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EFFECT OF ATLAS CEDAR ESSENTIAL OIL AGAINST PATHOGENIC MEDICAL BACTERIAL STRAINS - IN VITRO TEST

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Abstract

The *Atlas cedar* or *Cedar Atlas*is one of the dominant plants in Morocco, it is interesting to know its therapeutic virtues, in order to replace synthetic products with bioactive molecules which are based on plants. This work consists in evaluating the antibacterial activity of the essential oil of spontaneous *Cedar Atlas* collected in the region of Ifrane (Morocco). After extracting the essential oil from the aerial part of *Cedar Atlas* by hydrodistillation using an "Alambic" pilot extractor, the chromatography analysis was carried out by gas chromatography coupled with mass spectrometry for the determination of chemical compositions. The results showed that the essential oil of *Cedar Atlas*is characterized by the presence of α-himachalene (19.47%), α-longipinene (21.26%), β-himachalene (28.20%), These three major compounds were obtained with a percentage of (68.93%). On the other hand, the antimicrobial power of *Cedar Atlas* essential oil has been studied *in vitro* against medical bacterial strains: Three Gram-negative bacteria, namely *Escherichia coli* (ATCC 4157), *Klebsiella pneumoniae* (ATCC 4352), and *Bordetella bronchiseptica* (ATCC 4617). And Three Gram-positive bacteria namely *Micrococcus luteus* (ATCC 533), *Bacillus cereus* (ATCC 10876), and *Enteroccocus faecium* (ATCC 6569). The bacterial strains tested were found to be sensitive to the essential oil studied, and therefore *Cedar Atlas*to show a very effective and remarkable bactericidal activity. In short, all of these results obtained only constitute a first step in the search for biologically active substances of natural origin.

Keywords: *Cedrus atlantica, essential oils, chemical composition, GC-MS, antimicrobial activities.*

Introduction

The climate of Morocco is characterized by sunny and mild weather which contributes to the formation of a biodiversity very rich in aromatic and medicinal plants which represent an important reservoir of products, in particular essential oils, having various activities, essential oils are used in alternative medicine for a very long time, and thus the use is linked to their various biological activities recognized for example as antifungals, antibacterials, anti-insects or antivirals **[1-3]**.

The *Atlas cedar* or *Cedar Atlas* is one of the dominant plants in Morocco, it is the noble essence emblematic of the *Atlas* and Rif mountains, appreciated for its technological, ecological and biogeographic values **[4]**. On the economic level, a large part of neighboring Moroccan families live directly from the resources offered by the cedar groves: firewood, construction wood, rangelands ... Ecologically, the cedar organizes very important forest ecosystems it occupies a remarkable, if not at the forefront in the Moroccan forest landscape **[5]**. As for its biogeographical value, it is linked to the fact that it represents an ancient genus among us, the appearance of which dates back to the Lower Cretaceous **[6]**. The *Atlas cedar* is a mountain species, occupies areas of unequal importance and spontaneously forms a single geographical block in North Africa represented by the Moroccan Atlas (130,000 ha), the Moroccan Rif (15,000 ha) and the Algerian Atlas (40,000 ha). In Morocco, the *Atlas cedar* has been the subject of several bioecological and phytosociological studies **[7-8]**.

In other hand, antimicrobial agents, including medical preservatives and organic acids, have been used to inhibit medical bacteria and treat certain bacterial diseases **[9]**. Many natural compounds found in edible and medicinal plants and herbs have been shown to have antimicrobial functions and may serve as a source of antimicrobial agents against medical pathogens **[10-14]**. Phenolic compounds and their subclasses, such as coumarins, flavonoids, and essential oils, have antimicrobial function. The antimicrobial activity of herbs and essential oils is well recognized **[15]**. The essential oils of thyme, cinnamon, bay leaf and cloves are known to possess antimicrobial activity **[16-18]**. Essential oils and their constituents have been widely used in several pharmaceutical and medical preparations, despite the difficulties of insertion according to world standards **[19]**. Many of these compounds are classified as generally recognized as safe. Since pathogens of medical origin were rarely included in previous antimicrobial studies on constituents of essential oils, six pathogenic strains, *Escherichia coli* (ATCC 4157), *Klebsiella pneumoniae* (ATCC 4352), *Bordetella bronchiseptica* (ATCC 4617), *Micrococcus luteus* (ATCC 533), *Bacillus cereus* (ATCC 10876), *Enteroccocus faecium* (ATCC 6569), were used in this study to assess the antibacterial activity of the essential oil of atlas cedar available in the Middle Atlas region. using an in vitro assay. This essential oil was chosen because it has already been shown to possess antibacterial and antifungal activities against many plant and medical microorganisms **[20]**.

Material and methods

Plant Material

Aerial parts of Atlas cedar amounting to 10 kg were collected in Ifran National Park (Morocco). The identification of the plants was made by Dr Ainane (EST-Khenifra) and the specimen was deposited in the herbarium of the "biotechnology and analysis" department with the code DBA-2021-45.

Essential oil extraction

The extraction of the essential oil was carried out by hydrodistillation using an "Alambic" pilot extractor for 2 hours, in a laboratory at EST-Khenifra. The amount of 10 kg of dry plant material was used with a volume of 50 liters of water. The temperature of the device is set at 100 ° ± 5 ° C, to promote maximum evaporation of water and essential oil without destroying it. This method is recommended by the European Pharmacopoeia. The recovered oil is dehydrated with sodium sulfate (Na_2So_4), then weighed to calculate the yield, then and stored in opaque and hermetically sealed bottles at 4 ° C ± 1. This essential oil will be studied and analyzed. The yield was calculated according to the formula: R% = (MHE / MVS) x 100. MHE: mass of the essential oil and MVS: mass of the dry plant material. The two masses are expressed in the same unit **[21]**.

Chemical identification

The analyzes were carried out using a Hewlett Packard type 5941 chromatograph, equipped with a silica capillary column (25 m × 0.20 mm internal diameter) packed with polydimethylsiloxane. The carrier gas is helium with a flow rate of 0.6 ml / min. The temperatures of the injector and the detector are respectively 220 and 250 °C. The temperature is programmed at 50 °C for 3 min and then increased to 250 °C at a rate of 3 °C / min. Mass spectra were recorded by a quadrupole type detector and ionization was performed by electron impact at 70 eV. Volatile compounds were identified by their mass spectrum and their relative retention index IR, calculated from the retention times of the separated compounds and linear alkanes **[22]**.

Antimicrobial activities

The antimicrobial activities of essential oil of Atlas cedar was studied *in vitro* against bacterial strains: Three Gram-negative bacteria, namely *Escherichia coli* (ATCC 4157), *Klebsiella pneumoniae* (ATCC 4352), *Bordetella bronchiseptica* (ATCC 4617). And three Gram-positive bacteria namely *Micrococcus luteus* (ATCC 533), *Bacillus cereus* (ATCC 10876), *Enteroccocus faecium* (ATCC 6569). These bacterial strains were provided by the local laboratory of the Superior School of Technology-khenifra, after having been purified and identified.

The diameters of the zones of inhibition of the different fractions of EO were determined by the method of Ainane et al. (2014) **[23]**, from 24-hour cultures (10^5 -10^6 CFU / mL). Flood seeding was done from the obtained inoculum, solubilizing the colonies in sterile distilled water and agar. The obtained inoculum (1 mL) is poured into Petri dishes containing Mueller Hinton's agar. The excess inoculum was subsequently aspirated, and the dishes were dried in an oven (37 ° C). After 15 min of drying, the wells were cut out using Pasteur pipettes (6 mm thick end). Then, each EO fraction (50 µL) and gentamycin (50 mL) (control) were distributed into each well. After diffusion, the cultures were incubated in incubators at 37 ° C for 24 h. The inhibition halos were measured with a caliper. The activity is considered zero for a diameter of the inhibition zone less than or equal to 8 mm; low for a diameter of the inhibition zone between 8 and 14 mm, medium for a diameter of the inhibition zone between 14 and 20 mm; strong for a diameter of the inhibition zone greater than or equal to 20 mm.

A medium consisting of sterile Mueller Hinton broth and a sterile solution of tween 80 was prepared in order to obtain a homogeneous distribution of the EO fractions in the medium, and to maximize their miscibility in the medium. For the determination of the MIC, 10 test tubes were used. The first eight were used to prepare concentration ranges (80; 40; 20; 10; 5; 2.5; 1.25 and 0.625 mg / mL) of each EO fraction; the last two containing the positive and negative controls. In the test tubes containing each fraction of EO and the positive control, 10 µL of the inoculum was introduced. After 24 hours incubation of the tubes at 37 ° C, the MIC corresponding to the lowest concentration of essential oil capable of inhibiting bacterial growth after 18 to 24 hours of contact, was determined **[24]**. The concentration ranges used for the determination of the MIC were used to measure the CMB. Samples were taken from the control tube and from each of the tubes without a bacterial pellet, then deposited "streaked" on MHA agar. The inoculated dishes were incubated for 24 h at 37 °C.

Results and discussion

Essential oil

The average yield of *Cedar Atlas* essential oil was calculated according to the dry plant matter obtained from the aerial parts of the plant studied. The value found is 1.36% (v/w). The analysis of the results of the chemical composition carried out by gas chromatography coupled with the mass spectrometry of the essential oil of *Cedar Atlas* studied is mentioned in Table 1. Analysis of the results of this table showed the chemical composition of the essential oil of *Cedar Atlas*. The main majority compounds identified are: α-Himachalene (19.47%), α-Longipinene (21.26%), β-Himachalene (28.20%), The majority compounds were obtained with a percentage of (68.93%).

The results of Satrani et al. (2009) **[25]** indicate that E-α-atlantone (28.75%) and β-himachalene (14.62%) are the main constituents of the essential oil of *Cedar Atlas* sawdust. According to Ainane et al. **[26]**, the chromatographic analysis of essential oil of the aerial part of *Cedar Atlas* collected in the Khenifra region shows that β-himachalene is the major constituent of this essential oil with a content of approximately 31.24%, followed by α-himachalene (15.63%) and γ-himachalene (14.46%). The other constituents are detected in average percentage: (E) -Atlantone (4.30%), γ-Dehydro-arhimachalene (3.67%), Deodarone (2.75%), oxide (+) - βHimachalene (2, 30%), α-dehydro-ar-himachalene (2.06%), allohimachalol (1.66%) and aromandendrene (1.55%). The content of the rest of the constituents is often less than 1%. The results obtained by Derwich et al. (2010) **[27]**, of the essential oil of the aerial parts of *Cedar Atlas* collected in the Middle Atlas, mountainous region of Morocco shows that the majority compound was α-pinene (14.85%) followed by himachalene (10.14%), β-himachalene (9.89%), σ-himachalene (7.62%), cis-α-atlantone (6.78%), himachalol (5.26%) and α-himachalene (4.15%), germacrene D (3.52%), β-caryophyllene (3.14%), cadinene (3.02%), β -pinene (2.35%), humulene (2.30%) and copaene (2.26%). It can therefore be concluded that the chemical composition or the percentage of constituents of essential oils differs depending on the part of the plant subjected to extraction. Other studies by Slimani et al. (2014) **[28]**., have also shown that the chemical composition of the essential oils of *Cedar Atlas* varies considerably depending on the geographical areas, the harvest period and the age of the plant.

Antimicrobial activities

The results of the antibacterial evaluations of all the methods applied to the essential oil of *Cedar Atlas* are expressed in terms of diameters (Φ) of zones of inhibition measured around the discs, of the minimum inhibitory concentration (MIC), of the minimum bactericidal concentration (MBC) and ratio (MBC/MIC). All the values obtained are listed in Table 2. From the results obtained, it can be concluded that the essential oil of *Cedar Atlas* possesses interesting antimicrobial activities against the studied strains. The diameters of zones of inhibition Φ obtained from the disk diffusion method vary from 20.01 mm to 8.00 mm. The strains *Escherichia coli* (ATCC 4157), *Klebsiella pneumoniae* (ATCC 4352), and *Bordetella bronchiseptica* (ATCC 4617) are extremely sensitive and therefore possess strong activity. While *Micrococcus luteus* (ATCC 533), *Bacillus cereus* (ATCC 10876), *Enteroccocus faecium* (ATCC 6569) are sensitive and possess moderate acceptable activities.

The minimum MIC inhibitory concentrations vary from 56.35 to 156.54 µL / mL and the minimum bactericidal MBC concentrations vary from 64.80 to 168.30 µL / mL. In general, therefore, the essential oil of *Cedar Atlas* exhibits remarkable activities with respect to all the strains studied.

According to the MBC/MIC ratio, *Cedar Atlas* essential oil has bactericidal effects against all the strains studied (Table 3). In general, it has also been established in numerous studies that the activity of an essential oil is related to the majority compounds and the possible synergistic effects between the constituents.

Conclusion

In this work we studied the chemical composition and the antimicrobial activity of the aerial part of *Cedar Atlas* essential oil collected in the region of Ifrane-Morocco. Forty-nine chemotypes were identified in the crude oil. The results showed that the essential oil is characterized by the presence of α-himachalene (19.47%), α-longipinene (21.26%), β-himachalene (28.20%). These three major compounds were obtained with a percentage of (68.93%). The antimicrobial efficacy of *Cedar Atlas* essential oil is remarkable against the six bacteria tested. The results we obtained confirm that the essential oil of this species has a very important antimicrobial power, this activity is mainly due to the major constituents. On the other hand the factors that influence the active molecules are numerous, the variability can be explained by the different conditions such as the geographical location, the climate, the attitude, the part used, and the choice of the extraction method. All of its results suggest prospects for formulation research based on *Cedar Atlas* essences in place of certain synthetic preservatives in the medical fields.

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Table 1. Chemical composition of the essential oil of *Atlas Cedar*.

Pic	RT	Composants	(%)
1	7.75	2-Hydrazinoethanol	0.08
2	7.83	2-(1-Hydroxyethyl)norbornadiene	0.15
3	7.93	3-Carene	0.07
4	7.98	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	0.08
5	8.03	trans-α-Ocimene	0.05
6	8.44	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	0.07
7	8.48	Camphene	0.03
8	9.20	α-Pinene	0.07
9	9.34	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.15
10	10.87	Benzene, 1-methyl-3-(1-methylethyl)-	0.04
11	10.98	D-Limonene	0.06
12	11.60	Eucalyptol	2.48
13	14.95	Bicyclo[3.1.1]hept-2-en-6-one, 2,7,7-trimethyl-	0.05
14	15.13	4-Acetyl-1-methylcyclohexene	0.54
15	15.78	(+)-2-Bornanone	0.50
16	16.43	endo-Borneol	0.08
17	17.09	trans-Chrysanthenyl acetate	0.02
18	17.30	α-Terpineol	0.11
19	19.23	Pulegone	0.07
20	22.75	α-ylangene	0.08
21	24.00	Isolongifolene, 4,5-dehydro-	0.70
22	24.57	β-Cadinene	0.51
23	24.79	Valencene	0.36
24	25.41	Himachala-2,4-diene	0.30
25	**26.71**	**α-Himachalene**	**19.47**
26	27.55	Longifolene-(V4)	11.18
27	**28.66**	**β-Himachalene**	**28.20**
28	**28.89**	**α-Longipinene**	**21.26**
29	28.95	Isolongifolene, 4,5,9,10-dehydro-	1.24
30	29.06	δ-Cadinene	2.70
31	29.21	Neoisolongifolene, 8,9-dehydro-	0.99
32	29.31	Benzene, 1-(1,2-dimethyl-3-methylenecyclopentyl)-4-methyl-, cis-	2.39
33	29.45	γ-Himachalene	0.54
34	29.58	α-Calacorene	0.67
35	29.73	Cadala-1(10),3,8-triene	0.05
36	30.08	γ-Muurolene	0.05
37	30.77	3-Isobutyl-4,5-dimethyl-3H-isobenzofuran-1-one	0.35
38	31.41	Longiborneol	0.13
39	31.91	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	0.43
40	32.10	Aristolene epoxide	0.30
41	32.21	4-epi-cubedol	0.33
42	32.31	Cedrene	0.10
43	32.63	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	0.08
44	33.28	Cubenol	0.36
45	33.39	α-Guaiene	0.01
46	33.98	Tumerone	0.16
47	34.30	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)-	0.55
48	34.54	6-(1,3-Dimethyl-buta-1,3-dienyl)-1,5,5-trimethyl-7-oxa-bicyclo[4.1.0]hept-2-ene	0.14
49	36.31	6-Isopropenyl-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	0.38
Total			**98.71**

Table 2. Parameters of antibacterial activities of essential oil of *Atlas Cedar.*

Strains	Applied methods	*Cedrus atlantica*
Escherichia coli	Φ (mm)	20.01 ± 0.02
	MIC (µL/mL)	56.35
	MBC (µL/mL)	64.80
	MBC/MIC	1.14
Klebsiella pneumoniae	Φ (mm)	19.55 ± 0.21
	MIC (µL/mL)	92.34
	MBC (µL/mL)	101.99
	MBC/MIC	1.10
Bordetella bronchiseptica	Φ (mm)	15.49 ± 0.13
	MIC (µL/mL)	132.66
	MBC (µL/mL)	163.84
	MBC/MIC	1.23
Micrococcus luteus	Φ (mm)	9.85 ± 0.62
	MIC (µL/mL)	65.34
	MBC (µL/mL)	71.49
	MBC/MIC	1.09
Bacillus cereus	Φ (mm)	11.11 ± 0.87
	MIC (µL/mL)	86.50
	MBC (µL/mL)	99.01
	MBC/MIC	1.14
Enteroccocus faecium	Φ (mm)	8.00 ± 0.11
	MIC (µL/mL)	156.54
	MBC (µL/mL)	168.30
	MBC/MIC	1.07

Table 3. Bactericidal or bacteriostatic character of the essential oil of *Atlas Cedar*.

Strains	*Effect*
Escherichia coli	Bactericidal
Klebsiella pneumoniae	Bactericidal
Bordetella bronchiseptica	Bactericidal
Micrococcus luteus	Bactericidal
Bacillus cereus	Bactericidal
Enteroccocus faecium	Bactericidal

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