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ESSENTIAL OIL OF ALEPPO PINE NEEDLES : ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES

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Abstract

Pine needles provide a natural and widely dispersed matrix for long-term monitoring of semi-volatile chemical substances in the atmosphere. In the light of the above efficiency and sustainably converting these plants into the production of the oil extracted from its seeds. This study aims to extract essential oil from Aleppo pine needles (*Pinus halepensis*) harvested in the region of Fez and investigate its antibacterial and antioxidant properties. The essential oil was extracted using hydrodistillation. The DPPH technique was used to determine antioxidant activity. *Staphylococcus aureus* and *Escherichia coli* strains were used to investigate the antibacterial activity of this oil: Chemical analysis (GC / MS) of the essential oil derived from the aerial sections of dried, crushed, and sieved Aleppo pine needles harvested in the region Fez, have proven that it contains 21 constituents, representing 100% of the identified components of the essential oil. The majority compounds in decreasing order are: *Caryophyllène* (32.97 %); *α-Pinene* (13.72 %); *β-Pinène* (11.02 %); *α-Humulène* (7.86 %); *Cembrene* (6.30 %); *trans-β-Ocimene* (5.31 %); *1R-α-Pinene* (5.00%) et *Isovalérate de β-phényléthyle* (4.43%), with a total percentage of 86.44%; an anti-oxidant activity and an antibacterial activity against the studied strains.

Keywords: *Aleppo pine; Essential oil; chemical composition; antibacterial activity; antioxidant activity.*

Introduction

Morocco has an immense ecological diversity due to its biological, climatic, and geographical position thus providing a collection of key indigenous medicinal plants used to treat human illnesses, it occupies a special place in the Mediterranean countries, that have a strong medical tradition and a great traditional heritage based on medicinal plants [1, 2]. Plants produce a vast range of phytochemical secondary metabolites, which are the source of an infinite number of physiologically active substances, such as phenols, alkaloids, flavonoids, amino acids, and terpenoids, which are employed in a variety of studies and applications [10] They have strong antimicrobial activity [2, 10] and are a source of numerous antioxidants that serve as active oxygen scavengers [3]. These natural metabolites open up a plethora of possibilities for future medication development. Indeed, oxidative stress caused by oxygen free radicals is the root cause of a wide range of degenerative diseases, including stomach ulcers, cancer, atherosclerosis, and others. Essential oils are organic plant metabolites that are extracted by physical means. They're mostly found in the plant's bark, leaves, fruits, roots, and other sections [4, 5]. Essential oils also have antimicrobial and antioxidant properties [6]. Pine essential oil is a non-wood forest product among them. It is a complex mixture of bioactive compounds such as monoterpene hydrocarbons and -pinenes, which are widely recognized as antimicrobial substances (antifungal, antibacterial, and antiviral), as well as antioxidant, anticancer, and genotoxic properties [7,8]; the two monoterpenes exist naturally in two enantiomeric forms: dextrorotatory (1R)-(+)-and/or levorotatory (1S)-(-) from. Pinenes are frequently employed as solvents in the industry [9, 10]. Pine oil has been utilized as a fragrance in cosmetics, food, and beverage flavoring additives, flavoring agents in a variety of household items, and intermediates in the synthesis of chemical perfumes [11, 12]. The medical use of essential oils has piqued the interest of scientific communities in recent years, and their antioxidant activity has become the subject of research [6]. This study aims to grow Aleppo pine needles to extract the essential oil and analyze its antioxidant and antibacterial properties from pine trees to discover new sources of natural antioxidants in food formulations and pharmaceuticals [13]. Like many other conifers, the Aleppo pine is an important forest tree for European forestry. It has needle-shaped, more or less thorny leaves that are frequently dark green and persistent. Pines are distinguished by their great dimorphism of twigs, which sets them apart from other species. We're interested in examining some of this plant's actions because of its abundance in Morocco and the previously tested qualities. The goal of this research is to provide the findings of valuing Aleppo pine by essential oil extraction and antioxidant and antibacterial activity testing.

Materials and Methods

Essential oil of Aleppo pine needles

In April 2021, pine needle samples were gathered in the Dhar El Mahraz area of Fez, Morocco (Figure 1)

The needles of the Aleppo pine were detached from the branches, dried for a week in amber and 48 hours at 40 ° C in an oven, then crushed and sieved in a sieve with a diameter of 2mm. Then 500g of the obtained powder was introduced into a Bicol hydro distillation flask to which was added a liter of distilled water. The mixture was brought to a boil using a flask heater for 4 hours until the distillate was completely clear. During this step, the water vapor was condensed and then separated into two phases, organic and oily. The aqueous phase is extracted with diethyl ether to increase the volume of the produced essential oil. After evaporation of the solvent, the second organic phase is obtained and combined with the first one. Finally, anhydrous sodium sulfate was used to dry the essential oil (Na_2SO_4). Then it was stored at 4 ° C for subsequent use. For ecological and economic reasons, all the organic solvents used during the extraction were recycled by a rotary evaporator to avoid any pollution and to be used for other extractions [14].

Gas chromatography coupled with mass spectrometry (GC / MS) type (TQ8040 NX) (Shimadzu brand), connected with a triple quadrupole ionization detector, and was used to analyze the essential oil of Aleppo pine. The sample analysis conditions were as follows: Column: RTxi- 5 Sil MS non-polar capillary - 30m x 0.25mm ID x 0.25 µm; Carrier gas: Helium; Mode: splitless; injection

volume: 1 Microliter (µL); Dilution solvent: Methanol; Injector temperature: 250 ° C; Detector temperature: 280 ° C; source temp ion: 200 ° C; Temperature program: 50 ° C (2min) - 5 ° C / 2min-160 ° C; Pressure: 37.1 kPa; analysis time: 50min; Program: opening of the split at 2.5 min. The identification of the molecules constituting the essential oil of pine needles was carried out by comparing the retention index of different compounds with the retention index given in the literature. The essential oil of pine needles was valued by testing two activities: antioxidant and antibacterial [15].

DPPH test

The antioxidant activity in vitro The scavenging ability was measured and evaluated the power of the DPPH radical (1,1-Diphenyl-2-picryhydrazyl) or 0.1mL of oil tested at different concentrations (0.01; 0.02; 0.04; 0.06; 0.08; 0.1%) was mixed with 750 µL of a methanolic solution of DPPH and 650 µL of methanol.

The resultant mixture was incubated for 30 minutes at room temperature in the dark, then the absorbance or wavelength of 517 nm was measured. The following equation was used to compute the % inhibition:

$$I(\%) = \frac{(A_0 - A_s)}{A_0} \times 100$$

Where A_0 is the negative control's absorbance and A_s is the sample's absorbance.

The result was evaluated by comparison with a reference control; butylated hydroxytoluene (BHT), and by calculating the essential oil concentration that causes a 50 % inhibition of the DPPH radical (IC_{50}). This parameter is determined by extrapolation from the curve relating to the percentage of inhibition **(I%)** as a function of the essential oil concentration [16].

Antibacterial activity

The antibacterial activity of the essential oil of A.P.N has been tested against two multi-resistant pathogenic strains, Staphylococcus aureus and Escherichia coli. These strains were isolated from foodstuffs, identified on the basis of Gram staining, classical morphological and biochemical characteristics and preserved in glycerol at -80°C [17].

Using a Pasteur pipette, identical colonies of the bacterial strains were scraped onto nutritional agar and sealed for 24 hours. A total of 10 mL of sterile saline solution was expelled. After homogenizing the bacterial culture, the opacity was reduced to 0.5 Micro Farlane, which corresponds to 10^6 CFU mL-1. The suspension was then diluted until it contained 10^8 CFU mL-1 inoculum.

Antibacterial activity was carried out by the method of diffusion on disks in agar medium, i.e., Vincent's method, inspired by that of antibiograms, has been generalized to essential oils (Cornet, 1981) [18]. This method makes it possible to predict with certainty the in vitro effectiveness of essential oils and antibiotics. It is in fact a qualitative evaluation of the activity. The quantitative aspect will then be estimated by determining the minimum inhibitory concentration (MIC) of the oil that shows activity against tested strains larger than 12 mm in diameter. Each strain was subcultured in 2 mL of BHI broth and incubated at 37°C for 2.5 to 3 hours. Subsequently, 20 mL of Muller Hinton agar medium was poured per Petri dish. Once the agar was cooled, the bacterial inoculum was inoculated by swabbing technique. After 5 min, on the surface of each plate, a 6-mm diameter sterile filter paper disk was aseptically placed, to which 10 µL of the essential oil was added. At the same time, a blank essential oil control was prepared. The dishes were allowed to stand for 1 hour at 4°C and then inverted and incubated at 37°C for 18 to 24 hours. After incubation, the inhibition diameter was measured in millimeters including the disc.

The culture media used were prepared according to NM 08.0.155 (2006). Before using these media, they were checked for pH and sterility [19].

Results and discussions

Chemical composition of essential oil

The essential oil extracted from *pine* needle powder by hydrodistillation is white, transparent, and has a strong odor. The extraction yield is important; it is 0.65%. Analysis of this essential oil by chromatography (GC / MS) reveals the presence of 21 compounds, 8 of which are predominantly

presented in descending order: are *Caryophyllène* (32.97 %); *α-Pinene* (13,72 %); *β-Pinène* (11.02 %); *α-Humulène* (7.86 %); *Cembrene* (6.13 %); *trans-β-Ocimene* (5.31 %); *1R-α-Pinene* (5.00 %) et *Isovalérate de β-phényléthyle* (4.43 %), with a total percentage of 86.44%. The content of the rest of the constituents is low (Table 1).

The comparison of the composition of the essential oil of A.P.N obtained by "hydro distillation" of the needles of the same species studied previously [20, 21, 22, 23, 24] shows variable compositions from one country to another and from one region to another, most probably due to environmental conditions, such as altitude, humidity, thermal amplitude as well as the type of soil, their mineral richness, the nature of the plant (wild or planted), the period of its harvest and its method of extraction. The common compounds between the different countries and the two regions of Morocco, to varying degrees, are α-pinene and myrcene. The majority of compounds of the essential oil of Algerian origin consist mainly of Caryophyll oxide, Thumbergol, and Humulin oxide. While that of Italian origin, the majority of compounds are α-pinene, The myrcene, Sabinene, and α-Terpinolene. For Turkey, it is rich in Caryophyll oxide, Caryophyllin, α-pinene, The myrcene, and 3-Carene. Concerning the essential oil of P. A of Greece, it contains α-pinene, The myrcene, and 3-Carene in addition to the common compounds.

The present literature review shows that monoterpenes, due to their numerous sources, can be present at very variable and potentially high concentration levels in indoor environments. These levels can also increase following renovation works or the use of products rich in these compounds **[25]**. Among the dominant compounds in the Aleppo pine essential oil is Caryophyllene; α-Pinene and β-Pinene. Table 3 gathers the identity of the major compounds of the essential oil of A.P.A in terms of crude formula, structure, characteristics, composition and properties. It also relates other sources or products rich in these elements.

Antioxidant activity

The capacity of A.P.N essential oil to trap the free radical DPPH, which is produced in solution by the donation of a hydrogen atom or an electron, was measured (Figure 2) demonstrate that the proportion The amount of free radical inhibition increases as the content of BHT or P.A.N essential oil increases Show that the essential oil and the positive control have a significant difference (BHT). For low doses, the inhibition index of the control is slightly higher than that of the essential oil of A.P. The efficacy of the essential oil of A.P.N is the same as that of BHT at a concentration of 0.8 mg / mL. Extrapolating from the curve in (Fig3), the concentrations that cause 50% inhibition (IC_{50}) reveal that the antioxidant potential of BHT remains stronger than that of the Aleppo pine essential oil. (Table 4). The results thus obtained prove that Aleppo pine essential oil possesses antioxidant activity in vitro[. This ability to reduce free radicals seems to be due to its chemical profile, which is particularly rich in *Caryophyllene* (32.97 %); *α-Pinene* (13.72 %); *β-Pinene* (11.02 %); *α-Humulene* (7.86 %); *Cembrene* (6.13 %); *Trans-β-Ocimene* (5.31 %); *1R-α-Pinene* (5.00 %) et *Isovalerate de β-phenylethyle* (4.43 %).

Previous research on the antioxidant activity of essential oils of *P. halepensis* has generally shown their strong antioxidant activity [27].

Antimicrobial activity

The results of the antibacterial evaluations were in terms of essential oil of Aleppo pine needles (Figue 3) diameters (Φ) of the zones of inhibition measured around the discs of the strains studied Staphylococcus aureus and Escherichia coli and revealed a negative effect against the Gram-positive bacteria from the essential oil of *P. A*: staphylococcus aureus. While Gram-negative bacteria: Escherichia coli, exhibits moderate and acceptable antibacterial activity. The diameter of the inhibition zones Φ obtained by the disk diffusion method is 7 mm.

The antibacterial activity of P. *Monocytogenes, Enterococcus faecalis, Pseudomonas aeruginosa, Acinetobacter baumaniii, Citrobacter freundii*, and *Klebsiella* pneumoniae was discovered in a study of the literature [28]. These findings show that pine essential oil could be a novel natural antibacterial source for the pharmaceutical and food industries.

Conclusion

The study on the valorization of *Aleppo pine* needles (A.P) shows that this plant is abundant in Morocco and not valued until now. The essential oil extraction yield by hydrodistillation is 0.65%. It is composed of 21 constituents, 8 of which are in large proportion and belong to the sesquiterpene family characterized by their antioxidant, antifungal, antiseptic, anticancer, and antibacterial effects. It is mainly rich in Caryophyllene (32.97%), α-Pinene (13.72%), and β-Pinene (11.02%). The tests of antioxidant and antibacterial effect of essential oil of Aleppo pine needles record, on the one hand, its antioxidant power and on the other hand a difference of antibacterial power. The latter was revealed only against the strain Escherichia coli and not on that of Staphylococcus aureus. All of the results obtained in vitro only constitute a first step in the search for natural biologically active substances of the essential oil studied. More research is needed to develop formulations that harness the antibacterial and antioxidant characteristics of Alpine pine needle essential oil in the food, pharmaceutical, cosmetic, and agricultural industries.

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Table 1. The chemical make-up of the essential oil extracted from Aleppo pine needles.

Pic	Compound	R. Time	%
1	**α-Pinene**	7.805	12.5
2	**1R-α-Pinene**	8.035	3.68
3	Sabinene	8.990	1.21
4	4(10)-Thujene	9.146	1.17
5	β-Pinene	9.417	13.74
6	**Trans-β-Ocimene**	11.033	6.65
7	4-Isopropylidene-1-cyclohexene	12.319	4.32
8	Copaene	20.788	1.69
9	**Caryophyllene**	22.044	15.87
10	α-Bisabolene	22.673	1.53
11	**α-Humulene**	22.879	9.19
12	**Isovalérate de β-phényléthyle**	23.359	7.89
13	α-Murolene	23.889	1.39
14	α-Cadinene	24.375	1.83
15	α-Bisabolene	24.908	1.28
16	Caryophyllene oxide	26.208	0.85
17	Guaiol	26.599	0.89
18	Caryophyllene-(I1)	28.288	0.61
19	**Cembrene**	34.841	9.84
20	Cembrene A	35.485	1.22
21	Thunbergol	37.296	2.69

Table2. The main components of essential oils of Aleppo pine needles from different origins previously reported.

Compounds (%)	Morocco	Algeria [20]	Italy [21]	Morocco [22]	Turkey [23]	Greece [24]
Caryophyll oxide	-	52.00	-	-	07.80	-
Thumbergol	-	09.00	-	-	-	-
Humulin oxide	-	07.20	-	-	-	-
Caryophyllin	32.97	-	-	-	11.20	-
α-humulene	7.86	-	-	-	-	-
Remegram	-	-	-	-	-	-
α-pinene	13.72	-	18.10	23.30	47.10	13.40
β-Pinene	11.02	-	-	-	-	-
1R-α-Pinene	05.00	-	-	-	-	-
The myrcene	-	-	27.90	16.30	06.30	06.60
Cembrene	06.13	-	-	-	-	-
Sabinene	-	-	09.40	-	-	-
Trans-β-Ocimene	05.31	-	-	-	-	-
α-Terpinolene	-	-	09.90	-	-	-
3-Carene	-	-	-	-	-	06.90
Limonene	-	-	-	-	-	05.00

Table 3. Identity sheet of the majority compounds of Aleppo pine needles **[26].**

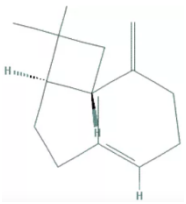
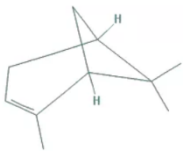
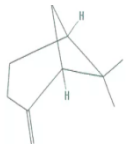
Major compounds	Gross formula and structure	Characteristics	Other E.O. rich in these molecules	Composition (%)	Properties
Caryophyllene	Gross formula: $C_{15}H_{24}$ - Other name: β-caryophyllene and trans-caryophyllene chemical structure: 	Biochemical family: sesquiterpene carbides -Molar mass: 204 g/mol -Boiling temperature: 262 to 264°C -Flashpoint: 96°C	- Cinnamon -Cade -Carrot -Clove -Helichrysum of Madagascar -True Lavender -Lavender Fine -Lemon Balm -Black Pepper -Savory Thyme Ylang -Ylang	-3 à 5 -3 à 9 -3 à 10 -2 à 14 -3 à 12 -< 6 -2 à 7 -2 à 23 -8 à 28 -2 à 9 -5 à 19	- Anti-infective -Antifungal -Bactericide -Antihistamine -Ulceroprotective -Anti-inflammatory -Spasmolytic -Calming -Antioxidant
α-Pinene	Gross formula: $C_{10}H_{16}$ Other name: 2.6.6-Trimethylbicyclo(3.1.1)-2-hept-2-ene chemical structure: 	-Biochemical family: Monoterpenic carbides -Molar mass : 136 g/mol -Boiling temperature: 156°C -Flash point: 32°C	-Turpentine -Green Myrtle -Cypress of Provence -Incense -Scots Pine -Juniper -Angelica -Cistus -Rosemary Verbenone -Red Myrtle -Hemlock -Rosemary with Camphor -Pistachio Mastic -Black Spruce -Douglas Fir -Goldenrod -Nutmeg -Balsam Fir -Rosemary Cineole -Siberian Fir -Eucalyptus Globulus - Black Pepper -Niaouli -Coriander Seeds -Galbanum -Lavender Leaf Sage -Noble Laurel -Greenland Cedar -Roman Chamomile	-70 à79 -45 à 68 -40 à 65 -38 à 58 -37 à 53 -34 à 54 -17 à 30 -18 à 50 -15 à 40 -19 à 28 -16 à 23 -15 à 27 -12 à 28 -12 à 22 -10 à 20 -10 à 20 -10 à 18 -9 à 19 -9 à 17 -9 à 13 -6 à 22 -5 à 19 -5 à 15 -5 à 10 -4 à 21 -3 à 11 -3 à 10 -2 à 10 -1 à 10	- Antibactérienne -Antifongique -Antivirale -Antiseptique atmosphérique -Anti-oedemateuse -Expectorante
β-Pinene	-Gross formula: $C_{10}H_{16}$ -Other name: 6,6-Dimethyl-2-methylenebicyclo(3.1.1)heptane chemical structure: 	-Biochemical family: Monoterpenic carbides -Molar mass: 136 g/mol -Boiling temperature: 166°C -Flashpoint: 36°C	-Galbanum -Balsam Fir -Douglas Fir -Scots Pine -Turpentine -Helichrysum of Madagascar -Black Pepper -Lemon -Neroli -Nutmeg -Saro -Rosemary Cineole -Annual Tansy -Marjoram	-40 à 88 -28 à 38 -20 à 28 -15 à 41 -13 à 19 -8 à 16 -8 à 18 -7 à 17 -7 à 17 -5 à 12 -5 à 10 -2 à 10 -⩽10 -⩽6 1	-Antibacterial -Antifungal -Antiviral -Atmospheric antiseptic -Anti-oedematous -Expectorant

Table 4. The antioxidant activity of Aleppo pine needles essential oil, represented as IC_{50} (mg/mL)

Sample	IC_{50} (mg/mL)
BHT	0.12
Essential oil	0.41

Figure 1. Geographical origin of the sample of Aleppo pine needles branches.

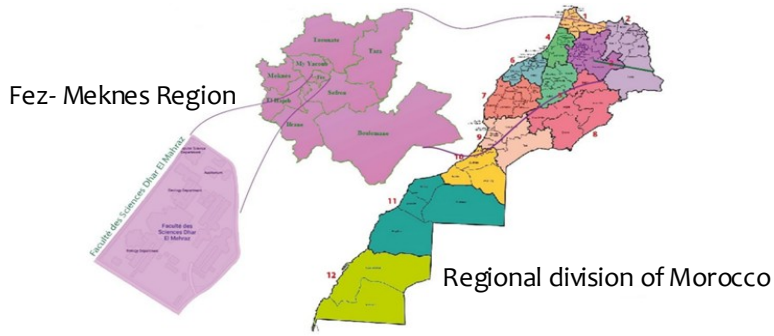


Figure 2. Antioxidant profile of BHT and essential oil of Aleppo pine needles.

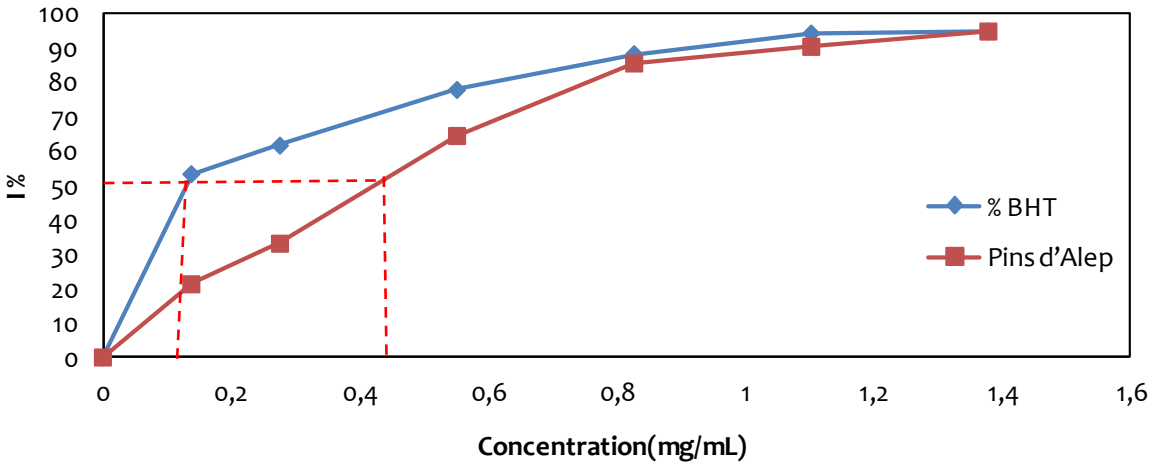
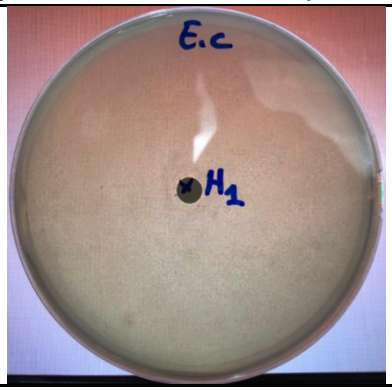
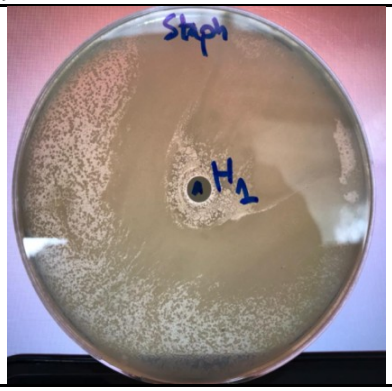


Figure 3. The anti-bacterial power against two strains *Staphylococcus aureus* and *Escherichia coli*.



a- Staphylococcus aureus



b- Escherichia coli