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Chemical composition, antibacterial and antifungal activities of the *Cedrus atlantica* (Endl.) Manettiex Carrière seeds essential oil

Rachid Rhafouri[1,2], Badr Strani[2], Touria Zair[3], Mohamed Ghanmi[2], Abderrahman Aafi[2], Mohamed El Omari[1] and Amar Bentayeb[1,*]

[1]Equipe Physico-chimie de la Matière Condensée, Faculté des Sciences, Université Moulay Ismaïl, Meknès, Maroc

[2]Centre de Recherche Forestier, Rabat, Maroc

[3]Chimie des Molécules Bioactives et Environnement, Faculté des Sciences, Université Moulay Ismaïl, Meknès, Maroc

Abstract: The Atlas cedar is an endemic species of the African North Mountains. The goal for which this work was conducted is to determine the chemical composition and to study the antibacterial and the antifungal activity of the hydrodistilized essential oil from both the winged and wingless seeds of the High Atlas *Cedrus atlantica* (Morocco). The essential oil is analyzed by gas chromatography and gas chromatography mass spectrometry. The essential oil yields of winged as well as wingless seeds were respectively 2.6% and 3.6%.The main constituents of the cedar wingless seeds are the α-pinene, the manool, and the bornyl acetate; whereas, the major constituents of the cedar winged seeds are the manool and the α-pinene. The antibacterial and antifungal activities of the essential oils were tested on four bacteria, three molds and four fungi of wood rot. The fungal strains tested were revealed more sensitive to the essential oil studied than the bacterial strains.

Keywords: *Cedrus atlantica*; Hydrodistillation; Essential oil; Chemical composition; Bioactivity.

Introduction

The genus *Cedrus*, belonging to the Pinaceae family, is considered as the oldest after the *Pinus*genus[1]. It includes four species[2,3], which occupy unequal surfaces at mountain stage of the mediterrano-himalayan region. These four species are: *Cedrus atlantica, Cedrus libani, Cedrus brifolia* and *Cedrus deodora*.

The Atlas cedar is an endemic species of North Africa Mountains (Morocco, Algeria). In Morocco, the Atlas cedar occupies an area of 132,000 hectares divided into two blocks of unequal importance: the Rif; the Moroccan Middle and High Atlas[4], presenting an altitudinal ranging from 1500m to 2600m.

It is a tall tree, ranging from 40m to 60m of height. The cedar is a monoecious species, whose male organs are kittens and female ones are inflorescences, which develop gradually in cone lets then in cones. The seed is resinous and tender[5]. It comprises at least one resin canal pocket on each side; the breaking of this pocket hinders definitely the seed's germination.

*Corresponding author: Amar Bentayeb

E-mail address: abentayeb2002@yahoo.fr

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The cedars represent a forest fortune of primary importance, from both the ecological and economical strict point of view. From the chemical point of view, several studies have been realized; especially, on needles essential oils[6] and cedar sawdust[7,8].

The essential oil of cedar wood is antiseptic, healing, antifungal, purifying and relaxing. It is used in perfumery and in cosmetology. Also, it is prescribed against dermatitis and skin inflammation[9]. The cedar essential oils are useful in perfumery and in cosmetics, thanks to the himachalenes which they contain[10].

A single study, on the chemical composition of the cedar seeds essential oils, was made in Algeria[11]. It showed that these oils have a high content in α-pinene and in manool. In Morocco, no work has been done on the chemical profile of the Atlas cedar seeds essential oils. In addition, the chemical composition study of essential oils of both winged and wingless seeds, as well as the determining of their antimicrobial activity were never investigated. Therefore, the objective of our work is to characterize the chemical composition and to study the antibacterial and antifungal spectrum activity of Moroccan High Atlas cedar seeds essential oil. This study will allow us to understand better and valorize the *Cedrus atlantica* speciesre productive organs.

Experimental Section

Plant material

The *Cedrus atlantica* cones were collected by Azrou's Regional Station Seed (Middle Atlas), during the month of September 2012, in a region of the High Atlas (Bouadil). The collection of the cones was performed on cedar trees growing in natural stands. After isolating the seeds from cones, they are stored in plastic bags in a refrigerator at 3°C. Our study sample is consisted of bothwinged and wingless seeds.

Studied microorganisms

Four bacteria (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Micrococcus luteus*): bacterial strains which are lots ATCC (*American Type Culture Collection*). They are maintained by subculture on nutrient agar favorable to their growth during 24 h in the obscurity and at 37°C.

Seven fungi of which three molds (*Aspergillusniger, Penicilliumdigitatum and Penicilliumexpansum*) are known by their high degrees to contaminate the foodstuffs and by their pathogenicities. For other fungi, they are fungal species, responsible of brown and white rot wood. They were chosen for the considerable damage which they cause to timber and derived products (*Gloeophyllumtrabeum, Coniophoraputeana, Poria placenta, Coriolusversicolor*).

The fungal strains and molds belong to the Mycotheque Collection of Microbiology Forestry Centre (Rabat, Morocco) Laboratory. They are regularly maintained by transplanting on the nutrient environment PDA (*Potato Dextrose Agar*).

Extraction of essential oils

The cedar seeds essential oils were obtained by hydrodistillation during 210 min of extraction in a Clevenger type apparatus[12]. The yield samples of essential oil was determined after three hydrodistillations of 100g dry material, and the moisture calculation by drying 5g of each sample during 4 h in a stove at 102°C. The obtained essential oil is dried on anhydrous sodium sulfate, and conserved at an obscurity of 4°C temperature.

Chromatographic analysis

The gas chromatography (GC) analysis were performed using a Hewlett Packard Gas Chromatographer (HP 6890) with electronic pressure control, equipped with a HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm), a FID detector set at 250°C and using a H_2/Air mixture, and a *split-splitless* injector set at 250 °C. The injection mode was split (split ratio: 1/50, flow rate: 66 ml min^{-1}) and the injected volume was about 1 µl. Nitrogen was used as carrier gas with a flow rate of 1.7 $ml.min^{-1}$. The column temperature was programmed from 50 to 200 °C at a heating rate of 4°$C.min^{-1}$, during 5 min. The apparatus was controlled by a "Chemstation" computer system.

The gas chromatography/mass spectrometry (GC/MS) analysis were performed by a Hewlett-Packard Gas Chromatographer (HP 6890) coupled with a mass spectrometer (HP 5973). Fragmentation was performed by electron impact at 70 eV. The column used was HP-5MS (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas is helium whose flow is fixed at 1.5 $ml.min^{-1}$. The injection mode was split (split ratio: 1/70, flow rate112 ml min^{-1}). The column temperature was programmed from 50 to 200 °C at a heating rate of 4 °$C.min^{-1}$, during 5 min. For the chromatographic analysis, essential oils were diluted in methanol (1/20 v/v).

The identification of the components is based on the comparison of their mass spectra (GC/MS), respective with spectra of the library (NIST 98), of the bibliography (Adams, 2007)[13] and on the basis of calculation of Kovats indices. Indeed, the index system is based on a notion of relative retention. It compares the retention of whatever product to that of a linear alkane. This system is applicable in gas chromatography to all compounds on all columns. By definition, it assigns an index of 800 to the linear alkane in C_8 (n-octane), 1000 to C_{10} linear alkane (n-decane), and this, whatever the stationary phase, the length of column, the flow rate or the temperature. The KI are determined by injecting a mixture of C_9 to C_{24} alkanes in the same operating conditions[14]. They are calculated from the following equation:

$$Ik=\left[\frac{TR_x-TR_n}{TR_{n+1}-TR_n}+n\right]\times 100$$

Where in TR_x is the retention time of the solute x, TR_n and TR_{n+1} are the retention times of linear alkanes to n and n + 1 carbon atoms and which frame the peak of the solute. The retention index KI or a compound A is independent from the flow rate, of the column length and of the injected amount (within a certain limit). The retention index of a compound A depends on the stationary phase and temperature.

In general, the technique of KI is widely used to identify the usual essential oils compounds, but it is insufficient to determine the total chemical composition. The IK tables specific to each product are proposed in the literature. They were developed using analyzes on different types of columns. These benchmark indices are compared to those calculated from our samples.

Microbiological procedure

The minimum inhibitory concentrations (MIC) of the essential oils were determined according to the method reported by Remmal et al[15], also by Satraniand al[16]. Because of the essential oil immiscibility with water and, therefore, to the cultural environment, anemulsification was realized thanks to an agar solution at 0.2%. It allowed to obtain, in the middle, a homogeneous distribution of essential oils and to make the higher maximum of compound/germ contact. Dilutions are prepared at $1/10^e$, $1/25^e$, $1/50^e$, $1/100^e$, $1/200^e$in this agar solution.

In test tubes, containing each 13.5 ml of solid environment TSA (*Tryptic Soy Agar*) for bacteria, and the PDA (*Potato Dextrose Agar*) for fungi, sterilized at the autoclave during 20 min at121°C and cooled at45°C, we add aseptically 1.5 ml of each dilution so as to obtain the final concentrations of 1/100, 1/250, 1/500, 1/1000 and 1/2000 (v/v). We shake the tubes to disperse properly the essential oil in the cultural environment before pouring them into Petri dishes. Witnesses, containing the cultural environment and agar solution at 0.2% alone, are equally prepared. The seeding is done by streaking with the help of a calibrated platinum loop to withdraw the same inoculums volume.

This latter is presented in the form of culture broth of 24 h for bacteria and in the form of a suspension in physiological water of spores resulting from a culture of 7 days in the PDA for fungi. The seeding is done, for these latters, by the fragments deposition of $1cm^3$of diameter, taken from the periphery of a mycelia mat, and originating from a 7 days culture in the malt extract. The incubation is done at 37°C during 24 h for bacteria, and at 25°C during 7 days for fungi. Each test is repeated three times.

Results and Discussion

Yield and chemical composition

The Atlas cedar winged and wingless seeds have provided respectively a yield of essential oil about 2.6% and 3.6%. Cedar wingless seeds have provided a yield of essential oil, more important than that of winged seeds. This can be explained by the difference of weight between the winged seeds and the wingless ones, knowing that the wings are devoid of resin canal pockets rich in essential oil. However, these yields provided by the seeds are higher than those obtained by the needles (1.8%)[6], and sawdust cedar (2.4%)[7].

The study led by Boudarene and al[11] on the cedar seeds, originated from Algeria, have provided a yield much lower and which have not exceeded 1.2%,by the same method of distillation.

The cedar seeds essential oils analysis, using the technics GC and GC/MS (**Figure 1**) have permitted to identify 27 compounds for wingless seeds and only 9 compounds for the winged seeds, representing respectively about 97.36 % and 99.41% of these oils total chemical composition (**Table 1**).

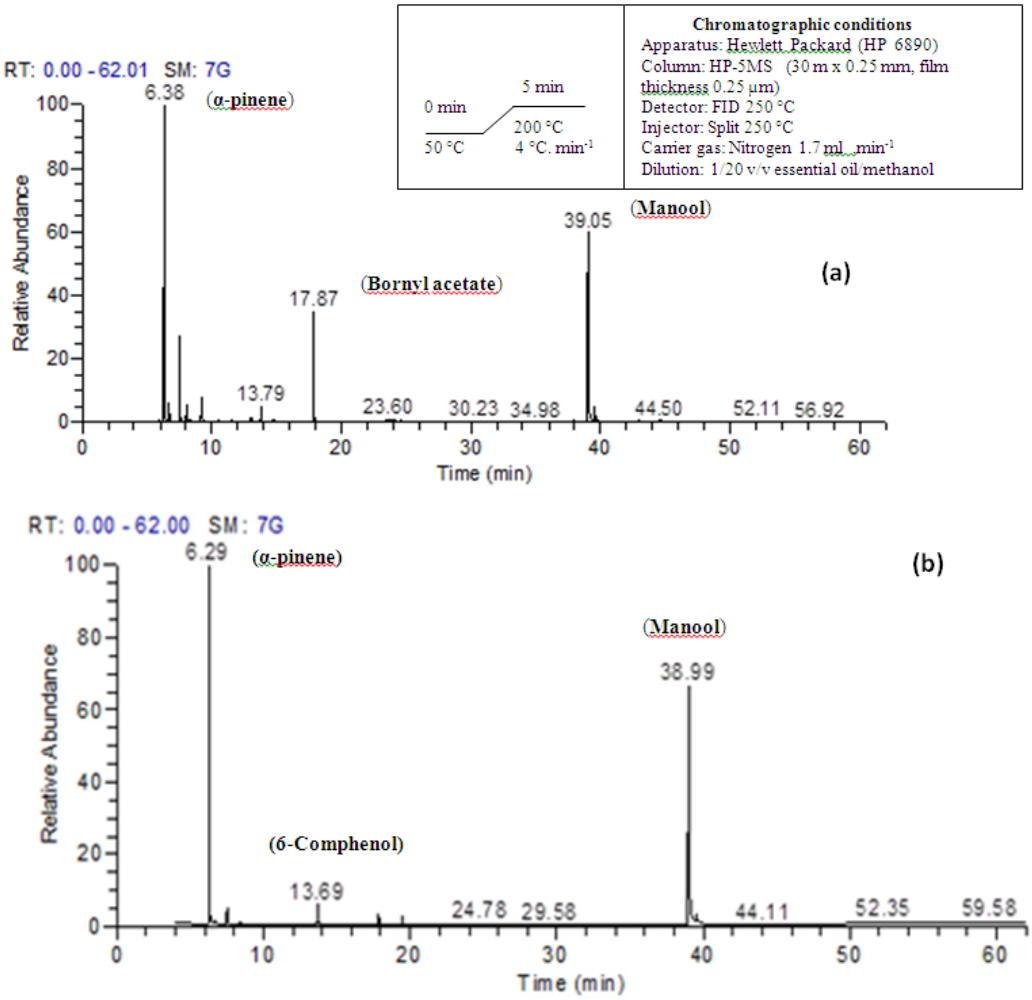

Figure 1. Chromatographic profiles of Cedrus atlantica wingless **(a)** and winged **(b)**seeds essential oil of Morocco.

The major constituents of the wingless seeds essential oil are the α-pinene (46.16 %), the manool (25.47 %), the bornyl acetate (10.18%), the β-pinene (5.95%), and the α-terpinene (2.71%). Those of winged seeds are the manool (49.02%), the α-pinene (40.82%), 6-comphenol (2.52%), and the β-pinene (2.13%).

Thus, we can conclude that the rate of manool in the wingless seeds essential oil (25.47 %) have completely doubled in the gasoline winged seeds (49.02 %). However, we note the decrease in the α and β-pinene content and bornyl acetate in the cedar winged seeds essential oil. Also, we observe that the relative percentages of the α-terpinene (2.71%), myrcenol
(1.20%), cis-linalool oxide (0.48%) and the cis-hydrate sabinene (0.34%) were detected only in the essential oil of Atlas cedar wingless seeds.

This chemical analysis of the two samples of essential oils from the same source has permitted to identify 18 more compounds for wingless seeds of which the percentage is ranging from 0.03 % to 2.71 %. This difference can be explained: i) on one hand, by the fact that the performed hydrodistillation on wingless seeds has permitted a direct contact and permanent between the water steam and resiniferous pockets seeds, which has favourised the entrainment of even minor the constituents, such as ortho-cymene, α-terpinene, and myrcenol. Thus, the absence of these 18 components in the gasoline wingless seeds was offset by the

increase in the rate of other components, such as the manool and the 6-camphenol. ii) on the other hand, the presence of the wing has increased the seed's weight in the sample to be distilled which has resulted the decrease in the number of secretory cells of the essential oils. This had as a consequence, the reduction of the components rate in the essential oil winged seeds, which become at the state of trace undetectable by the analyzing unit. Knowing that, preliminary distillation tries of wings only have shown the absence of essential oils in these latters.

Table 1. Chemical composition of the wingless and winged seeds essential oil of Moroccan *Cedrus atlantica*.

Kovats Indices (KI)	Compound	Wingless seeds (-Ws) oil percentage	Winged seeds (+Ws) oil percentage
926	Tricyclene	0.04	-
939	α-pinene	46.16	40.82
953	Comphene	1.19	0.45
980	β-pinene	5.95	2.13
991	Myrcene	0.11	0.40
1018	α-terpinene	2.71	-
1022	ortho-cymene	0.03	-
1062	γ-terpinene	0.05	-
1068	Cis-sabinene hydrate	0.34	-
1074	Cis-linalooloxide	0.48	-
1103	6-Comphenol	0.15	2.52
1118	Myrcenol	1.20	-
1142	α-terpineol	0.14	-
1183	p-cymen-8-ol	0.20	-
1217	Trans-carveol	0.03	-
1285	Bornylacetate	10.18	1.82
1334	1-phenyl pentan-3-one	0.06	1.14
1499	β-Himachalene	0.07	-
1511	α-dehydro-ar-Himachalene	0.07	-
1610	β-himachaleneoxide	0.08	-
1697	Z,EFarnesol	0.09	-
2010	13-epi- manoyloxide	0.26	-
2055	Manool	25.47	49.02
2077	Abietadiene	1.72	1.11
2235	7-α-hydroxy-manool	0.34	-
2283	4-epi-Abietal	0.16	-
2391	Abietol	0.08	-
Total		97.36%	99.41%

The study of the chemical composition of the Algerian Atlas cedar seeds essential oil (region Ouled Yacoub (OY) and Tala Guilef (TG)), carried out by Boudarene and al (2004)[11], has given as major compounds respectively for the OY and TG regions, the α-pinene (37.1 and 5.5%), the β-pinene (8.06 and 1.8%), the myrcene (3.6 and 0.6%), the limonene (2.5 and 0.6%), the bornyl acetate (5.4 and 4%), the (E)-β-farnesene (6.8 and 1.9%), and the

manool (8.3 and 20.7%). These results show that there is a significant difference in the chemical composition of the Algeria's cedar seeds essential oil, region of Tala Guilef, and that of Moroccan High Atlas. Indeed, we see that the Moroccan Atlas cedar seeds essential oil present a high rate in some compounds compared to that of Algeria such as: respectively, the α-pinene (46.16 against 5.5%), the manool (25.47 against 20.7%) and the bornyl acetate (10.18 against 4%). However, the Algerian cedar seeds essential oil is characterized, especially, by the presence of (E)-β-farnesene (1.9%) and limonene (0.6%).

The observed difference between the chemical composition of the essential oil of the Moroccan Atlas cedar seeds and those of Algeria could be due to the difference of both climatic and geographical factors, such as the altitude and the soil type[17-19].

The chemical profile of Moroccan Atlas cedar's winged seeds essential oil is characterized by a high rate of 49% manool. To our knowledge, rare are the plants which present as high a rate in this terpene alcohol.

Several studies have shown that manool is a useful intermediate component in the hemisynthesis of the relevant compounds in perfumery such as, the amber acetal[20,21] and also of some bioactive molecules like, the agelasine D[22]. On the basis of these results, the use of cedar seeds essential oil, characterized by a high rate of this compound terpene, thus turns out being important in various fields.

Bioactivity

The obtained results of the antibacterial and antifungal activity study of the Atlas Cedar winged or wingless seeds essential oil originated from Morocco, are presented in **Table 2** below.

Table2. Antibacterial and antifungal activities of Moroccan Atlas cedar's winged and wingless seeds essential oil.

Concentrations (v/v)	1/100		1/250		1/500		1/1000		1/2000		W	
	-Ws	+Ws	-Ws	+Ws	-Ws	+Ws	-Ws	+Ws	-Ws	+Ws	-Ws	+Ws
Bacteria												
E. coli	-	-	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+
M. luteus	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+
Wood rot fungi												
G. trabeum	-	+	-	+	+	+	+	+	+	+	+	+
P. placenta	-	+	-	+	+	+	+	+	+	+	+	+
C. puteana	+	+	+	+	+	+	+	+	+	+	+	+
C. versicolor	+	-	+	-	+	-	+	+	+	+	+	+
Molds												
A. niger	+	+	+	+	+	+	+	+	+	+	+	+
P. expansum	+	+	+	+	+	+	+	+	+	+	+	+
P. digitatum	+	+	+	+	+	+	+	+	+	+	+	+

+: Growth, -: Inhibition, W: Witness, +Ws: Winged seeds, -Ws: wingless seeds.

Following the outcome of our biologic tests, we see that the Atlas cedar seeds essential oil has manifested various antibacterial and antifungal characteristics depending on tested microorganisms.

Indeed, the antibacterial tests of the two oils have shown that only *E. coli* was sensitive to the two essential oils and was inhibited to the concentration of 1/100 v/v.

The essential oil of the winged seeds has shown an inhibiting effect vis-à-vis of an only wood rot fungi, *C. versicolor,* at the concentration of 1/500 v/v.

We also note that the two wood rot fungi *G. trabeum* and *P. placenta* were inhibited at a concentration of 1/250 v/v vis-à-vis to the Atlas cedar wingless seeds essential oil.

Thus, all tested molds have resisted to the cedar seeds essential oils, at even a highest concentration.

The antimicrobial activities of these essential oils observed vis-a-visof some germs, could be attributed either to their chemical profile, characterized by its main constituents, especially the manool and the α-pinene. In fact, several studies have shown that the manool is endowed of an antibacterial ability against *Staphylococcus aureus* and *Stenotrophomonasmaltophilia*, as well as antifungal activity against *Candida albicans*[23,24]; either to the synergy phenomenon between several volatile components whose interactions may be the origin of an activity much more pronounced than that expected for the majority of compounds, as it is proved by several studies[25-31].

Generally, the two Atlas cedar winged and wingless seeds oils have exerted a weak fungicidal and bactericidal activity against tested fungi and bacteria. This is due to their chemical profiles rich in hydrocarbons terpene[32] and poor in phenols terpene[33].

Conclusion

The obtained oils, of Moroccan *Cedrus atlantica* from winged and wingless seeds, have respectively shown the presence of 9 and 27 compounds, which corresponds to 99.41% and 97.36% of the oils totality. The yields of these oils were 2.6 % and 3.6%.

The majority of winged and wingless seeds compounds are presented with variable percentages: respectively, 46.16% and 40.82% for the α-pinene; 25.47% and 49.02% for the manool and 5.95 % and 2.13% for the β-pinene. The bornyl acetate with 10.18% and the α-terpinene with 2.7%are pretty important components in the winged seeds essential oil; whereas, the 6-comphenol with 2.52% is the most present in the wingless seeds essential oil.

The antimicrobials tests of the two essential oils of seeds have shown a weak activity against tested bacteria and fungi. Only few microbial strains such as *C. versicolor*, *G. trabeum*, *P. placenta,* and *E. coli,* were sensitive to cedar seeds essential oils.

With a higher content of about 50% of manool, the essence of cedar winged seeds could constitute a source in this component used in the synthesis of the principal active, at a high added value used in various industrial fields.

The results of this study (chemical analysis and antimicrobial activity), of the Moroccan *Cedrus atlantica* seeds essential oil, will allow a better knowledge of this reproductive organ and contribute to give a more value to the Atlas cedar in general.

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