by the presence of monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes. Sesquiterpene hydrocarbons (75.43%) were the major class of compounds followed by monoterpene hydrocarbons (4.63%). The major constituents of the monoterpene hydrocarbons were myrcene (2.07%) and α-pinene (1.29%). Sesquiterpenes hydrocarbons were dominated by β-caryophyllene (30.9%) and β-selinene (13.45%).

According to the literature, monoterpenes and sesquiterpenes are the components found to occur in higher quantities in essential oils from *P.* species[13]. Up to now, there are only few data related to the chemical composition of *P. pinaster* essential oils. Our results are in agreement with those reported by Dob *et al.*[3] and Meullemiestre *et al.*[14], in which β-caryophyllene was indicated as a major component in the essential oils of *P. pinaster* collected respectively from Algeria and France. However, various researchers obtained different chemical compositions for this species. Macchioni *et al.* showed that α-pinene and β-pinene are main components in *P. pinaster* essential oil from Italy[15]. Also, Zolfaghari and Iravani reported α-pinene as the major compound in *P. pinaster* essential oils from Iran[16]. This variability observed in the composition of essential oils can be attributed to the different geographical sources, the climate, the harvesting seasons, the genotype, the drying procedure, the distilled part of the plant and also at the technique for processing[17-19].

Our results indicated that the Gram-positive strains of bacteria were more sensitive to the essential oils. This can be attributed to the absence of an outer lipopolysaccharide layer in Gram-negative bacteria that provides a resistant barrier[20,21].

The antibacterial and antifungal activities vary with the species used. Many studies have reported the antibacterial activity of essential oils against Gram-positive and Gram-negative bacteria[22,23] and have confirmed their antifungal activity[24].

Essential oils are composed of numerous different chemical compounds, and their antimicrobial activity might be attributed to changes in the chemical components[25]. Several investigations in the essential oil activity of Pinaceae reported a weak antimicrobial effect of this spices. Previous work showed that the essential oil from *Pinus densiflora* had moderate antimicrobial activity[26]. Also, Hmamouchi *et al.* reported that essential oils from *P. pinaster* and *Pinus pinea* oils have a weak antibacterial activity[27].

According to the present study, the essential oils from the *Pinus* needles did not inhibit the growth of *Aspergillus flavus* and *Aspergillus niger*. This can be due to the composition in main compound of essential oils. In fact, antimicrobial activity of an essential oil is attributed mainly to its major components[7]. Therefore, antimicrobial, activities may vary, based on the variations in the chemical composition[28]. It has been reported that components with phenolic structures such as carvacrol, eugenol and thymol, were highly active against microorganisms[22-29], these phenolic compounds were not found in the essential oil of the *Pinus* investigated in our work. The low activity of this essential oil is probably due to its lack of phenolic contents.

Karapandzova *et al.* showed that the antifungal activity of *Pinus radiata* essential oil can be caused by the higher amount of α-pinene and β-pinene[30]. In agreement with these results, Krauze-Baranowskaa *et al.* found that the antifungal activity of oil from *Pistacia lentiscus* was due to the high concentration of α-pinene[31]. In other hand, Sonibare *et al.* reported no activity of essential oils of *Pinus caribaea* with a high amount of β-phellandrene (67.9%) and β-caryophyllene (10.2%) against several microorganisms as *Candida albicans, Bacillus subtilis, Staphylococcus typhi, Bacillus aureus* and *Proteus mirabilis* bacteria[32]. Our study produced results which corroborate with these observations. In fact, the essential oil evaluated in this work is characterized by a low amount of α-pinene that may be responsible for the lack of activity against the tested microorganisms.

It can be concluded from the present findings that the essential oil of *P. pinaster* from Algeria had a weak antibacterial activity. The variation of the chemical composition affected the antimicrobial activity of the oil. The most sensitive bacteria against *P. pinaster* needle essential oils were *S. aureus* and *B. subtilis*. Using GC-MS, twenty-three components were identified. Sesquiterpene hydrocarbons were the main components in the oil followed by monoterpene hydrocarbons. Sesquiterpene hydrocarbons were dominated by β-caryophyllene and β-selinene. Further research should be done to evaluate the spectrum of activity of maritime pine essential oils.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Essential oils are volatile compounds formed by aromatic plants as secondary metabolites and well known for their antimicrobial properties that could be used to control food spoilage and pathogenic bacteria. This study was conducted to determine the antimicrobial activity and chemical composition of essential oils of *P. pinaster*.

Research frontiers

This paper investigated the antimicrobial activity and chemical composition of essential oils of *P. pinaster*.

Related reports

In this present investigation, the authors have followed standard protocols to obtained the chemical composition of the essential oils which were analysed by GC-MS technique. The antimicrobial potential has been tested against six microorganisms performing the disc diffusion assay. The results suggest that the essential oil from *P. pinaster* can be used as an antibacterial agent.

Innovations and breakthroughs

In my opinion, there is no work for antimicrobial activity of essential oil from *P. pinaster*. The paper serves as the first hand information on the fact of this plant.

Applications

This research report that the essential oil from *P. pinaster* can be used as an antibacterial agent and could be a new potential source as natural antimicrobial applied in pharmaceutical and food industries.

Peer review

This a good study in which the authors evaluated antimicrobial activity of essential oil from *P. pinaster*. The research could be useful in pharmaceutical and food industries.

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