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Phytochemical composition of cedar tar of the atlas and it's *in vitro* antifungal activity against *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis*

Sanae Masmoudi[1]*, Abdelmjid Aiboud[2], Laila Chaoui[3], Fatima Zahra Milouk[4], Ahmed Moussaif[2], Aboubaker El Hessni[5] and Abdelhalim Mesfioui[5]

[1]Higher Institute of Nursing Professions and Health Technologies, Rabat, Kenitra Annex, Morocco
[2]National Center for Energy Sciences and Nuclear Techniques, Rabat, Morocco
[3]Laboratory of Physical Chemistry and Biotechnologies of Biomolecules and Materials, University Hassan II Casablanca, FSTM, Mohammedia, Morocco
[4]Higher Institute of Nursing Professions and Health Technologies, Fes, Morocco
[5]Biology and Health Laboratory - Faculty of Sciences, Ibn Tofail University, Kenitra, Morocco

Abstract: The objective of this study was to identify the major compounds present in Cedar tar obtained by distillation of *Cedrus atlantica* wood from the Taza forest (Morocco) and to evaluate its antidermatophytic activity *in vitro* against the three strains of dermatophytes most widespread in Morocco, considered the main prevailing causes of fungal infections of the skin, hair and nails. GC/MS analysis revealed that cedar tar is composed mainly of hydrocarbon sesquiterpenes and oxygenated sesquiterpenes, with nine major compounds identified, including α-Cedrene, β-Cadinene, γ-Cadinene, β-Himachelene, α-Turmerone, β-Turmerone, Ar-tumerone, α-Atlantone and Himachalol. The evaluation of antifungal activity was carried out by the micro dilution technique. The MIC values found were 100µg/mL, 2µg/mL and 0.1µg/mL on *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis* strains respectively. The observed strong antifungal activity of cedar tar is attributed to the prevalence of oxygenated and hydrocarbon sesquiterpenes, known for their established antidermatophytic properties. This study highlights the potential of the Atlas Cedar tar as an effective antifungal agent for the treatment of superficial mycoses, particularly dermatophytoses.

Keywords: Cedar tar*, Cedrus atlantica,* dermatophytes, antifungal activity, sesquiterpene

INTRODUCTION

Dermatophytosis, a prevalent global infectious ailment, imposes chronic morbidity, particularly in developing nations (Ramaraj *et al*., 2016). This condition, caused by keratin-requiring fungi called dermatophytes, has witnessed an escalating incidence worldwide, especially in developing regions (Moriarty *et al*., 2012).

The treatment of dermatophytosis involves prolonged use of topical and/or oral antifungal drugs. Antifungal resistance represents a major clinical challenge to clinicians responsible for treating invasive fungal infections due to the limited arsenal of systemically available antifungal agents. In addition current drugs may be limited by drug-drug interactions and serious adverse effects/toxicities that prevent their prolonged use or dosage escalation (Nathan, 2017). Plants, synthesizing diverse active compounds with antibacterial and antifungal properties, emerge as promising sources for novel antimicrobial agents (Diba *et al*., 2018; Umamaheswari *et al*., 2023).

The World Health Organization estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population (Nayan *et al*., 2011). Morocco, by its biogeographical position, possesses a very rich ecological and floristic diversity, constituting a true plant genetic reserve, with a high level of endemic plants belonging to different botanical families (Zaher *et al*., 2018), with a very rich flora, which contains a large number of rare, endemic, or very remarkable species (Najem *et al*., 2019).

Cedrus atlantica (Atlas cedar) is a species endemic to the mountains of North Africa. In Morocco, the Atlas cedar occupies an area of 132,000 hectares (Ez Zoubi *et al.*, 2017). It has been investigated in several studies in regard to different bio-functions including antimicrobial (Zrira and Ghanmi, 2016).

In the context of this work, we have been interested in the wood tar of *C. atlantica* obtained by dry distillation. Dry distillation of wood (pyrolysis) is the process in which wood is heated (not burned) to form coal and vapors, these vapors are condensed to form a brownish liquid, this liquid is called wood tar and it is a complex and unstable mixture of many compounds (Jindal and Jha, 2016).

Cedar tar, referred to as "Gatran" in the Maghreb. With a longstanding history in traditional medicine, either alone or in combination with other products, cedar tar has been used to treat various dermatological conditions such as eczema (Lahsissene *et al*., 2009).

**Corresponding author:* e-mail: sanaemasmoudi@yahoo.fr

The aim of this study was to identify the major compounds of *Cedrus atlantica* tar of the Atlas and to demonstrate its antidermatophytic activity *in vitro* against three strains of the most common dermatophytes in Morocco through the determination of MICs and the antifungal structure-activity relationship of the major compounds.

MATERIALS AND METHODS

Plant material

Cedar tar of the Atlas is produced in traditional stone ovens by dry distillation of *Cedrus atlantica* a conifer of the Pinaceae family native to the Atlas Mountains of North Africa. The Atlas cedar tar we used in this study was purchased from a traditional producer located in Taza region is located in northern Morocco, bordering the Mediterranean Sea to the northeast, situated at approximately 34.22° N latitude and 4.01° W longitude.

Phytochemical characterization of the major compounds

Mass Coupled Gas Chromatography (GC/MS) analysis of Cedar tar was performed using a 30-meter Agilent Technologies 6890N column (Wilmington, DE, USA). HP-5, 0.32mm id, 0.25µmde diameter. The carrier gas used was helium with a flow rate of 1mL per minute. The injection temperature was set at 275°C. The temperature profile was programmed as follows: 80°C for 2 min, with a slope of 4°C /min up to 280°C, then a plateau at 280°C for 10min. The injection volume was 1µl prepared in acetone (Lindborg, 2008).

In vitro antifungal activity of cedar tar

Stains

In vitro antidermatophyitic activity of Cedar tar of the Atlas was tested on the three strains of the most common dermatophytes in Morocco (Iourdane *et al.*, 2017). It concerns mainly the strain of *Trichophyton rubrum*, *Microsporum canis* and *Trichophyton mentagrophytes*. These strains were isolated and identified by the Firdaous Medical Analysis Laboratory in Kenitra City from patients with clinical suspicion of onychomycosis or *Tinea capitis*.

Preparation of inoculums

The inoculum suspension for each dermatophyte strain was prepared from the 10-day cultures grown on PDA at 28°C. The fungal colonies were covered with approximately 10mL of sterile saline solution supplemented with 1% Tween 80 and the suspension was prepared by scraping the surface with the tip of a sterile loop. The resulting mixture of conidia and hyphal fragments was collected and transferred to sterile tubes and left for 15 to 20 minutes at room temperature to sediment the heavy particles. The optical density of the suspensions containing conidia and hyphal fragments was read at 530 nm. The suspension is adjusted by successive dilutions in phosphate-buffered saline (PBS) to achieve a transmittance of 65 to 70% (~10^5-10^6 CFU/mL-1) (Aktas *et al.*, 2014).

Determination of the minimum inhibitory concentration (MIC)

The liquid dilution method was used to determine the extract concentration to be used for further analysis and to determine the MIC. 20µL of each inoculum suspension was inoculated into a tube containing 10mL of Sabouraud dextrose broth. Then, 100µL of the inoculated culture medium of the suspension was poured into each of the wells of micro dilution plates 96 and simultaneously in the same well was added 100µL of each extract with increasing concentration (from 0.01 to 100µg/mL). The first well contains no antifungal agent and serves as a control. The micro titer plates were incubated in the dark at 28°C and the results were read after 72-96 hours (Fernández-Torres *et al.*, 2003 and Ogba *et al.*, 2023).

Tests were performed in duplicate. Further, The MIC was recorded spectrophotometrically at 530nm using a UNICAM UV 500 spectrophotometer. MICs were defined as the first concentration at which an 80% or more reduction was measured spectrophotometrically (Tiwari *et al.*, 2011).

STATISTICAL ANALYSIS

The statistical analysis of the results was carried out using the student test.

RESULTS

Identification of major compounds

The identification of the major compounds was carried out by comparing the mass spectra with the reference spectra of the NIST standard database as shown in fig. 1 and table 1. We note that Cedar tar of the atlas is mainly composed of hydrocarbon sesquiterpenes (α-Cedrene, β-Cadinene and γ-Cadinene) and oxygenated sesquiterpenes (β-Himachelene, α-Turmerone, β-Turmerone, Ar-tumerone, α-Atlantone and Himachalol).

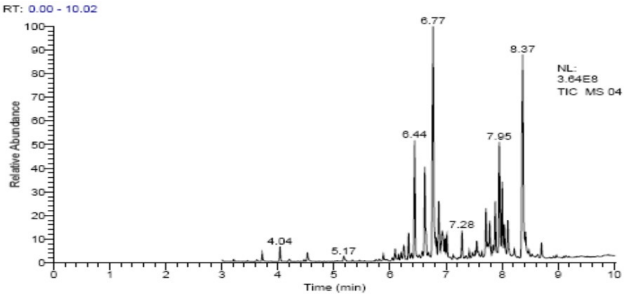
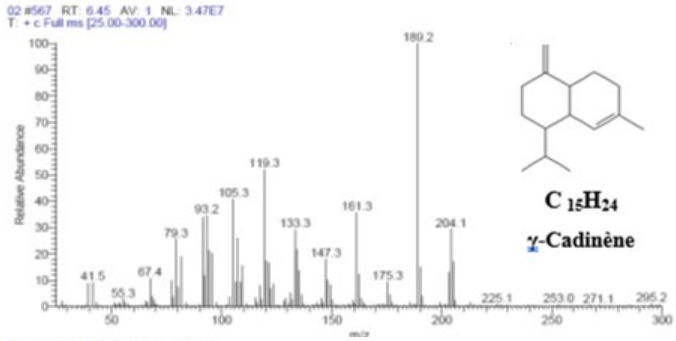
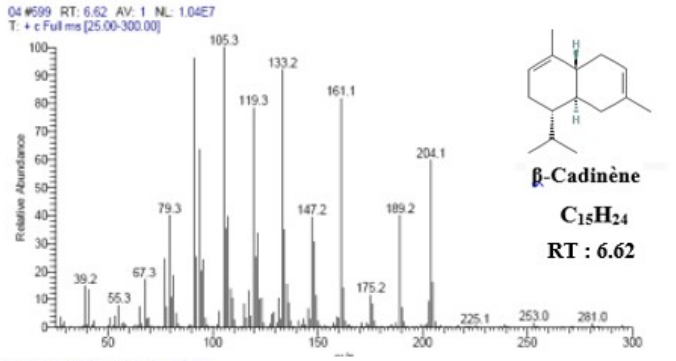
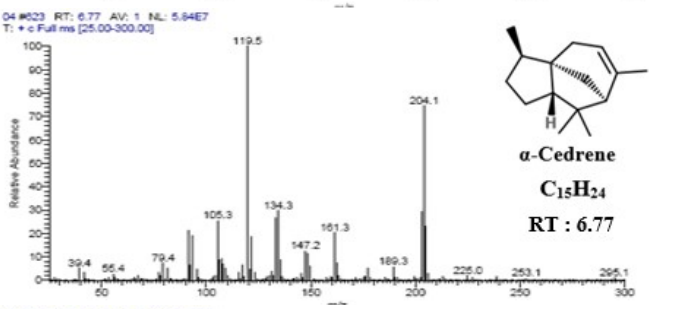
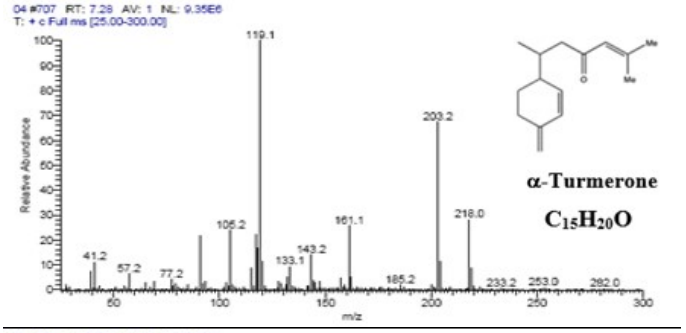
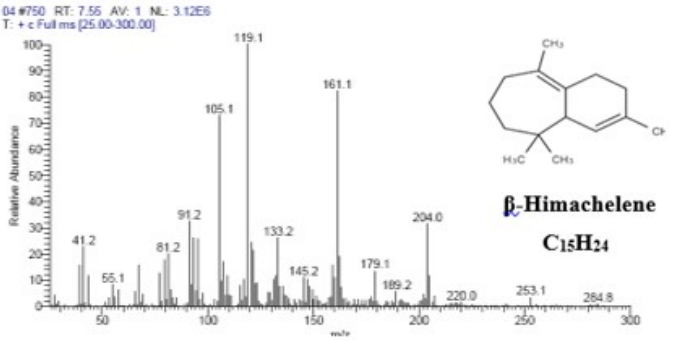


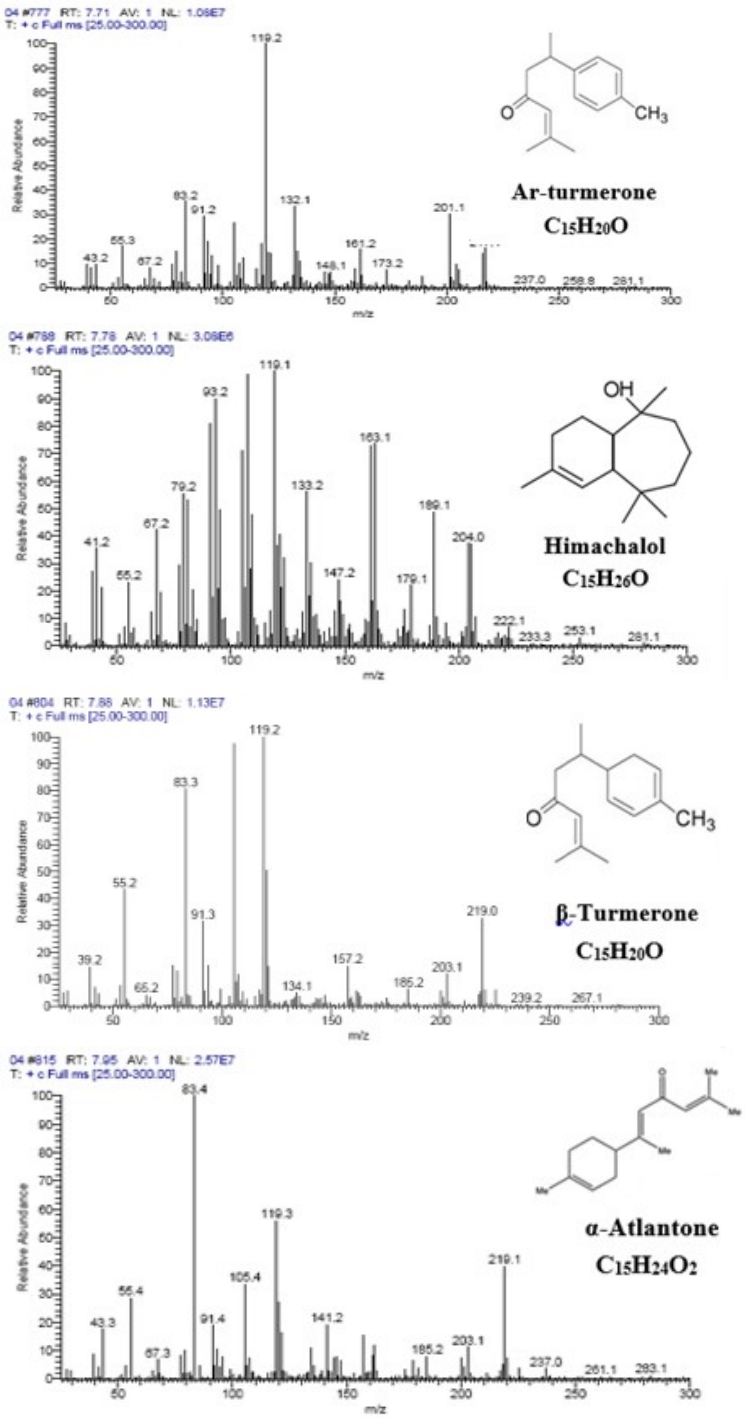
Fig. 1: GC chromatogram of Cedar tar of the Atlas.

MICs of cedar tar of the atlas against trichophyton rubrum, Trichophyton mentagrophytes and micros porum canis

The results obtained showed that cedar tar exhibited moderate to high antidermatophytic activity against the three dermatophyte strains tested.

Mass spectrum	RT (min)	Percentage composition (%)
	6 :45	4.3
	6.62	3.8
	6.77	10.8
	7 :28	2.1
	7 :56	21.6

Continue…



7 :71	2.2
7 :78	1.9
7 :88	2.5
7 :95	15.7

Fig. 2: Mass spectrum of major compounds identified in Cedar tar of the Atlas.

Table 1: Inhibition percentages of Cedar tar against *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis*

Cedar Tar Concentration / Dermatophyte strains	0.01 g/mL	0.1µg/mL	2µg/mL	10 µg/mL	40 µg/mL	100µg/mL
Trichophyton rubrum	72.13+0.22%	72.71+0.13%	74.74+0.26%	75.14+0.44%	78.77+0.26%	81.27+0.08%*
Trichophyton mentagrophytes	76.02+0.13%	77.64+0.3%	82.38+0.18%*	84.68+0.23%*	88.05+0.42%*	97.39+0.44%*
Microsporum canis	75.57+0.36%	87.41+0.14%*	96.93+0.18%*	97.70+0.33%*	97.70+0.25%*	97.70+0.16%*

* Statistically significant ($P < 0.05$)

As shown in table 1, Cedar tar of the Atlas showed a strong inhibitory effect against *Microsporum Canis* and *Trichophyton mentagrophytes* with a MIC of 0.1µg/mL and 2µg/mL respectively, otherwise Cedar tar showed moderate antidermatophytic activity against *Trichophyton rubrum* with a MIC of 100µg/mL.

DISCUSSION

The analysis of Cedar tar showed that it is composed mainly of hydrocarbon sesquiterpenes (cadinene, α-Cedrene and β-Himachelene) and oxygenated sesquiterpenes (β-Himachelene, α-Turmerone, β-Turmerone, Ar-tumerone, α-Atlantone and Himachalol). These results confirm those of other authors. Mohamed and collaborators, cited that the analysis of various samples of cedar oils from the Middle Atlas region of Morocco showed that it presents similar chemical composition with α, β-and γ-himachalene, deodarone and (E)-α-atlantone as dominant components representing approximately70% of these oils. Other components such as δ-cadinene, 1- epi-cubenol, himachalol, (E)-γ-atlantone and (Z)-α-atlantone were present only at levels of 1-4%, representing 10-20% of the oils. (Mohamed *et al.*, 2004). Anne M N and collaborators, cited that cedar tar obtained from the cedar forests of Ouiouane, Middle Atlas (Morocco) is composed mainly α-himachalene, β-himachalene, γ-himachalene and (Z)-α-atlantone which represent about 60% of the total composition of cedar tar (Anne *et al.*, 2015). Yusuf Kurt and collaborators, cited that Cedar tar manufactured by traditional process is composed mainly of β-Himachelene and Tumérone (Yusuf *et al.*, 2008). Other studies showed that Himachalol and β-Himachalene appear as specific markers of *Cedrus atlantica* and only the leaves of the cedar tree produce cadinene (Saab *et al*., 2005).

In general, the antifungal and antimicrobial activity of essential oils is mainly attributed to the presence of sesquiterpenes (Dorman *et al.*, 2000). Sesquiterpenoids are the most abundant natural products, with various activities and excellent prospects in drug development. with abundant structural skeleton types and a wide range of bioactivities, they are considered good candidates to be antibacterial and antifungal agents (Hang-Ying Li *et al*, 2022). The antidermatophytic activity of the sesquiterpenes identified in this study has been cited in numerous studies.

The antifungal activity of the essential oil extracted from cedar wood was first demonstrated in a study conducted by Clark AM and collaborators. This activity was mainly attributed to α-cedrene and ß-cedrene (Clark *et al.*, 1990). The antifungal activity of cadinene has been reported in several studies. δ-cadinene was reported as the main antifungal compound in *Cryptomeria japonica* essential oil, mainly against *Trichophyton rubrum* (Kim *et al.*, 2012).

Also, Matasyoh J.C. and collaborators showed that the essential oil obtained from the leaves of *Hyptis ovalifolia* at a concentration of 31.2µg mL-1 was highly effective against *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*. This activity has been attributed to the presence of sesquiterpenes, mainly γ-cadinene (Matasyoh *et al.*, 2014). It was reported in another study conducted by Daoudi and colleagues that beta-himachalene has antifungal properties against Botrytis chinera (phytopathogenic fungus) (Daoubi *et al.*, 2005).

Regarding the antidermatophytic activity of the oxygenated sesquiterpenes identified in this study, it was reported that Ar-Turmerone is the major compound in turmeric oil (about 70% of the total composition), it was responsible for the antidermatophytic activity against several dermatophytes (*Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum gypseum and Epidermophyton floccosum*) at a concentration between 1.56 and 6.25µg/mL, this in comparison with ketoconazole (Jankasem *et al.*, 2013).

In another study conducted by Chaudhary, A and collaborators, it was cited that atlantone and α-(E)-Atlantone were active against most fungi, (E)-(2S,3S,6S)-atlantone-2,3,6-triol showed an antifungal activity only against *Trichophyton rubrum* with a MIC value *of* 125µg/mL (Chaudhary *et al.*, 2012). The antifungal activity of himachalol has been reported mainly against *Aspergillus fumigatus*, the causative agent of invasive aspergillosis with a MIC value of 46.4µg/mL (Zafar *et al.*, 2000).

Regarding their carbon skeletons, sesquiterpenoids with antibacterial and antifungal activity mainly include bisabolane, guaiane, eudesmane, eremophilane, carotane, lindenane, germacrane, cadinane, farnsane, chamigrane, pseudoguaiane, drimane, aromadendrane, cuparane, daucane, illudalane, oplopanane, picrotoxane, rhodolaurane and other types (Delmondes *et al.*, 2020).

The main mechanisms of antifungal effects of sesquirerpenes involve transport of interfering substances, yeast-hypha transition, host immunity, redox and others (Houst *et al.*, 2020). The hydrophobicity of some sesquiterpenoids disturbs the cytoplasmic membrane or compounds in it, such as some classes of proteins, increasing the ionic permeability and causing cytoplasmic extravasation and as consequence, cellular lysis, as well as interfering with the activity of the respiratory current and energy production (Hang-Ying Li *et al*, 2022)

Lipophilic sesquiterpenoids can destroy the membrane and cause ion leakage in the membrane. The results showed that the action mode of β-caryophyllene is to damage the cell membrane and produce non-selective pores, causing the leakage of substances in the cells and finally causing cell death (Moo *et al.*, 2020).

While our study contributes valuable insights into the effectiveness of the *in vitro* antifungal activity of cedar on the three most common strains of dermatophytes in Morocco, it is important to acknowledge certain limitations that may affect the interpretation of our findings such as the lack of *in vivo* experiments and clinical trials.

CONCLUSION

In conclusion, This study revealed that cedar tar presents a robust antifungal effect *in vitro* against the three most common strains of dermatophytes including *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis* with MICs100µg/mL, 2µg/mL et 0.1µg/mL respectively, highlighting its potential as a natural and effective therapeutic agent.

The antidermatophytic activity of Cedar tar is attributed to the predominance of oxygenated and hydrocarbon sesquiterpenes mainly γ-Cadinene, Ar-Turmerone and β-himachalene which, according to several authors, have shown strong antidermatophytic activity against *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis* and *Microsporum gypsum*.

This means that Cedar tar of the Atlas can be used as an effective antifungal agent in the treatment of superficial mycoses. Understanding the potential of cedar tar as an antifungal agent is not only relevant to advancing natural product-based therapies, but also to addressing the pressing problem of antifungal drug resistance.

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