

# Repellent properties against four Ascomycota species and *Reticulitermes flavipes* of acetonic extractives from *Cedrus atlantica* sapwood to inner heartwood fractions

Kévin CANDELIER<sup>1,2</sup>  
Christian JAY-ALLEMAND<sup>3</sup>  
Rebecca DIJOUX<sup>4</sup>  
Romain DUCRUET<sup>4</sup>  
Emma KIENY<sup>4</sup>  
Dorian AZNAR<sup>3,5</sup>  
Caroline CAYZAC<sup>5</sup>  
Luc P. R. BIDEL<sup>6</sup>

<sup>1</sup> CIRAD  
Research Unit BioWooEB  
34000 Montpellier  
France

<sup>2</sup> BioWooEB, Univ. Montpellier,  
CIRAD  
Montpellier  
France

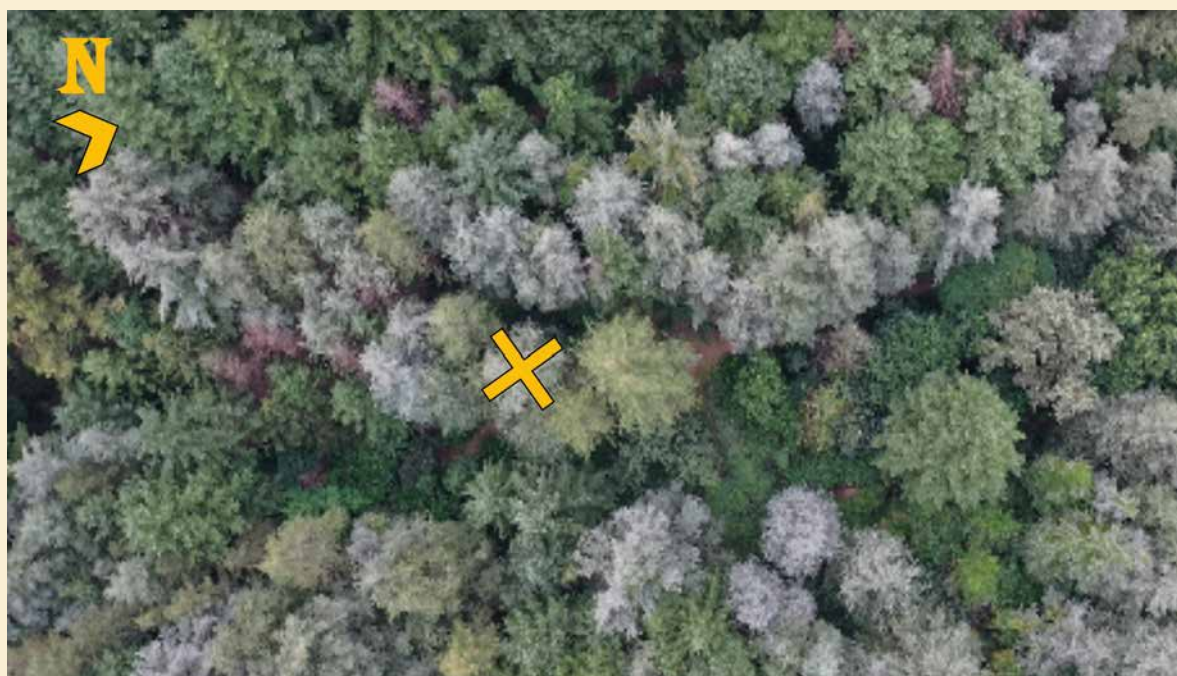
<sup>3</sup> UMR IATE, University of  
Montpellier, Institut Agro, INRAE  
France

<sup>4</sup> Master in Wood Sciences,  
University of Montpellier  
France

<sup>5</sup> Antofenol  
Place Eugène Bataillon  
34090 Montpellier  
France

<sup>6</sup> UMR AGAP, University of  
Montpellier, CIRAD, INRAE,  
Institut Agro  
France

**Auteur correspondant /  
Corresponding author:**  
Kévin CANDELIER –  
[kevin.candelier@cirad.fr](mailto:kevin.candelier@cirad.fr)



**Photo 1.**  
Location of the studied *Cedrus atlantica* tree, harvested in the forest of Avèze (Gard, Mediterranean Basin, France). Aerial optical photography.  
Photo © Master Sciences du Bois de l'Université de Montpellier.

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## RÉSUMÉ

**Propriétés répulsives contre quatre Ascomycètes et *Reticulitermes flavipes* des extractibles acétoniques de *Cedrus atlantica* extraits des fractions de l'aubier au duramen.**

*Cedrus atlantica* est une essence ligneuse présente dans le sud de la France dont la conservation, dans le contexte du changement climatique, est potentiellement à privilégier dans les années à venir. Cette essence présente également un intérêt écologique et socio-économique majeur dans la région du bassin méditerranéen, où elle est principalement reconnue pour son bois durable, mais aussi pour les propriétés chimiques potentiellement intéressantes de son huile essentielle. L'intérêt de recherches sur la formation du duramen et les propriétés de ses composés extractibles est donc certain, bien que très peu d'études aient été menées jusqu'à présent. La distribution radiale des extractibles aqueux et acétoniques de cette essence (écorce, aubier, bois de transition, bois de cœur externe et bois de cœur interne) sélectionnée pour cette étude a été analysée à différentes hauteurs de l'arbre. Des analyses par chromatographie liquide à haute performance (HPLC) ont été réalisées, en particulier pour caractériser les composés flavonoïdes dans ces fractions extractibles. La variation radiale de la composition d'extractibles obtenue met en évidence le phénomène de formation du bois de cœur. Des hypothèses ont été émises quant aux voies métaboliques impliquées dans le processus de formation du duramen du cèdre, en se basant notamment sur l'occurrence et l'évolution radiale de la (-)-catéchine, de la (+)-taxifoline et des composés flavoniques. Les activités fongicides et répulsives contre les termites des extraits ont ensuite été testées. Les extraits eau/acétone de l'individu de *Cedrus atlantica* étudié ont montré une forte activité répulsive contre les termites et une activité antifongique modérée contre les pathogènes des cultures et des fruits. Bien que l'échantillonnage pour cette étude était limité à un seul arbre, les résultats indiquent un potentiel de valorisation des extraits du cèdre de l'Atlas comme moyen de protection du bois et/ou comme produits de biocontrôle contre les pathogènes des cultures agricoles ligneuses. D'autres tests et analyses chimiques seront effectués pour confirmer ces résultats préliminaires intéressants.

**Mots-clés :** antifongique, antitermites, extractibles acétoniques, *Cedrus atlantica*, flavonoïdes, duramenisation, HPLC, propriétés répulsives.

## ABSTRACT

**Repellent properties against four Ascomycota species and *Reticulitermes flavipes* of acetonic extractives from *Cedrus atlantica* sapwood to inner heartwood fractions**

*Cedrus atlantica* is a tree species present in the south of France which, in the context of climate change, could become a conservation priority in the coming years. This tree species is also of considerable ecological and socio-economic interest in the Mediterranean Basin area, where it is mainly recognized for its durable timber, and also for the potentially interesting chemical properties of its essential oil. Studies of its heartwood formation process and the properties of its extractive compounds are therefore of interest, although very few have been conducted so far. The radial distribution of water/acetone extractives within the Atlas cedar tree (bark, sapwood, transition wood, outer heartwood and inner heartwood) selected for this study was screened at different tree height levels. High-performance liquid chromatography (HPLC) analyses were performed, especially to characterize flavonoid compounds in these extractive fractions. The radial variation of the extractive composition obtained highlights the phenomenon of heartwood formation. Assumptions were put forward as to the metabolic pathways involved in the heartwood formation process of cedar wood, based in particular on the occurrence and radial evolution of (-)-catechin, (+)-taxifolin and flavan compounds. The fungicidal and termite-repellent activities of the extracts were then tested. The water/acetone extractives from the *Cedrus atlantica* individual studied showed strong termite repellent activity and moderate antifungal activity against crop and fruit pathogens. Even though the sampling in this study was limited to a single tree, the results point to a potential for commercial use of Atlas cedar extractives as wood preservatives and/or as biocontrol products against pathogens of ligneous agricultural crops. Additional tests and chemical analyses will be carried out to confirm these interesting preliminary results.

**Keywords:** antifungal, termite-repellent, acetonic extractives, *Cedrus atlantica*, flavonoids, heartwood formation process, HPLC, repellent properties.

## RESUMEN

**Propiedades repelentes para cuatro especies de Ascomycota y *Reticulitermes flavipes* de los compuestos acetónicos extraídos desde la albura hasta el duramen interno de *Cedrus atlantica***

El *Cedrus atlantica* es una especie arbórea presente en el sur de Francia cuya conservación, en el contexto del cambio climático, podría convertirse en una prioridad en los próximos años. Esta especie arbórea también reviste un gran interés ecológico y socioeconómico en la cuenca mediterránea, donde se la reconoce principalmente por la madera duradera y también por las propiedades químicas potencialmente útiles de su aceite esencial. Por ello, resultan interesantes los estudios sobre la formación de su duramen y las propiedades de sus extractos, aunque hasta ahora se han realizado muy pocos.

La distribución radial de los extractos agua-acetona del cedro del Atlas (corteza, albura, madera de transición, duramen externo y duramen interno) seleccionado para este estudio se examinó en diferentes niveles de altura del árbol. Se realizaron análisis de cromatografía líquida de alta resolución (HPLC), especialmente para caracterizar los compuestos flavonoides de estas fracciones extractivas. La variación radial de la composición extractiva obtenida demuestra el interés del fenómeno de formación de duramen. Se propusieron hipótesis como las vías metabólicas implicadas en el proceso de formación del duramen de la madera de cedro, basadas en particular en la aparición y evolución radial de (-)-catequina, (+)-taxifolina y compuestos flavanos. A continuación, se probaron las actividades fungicidas y repelentes a las termitas de los extractos. Los extractos agua-acetona del individuo de *Cedrus atlantica* estudiado mostraron una fuerte actividad repelente a las termitas y una moderada actividad antifúngica contra patógenos de cultivos y frutas. Aunque el muestreo en este estudio se limitó a un solo árbol, los resultados apuntan a un potencial uso comercial de los extractos del cedro del Atlas como conservantes de la madera y/o como productos de biocontrol contra patógenos de cultivos agrícolas leñosos. Se realizarán pruebas y análisis químicos adicionales para confirmar estos interesantes resultados preliminares.

**Palabras clave:** antifúngico, repelente a las termitas, extractos acetónicos, *Cedrus atlantica*, flavonoides, proceso de formación del duramen, HPLC, propiedades repelentes.

## Introduction

*Cedrus atlantica* Manetti (Pinaceae) is endemic to the western Mediterranean areas (Mhirit and Blerot, 1999). Atlas cedar (*Cedrus atlantica*) was introduced in French forests (in the south area) in the mid-19th century. Cedar is one of the most successful cases of tree species acclimation around the French Mediterranean Rim (Courbet *et al.*, 2012). In fact, many tree populations still survive and regenerate. Even though these Cedar plantations rarely had any thinning or pruning, they showed good climatic adaptation, reflected by a reasonable growth rate in many European regions. These are mainly located around the Mediterranean Basin, such as Southern France, Italy, Bulgaria and Hungary (Brunetti *et al.*, 2001; Rabhi *et al.*, 2018).

Atlas cedar is of great socio-economic and ecological relevance, as it is mainly known for its natural resistance against biological pathogens, thus providing sustainable timbers (Mhirit and Blerot, 1999). Its wood exhibits great decay and termite resistance and good physical and mechanical properties (El Azzouzi and Keller, 1998; Fidah *et al.*, 2016; Gérard *et al.*, 2017) and is commonly used for furniture, millwork and veneer. Its odour and volatile compounds make it particularly popular for clothing storage units due to its repellent activity toward insects, such as *Tinea pellionella* (Medha *et al.*, 2021). Primary processing of these woods can generate by-products estimated at 30%. Generally, these residues are valorised by the trituration industry (paper pulp, particle panel). However, most of the residues are traditionally used as a fuel, agents for cleaning of the industrial building, as litter for the poultry or the cattle (Hammani and Mourad, 2019). A step forward, the maximization of the valorisation potential of Atlas cedar residues relies on the production of valuable amounts of extractive compounds (Aberchane *et al.*, 2004; Bourkhiss *et al.*, 2015).

The Atlas cedar is also used to produce cedar oil, which is currently sold for medicinal as well as cosmetics and fragrances purposes. The Cedar essential oils were highly investigated and a past study highlighted their antioxidant, antifungal, antiparasitic, anti-inflammatory, and antiseptic activities (Jaouadi *et al.*, 2021). However, there is still a lack of knowledge on the chemical composition of these extractives (which are often formed during heartwood formation). The conversion of sapwood into heartwood is associated with chemical and physical modifications affecting the quality of the wood and often conditioning its possibilities of use (Rabhi *et al.*, 2014). This secondary changes in wood (Hillis, 1987) is associated to the natural aging of cells, the accumulation of air in vascular tissue, the devitalization of the parenchyma by micro-organisms or the mortality due to the accumulation of polyphenols reaching a toxicity threshold (Polge, 1982). Taylor *et al.* (2002) estimated that the heartwood formation is accompanied by a change in metabolic activity, the formation of extractives and the death of parenchyma cells, resulting in the transformation of sapwood into heartwood. Extractives are composed by low molecular weight compounds located in the porous structure of the xylem. Most of them are synthesized

during the heartwood formation process (duraminization), that transforms non-structural carbohydrates (starch, sucrose, glucose and fructose) accumulated in wood into various chemical structures of phenolics (phenolic acids, flavonoids, naphthoquinones, etc.) (Label *et al.*, 2000; Beritognolo *et al.*, 2000; Mbakidi-Ngouaby, 2017). These molecules are then responsible for several wood properties such as odour, colour, acoustic properties and natural durability (Burtin *et al.*, 1998, 2000; Brémaud, 2006; Niamké *et al.*, 2012; Candelier *et al.*, 2020).

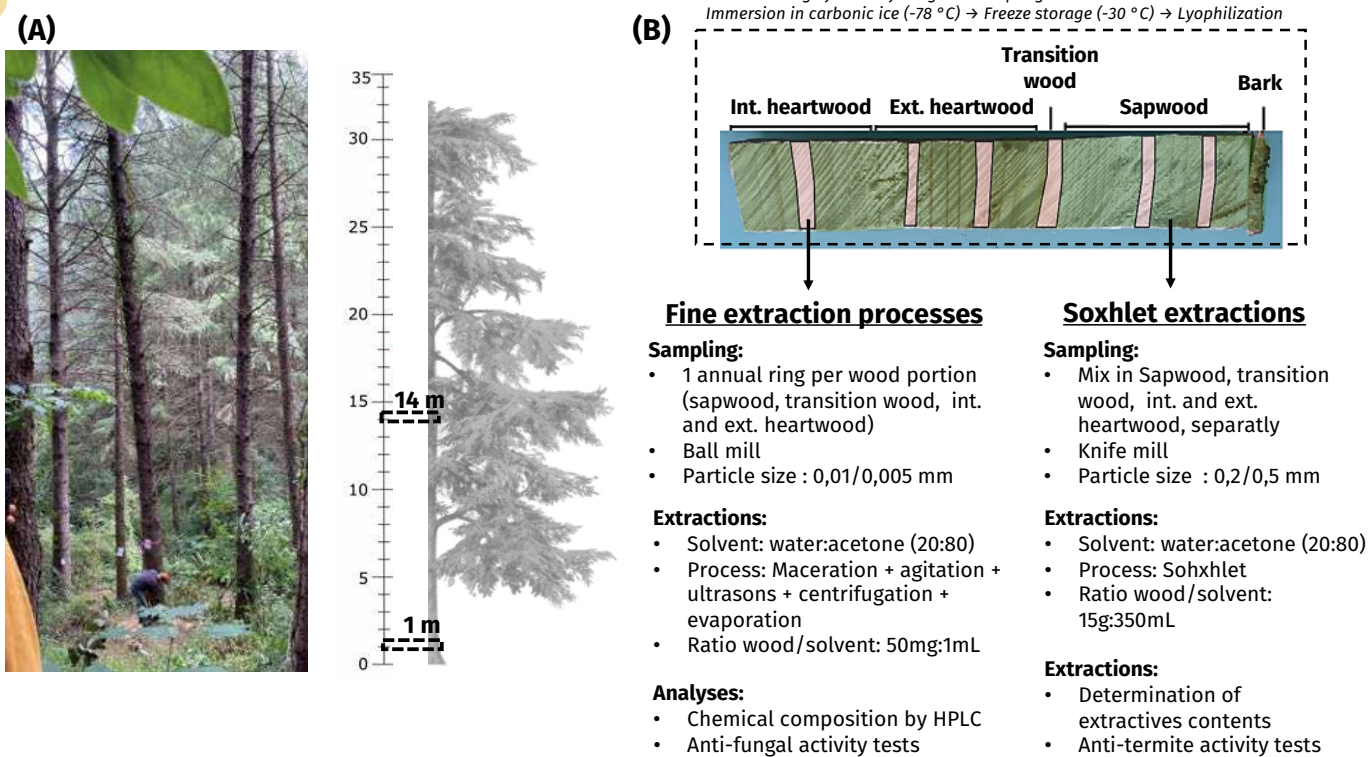
To better understand the heartwood formation process in Atlas cedar, content and chemical composition of wood extractives were determined across the diameter of the tree trunk. Assessing variation of the extractive chemical composition between stem tissues (bark, sapwood, transition, outer and inner heartwood) should offers significant insights into the heartwood formation process of Atlas cedar tree, as well as the variation of its durability properties. In fact, several chemical and enzymatic reactions occur during the heartwood formation process, changing or generating some new extractive compounds from sapwood to heartwood, affecting in turn the final wood properties, such as natural durability. Today, the heartwood formation process is still unknown for most of the timber species. However, the study of this biochemical process is of growing interest in order to better understand the source and the evolution of wood properties. In this study, the chemical composition of Atlas cedar wood extractives for potential further applications, such as wood preservatives or antifungal compounds, was investigated. For this purposes, water/acetone extractive contents, high performance liquid chromatography (HPLC) characterization, antifungal and anti-termite activities of the extractives, from bark, sapwood, transition wood, outer and inner heartwood fractions were evaluated against four Ascomycota species, *Botrytis cinerea* Pers., *Mycosphaerella graminicola* J. Schröt., *Penicillium digitatum* (Pers.) Sacc., *Venturia inaequalis* (Cooke) G. Winter, and one termite species, *Reticulitermes flavipes*.

## Material & Methods

### Wood sampling and preconditioning

As part of a student's project of the Master in Wood Sciences of Montpellier, France (Master in Wood Sciences, 2022), a comprehensive characterization (carbon balance, physical and mechanical properties, natural durability, energy properties, etc.) was carried out on one Atlas cedar (*Cedrus atlantica* M.) tree. However, the present study presents only the work carried out on the composition of the extractive fractions and their respective biological activities.

As specified in figure 1A, and due to the limited access to the resource, only one Atlas cedar (*Cedrus atlantica* M.) was selected. This tree was harvested in a forest plot in the commune of Avèze, in the Gard department, Medi-



**Figure 1.** (A) Description of the wood sampling scheme within the *Cedrus atlantica* tree and (B) selection of the wood samples for the respective analyses and activity tests. Pink bands correspond to wood samples used for fine extraction processes, LC-MS analyses and antifungal bioassays. Green bands correspond to wood samples used for Soxhlet extraction processes, extractive contents determination and anti-termite activity tests.

terreanean Basin, France (latitude: 43° 58' 20" N, longitude: 03° 36' 03" E, altitude: 250 m), in September 2021. The stand is composed by the following tree species: Atlas cedar (*Cedrus atlantica* sp.), Douglas fir (*Pseudotsuga menziesii*) and Corsican pine (*Pinus nigra* subsp. *laricio* var. *corsicana*). The global stand density is around 500 trees/ha, with an Atlas cedar tree density of 350 trees/ha. The 53 years old harvested Atlas cedar tree measured 32 meters high and its diameter at 1.30 m from the ground level (DBH) was 48,4 cm.

Given that only one tree was sampled, precautions were taken to select an individual representative of the stand (age, size and diameter, sanitary status, closed forest environment) and comparable to those previously studied (Fidah *et al.*, 2016; Salhi *et al.*, 2020). However, the findings of this study must be taken with extreme precautions, as they do not reflect the intra-specific diversity of Atlas cedar wood. Supplementary analyses on different trees are required to substantiate these following preliminary results.

### Extraction processes

#### Fine extraction process

Two wooden disks were collected at 1 m and 14 m above the ground level (figure 1A) along the trunk. The first one was used for a comprehensive study on the chemical composition and biocidal activities of each extractive fraction. The second one was only used to assess differences

between the stem base and higher up the stem (axial variation).

Annual rings present within sapwood, transition wood, inner and outer heartwood fractions (figure 1B) were firstly manually fragmented into chips and then grounded, separately, using a ball mill (Retsch MM 200, Retsch GmbH, Haan, Germany), during 2 min at 30 ball turns/second, to obtain about 500 mg of powder samples (from each wood part) with a particle size ranged from 0,005 to 0.01 mm. After a desiccator-conditioning step, 50 mg of each powder sample (3 replicates) was then placed into an Eppendorf tube (capacity of 2 mL). One milliliter of cold extractive solvent (water/acetone, 20/80, v/v at 3 °C) was added to each powder sample, which was then released in maceration for 12 h on a medium level agitation table VWR. The solutions were subsequently submitted to an ultrasonic bath (Branson 5510, Branson Ultrasonics Corp, Danbury, USA) for 20 min, before being centrifuged for 15 min at 3,290 g, after which the solvent was evaporated with an Eppendorf Concentrator plus (2 h at 45 °C). The whole process (including solution, maceration, agitation, centrifugation and evaporation steps) was repeated a second time on the same sample to optimize the amount of soluble compounds extracted.

#### Soxhlet extraction process

The experimental protocols employed for the determination of extractive contents were slightly adapted from the

procedures described by Rowell *et al.* (2013).

Bark, sapwood, outer and inner heartwood fractions of Atlas cedar tree were extracted from the wood disc collected at 1 m from ground level (figure 1A). Then, all samples were crushed and sieved in order to get particle sizes ranging from 0,2 to 0,5 mm. After a drying step at  $103 \pm 2$  °C up to mass stabilization ( $m_0$ ), the sawdusts (15 g, in dry basis) were separately extracted in a Soxhlet with a acetone:water (80/20, v/v) solution for 6 h following by 12 h of maceration, then dried at  $103 \pm 2$  °C for 48 h to obtain the anhydrous mass ( $m_1$ ). Soxhlet extraction processes were triplicated. The extractive content of each wood sample was determined by the following Equation (1):

$$\text{Ext}_{ac}(\%DW) = [(m_0 - m_1) / m_0] \times 100 \quad (1)$$

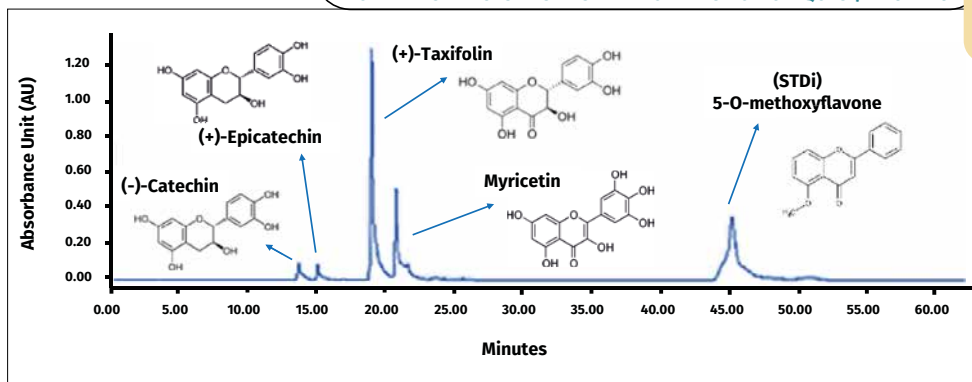
#### Chemical composition by HPLC analyses

Before being analyzed by HPLC, solid extractive fractions were diluted in 500  $\mu$ L of methanol:water (80/20, v/v) solution and then submitted to an ultrasonic bath system for 20 min. This extractive solution was divided into two parts: 150  $\mu$ L was devoted to HPLC analyses and 350  $\mu$ L was devoted to antifungal activity tests.

The method used for the HPLC analyses was based on the one developed by Niamek *et al.* (2011). They were performed with a Kontron apparatus (Entraigues, France) using an C18 column ODB 5  $\mu$ m RP-18, 250 $\times$ 4.6 mm. All analyses were conducted with a flow rate of 0,8 mL/min, at a pressure of 257 bars and at temperature of 30 °C.

The mobile phase was composed by water/formic acid (99/1, v/v) and acetonitrile solvent. The initial concentration of the mobile phase was 95:5 water-formic acid:acetonitrile till to reach 100 % acetonitrile after 60 min. The chemical analyses were focused on the flavonoid compounds, for their antifungal and anti-termites' activities (Orhan *et al.*, 2010; Ohmura *et al.*, 2000). Indeed, the flavonoid substances exhibit toxic and repellent properties against termites through feeding deterrent mechanisms (Ohmura *et al.*, 2000; Verma *et al.*, 2009), and they are also effective anti-fungal agents against a wide range of pathogenic organisms (Aboody and Mickymaray, 2020).

The HPLC detection was fixed at 290 nm (Dulac, 2011). Each compound was characterized by its retention time (min), peak area ( $\mu$ V $\cdot$ sec.), peak intensity ( $\mu$ V) and the wavelength of maximum absorbance of the molecule (nm). The (-)-catechin, (+)-epicatechin, (+)-taxifolin and myricetin were identified by comparison with authentic compounds analyzed separately by HPLC (figure 2) giving their retention times and absorbance spectra from 220 to 620 nm. 5-O-methoxyflavone was also used as internal standard (STDi). Each



**Figure 2.**

HPLC chromatogram of the control solution used for identification of the major flavonoid compounds present in *Cedrus atlantica* wood samples. The absorbance (AU) was recorded at 290 nm, and the concentration of each components was 10<sup>-3</sup> mol/L.

component concentration in this reference solution was 10<sup>-3</sup> mol/L. The chemical analyses were focused on diverse flavonoids, known for their antifungal and anti-termite activities (Orhan *et al.*, 2010; Ohmura *et al.*, 2000).

#### Antifungal activity tests

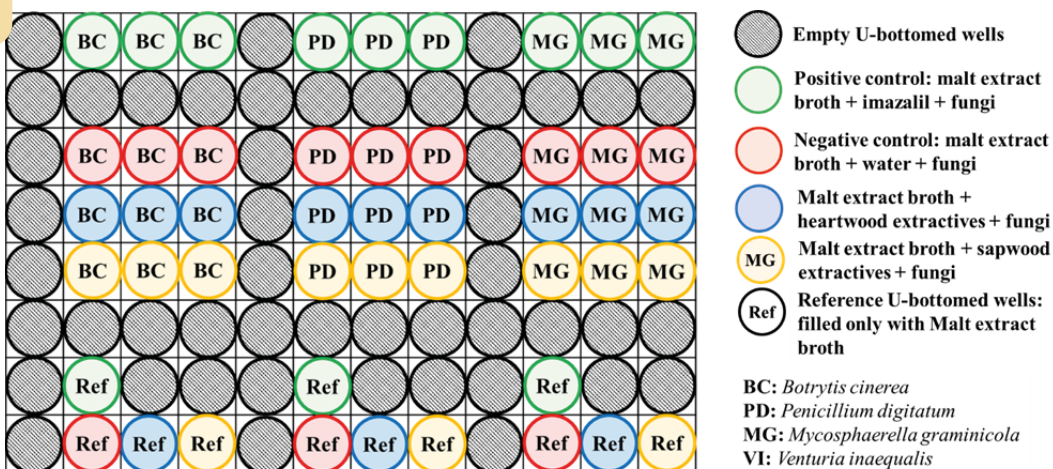
The antifungal activities against the following pathogens were tested by Antofenol Company<sup>1</sup>:

- *Botrytis cinerea* Pers. (fungus that can infect and destroy ornamental plants, as well as a wide range of fruits and vegetables, such as the grapevines, tomatoes and strawberries);
- *Mycosphaerella graminicola* J. Schröt. (fungus causing septoria leaf blotch of wheat);
- *Penicillium digitatum* (Pers.) Sacc. (fungus that can infect and destroy post harvesting citrus fruits);
- *Venturia inaequalis* (Cooke) G. Winter (fungus that can infect and destroy apples in the field).

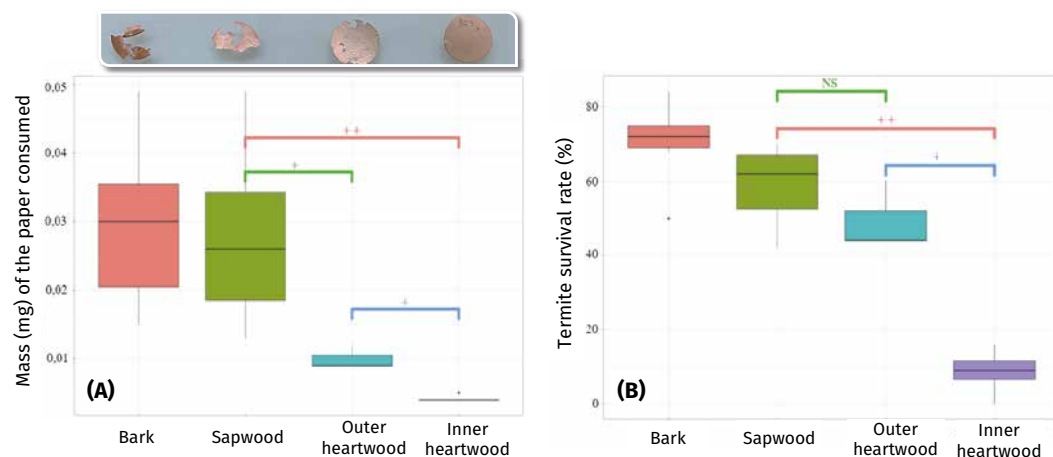
Firstly, the 350  $\mu$ L of extractive solution were evaporated to obtain dried extractive fraction (Eppendorf Concentrator plus, 45 °C, 4 h). Due to the low amount of each extractive fraction, all extractive samples from each type of wood, sapwood (11 mg) or heartwood (12 mg), were added together, and then diluted in an ethanol/water (8/92, v/v) solution to reach a concentration of 2% (in dry weight).

The antifungal bio-assays were carried out using a broth micro-dilution (Hadacek and Greger, 2000) with sterile, disposable microtitre plates with 96 U-bottomed wells (Elisa type from Corning Inc., Corning, NY, USA) (figure 3). Extractive solutions were blended with 40 g/L of the malt extract broth. Stock solutions were used at varying concentrations. The commercial biocide Imazalil (PESTANAL<sup>®</sup>, analytical standard, Merck, Germany) was applied as the positive control, and sterilised milliQ water as the negative control. The plates were incubated in a dark room at 21 °C and 70% humidity. In addition, U-bottomed wells filled with culture medium only (malt extract broth) were used as a reference for the optical density (OD) measurement performed at 800 nm, in order to determine the fungi development after 4 days. The higher the optical density, the more the fungus has grown with an optical density equalling to zero indicating a complete inhibition of the fungus growth.

<sup>1</sup> «Microbiological Testing and Homologation» Branch, University of Montpellier, Montpellier, France. More information can be found on the following website: <http://www.antofenol.com/fr>

**Figure 3.**

Scheme of one part of the antifungal bioassays carried out on acetonic extractives from *Cedrus atlantica* sapwood and heartwood samples.

**Figure 4.**

Effectiveness of acetonic extracts from bark, sapwood, outer heartwood and inner heartwood of *Cedrus atlantica*, against *Reticulitermes flavipes*. (A) Mass (mg) of paper consumed by the termites and (B) termite survival rate, associated to the visual aspect of the samples after 21 days of termite's exposure. ++ and + denote significant difference,  $p < 0.05$  and  $p < 0.01$  respectively. Non-significant differences (NS) are also highlighted. To be noted that these results were the mean values of three replicates for each extractives modalities, coming from a single *Cedrus atlantica* tree.

### Anti-termite activity tests

The anti-termite activities of each Atlas cedar wood extractive fraction (bark, sapwood and both heartwood parts), obtained through Soxhlet process, were assessed by rapid screening tests.

Anhydrous cellulose paper (Whatman™, CAT n° 1001-325 – Grade 1, United Kingdom) measuring 2,5 cm in diameter was weighed ( $m_2$ ) and then impregnated with 70  $\mu$ L of the diluted extracts in acetone ( $C = 2\%$  and  $4\%$ , m/m), air

dried ( $20 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$  and  $65\% \pm 5\%$ ) for 2 hours ( $m_3$ ), then placed in the centre of a Petri dish (5,5 cm diameter).

Fifteen grams of wet sand (4 vol. sand/1 vol. water) was disposed evenly around the paper and 50 termite workers (*Reticulitermes flavipes*, ex. *santonensis*) were incorporated to each test device. Three replicates were performed for each diluted wood extract. A comparable protocol is detailed and illustrated in a previous work (Candelier *et al.*, 2020).

Three papers impregnated with acetone only were subjected to the same tests in order to check the impact of the solvent on termite activity. Tests with acetone only were also considered as a termite virulence control. For each test facility, the paper samples were placed on a plastic grid avoiding direct contact with sand humidity. Lastly, three diet control set-ups containing only 15 g of wet sand and 50 termites were used to check termite survival without any feeding possibilities/without any trophic sources. All test set-ups were kept at  $27 \text{ }^\circ\text{C}$  and  $> 75\%$  RH.

Every two days, each test set-up was observed to check sand humidity and to keep track of termite behaviour and activity. Water was added when needed. When all termites contained in the diet control set-ups had died, the test was stopped (maximum duration of 21 days). The termite survival rate was then determined, the anhydrous mass of the cellulose papers was measured ( $m_3$ ) and the Weight Losses ( $WL_{\text{term}}\%$ ) due to termite degradation were calculated by the following Equation (2):

$$WL_{\text{term}}(\% \text{ DW}) = [(m_2 - m_3) - m_2] \times 100 \quad (2)$$

### Statistical analyses

The statistical tests performed to assess the anti-termite and anti-fungal activity of the various tested cedar extractives were carried out with RStudio® software [version 2021.09.2+382, RStudio Inc.]. These statistical tests are all non-parametric tests, consisting in a comparison of median values (Wilcoxon test). The results of the tests are specified in figure 4 for the anti-termite activities and in figure 5 for the antifungal activities.

## Results and Discussion

### Extractive contents

Table I shows the extractive contents of the studied Atlas cedar wood, by acetone/water soxhlet extractions, from bark, sapwood, outer and inner heartwood. The highest extractive rate was recorded in the bark fraction (24%), followed by heartwood (2.8% for inner and 2.7% for outer) and sapwood (2.2%) samples. In agreement with previous studies, acetone and water extractive contents of the bark fraction were significantly different from those of trunk and knot wood sections (Kebbi-Benkeder *et al.*, 2015; Heim *et al.*, 2022). In addition, previous studies also highlighted that the bark fraction contains higher levels of extractives than both sapwood and heartwood (Rowe and Conner, 1979). The extractive contents gradually increased from pith towards the heartwood-sapwood border, with no saturation at mature age (Gierlinger and Wimmer, 2004). These findings from the studied cedar tree confirm the lower extractive contents found in sapwood as compared to outer and inner heartwood.

### Anti-termite activity

The control papers, impregnated with acetone, were completely eaten (0.045 g) by termites during the termite exposition tests. Moreover, the mean value of termite's survival rates was  $90.2 \pm 3.7\%$ , meaning that acetone had no impact against termite attack.

The masses of the paper samples consumed by termites are not significantly different for bark and sapwood extractive impregnations. However, the degradations caused by termites are significantly lower for the paper samples impregnated with extractives from outer and inner heartwood, with samples impregnated with inner heartwood extractives being only marginally degraded (figure 4A). The pictures in figure 4 illustrate the striking differences observed between extractive fractions after 21 days of termite's exposure.

Termite survival rate revealed the impact on termite biology. Even though the termite survival rate was not significantly different for bark, sapwood and outer heartwood extractives, it was significantly lower for the inner heartwood extractives samples (figure 4B).

These results suggest that acetone extracts of the studied Atlas cedar heartwood contain molecules with anti-termite activity. In addition, the observations carried

**Table I.**

Mean values (and standard deviation) of extractives contents of *Cedrus atlantica* wood, at 1 m above ground level, and according to the wood position within the tree, obtained by Soxhlet extractions.

<i>Cedrus atlantica</i> Wood disc located at 1 m DHB level	Bark	Sapwood	Outer heartwood	Inner heartwood
Acetonic extractive contents (% in dry basis)*	24 ± 2.6	2.2 ± 0.05	2.8 ± 0.9	2.7 ± 1.1
* To be noted that these results were the mean values of three replicates coming from a single <i>Cedrus atlantica</i> tree.				

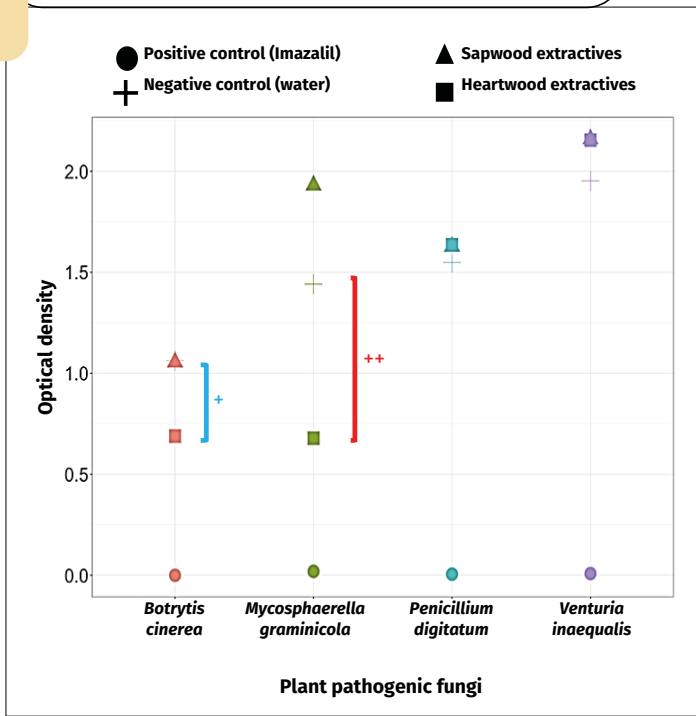
out every two days during the anti-termite activity tests, showed that the termites submitted to the paper samples impregnated with inner heartwood extractives and those from the control diet device without any source of feeding died simultaneously. It can therefore be assumed that the cedar heartwood extractives possess termite repellent properties. Comparable repellent activity and resistance to termite attacks of *Juniperus oxycedrus* heartwood, a species closely related to *Cedrus atlantica*, were also highlighted by Ouair *et al.* (2022).

### Antifungal activity

Figure 5 shows the optical density values recorded at 800 nm wavelength on the antifungal test devices, according to the extractive fractions and the fungal species. The higher the optical density, the more the fungus has grown. An optical density equal to zero indicates a complete inhibition of fungus. Acetone extractives of *Cedrus atlantica* sapwood did not affect significantly the four fungal growths. However, acetone extractives of the studied cedar heartwood inhibited the development of *Mycosphaerella graminicola* fungi significantly.

Different concentrations of extractive formulations should be tested to confirm these preliminary results. Fidah *et al.* (2016) highlighted that the durability class of *Cedrus atlantica* wood was positively correlated with the bioactivity of sawdust essential oil against lignivorous fungi. By performing bioassays, Fidah *et al.* (2016, 2017) highlighted that both *Tetraclinis articulata* and *Cedrus atlantica* woods, ranking from durable to very durable in natural durability classes (Fidah *et al.*, 2015, 2016), possess extractive compounds with strong activity against wood-destroying fungi.

Thus, it could be interesting to carry out supplementary tests of acetone extractives from Atlas cedar heartwood against wood-destroying fungi, used to determine the natural durability of wood in European standard (EN 350, 2016). These further tests on wood-degrading fungi could confirm that the sawdust from *Cedrus atlantica* heartwood may be a valuable source of active molecules for wood preservatives.



**Figure 5.** Effectiveness of acetonics extracts from bark, sapwood, inner heartwood and outer heartwood of *Cedrus atlantica*, against four different fungal crops and fruits pathogens, after 4 days of fungi's exposure. ++ and + denote significant difference,  $p < 0.05$  and  $p < 0.01$  respectively. To be noted that these results were the mean values of three replicates for each extractives modality, coming from a single *Cedrus atlantica* tree.

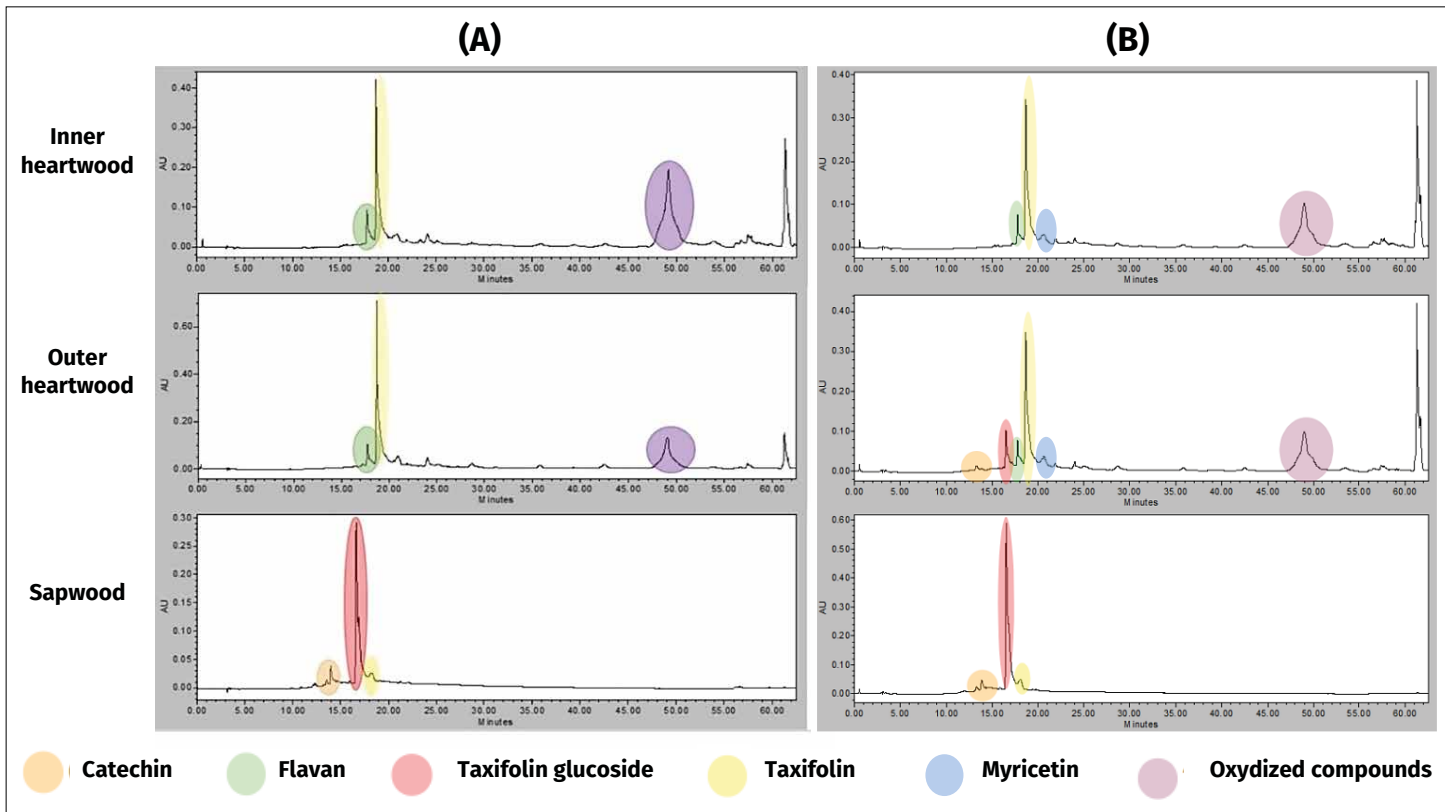
**Chemical composition of acetonics extracts**

The usual chromatograms from HPLC analyses of the major flavonoid compounds of sapwood, outer and inner cedar heartwood extractives by the acetone/water extraction process are illustrated in figure 6.

Preliminary HPLC analyses highlighted the presence of (-)-catechin, taxifolin glucoside (Hergert and Goldschmid 1958) and (+)-taxifolin, in sapwood. (-)-Catechin is not present in outer and inner heartwood. However, (+)-taxifolin strongly increases from sapwood to inner heartwood. In addition, inner and outer heartwood contain flavan-3-ols and other unidentified compounds (probably oxidized or methylated forms of diverse flavonoids), that are not observed in sapwood.

(-)-Catechin and (+)-taxifolin are the main compounds identified by HPLC analyses. These chemical analyses showed a decrease of (-)-catechin from the sapwood to the transition zone. Conversely, the amount of taxifolin increases from the transition zone to the heartwood. Thus, (-)-catechin appears to be transformed by hydroxylation and/or polymerised at the transition zone (sapwood/outer heartwood boundary) while (+)-taxifolin appears to be highly accumulated in the outer heartwood.

The heartwood formation process can explain the variation of (-)-catechin and (+)-taxifolin within the studied *Cedrus atlantica* tree. At the transition zone, the transformation of glycosylated taxifolin into (+)-taxifolin could be hypothesized. According to Mbakidi-Ngouaby (2017),



**Figure 6.** Compilation of the HPLC chromatograms of acetonics extracts from sapwood, outer heartwood and inner heartwood (A) from the wooden discs harvested at 1 m and (B) from the wooden discs harvested at 14 m above the ground level of the *Cedrus atlantica* tree.



(+)-taxifolin derives from carbohydrate monomers contained in the parenchyma wood cells. These monomers are metabolized through aromatic amino acids involving the shikimic pathway and then transformed in phenolic acids and flavonoids by diverse enzymatic activities such as PAL (Phenylalanine Amonia-Lyase), CHS (CHalcone Synthase) and CHI (CHalcone Isomerase). Therefore, (+)-taxifolin can be considered as the main molecule of the metabolic pathway of flavonoids (Thuan *et al.*, 2021) in cedar, which could be transformed in flavan-3-ols involving the activities of DFR (Dihydro-Flavonol 4-Reductase) and LAR (Leuco-Antocyanidin Reductase) enzyme (Beritognolo *et al.*, 2002; Liu *et al.*, 2021).

During the formation of heartwood, occurring in the transition zone (TZ), the amounts of phenolic compounds formed and accumulated in sapwood could be increased due to the activation of the phenolic metabolism, transforming sugars as starch, sucrose, fructose and glucose in flavonoids in the active cells (axial parenchyma and woody rays). However, others chemical and enzymatic processes occur before the death of cell through the three following different reactions (figure 7):

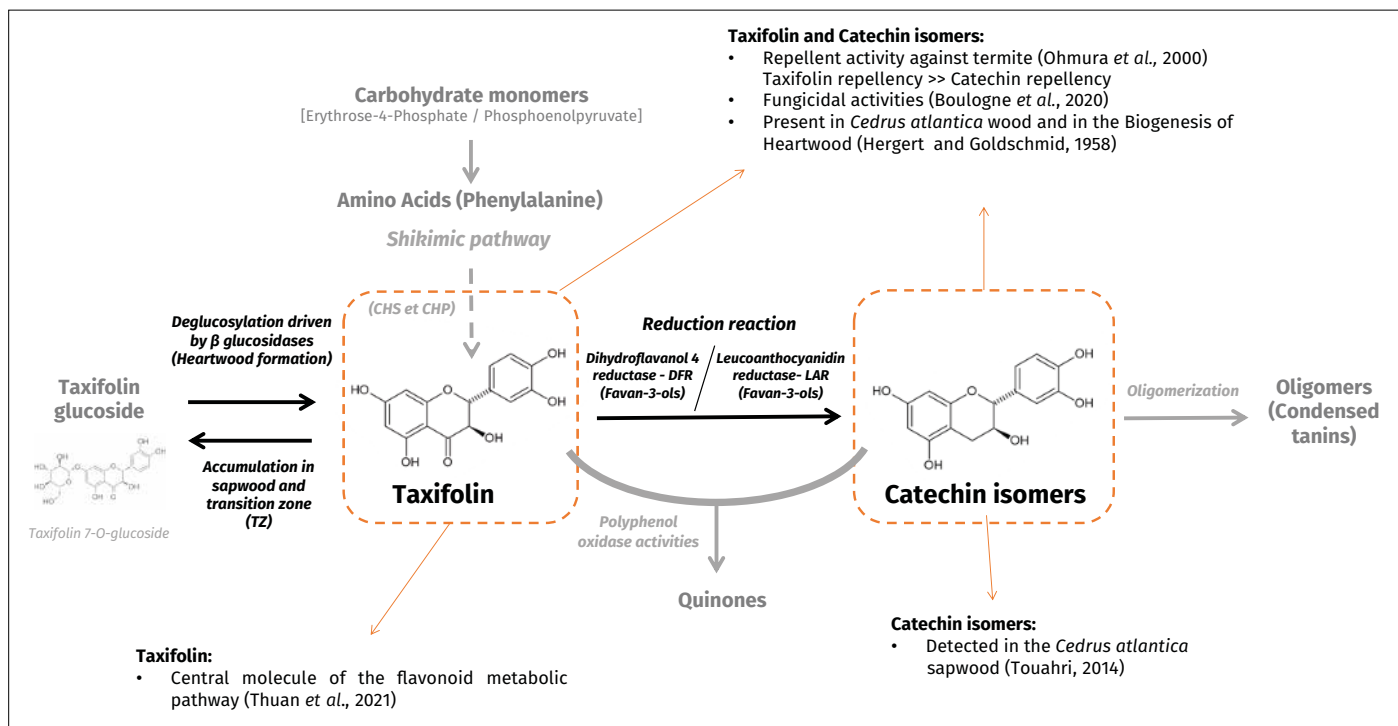
- (i) The deglucosylation of taxifolin glucoside driven by  $\beta$ -glucosidases releasing taxifolin (Auger-Rozenberg *et al.*, 1990, 1994);
- (ii) The oligomerization of catechin isomers and other monomers of flavan-3-ols;
- (iii) The oxidation of phenolic compounds involving polyphenol oxidases or peroxidases giving quinones with apolar properties and polymers.

All these reactions could explain their accumulation in heartwood (figure 7).

Finally, the distribution of (-)-catechin and (+)-taxifolin

in sapwood and heartwood could partly explain the difference in anti-fungal and anti-termite activities. Indeed, both (-)-catechin and (+)-taxifolin have anti-termite repellent properties (Ohmura *et al.*, 2000). Regarding the relationships between structure and activity, it was found that compounds possessing two hydroxyl groups at C-5 and C-7 in A-rings displayed a strong antifeedant activity (figure 7). In addition, these authors highlighted that (+)-taxifolin is more repellent than (-)-catechin. This agrees with the results of the activity tests performed on Soxhlet extraction showing a stronger effect of heartwood extractives (mostly (+)-taxifolin) than sapwood ones (mostly (-)-catechin) against fungi and termite attacks. Although carrying out HPLC analysis on extractives issued from the soxhlet extraction processes would have been more appropriated, it is very likely that the chemical signature of the different extractive fractions obtained from the two processes would be comparable. As both types of extraction processes used the same solvents (acetone:water, 80:20), each recovered extractive fraction should have a similar chemical signature, but possibly with varying amounts for a given molecule. In this sense, the chemical composition analyzed here is a first approach explaining the antifungal and anti-termite activities observed during bioassays using extractives from soxhlet process. Moreover, HPLC and LC-MS analyses of the different extractive's fractions obtained with the Soxhlet process will be considered in order to confirm that their chemical profiles are similar than those from the fine extraction method.

New investigations about the phenolic composition of wood should be developed through Liquid chromatography-mass spectrometry (LC-MS) analyses for identifying new phenolics, the derivatives from (-)-catechin and (+)-taxifolin, including oligomers formed in heartwood during the



**Figure 7.**

Putative reactions and roles of taxifolin and catechin isomers involved in the natural durability of *Cedrus atlantica* heartwood.

heartwood formation process in *Cedrus atlantica*. Then, the major chemical structures identified in heartwood could be tested alone or combined in order to better understand its protective effects.

## Conclusion

High performance liquid chromatography (HPLC) analyses of acetonic extractives from the studied *Cedrus atlantica* (Atlas cedar), sapwood, transition wood and heartwood, highlighted the role of two major flavonoid compounds, the (-)-catechin and the (+)-taxifolin, that could be involved in antifungal activities against four Ascomycota species, *Botrytis cinerea* Pers., *Mycosphaerella graminicola* J. Schröt., *Penicillium digitatum* (Pers.) Sacc., *Venturia inaequalis* (Cooke) G. Winter, or anti-termite activities against *Reticulitermes flavipes*.

The (-)-catechin is present only in sapwood, whereas the (+)-taxifolin is present in trace in sapwood and more substantially from transition wood to inner heartwood. These results allow a preliminary understanding of the heartwood formation process in Atlas cedar, based on (i) the loss of the (-)-catechin from the sapwood to the heartwood explained by oligomerization within the transition zone and (ii) the transformation of glycosylated taxifolin into the (+)-taxifolin in the transition zone. In this sense, the (+)-taxifolin and the (-)-catechin could be considered as central molecules in the flavonoid metabolic pathway, useful to assess the heartwood formation and protective process of Atlas cedar wood.

Acetonic extractives of Atlas cedar heartwood are repellent to termites and show a potential antifungal activity against the four Ascomycota phytopathogens.

These fungicidal and termicidal properties of the different wood parts of the studied Atlas cedar tree seem to be linked with their composition in (-)-catechin and (+)-taxifolin.

Additional tests and Liquid chromatography–mass spectrometry (LC-MS) analyses need to be carried out to confirm the potential valorization of *Cedrus atlantica* extractives in wood preservatives or in pest control towards crops and fruits pathogens. Moreover, HPLC and LC-MS analyses will be also performed on all the extractive fractions obtained with the Soxhlet process in order to confirm that their chemical profiles are similar than those from the fine extraction method. Finally, similar studies should be carried out on other *Cedrus atlantica* trees from various areas covering different climatic condition, and of different age, in order to confirm these interesting preliminary results and to evaluate the impact of growing conditions on heartwood formation process.

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## Data access

The detailed data obtained through this study and presented in this article are available in the “CIRAD Dataverse Portail” with the following reference: Candelier K., JAY-ALLEMAND C. DIJOUX R., DUCRUET R., KIENY E., AZNAR D., CAYZAC C., BIDEL L. P. R., 2023. Replication Data for: Repellent activities against four Ascomycota species and *Reticulitermes flavipes* of acetonic extractives from sapwood to inner heartwood fractions of *Cedrus Atlantica*. CIRAD Dataverse. <https://doi.org/10.18167/DVN1/LYGTS4>

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### Candelier et al. – Author's contributions

Contributor role	Contributor names
Conceptualization	L. Bidet, C. Jay-Allemand, K. Candelier
Data Curation	L. Bidet, C. Jay-Allemand, K. Candelier, R. Dijoux, R. Ducruet, E. Kienny
Formal Analysis	R. Dijoux, R. Ducruet, E. Kienny
Funding Acquisition	L. Bidet, C. Jay-Allemand, K. Candelier, C. Cayzac
Investigation	R. Dijoux, R. Ducruet, E. Kienny
Methodology	L. Bidet, C. Jay-Allemand, K. Candelier
Project Administration	L. Bidet, C. Jay-Allemand, K. Candelier, R. Dijoux, R. Ducruet, E. Kienny
Resources	L. Bidet, C. Jay-Allemand, K. Candelier, D. Aznar, C. Cayzac
Software	R. Dijoux, R. Ducruet, E. Kienny
Supervision	L. Bidet, C. Jay-Allemand, K. Candelier
Validation	L. Bidet, C. Jay-Allemand, K. Candelier
Visualization	R. Dijoux, R. Ducruet, E. Kienny
Writing – Original Draft Preparation	L. Bidet, C. Jay-Allemand, K. Candelier, R. Dijoux, R. Ducruet, E. Kienny
Writing – Review & Editing	L. Bidet, C. Jay-Allemand, K. Candelier

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Cirad - Campus international de Baillarguet,  
34398 Montpellier Cedex 5, France  
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