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ANTIBACTERIAL AND ANTIFUNGAL TERPENES FROM Pilgerodendron uviferum (D. DON) FLORIN.

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ABSTRACT

Ten sesquiterpenes (a-cubebene, copaene, α-caryophyllene, caryophyllene-4,5-epoxide, δ-cadinene, cubenol, epicubenol, torreyol, humulene-1,2-epoxide, 15-copaenol) and six diterpenes (hinokione, hinokiol, ferruginol, 6,7-dehydroferruginol, totarol, sugiol) were isolated and identified from *Pilgerodendron uviferum* (D. Don) Florin wood and bark.

The molecular structures were elucidated by spectroscopic methods. Twelve pure compounds were tested for antimicrobial activity against standard bacterial *(Staphylococcus aureus, Bacillus subtilis,*

Escherichia coli, Pseudomonas aureginosa, Streptococcus pyogenes) and fungal strains *(Ophiostoma piliferum, Rhizoctonia solani, Phragmidium violaceum, Fusarium graminearum, Pythium irregulare, Botrytis cinerea, Schizophyllum commune)*, as were crude total extracts, n-hexane, dichloromethane, and ethyl acetate partial extracts. All the compounds tested exhibited biological activity, with the most active being ferruginol, hinokiol, 15-copaenol, cubenol, torreyol, and n-hexane wood extracts and ethyl acetate bark extract.

INTRODUCTION

Pilgerodendron uviferum (D. Don) Florin (Ciprés de las Guaitecas) belongs to the Cupressaceae family (Coniferophyta division). Endemic to southern Chile and Argentina, its geographical distribution includes some 1600 km between 39° 36´ and 54° 20'S [1,2], making it the world´s southernmost native conifer. Exploitation for timber, grazing, and fire, however, have severely reduced *P. uviferum* populations [3]. Its two closest relatives are *Austrocedrus chilensis* (D. Don) Pic. Serm. et Bizarri (Ciprés de la Cordillera) and *Fitzroya cupressoides* (Molina) I. M. Johnston (Alerce).

The quality and durability of *P. uviferum*'s aromatic, yellow-orange wood is excellent and it resists rotting notably [4]. These properties render *P. uviferum*, therefore, a commercially valuable species in the manufacture of wharfs, naval constructions, poles, flooring, and furniture [4,5].

Plant secondary metabolites, synthesized in the cellular metabolism, accumulate like extractables in the wood´s resiniferous channels, xylem rays, and cellular levels [6]. Secondary metabolites seem to function principally by enhancing the plant´s resistance to microbial attacks. Different kinds of metabolites in Angiospermae and Gymnospermae extracts have been studied: fatty acids, hydrocarbons, alkaloids, flavonoids, lignans, phenols, terpenes, steroids, tannins, quinones, and resins [11]. Terpenes are common constituents of conifers [10].

Previous chemical studies in the Cupressaceae family have produced a series of compounds such as biflavones [7], monoterpenes, sesquiterpenes, diterpenes, flavonoids, diterpene lactones, sesquiterpene lactones, lignan lactone, and lignans [11,14,16]. Some of these compounds, especially the terpenes, showed antibacterial, fungicidal, and insecticidal activities [12,13,14]. Previous chemical studies on *P. uviferum* leaves, wood, and branches revealed ten sesquiterpenes, alkaloids were not found [8,9]. Biological antiproliferative activities of crude *P. uviferum* extracts have also been tested [15].

P. uviferum's wood is an interesting topic for further research. Well known for its durability and resistance to rotting, preliminary studies have shown several terpene type substances that could have inhibitory antimicrobial activities.

The fundamental objective of this study was to characterize the antibacterial and antifungal activities of the principal terpene compounds in *P. uviferum* wood and bark extracts, using a bioassay-guided fractionation procedure, and to relate these antimicrobial activities to the wood´s natural durability.

EXPERIMENT

2.1. Samples: Samples of *P. uviferum* wood and bark were collected in the Cordillera Pelada (X Region, Chile), in April 2000. The wood (3 kg) and bark (300g) were separated mechanically and were chopped.

2.2. Extraction: Both samples were extracted three times with methanol at 40°C for 48 h, resulting in a colorless oil; 230 g were obtained from the wood and 38 g from the bark. The crude total extracts were evaporated to dryness under vacuum conditions. Three successive extractions were then carried out on each crude extract with three solvents of increasing polarities (η-hexane, CH_2Cl_2, ethyl acetate). These three extracts were processed separately.

2.3. Terpene isolation and purification: The three wood and bark extracts were fractionated by column chromatography with silica gel, eluting with η-hexane, followed by mixtures of η-hexane and ethyl acetate (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 0:100). The different fractions eluting from the column were further separated and/or purified on preparative TLC plates (silica gel 60 F_{254}) using different solvents (η-hexane, CH_2Cl_2, ethyl acetate).

The η-hexane wood extract was applied to a silica gel column (200 g) and repeatedly eluted with mixtures of n-hexane and ethyl acetate of increasing polarities (100:0, 90:10, 85:15, 80:20, 70:30,

50:50, 0:100). The compounds obtained from the column were further separated and/or purified on preparative TLC plates (silica gel 60 F_{254}), yielding the followings compounds:

Compound 1 (α-cubebene). Amorphous compound: $[a]^{22}_D$ = -19.6° ($CHCl_3$; c 0.13). MS m/z (rel. int.): 204 [M]$^+$ (14), 189 (1), 175 (1), 161 (90), 147 (3), 133 (9), 119 (90), 105 (100), 91 (35), 77 (14), 55 (23). Compound 2 (copaene). Amorphous compound: $[a]^{22}_D$ = -6.8° (CHCl3; c 0.17). MS m/z (rel. int.): 204 [M]$^+$(17), 189 (4), 161 (100), 143 (4), 133 (12), 119 (94), 105 (99), 93 (61), 77 (23), 55 (35). Compound 3 (a-caryophyllene). Amorphous compound: $[a]^{22}_D$ = -14.8° ($CHCl_3$; c 0.20). MS m/z (rel. int.): 204 [M]$^+$ (4), 189 (2), 175 (2), 161 (3), 143 (16), 136 (3), 121 (25), 107 (15), 93 (100), 80 (39), 67 (22), 53 (22). Compound 4 (caryophyllene-4,5-epoxide). Mp 61 62°C. MS m/z (rel. int.): 220 [M]$^+$ (24), 189 (63), 175 (100), 159 (18), 145 (18), 133 (21), 117 (16), 105 (31), 91 (51), 79 (26). 67 (16), 55 (19). The ^{1}HNMR [17] and ^{13}CNMR [18,19] were similar to previously reported data. Compound 5 (δ-cadinene). Amorphous compound: $[\alpha]^{22}_D$= +75° ($CHCl_3$; c 0.12). MS m/z (rel. int.): 204 [M]$^+$ (47), 179 (55), 161 (100), 119 (57), 105 (47), 95 (53), 79 (40), 55 (42). The ^{13}CNMR [8] and ^{1}HNMR [25] were similar to previously reported data. Compound 6a (cubenol). Amorphous compound: $[a]^{22}_D$ -28° ($CHCl_3$; c 0.13). MS m/z (rel. int.): 222 [M]$^+$ (18), 221 (100), 205 (10), 189 (4), 161 (41), 137 (20), 121 (24), 105 (31), 91 (25), 77 (18), 55 (24). The ^{1}HNMR [21,22] and ^{13}CRMN [8] were similar to previously reported data. Compound 6b (epicubenol). Amorphous compound: $[\alpha]^{22}_D$ -96° ($CHCl_3$; c 0.14). MS m/z (rel. int.): 222 [M]$^+$ (60), 205 (28), 189 (7), 161 (58), 138 (25), 121 (27), 105 (35), 93 (32), 79 (25), 55 (28). The ^{13}CNMR [8] and ^{1}HNMR [21, 22] were similar to previously reported data. Compound 6c (torreyol): Mp 138-139C: $[a]^{22}_D$ -98° ($CHCl_3$; c 0.20). MS m/z (rel. int.): 222 [M]$^+$ (27), 204 (5), 179 (4), 161 (9), 135 (9), 121 (100), 108 (41), 105 (13), 93 (27), 81 (84), 67 (9), 55 (18). The ^{13}CNMR [8] and ^{1}HNMR [23] were similar to previously reported data. Compound 7 (humulene-1,2-epoxide). Amorphous compound: $[\alpha]^{22}_D$ -29.0° ($CHCl_3$; c 0.15). MS m/z (rel. int.): 220 [M]$^+$ (10), 207 (5), 202 (13), 187 (13), 177 (7), 162 (45), 159 (15), 147 (20), 135 (100), 119 (25), 107 (52), 93 (55), 79 (53), 71 (53), 55 (92). The ^{1}HNMR [20] and ^{13}CRMN [8] were similar to previously reported data. Compound 8 (15-copaenol). $[a]^{22}_D$ -28.6° ($CHCl_3$; c 0.2). MS m/z (rel. int.): 220 [M]$^+$ (18), 202 (9), 177 (84), 159 (45), 149 (20), 147 (31), 136 (37), 135 (95), 131 (23), 121 (40), 119 (27), 117 (33), 107 (30), 105 (75), 93 (75), 92 (33), 91 (100), 81 (35), 79 (49), 77 (31), 69 (22), 67 (28), 55 (41). The ^{1}HNMR [8] and ^{13}CRMN [8] were similar to previously reported data.

The compounds hinokione and hinokiol were isolated from the CH_2Cl_2 wood extract, using the same chromatograph, eluting, and TLC plate methods used for extracting h-hexane.

Compound 9 (hinokione). Mp 192-195 °C. $[a]^{22}_D$ + 224° ($CHCl_3$; c 0.66) MS m/z (rel. int.): 300 (M)$^+$ (100), 285 (30), 267 (15), 257 (22), 225 (19), 189 (52), 175 (38), 159 (22), 147 (39), 115 (17), 91 (17), 55 (41). Compound 10 (hinokiol). Mp. 240-242 °C. $[a]^{22}_D$ + 72.5° ($CHCl_3$; c 0.46). MS m/z (rel. int.): 302 (M)$^+$ (90), 287 (34), 269 (100), 255 (60), 227 (34), 213 (38), 189 (69), 175 (67), 147 (54), 133 (27), 115 (23), 91 (15), 55 (30).

The diterpenes totarol, ferruginol, 6,7-dehydroferruginol, and sugiol were isolated from the ethyl acetate bark extract, and were chromatographed on a silica gel column (80 g) and eluted repeatedly with h-hexane and ethyl acetate mixtures of increasing polarities (100:0, 90:10, 80:20, 70:30, 50:50, 0:100) and the compounds obtained from the column were further separated and/or purified on preparative TLC plates (silica gel 60 F_{254}).

Compound 11 (totarol). Mp 128-130 °C. $[a]^{22}_D$ = +41.2° ($CHCl_3$; c 0.71) MS m/z (rel. int.): 286 (M)$^+$ (44), 271 (100), 255 (2), 243 (7), 229 (7), 215 (7), 201 (52), 189 (31), 175 (83), 159 (16), 133 (11), 115 (10), 91 (7), 69 (31), 55 (18). The ^{1}HNMR [11] and ^{13}CRMN [11] were similar to previously reported data. Compound 12 (ferruginol). Mp 175 °C. $[a]^{22}_D$= +40.9° ($CHCl_3$; c 0.55) MS m/z (rel. int.): 286 (M)$^+$ (100), 271 (89), 255 (2), 229 (28), 215 (18), 201 (55), 189 (81), 175 (87), 159 (25), 149 (36), 133 (20), 115 (20), 91 (14), 69 (72), 55 (33). The ^{1}HNMR [11] and ^{13}CRMN [11] were similar to previously reported data. Compound 13 (6,7-dehydroferruginol). $[a]^{22}_D$ = -60.4° ($CHCl_3$; c 0.19) The ^{1}HNMR [11] and ^{13}CRMN [11] were similar to previously reported data. Compound 14 (sugiol). Mp 273-275 C. $[a]^{22}_D$ + 12.3° ($CHCl_3$; c 0.1). The ^{13}CRMN [24] were similar to previously reported data.

The sesquiterpenes and diterpenes isolated from wood and bark crude extracts are shown in Figs 1 and 2.

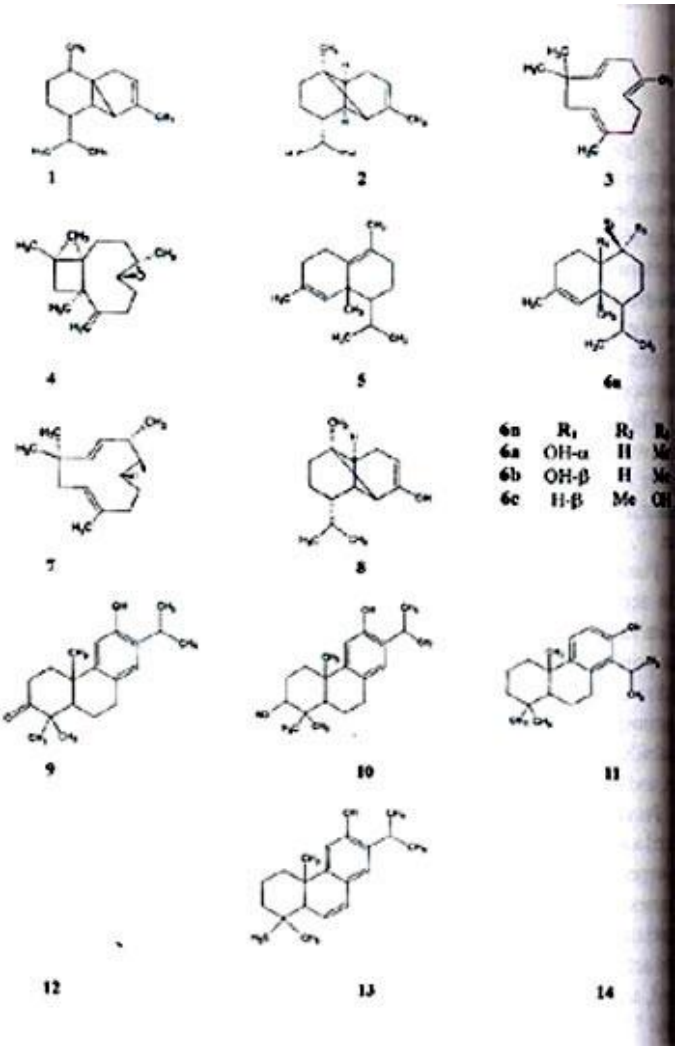
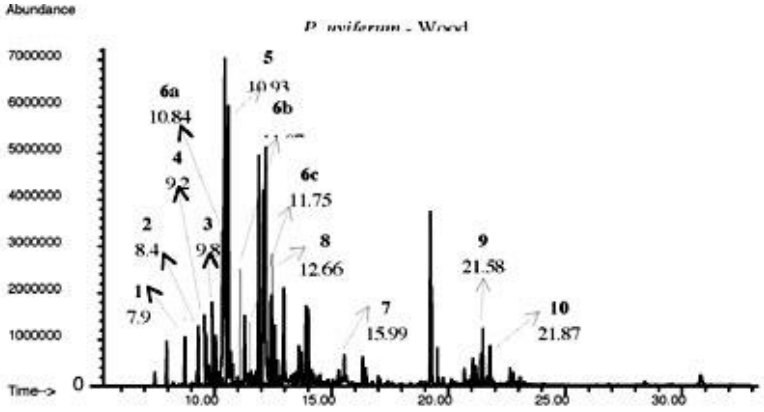
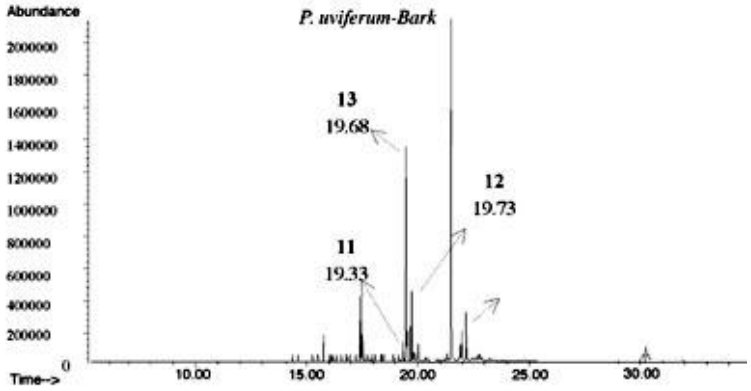


Fig 1. Sesquiterpenes and diterpenes isolated from P. Uviferum wood and bark, numbers refer to experimental part.



a) Crude total extract from *P. Uviferum* wood, numbers refer to experimental part.



b) Crude total extract from *P. Uviferum* bark, numbers refer to experimental part.

Fig 2. Crude total extracts chromathogram from *P. Uviferum* a) wood, b) bark.

2.4 Terpene identification: The isolated and purified compounds were characterised and identified as follows. Melting points were determined on a kofler block. Optical rotations $[a]^{22}_{D}$ were measured on a Carl Zeiss polarimeter, with chloroform as solvent. The NMR spectra (^{1}H and ^{13}C) were recorded by an AM-400 BRUKER spectrometer (400 MHz), and mass spectra were obtained with a 5972 series Hewlett Packard mass spectrometer. Gas chromatography with a mass selective detector at 70 eV was performed with a GC HP 6890-MS HP 5972 chromatograph, under the following conditions: Column HP-5: 30m x 0.25 mm x 0.25 µm; Temperature: 100 °C isothermal for five minutes, then a ten degree increase per minute up to 275 °C, after which this temperature was held constant for 20 minutes; Split injection: 100:1; Injector temperature: 275 °C; Detector temperature: 300 °C; and Helium Carrier.

2.5 Antimicrobial activity. Antimicrobial activities were determined from crude total wood and bark extracts, partial *P. uviferum* wood and bark fractions (h-hexane, CH_2Cl_2, ethyl acetate), as well as 12 compounds (four terpenes and eight sesquiterpenes) purified from the crude total extracts and partial fractions by means of a bioassay-guided fractionation procedure. Filter paper discs (6 mm diam., Whatman) with pure compounds were impregnated with 10 µl of a 10 µg/µl solution [11,16] of each compound and the filter paper discs of the crude total extracts and partial fractions were impregnated with 10 µl of a 40 µg/µl solution of each extract and placed in Petri dishes containing the test organisms (*Staphylococcus aureus* (ATCC6538p), *Bacillus subtilis* (ATCC6633), *Escherichia coli* (ATCCSI), *Pseudomonas aureginosa* (ATCC27953), *Streptococcus pyogenes* (isolated in Microbiology laboratory of the Universidad de Concepción). Cultures were incubated at 37 °C, and after 18 h, the diameter of inhibition zone was determined (mm). The antibacterial activity of the extracts and pure compounds was assessed using the disc-diffusion method. The mean value from at least four different experiments was used for the calculation, and each experiment was done in triplicate. The treatments were 22 diferents compounds evaluated with a completely randomized design. The treatments were subjected to a one-way analysis of variance (ANOVA) and means were compared with the Tukey HSD test (P=0.05).

Antifungal activities were determined with P. uviferum wood and bark crude total extracts and partial fractions using the disc-diffusion method. The cultures were incubated at 22°C for nine days. Mycelial growth diameters were measured daily and recorded as mean percentages (%) of growth. The fungal strains (*Ophiostoma piliferum, Rhizoctonia solani, Phragmidium violaceum, Fusarium graminearum, Pythium* irregulare, Botrytis cinerea, Schizophyllum commune), were identified by specialists from the Department of Botany of the Universidad de Concepción.

3. RESULTS AND DISCUSSION

We isolated and identified 10 sesquiterpenes (α-cubebene, copaene, a-caryophyllene, caryophyllene-4,5-epoxide, δ-cadinene, cubenol, epicubenol, torreyol, humulene-1,2-epoxide, 15-copaenol) and six diterpenes (hinokione, hinokiol, ferruginol, 6,7-dehydroferruginol, totarol, sugiol) from *P. uviferum* wood and bark. Furthermore, the compounds b-sitosterol and estigmasterol were identified via GC/MS.

The major compounds found in the bark were 6,7-dehydroferruginol, β-sitosterol, estigmasterol, and ferruginol, whereas the major compounds found in the wood were δ-cadinene, 15-copaenol, epicubenol, cubenol, 6,7-dehydroferruginol and humulene-1,2-epoxide. Ferruginol, 6,7-dehydroferruginol and sugiol were present in the wood as well as in the bark (Table1).

Table 1. Proportion (%) of pure compounds present in *P. uviferum* wood and bark, relative to the total crude extract.

Compounds	Bark extracts (%)	Wood extracts (%)
totarol	1.84	-
6,7-dehydroferruginol	21.99	5.03
ferruginol	3.21	2.08
sugiol	2.03	0.90
hinokiol	-	0.39
hinokione	-	0.70
α-cubebene	-	0.37
copaene	-	1.17
α-caryophyllene	-	2.04
caryophyllene-4,5-epoxide	-	1.74
15-copaenol	-	9.57
δ-cadinene	-	16.53
cubenol	-	5.41
epicubenol	-	8.85
torreyol	-	1.85
humulene-1,2-epoxide	-	4.33
β sitosterol	11.53	0.85
estigmasterol	6.59	-4.33

All 12 pure compounds examined (8 sesquiterpenes and four diterpenes) showed biological activity against at least one of the five test species (*Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aureginosa, Streptococcus pyogenes*), with the inhibition diameter varying between 6 and 32 mm (Table 2).

Table 2. The mean diameter (mm) of the bacterial activity, and mean percentages (%) of growth inhibition of the antifungal activity of crude total extracts, partial extracts, and pure compounds obtained from wood and bark, including streptomicina, copper oxide and a control. Filter paper disc = 6mm diameter.

Compounds	Bacteria Strains Mean diameter (mm)					Fungal Strains Mean percentages (%)						
	B. subtilis	*S. aureus*	*S. pyogenes*	*E. coli*	*P. Aureginosa sp.*	*F. Violaceum*	*O. piferum*	*R. solani*	*P. irregulare*	*F. graminearum*	*B. cinerea*	*S. commune*
Total wood	7 (ab)	7(a)	9 (b)	6 (a)	11 (c)	11 (d)	35(c)	18(e)	16(e)	33(c)	10(c)	18(c)
n-hexane	9 (cde)	11 (d)	8 (b)	6 (a)	15 (d)	14 (e)	40 (d)	18 (e)	13 (d)	28 (d)	28 (e)	20 (d)
CH2CL2	6 (ab)	9 (bc)	9 (b)	6 (a)	11 (c)	8 (c)	0 (a)	5 (b)	10 (c)	0 (a)	16 (d)	0 (a)
AcOet	7 (ab)	6 (a)	6 (a)	6 (a)	9 (b)	3 (b)	0 (a)	5 (b)	5 (b)	0 (a)	8 (b)	0 (a)
α cubebene	6 (ab)	6 (a)	6 (a)	6 (a)	12 (c)	-	-	-	-	-	-	-
copaene	6 (ab)	22 (e)	6 (a)	6 (a)	8 (b)	-	-	-	-	-	-	-
cubenol	9 (cd)	9 (bc)	6 (a)	6 (a)	11 (c)	-	-	-	-	-	-	-
15 copaenol	9 (cd)	12 (d)	6 (a)	6 (a)	12 (c)	-	-	-	-	-	-	-
humulene-1,2-epo	9 (cdf)	8 (b)	6 (a)	6 (a)	6 (a)	-	-	-	-	-	-	-
α cariofilene	7 (ab)	9 (bc)	6 (a)	6 (a)	6 (a)	-	-	-	-	-	-	-
δ-cadinene	6 (ab)	9 (bc)	6 (a)	6 (a)	6 (a)	-	-	-	-	-	-	-
torreyol	9 (cdf)	9 (bc)	6 (a)	6 (a)	8 (b)	-	-	-	-	-	-	-
hinokione	6 (ab)	9 (bc)	6 (a)	6 (a)	11 (c)	-	-	-	-	-	-	-
hinokiol	10(de)	10 (cd)	6 (b)	6 (a)	9 (b)	-	-	-	-	-	-	-
Total bark	6 (a)	12 (d)	6 (a)	6 (a)	6 (a)	0 (a)	0 (a)	13 (d)	10 (c)	0 (a)	0 (a)	0 (a)
n-hexane	6 (ab)	7 (a)	6 (a)	6 (a)	8 (b)	0 (a)	0(a)	5 (b)	15 (e)	0 (a)	0 (a)	0 (a)
CH2CL2	6 (a)	6 (a)	9 (b)	6 (a)	8 (b)	0 (a)	0 (a)	10(cd)	15 (e)	0 (a)	0 (a)	0 (a)
AcOet	11 (ef)	12 (d)	8 (b)	11 (b)	8 (b)	0 (a)	15 (b)	10 (c)	13 (d)	0 (a)	13 (c)	8 (b)
ferruginol	11 (f)	12 (d)	9 (b)	6 (a)	11 (c)	-	-	-	-	-	-	-
sugiol	8 (bc)	6 (a)	6 (a)	6 (a)	12 (c)	-	-	-	-	-	-	-
streptomicina	31 (g)	32 (f)	31 (c)	31 (c)	31 (c)	-	-	-	-	-	-	-
Control	6 (a)	6 (a)	6 (a)	6 (a)	6 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)	0 (a)
Copper oxide	-	-	-	-	-	0 (a)	0 (a)	0 (a)	5 (b)	30 (b)	43 (f)	0 (a)

Within columns, the mean diameter followed by the same letter indicates no significant difference at P=0.05.

The crude wood extract showed greater antibacterial activity than the crude bark extract, although the ethyl acetate extract of bark had a higher specific activity in bacteria. The crude wood and n-hexane wood extracts showed the highest antifungal activity (Table 2). The ethyl acetate bark extract´s antifungal activity was not only high, it was also the only extract active against the Gram-negative bacterium, E. coli. It is important to note that this extract concentrated the greatest diterpene proportions (6,7-dehydroferruginol, ferruginol, sugiol, totarol). The h-hexane wood extract was highly activity against bacteria and fungi, and this was the extract from which the 10 sesquiterpenes were purified and identified.

The sesquiterpenes with the highest antibacterial activity were 15-copaenol, cubenol, and torreyol. The presence of an OH group, which is an efficient uncoupler of the bacterial plasma membrane creates instability and breaks the membrane´s phospholipid-sterol interactions and is often lethal [27].

Among the isolated diterpenes, the most active compounds were ferruginol and hinokiol; sugiol and hinokione were less active. This is in agreement with previous reports associating the of wood´s resistance with high concentrations of phenolic diterpene compounds [11,16].

Overall the phenolic group joined to a single hydroxyl group confers lipophilicities and acidity, important factors in antifungal activity, to the molecule [26]. Assayed for antimicrobial activity, the phenolic diterpenes with more hydroxyl groups showed greater activity over bacteria and fungi [11,16,28], as reflected in this study´s results. A mevalonate pathway synthesizes the phenolic diterpenes and the aromatic ring is originally derived from a shikimate pathway. Phenolic compounds, however, are principally synthesized by a shikimate pathway [29].

The screening results of sesquiterpenes and diterpenes agree with the general structural requirements necessary for antifungal activity. The presence of at least one hydroxyl groups, a degree of lipophilicity, and the degree of molecule conjugation are especially important factors to consider [26].

The major compounds in the bark were the diterpenes 6.7-dehydroferruginol, ferruginol, and sugiol. These were mainly concentrated in the bark´s ethyl acetate extract, which contained the highest activity against most bacterial strains. In contrast, the major compounds in wood were sesquiterpenes, extracted mainly by n-hexane, which had the highest activity against most fungal strains. The wood´s n-hexane extract also contained several compounds like 15-copaenol, cubenol, torreyol, copaene, and a-cubebene with important biological activities against several bacteria. These screening results seem to imply that

Pilgerodendron uviferum´s resistance to microbial attack, in both wood and bark, is due to sesquiterpenes and diterpenes. Based on the purified standards, it should now be possible to quantify the levels of antimicrobial substances in P. uviferum wood and bark in order to test this.

4. CONCLUSIONS

The total crude wood and bark extracts had inhibitory biological activity against bacteria and fungi. The sesquiterpenes with the highest antibacterial activity were 15-copaenol, cubenol, and torreyol, which were found in the η-hexane partial wood fraction. The diterpenes with the highest antibacterial activity were hinokiol from the η-hexane wood fraction and ferruginol from the ethyl acetate bark fraction.

P. uviferum wood and bark extracts have secondary metabolites with inhibitory biological activity against bacteria and fungi, and it may be possible to correlate the presence of these substances with the natural resistance of *P. uviferum* wood and bark against microbial attack.

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