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Valorization of the Sabinas Forest Waste (*Juniperus phoenicea* and *Juniperus thurifera*) as Source of Biopesticides

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Abstract

Objectives: The potential forestry use of *Juniperus phoenicea* and *Juniperus thurifera* pruning woods is studied by analyzing the composition of the woods and testing the biological activities of the corresponding components with the ultimate target of finding new biopesticides. **Methods:** The air-dried wood from each plant was crushed and subjected to hydrodistillation, and the residue was extracted with a Soxhlet apparatus using different solvents. The corresponding extracts were fractionated, and their composition were studied by gas chromatography-mass spectrometry and nuclear magnetic resonance techniques. The ixodicidal and antifungal activities of the different samples obtained were evaluated. **Results:** The fraction oxygenated of the essential oil from both *J. thurifera* (cedrol >60%) and *J. phoenicea* shows a remarkable bioactivity as antitick with EC_{50} values of 3.4 μg/mg and 10 μg/mg, respectively. Cedrol and methyl hinokiate, present in the hexane extract *J. thurifera*, show a potent antifungal effect *against Aspergillus niger* with EC_{50} values of 45.99 and 52.23 μg/mL, respectively. **Conclusions:** Pruning woods from these species proved to be renewable and easily accessible sources of bioactive natural products such as cedrol, thujopsene, nootkatone, and totarol.

Keywords

Cedrol, sesquiterpenes, diterpenes, essential oils, bioplaguicides, waste valorization, *Juniperus phoenicea*, *Juniperus thurifera*

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Introduction

Nowadays, synthetic pesticides are the main method used in crop protection. However, their excessive use have increased pesticide resistance, environmental contamination, and toxicity and affected human health.[1] This problem has led to restrictions on the use of synthetic pesticides being implemented worldwide.[2] One solution lies in developing the use of pesticides of natural origin, known as biopesticides.[3] These can be essential oils, non-volatile extracts, essential oil hydrolates, or pure natural molecules.[4–7]

However, since forests require maintenance through periodic pruning, a large mass of residues is generated, which constitutes a sustainable natural resource of interest as a supply of biopesticide ingredients.[8] Juniper forests, mainly composed of *Juniperus phoenicea* or *Juniperus thurifera*, can be found throughout the Mediterranean basin such as in Spain, Morocco, Algeria, Turkey, or Greece.[9–11] The largest juniper forest in Europe is located in Spain, with an area of about 30 000 hectares.[12] *J. phoenicea* is a shrub up to 8 meters high while *J. thurifera* is a tree that reaches 20 m in height.[9] These two species have medicinal applications and have been reported to alleviate diseases such as diarrhea, arthritis, diabetes,[13] and to cure ulcers.[14,15] In addition, essential oils (EOs) of *J. phoenicea* and *J. thurifera* have shown antimicrobial, antifungal, and cytotoxic activity,[9,15–17] and EOs from leaves of *J. thurifera* have been reported to be used for pest control. Thus, these oils were reported to be active as insecticide against *Acanthoscelides obtectus*, *Tribolium castaneum*, and *Sitophilus oryzae*. They also presented antifungal activity against *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium*

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solani, *Rhizoctonia solani*, *Verticillium dahlia,*[17] *Penicillium digitatum*, *Penicillium expansum,* and *Aspergillus niger*[18] or anti-tick activity against *Hyalomma aegyptium.*[17]

The chemical composition of the different aerial parts (organs) of *J. phoenicea* and *J. thurifera* has been previously reported. The major components from the EO of the leaves of *J. phoenicea* leaves are the monoterpenes α-pinene and phellandrene.[11,15,19,20] When considering the European and African varieties of *J. thurifera*, a remarkable change in the composition was noticed. Thus, the essential oil from the leaves of the European variety is rich in limonene (>50%),[21,22] whereas the African variety contains more than 40% of sabinene and α-pinene.[19,23]

With regard to the composition of the arcestides, the EO of *J. phoenicea* arcestides is rich in α-pinene with a proportion ranging between 33% and 88%,[15,20,24] whereas the EO of *J. thurifera* arcestides presents a significant disparity, with the European variety being rich in limonene,[25] and the African variety containing mostly mentha-6,8-diene, β-pinene, elemol, and 4-terpineol.[16]

Significant differences are also observed in the wood, with the EO of the wood of *J. thurifera* (European variety) being abundant in sesquiterpenes such as cedrol (>40%), thujopsene, widdrol and α-cedrene (>10% each),[9] while *J. phoenicea* (var africana) is rich in α-pinene (over 50%) and δ−3-carene (14.5%).[26]

In this work, the potential forestry use of *J. phoenicea* and *J. thurifera* pruning waste is studied by analyzing the composition of the woods and testing the biological activities of the corresponding components against some pests, with the ultimate target of finding new biopesticide ingredients.

Results and Discussion

Essential Oils, Hydrolates, and Extracts of Sabinas Wood

Essential Oils. The different parts obtained from the pruning of Sabinas, namely, arcestides (berries), leaves, stems, and branches were separated. The branches–stems of each species are finely crushed, and then the sawdust is subjected to steam distillation to provide the corresponding EOs and hydrolates. The latter are extracted over activated charcoal and then eluted with ethyl acetate (EtOAc) to obtain the hydrolate extracts. The resulting plant biomass residues were subjected to Soxhlet extraction with hexane (H) and EtOAc successively to obtain the corresponding extracts. The yield of the essential oil obtained from *J. phoenicea* was higher than that produced collected from *J. thurifera* (0.52% vs 0.31%), Considering non-volatile extracts, the quantities extracted from *J. thurifera* doubled those obtained from *J. phoenicea* (Table 1). On the other hand, the yields of the hydrolate extracts proved to be very low.

Each essential oil was then fractionated into two parts by fast elution column chromatography, firstly with hexane to obtain a hydrocarbon fraction (HF), and then with diethyl ether to obtain an oxygenated fraction (OF) (Scheme 1).

The chemical composition of EOs and of the resulting HF and OF fractions was analyzed by GC-MS (Tables 1 and 2). The chromatograms show that in EO of *J. phoenicea* wood (**Jp-EO**) (Table 2) sesquiterpenes are the major constituents, representing more than 95% of the total composition of the EO (Table 1), with cedrol (**3**) (29.21%), thujopsene (**2**) (18.46%), and nootkatone (**4**) (6.45%) being predominant. After fractionation, the hydrocarbon fraction (**Jp-EO-HF)** contains four major components representing about 50% of the total weight, whereas the oxygenated fraction **Jp-EO-OF** is rich in **3** (42.67%) and **4** (11.86%).

Table 1. GC-MS Analysis of *J. phoenicea* EO and Fractions.

Compound[a]	Rt	EO	HF	OF
Longifolene	13.26	1.25	5.45	-
α-Cedrene (**1**)	13.43	**2.78**	**11.09**	-
β-Caryophyllene	13.55	1.03	6.25	-
Thujopsene (**2**)	13.82	**18.46**	**48.50**	-
β-Chamigrene	14.84	1.03	2.58	-
Elemol	16.26	3.03	-	5.77
Allocedrol	17.17	1.74		3.78
Cedrol (**3**)	17.46	**29.21**	-	**42.67**
β-Eudesmol	18.40	2.07	-	4.17
α-Bisabolol	18.93	2.98	-	6.13
Nootkatone (**4**)	21.31	**6.45**	-	**11.86**

aComponents with percentages higher than 1%.

The EO of *J. thurifera* (**Jt-EO**) (Table 2) also contains sesquiterpenes as major constituents (up to 98%). Among these, cedranes are by far the most abundant, with cedrol (**3**) and α-cedrene (**1**) representing 47.82% and 12.42% of total extract, respectively. Additionally, significant proportions of thujopsene (**2**) (10.21%) were also found. On the other hand, the **Jt-EO-HF** fraction is rich in **1** and **2,** with percentages exceeding 20% for these compounds. This fraction also contains significant proportions (around 10%) of γ-muurolene (**5**) and β-himachalene (**6**). Finally, the sesquiterpene alcohol cedrol **3** represents more than 62% of the oxygenated fraction (**Jt-EO-OF)**.

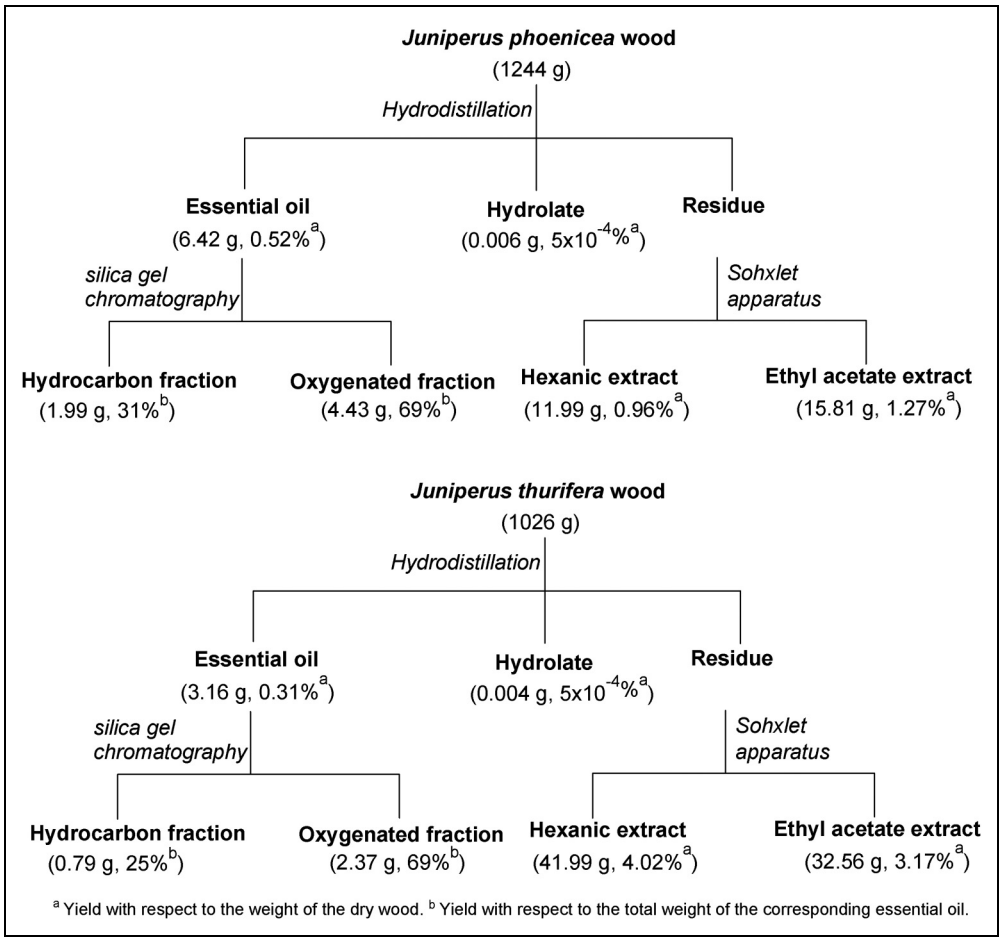
Noteworthy, there are three major compounds for both EOs, namely, cedranes **1** and **3** and thujopsene **2**. In addition, nootkatone **4** is found in the EO of *J. phoenicea* (Figure 1).

Cedrene (**1**) and thujopsene (**2**) present microbial activity.[9] Additionally, thujopsene shows pesticidal activities against the insect *Cullex pipiens pallens,*[27] and its autooxidation products show great activity as antifungal or antitermite.[28]

Cedrol (**3**) is of great interest due to its multiple applications with sedative effect,[29] hair growth,[30] anti-inflammatory,[31] or as a potent antimicrobial against Gram-positive and Gram-negative bacteria.[9] It is also a potent compound for pest control, being active as a tick killer against *Ixodes scapularis*[32] or insecticide against the rice weevil (*Sitophuilus oryzae*).[33]

Finally, nootkatone (**4**) is an insect antifeedant and ixodicidal agent.[6]

Hydrolates. The composition of the hydrolates of both species presents a higher proportion of oxygenated terpene components (Table 3). The sesquiterpenes cedrol **3** and nootkatone



Scheme 1. Fractionation of *J. phoenicea* and *J. thurifera* branches wood. Weights and yield are given in parentheses.

Table 2. GC-MS Analysis of *J. thurifera* EO and Their Fractions.

Compound[a]	Rt	EO	HF	OF
Di-*epi*-α-cedrene	12.81	1.15	3.63	-
α-Cedrene (**1**)	13.43	**12.42**	**26.16**	-
γ-Muurolene (**5**)	13.53	**3.64**	**12.32**	-
Thujopsene (**2**)	13.82	**10.21**	**24.24**	-
β-Himachalene (**6**)	15.29	**2.95**	**9.78**	-
Cuparene	15.39	1.29	4.35	-
Cedranoxide	16.20	3.55	-	6.33
Allocedrol	17.17	1.90	-	3.83
Cedrol (**3**)	17,46	**47.82**	-	**62.97**
3-*Epi*-cedrenal	18.22	2.27	-	4.53

aComponents with percentages higher than 1%.

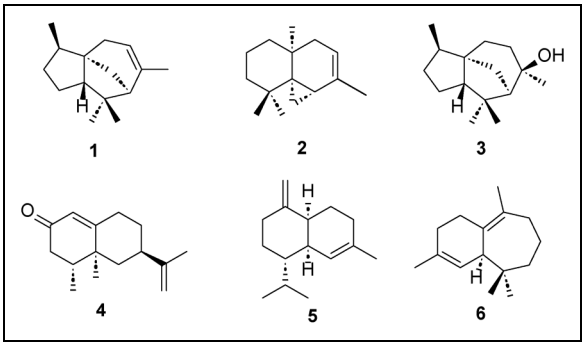


Figure 1. Major constituents identified.

4 (6.50% and 7.52%, respectively) are the more abundant constituents in *J. phoenicea*, whereas cedrol **3** is again the major component of the hydrolate extract of *J. thurifera* (24%). In addition, a significant presence of oleamide is relevant in both species, representing about 15% of the extract.

Extracts. We continued this study by analyzing the volatile components of the hexane and EtOAc extracts of these species by GC-MS. (Table 4). A significant presence of the sesquiterpene cedrol (**3**) was noticed in all the samples analyzed—its yield in the two extracts of *J. thurifera* exceeded 35%. The *J. phonenica* extracts contained a noteworthy proportion of nootkatone, which supposes more than 30% of the total amount in the EtOAc extract (**Jp-EtOAc**). In addition, the hexane extract of *J. phoenicea* (**Jp-Hex**) contains a significant proportion of diterpenes, the most abundant being totarol (**7**) (35%).

Table 3. GC-MS Analysis of Juniperus species Hydrolates Organic Fractions.

Compound[a]	Rt	Jp-HD	Jt-HD
p-Cymen-8-ol	8.25	1.06	2.72
trans-Carveol	9.02	3.12	-
6,6-Dimethylhepta-2,4-diene	9.72	-	1.05
Citral	10.14	1.81	-
Not identified	12.85	7.47	-
1-(4-Bromobutyl)-2-piperidinone	15.11	1.04	-
Cuparene	15.38	1.05	-
Viridiflorol	16.15	1.11	1.17
Eicosane	17.17	1.10	-
Widdrol	17.35	2.97	-
Cedrol (**3**)	17.40	**6.50**	**24.31**
γ-Eudesmol	18.01	-	1.06
3-*epi*-Cedrenal	18.21	-	1.11
Cyclopentanone, 33,4-trimethyl-4-(4-methylphenyl)-	18.26	-	1.38
γ-Himachalene	19.49	1.54	1.57
Widdrenal	19.57	1.45	-
Nootkatone (**4**)	21.30	**7.52**	-
Not identified	21.95	2.71	12.71
Palmitic acid, methyl ester	23.25	1.36	1.06
Oleamide	30.13	**16.54**	**14.62**

aComponents with percentages higher than 1%.

Totarol (**7**) (Figure 2) is a phenolic diterpene which has been reported to be involved in the biosynthesis of podolactones.[34] In addition, totarol presents significant antimicrobial activity against *Bacillus subtilis* or *Staphylococcus aureus,*[35,36] fungicidal[37] and has been reported as nematicidal, like ferruginol (**8**), against *Caenorhabditis elegans.*[38] Moreover, **7** presents a great pharmacological activity such as antitumoral or hyperchloresterolemic.[39]

Considering that, to the best of our knowledge, the composition of the hexane extracts of the branches wood of these species has not been studied, we addressed the phytochemical study of these hexane extracts by separating their main components using column chromatography and confirming the structures of the isolated natural products by nuclear magnetic resonance (NMR). Compound **7** was the major component of the hexane extract of *J. phoenicea* (**Jp-Hex**). Remarkably, this diterpene represents 20% of the total extract. Also worthy to be underlined was the relevant proportion of the acaricide nootkatone (14%). Minor proportions of cedrol (**3**) and *cis*- and *trans*-communic acids (**9a**-**b**) were also detected.[40]

The hexane extract *J. thurifera* (**Jt-Hex**) was column chromatographed on silica gel producing six main fractions. Subsequent esterifications and further separations of these fractions led to the isolation of seven compounds (Figure 3), five of them being sesquiterpenes (cedrol (**3**),[41] methyl hinokiate (**10**), widdrol (**11**),[42] 8*S*-14-cedrandiol (**13**)[43] and **14,**[44] and two of them diterpenes (methyl *cis*-communate (**9**)[40] and isocupresic acid (**12**)).[45] These compounds have been identified by analysis of their ^{1}H and ^{13}C-NMR spectra and by comparison of these data with those reported in the literature.

The diterpenes *cis* and *trans*-communic acids (**9a**-**b**) show a great antimicrobial activity, are important precursors of other bioactive diterpenes such as totarol (**7**)[46] and present antitumoral activity.[47] The sesquiterpene methyl hinokiate (**10**) is a potent antimicrobial against *Corynebacterium xerosis* and *Staphylococcus epidermidis.*[48] Isocupreic acid (**12**) presents antitumoral activity[47] and has been reported as a potent abortive.[49]

Biological Activity

The antifungal effects (spore germination inhibition against *A. niger*) are shown in Table 5. The essential oil **Jt-EO** (EC_{50} 126 μg/mL) and the organic extract **Jt-Hex** (EC_{50} 146 μg/mL) from *J. thurifera* wood were the strongest antifungals ($EC_{50} < 50$ μg/mL), with the fraction **Jt-EO-OF**, rich in cedrol **3**, being the most active. Cedrol (**3**), isolated from *J. thurifera,* has been included for comparison purposes (EC_{50} values against *A. niger* of 46 μg/mL).

The EOs and extracts were tested against the tick *Hyalomma lusitanicum* (Table 6). Among the EOs, the most active was **Jp-EO** (EC_{50} 17 μg/mg). In both species (Jp and Jt), the **EO-OF** fractions were more active than the parent EO, with **Jt-EO-OF** (cedrol **3** 67%, EC_{50} of 3.4 μg/mg), followed by **Jp-EO-OF** (**3**, 43%, EC_{50} 10 μg/mg), **Jt-EO-HF** (α-cedrene **1**, 26%, thujopsene **2**, 24%, EC_{50} 14 μg/mg), and **Jp-EO-HF** (**2**, 43%, nootkatone **4**, 12%, EC_{50} 21 μg/mg) (Table 6). The known ixodicidal compound nootkatone (**4**), a component of **Jp-EO** (6.5%) and **Jp-EO-OF** (12%), has been included for comparison purposes (EC_{50} 4 μg/mg).[6]

As previously mentioned, *J. phoenicea* essential oil (**Jp-EO**) and fractions were characterized by cedrol (**3**), thujopsene (**2**), nootkatone (**4**), and cedrene **1.** *J. thurifera* essential oil (**Jt-EO**) and fractions had cedrol (**3**), α-cedrene (**1**), thujopsene (**2**), γ-muurolene (**5**), and β-himachalene (**6**). Among the organic extracts (**Hex** and **EtOAc**), *J. phoenicea* contained varying amounts of widdrene, totarol **7,** nootkatone **4**, and cedrol **3**; while *J. thurifera* had cedrol (**3**) and α-cedrene **1**.

There are reports on the antifungal effects of *Juniperus* spp. essential oils, including *J. phoenica* (rich in α-pinene) against *Botrytis cinerea* mycelium[50] and hexane, ethanol, and methanol extracts (rich in cedrol, thujopsene, and widdrol) active on wood-rot fungi.[51,52]

Juniperus essential oils have been described as ixodicidal against several tick species,[53,54] including *J. thurifera* var. Africana (rich in sabinene, α-pinene, and γ-terpinene) against *H. aegyptium*[17] and *J. phoenica* (rich in α-terpinyl acetate, α-pinene, and germacene D) against *Ixodes ricinus.*[55] None of these oils were chemically similar to the ones studied here. Essential oils rich in some of these components have been described as ixodicidal. *Cupressus funebris* wood essential oil characterized by α-cedrene (**1**) (16.9%), cedrol (**3**) (7.6%), and β-cedrene (**5**) (7%) had repellency against nymphs of the ticks *Amblyomma americanum* and *I. scapularis.*[56] Tar (obtained by pyrolytic decomposition of the wood) from the Lebanon cedar

Table 4. Volatiles of *J. phoenicea* and *J. thurifera* from Organic Extracts (Hex and EtOAc). Estimated According to CG-MS.

		J. phoenicea Extracts		*J. thurifera* Extracts	
Compound[a]	Rt	Hex	EtOAc	Hex	EtOAc
(+)-Longifolene	13.26	1.53	-	-	-
α-Cedrene (**1**)	13.40	-	-	9.73	7.82
(-)-Thujopsene (**2**)	13.79	6.17	-	-	-
Widdrene	13.80	-	13.82	6.22	5.63
β-Himachalene (**6**)	15.29	-	-	2.51	1.59
Cuparene	15.38	-	-	1.17	-
γ-Muurolene	13.59	-	-	2.62	2.18
Cedranoxide	16.19	-	-	1.91	1.70
Allocedrol	17.17	-	-	1.04	-
Cedrol (**3**)	17.40	**8.16**	**10.95**	**36.48**	**34.93**
Epicedrol	17.67	-	-	1.05	0.72
3-*epi*-Cedrenal	18.21	-	-	1.97	1.89
β-Eudesmol	18.35	-	1.18	-	-
Not identified	18.57	-	-	2.94	2.24
Not identified	18.67	-	-	1.76	1.73
7-*epi-cis*-Sesquisabinene hydrate	18.93	-	-	3.62	-
ent-Germacra-4(15),5,10(14)-trien-1β-ol	19.41	1.53	2.50	1.46	1.35
Widdrenal	19.56	1.00	-	1.59	-
Unknown	19.57	-	1.54	-	1.85
Valerenal	21.15	-	-	1.31	1.02
Nootkatone (**4**)	21.30	**13.72**	**33.39**	-	-
Cryptomeridiol	21.43	-	2.15	-	-
1,5,9,9-Tetramethyl-2-methylene-spiro[3.5]non-5-ene	21.95	-	-	3.66	10.34
Aristolenepoxide	22.30	-	-	3.47	2.43
Widdrol	23.47	-	1.00	-	-
Silphinene	25.046	-	2.29	-	-
Unknown	29.14	3.34	-	-	-
Totarol (**7**)	29.53	**35.01**	2.48	-	-
Abieta-6,8,11,13-tetraen-12-yl acetate	29.70	5.12	2.01	-	-
Ferruginol (**8**)	29.78	4.32	-	-	-
Oleamide	30.16	-	1.83	-	1.36
Communic acid	30.24	1.04	-	-	-
Unknown	32.80	1.98	0.82	-	-
Unknown	32.92	1.89	1.96	-	-
Stigmasterol acetate	39.83	2.09	1.09	-	-

aComponents with percentages higher than 1%.

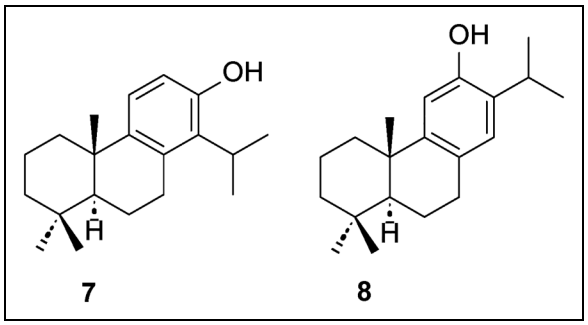


Figure 2. Aromatic diterpenes identified by GC-MS from **Jp-Hex**.

(*Cedrus libani*), characterized by β-himachalene (29.2%), was acaricidal and repellent against *Rhipicephalus sanguineus*.[57]

Additionally, some of these compounds (**1**-**7**) have reported fungicidal and ixodicidal effects. Cedrol (**3**) and thujopsene (**2**) showed strong inhibitory effects against wood-rot fungi.[52,58] Tick nymphs of *Ixodes scapularis* exhibited dose-dependent mortality when exposed to cedrol **3**[32] and nootkatone (**4**) is a well known ixodicidal against several tick species,[59,60] including *H. lusitanicum*.[6]

Conclusion

J. phoenicea essential oil (**Jp-EO**) and fractions were characterized by cedrol (**3**), thujopsene (**2**), nootkatone (**4**) and α-cedrene **1.** *J. thurifera* essential oil (**Jt-EO**) and fractions had cedrol (**3**), α-cedrene (**1**), thujopsene (**2**), γ-muurolene (**5**) and β-himachalene (**6**).

We have found that the hexane extracts from the branches wood of the two studied *Juniperus* species constitute an important source of cedrol, totarol, and nootkatone, respectively; with these natural products presenting an abundance

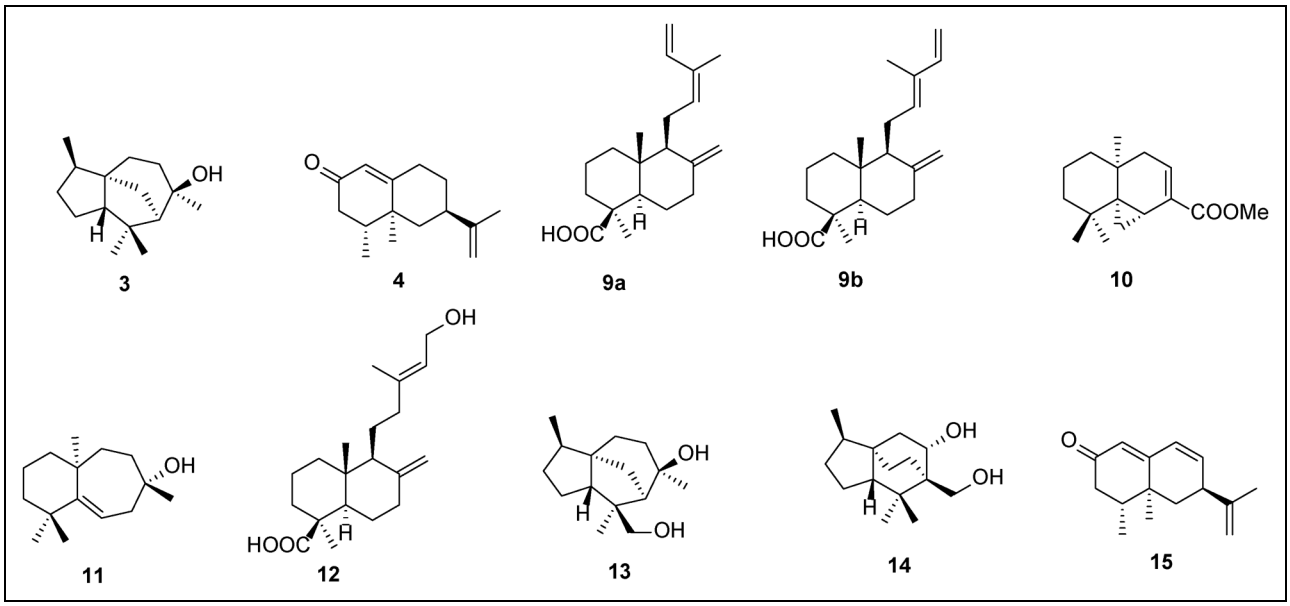


Figure 3. Compounds isolated from the hexane extracts from *J. phoenicea* (**Jp-Hex**) and *J. thurifera* (**Jt-Hex**).

Table 5. Antifungal Activity Against *A. niger*.

	A. niger spore germination (%)				
Sample	800 μg/mL	EC_{50} (μg/mL)	Fraction	800 μg/mL	EC_{50} (μg/mL)
Jp-EO	52.64 ± 4.3	≈800	**HF**	106.43 ± 4.75	>800
			OF	41.21 ± 2.97	522.17 (399.92-681.77)
Jp-Hex	74.94 ± 12.39	>800			
Jp-EtOAc	27.94 ± 2.89	293.96 (218.38-395.72)			
Jt-EO	38.47 ± 2.78	126.11 (76.39-208.21)	**HF**	57.88 ± 11.66	≈800
			OF	17.71 ± 0.39	<100
Jt-Hex	6.15 ± 0.53	146.61 (125.49-171.26)			
Jt-EtOAc	19.05 ± 1.87	180.21 (156.14-209.61)			
Cedrol		45.99 (30.89-65.55)			

Table 6. Ixodicidal Effects of *J. phoenicea* and *J. thurifera* Extracts (EOs and Fractions, Organic Extracts, Hex and EtOAc) Against *H. lusitanicum* larvae.

	H. lusitanicum larval mortality				
Sample	40 μg/mg	LD_{50}	Fraction	40 μg/mg	LD_{50}
Jp-EO	100	16.8 (14.5-19.1)	**HF**	100	21.2 (19.12-23.72)
			OF	100	10.3 (9.5-11.2)
Jp-HD	53.8 ± 3.5	>40			
Jp-Hex	0	>>40			
Jp-AcOEt	100	34.5 (31.9-37.24)			
Jt-EO	100	37.0 (16.3-20.9)	**HF**	100	13.8 (10.0-17.2)
			OF	100	3.4 (3.0-3.6)
Jt-HD	nt[a]				
Jt-Hex	100	14.0 (11.8-16.0)			
Jt-EtOAc	100	9.2 (6.3-11.6)			
Nootkatone[b]	100	4.02 (1.92-7.42)			

aNot tested. [b]From Galisteo *et al.*[6]

ranging from 50% to 14% with respect to the total weight of the extract.

The different samples obtained from the pruning of both *Juniperus* species showed antifungal and ixodicidal effects against *A. niger* and *H. lusitanicum*. The essential oil **Jt-EO** and the organic extract **Jt-Hex** from *J. thurifera* wood were antifungal, with the fraction **Jt-EO-OF**, rich in cedrol, being the most active, while the most ixodicidal EO was **Jp-EO**. Among the EO fractions, both **EO-OF** were more active against the tick than the parent EO.

Further work is already in progress, such as the study of the chemical composition of the EtOAc extracts of both species or the biological studies of all the isolated natural products.

All in all, this work proved that *Juniperus* prunings are renewable and easily accessible sources of pure sesquiterpenes cedrol, thujopsene or nootkatone, and diterpenes such as totarol, all of them presenting interesting bioactivities.

Part Experimental

- **Plant material:**

The wood of *Juniperus phoenicea* and *Juniperus thurifera* was collected in the Sierra de Albarracin, Teruel (Spain).

- **Extraction and fractionation:**

The wood from each plant was air-dried at room temperature for 48 h. Once dried, they were crushed and subjected to hydrodistillation with a Clevenger apparatus for 8 h.[9] After this time, the essential oil (**EO**) and the aqueous residue (hydrolate) were separated. The hydrolate extract (**HD**) was obtained following our protocol, by adsorption with activated charcoal and subsequent release with EtOAc.[61] Once the **EO** and **HD** samples were obtained, the residual wood was dried for 72 h at room temperature and extracted with a Soxhlet apparatus using hexane (**H Extract**) and ethyl acetate (**EtOAc Extract**) for 24 h each to obtain the corresponding extracts.

Each essential oil was partitioned by filtration through column chromatography of silica gel to afford a hydrocarbonated fraction (**HF**) eluted with hexane and the oxygenated fraction (**OF**) eluted with diethyl ether.

	Weight (g)	% Yield
J. phoenicea wood	1244	
EO (**Jp-EO**)	6.42	0.52
EO HF (**Jp-EO-HF**)	1.99	31
EO OF (**Jp-EO-OF**)	4.43	69
HD (**Jp-HD**)	0.006	5×10^{-4}
Hex extract (**Jp-Hex**)	11.98	0.96
AcOEt extract (**Jp-EtOAc**)	15.81	1.27
J. thurifera wood	1026	
EO (**Jt-EO**)	3.16	0.31
EO HF (**Jt-EO-HF**)	0.79	25
EO OF (**Jt-EO-OF**)	2.37	75
HD (**Jt-HD**)	0.004	4×10^{-4}
Hex extract (**Jt-Hex**)	41.29	4.02
AcOEt extract (**Jt-EtOAc**)	32.56	3.17

- EO analysis

The EOs and dichloromethane fraction of the hexanic and EtOAc extracts were analyzed by gas chromatography-mass spectrometry (GCMS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 μl) were carried out by an AOC-20i and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 μm film thickness) Teknokroma TRB-5 (95%) Dimetil-diphenylpolisiloxane (5%). Working conditions were as follows: split ratio (20:1), injector temperature 300 °C, temperature of the transfer line connected to the mass spectrometer 250 °C, initial column temperature 70 °C, then heated to 290 °C at 6 °C/min and a Full Scan was used (m/z 35-450). Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those found in the Wiley 229 and NIST 17 Mass Spectral Database. All extracts (4 μg/μl) were dissolved in 100% DCM for injection.

- Flash column chromatography of Jp-Hex:

A 2 g portion of **Jp-Hex** was subjected to separation by column chromatography on silica gel using a mixture of solvents (H and methyl *tert*-butyl ether MTBE) of increasing polarity to obtain 5 fractions.

- **Jp-Hex-A** (H-MTBE, 50-1). This fraction (107 mg, 5.4%) was constituted by a mixture of hydrocarbons.
- **Jp-Hex-B** (H-MTBE, 20-1). This fraction (406 mg, 20.0%) contained totarol (**7**).

Compound **7**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.02 (d, J = 8.5 Hz, 1H), 6.54 (d, J = 8.5 Hz, 1H), 4.47 (s, 1H), 3.32 (t, J = 7.2 Hz, 1H), 2.97 (dd, J = 17.1, 6.7 Hz, 1H), 2.84-2.71 (m, 1H), 2.25 (d, J = 12.8 Hz, 1H), 1.94 (dd, J = 13.2, 8.1 Hz, 1H), 1.80-1.56 (m, 3H), 1.49 (d, J = 13.5 Hz, 1H),1.40-1.37 (m, 6H), 1.34-1.22 (m, 3H), 1.20 (s, 3H), 0.97 (s, 3H), 0.94 (s, 3H), according to bibliography.[62]

- **Jp-Hex-C** (H-MTBE, 20-1). This fraction (36 mg, 1.8%) contained cedrol (**3**).
- **Jp-Hex-D** (H-MTBE, 10-1). This fraction weighed 614 mg, (30.7%). A 250 mg portion of this fraction was dissolved in 6 mL of benzene-MeOH (4-1) and 0.6 mL of trimethylsilyldiazomethane (TMSDM, 2 M in hexane) was added. After stirring for 30 min at room temperature, the reaction mixture was evaporated under reduced pressure.

The reaction crude was chromatographed on silica gel with mixtures of solvents (H) and EtOAc) of increasing polarity to afford 70 mg (H-EtOAc 20-1) containing the methyl esters of *cis*-communic acid (**9a**) and *trans*-communic acid (**9b**), and 162 mg (H-EtOAc 9-1) of a mixture of nootkatone (**4**) and 8,9-didehydronootkatone (**15**) in 2.25:1 ratio. A 10 mg portion of this mixture was subjected to semi-preparative HPLC using an isocratic elution (H-MTBE, 10-1) at a flow rate of 4 mL/min to give 2 mg of pure 8,9-didehydronootkatone (**15**).

Compound **9a**: Spectroscopic data match with those reported on bibliography.[40]

Compound **9b**: Spectroscopic data match with those reported on bibliography.[40]

Compound **15**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.23 (dd, J = 9.8, 2.7 Hz, 1H), 6.14 (d, J = 9.8 Hz, 1H), 5.77 (s, 1H), 4.87 (bs, 1H), 4.84 (bs, 1H), 3.08 (dm, J = 10.9 Hz, 1H), 2.47-2.32 (m, 2H), 2.09 (dq, J = 12.5, 6.4 Hz, 1H), 2.01 (dd, J = 13.0, 4.9 Hz, 1H), 1.78 (bs, 3H), 1.30 (t, J = 12.2 Hz, 1H), 1.09 (s, 3H), 1.01 (d, J = 6.8 Hz, 3H); according to bibliography.[6]

- **Jp-Hex-E** (H-MTBE, 5-1). This fraction (618 mg, 30.9%) was constituted by a mixture that could not be resolved.

● Flash column chromatography of Jt-Hex

A 3.5 g portion of **Jt-Hex** was subjected to separation by column chromatography on silica gel using a mixture of solvents (H and MTBE)) of increasing polarity to obtain six fractions.

- **Jt-Hex-A** (H-MTBE, 3-1). This fraction (590 mg, 16.8%) was constituted by a mixture of hydrocarbons.
- **Jt-Hex-B** (H-MTBE, 2-1) weighed 2270 mg (64.8%). A 200 mg portion of this fraction was dissolved in 5 mL of benzene-MeOH (4-1) and 0.45 mL of trimethylsilyldiazomethane (TMSDM, 2 M in hexane) were added. After stirring for 10 min at room temperature, the reaction mixture was evaporated under reduced pressure. The reaction crude was chromatographed on silica gel (H-MTBE, from 99-1 to 9-1) to give 58 mg of the so-called **Jt-Hex-B1** fraction and 102 mg of cedrol (**3**) (51%). **Jt-Hex-B1** (15 mg) was subjected to semi-preparative HPLC using an isocratic elution (H-MTBE, 99-1) at a flow rate of 4 mL/min to give 2 mg of the methyl ester of *cis*-communic acid (**9a**) and 5 mg of methyl hinokiate (**10**).

Compound **3**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.34 (s, 3H), 1.28 (s, 3H), 1.02 (s, 3H), 0.86 (d, J = 7.1 Hz, 3H). The spectroscopy data was compared to bibliography.[41]

Compound **10**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.54 (ddd, J = 7.2, 2.6, 1.4 Hz, 1H), 3.70 (s, 3H), 2.02 (dd, J = 8.9, 5.0 Hz, 1H), 1.86 (dd, J = 18.3, 2.6 Hz, 1H), 1,75-1,59 (m, 3H), 1,42-1,33 (m, 2H), 1,24-1,16 (m, 3H), 1.12 (s, 2H), 1.06 (s, 3H), 0.74 (dd, J = 9.0, 4.9 Hz, 1H), 0.64 (t, J = 5.0 Hz, 1H), 0.62 (s, 3H). ^{13}C-NMR (133 MHz, $CDCl_3$): δ 167.5, 133.1, 131.7, 51.7, 41.4, 40.0, 37.0, 34.7, 34.0, 31.5, 29.1, 28.4, 26.8, 19.4, 16.9, 11.5.

- **Jt-Hex-C** (H-MTBE, 2-1). (80 mg, 2.3%) was dissolved in 1.5 mL of benzene–MeOH (ratio 4:1) and 0.18 mL of trimethylsilyldiazomethane (TMSDM, 2 M in hexane) were added. After stirring for 10 min at room temperature, the reaction mixture was evaporated under reduced pressure and the reaction crude was chromatographed on silica gel (H–MTBE, from 99-1 to 9-1) to give 18 mg of **10** and 31 mg of **3**.
- **Jt-Hex-D** (H-MTBE, 1-1). This fraction (290 mg, 8.3%) was dissolved in 5.5 mL of benzene-MeOH (4-1 ratio) and 0.61 mL of trimethylsilyldiazomethane (TMSDM, 2 M in hexane) were added. After stirring for 10 min at room temperature, the reaction mixture was evaporated under reduced pressure and the reaction crude was chromatographed on silica gel (H-MTBE, 3-1) to give 57 mg de widdrol (**11**).

Compound **11**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.53 (dd, J = 9.0, 5.8 Hz, 1H), 2.52 (dd, J = 13.7, 5.8 Hz, 1H), 2.01 (dd, J = 13.7, 9.1 Hz, 1H), 1.79-1.24 (m, 10H), 1.24 (s, 3H), 1.22 (s, 3H), 1.11 (s, 3H), 1.10 (s, 3H). ^{13}C-NMR (133 MHz, $CDCl_3$): δ 154.3, 117.7, 73.0, 41.6, 40.0, 39.5, 39.4, 38.0, 36.8, 32.9, 32.0, 28.4, 26.6, 18.6. The data spectroscopy was compared to bibliography.[42]

- **Jt-Hex-E** (H-MTBE, 1-3) weighed 130 mg (3.7%). 15 mg of this fraction was subjected to semi-preparative HPLC using an isocratic elution (H-MTBE, 1-3) at a flow rate of 4 mL/min to give 3 mg of isocupressic acid (**12**) and 8 mg of 8S-14-cedranediol (**13**).

Compound **12**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.41 (td, J = 7.0, 1.4 Hz, 1H), 4.88 (s, 1H), 4.55 (s, 1H), 4.18 (d, J = 6.9 Hz, 2H), 2.45-2.41 (m, 1H), 2.23-2.13 (m, 2H), 2.02-1.82 (m, 5H), 1.69 (s, 3H), 1.60-1.41 (m, 5H), 1.35 (dd, J = 12.0, 3.1 Hz, 1H), 1.26 (s, 3H), 1.08 (td, J = 13.5, 4.2 Hz, 2H), 0.63 (s, 3H). ^{13}C-NMR (133 MHz, $CDCl_3$): δ 182.2, 147.9, 140.5, 123.1, 106.5, 59.4, 56.3, 55.5, 44.1, 40.4, 39.1, 38.7, 38.4, 38.0, 20.0, 26.1, 22.0, 19.9, 16.4, 12.8. The data spectroscopy was compared to bibliography.[45]

Compound **13**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.07 (d, J = 11.4 Hz, 1H), 3.31 (d, J = 11.4 Hz, 1H), 2-06-1.88 (m, 2H), 1.83 (d, J = 4.9 Hz, 1H), 1.76-1.62 (m, 4H), 1.57-1,45 (m, 3H), 1.43-1,35 (m, 2H), 1.34 (d, J = 1.0 Hz, 3H), 1.31-1,25 (m, 1H), 1.13 (d, J = 0.9 Hz, 3H), 0.87 (d, J = 7.1 Hz, 3H). ^{13}C-NMR (133 MHz, $CDCl_3$): δ 75.3, 69.1, 57.6, 53.8, 52.1, 49.5, 41.4, 41.1, 37.0, 35.5, 31.6, 30,9, 25.4, 23.8 15.6, according to bibliography.[43]

- **Jt-Hex-F** (MTBE) weighed 140 mg (4.0%). 15 mg of this fraction was subjected to semi-preparative HPLC using an

isocratic elution (H-MTBE, 1-3) at a flow rate of 4 mL/min to give 8 mg of **13** and 2 mg of **14**.

Compound **14**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.41 (ddd, $J = 9.6$, 5.9, 2.3 Hz, 1H), 3.87 (d, $J = 11.4$ Hz, 1H), 3.52 (d, $J = 11.5$ Hz, 1H), 2.13 (ddd, $J = 12.6$, 9.5, 3.1 Hz, 1H), 2.03-1.94 (m, 1H), 1.88 (ddd, $J = 14.3$, 11.2, 8.1 Hz, 1H), 1.52-1.21 (m, 6H), 1.13 (dd, $J = 12.5$, 5.9 Hz, 1H), 1.06-0,97 (m, 1H), 0.96 (s, 3H), 0.95 (s, 3H), 0.85 (d, $J = 7.2$ Hz, 3H). ^{13}C-NMR (133 MHz, $CDCl_3$): δ 71.4, 65.7, 53.2, 43.1, 40.8, 39.9, 35.2, 34.5, 30.5, 26.3, 23.8, 21.2, 19.7, 17.6, according to bibliography.[44]

- Bioassay
  - **Ixodicidal activity:** The ixodicidal tests were carried on with *H. lusitanicum* engorged female ticks as described.[6] Briefly, 25 mg of powdered cellulose were mixed with 50 µL of test solution (initial concentration of 20 mg/mL). Each test (20 larvae each) was replicated three times. Tick mortality was recorded after 24 h. The percent mortality was corrected according to Schneider–Orelli's formula.[63] Effective lethal doses (LC_{50} and LC_{90}) were calculated by Probit Analysis with 5 serial dilutions, (STATGRAPHICS Centurion XVI, version 16.1.02, Statgraphics Technologies, Inc., P.O. Box 134, The Plains, Virginia 20198, USA). Nootkatone (Sigma) was used as positive control based on its known ixodicidal activity including *H. lusitanicum*.[6]
  - **Antifungal Assay:** The antifungal activity of the essential oils was determined on *A. niger* spores (from ICA-CSIC, Madrid, Spain) as described.[63] The extracts (essential oils and hydrolate in 1% dimethyl sulfoxide, DMSO) were tested at 800, 400, 200, 100, and 50 µg/mL on spore suspensions (7.5×10^5 cells/mL in NaCl 0.9%) and Amphotericin B (Sigma), an antifungal used against aspergillosis (https://www.drugs.com/monograph/amphotericin-b.html), was used as a positive control (5 µg/mL).[64] Four replicates of each test in 96-well plates were incubated for 24 h (28 °C), then 25 uL of MTT (5 mg/mL) and menadione (1 mM) in RMPIMOPS were added, incubated for 3 h, and then 200 µL of acidic isopropanol (95% isopropanol and 5% 1 M HCl) were added to the plates after the removal of the medium and incubated for 30 min. The absorbance was read at 490 nm. The IC_{50} values (the effective dose to give 50% inhibition) were calculated by a regression curve of % spore germination inhibition on log dose (STATGRAPHICS Centurion XVI, version 16.1.02, Statgraphics Technologies, Inc., P.O. Box 134, The Plains, Virginia 20198, USA).

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Author Contributions

JFQM, AFB and AG-C wrote the first draft of the manuscript and formatted the last version. JFQM edited the manuscript. AFB and MFA revised the manuscript. AFB, JFQM, AG and HZ performed the analysis of the composition of the woods, including the isolation of pure compounds. JRL-M, MFA and AG-C performed the biological experiments. All authors have read and agreed to the published version of the manuscript.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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