ISOLATED LIGNANS OF *ARAUCARIA ARAUCANA* (MOLINA) K. KOCH PROVIDE WOOD PROTECTION AGAINST ATTACK BY THE XYLOPHAGOUS FUNGUS *PLEUROTUS OSTREATUS* (JACQ.) P. KUMM

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ABSTRACT

Araucaria araucana (Molina) K. Koch is an evergreen conifer endemic of Southern Chile and it is considered a sacred tree by the Pehuenche originary people. *A. araucana* is endangered in the red book. The knots wood of *A. araucana* are resistant to biological degradation, due to producing a high amount of lignans, even after the decomposition of the tree is possible to find its knots in the forest. In the present study the chemical composition of three wood tissue of *A. araucana*; knotwood, stemwood, and branches were analyzed by HPTLC-MS, GC-MS, and NMR. Three lignans were purified and identified as eudesmin (1), ((1*S*,2*R*,3*R*) 1,2,3,4-tetrahydrona phthalene-2,3-diyl) dimethanol (2) and secoisolarisiresinol (3). Folin-Ciocalteu, DPPH and resistance to biodegradation assays were evaluated to extracts from compressed wood zones. Eudesmin was identified as the principal lignan in knots with a 0.5%$^{w}/_{w}$ and outperformed the fungicide Nipacide® P511 in protecting wood against xylophage fungi *Pleurotus ostreatus* (Jacq.) P. Kumm.

Keywords: *Araucaria araucana*, lignan, knotwood composition, eudesmin, wood resistance, *Pleurotus ostreatus* (Jacq.) P. Kumm.

1. INTRODUCTION

In Chile, *Araucaria araucana* (Molina) K. Koch is a native conifer that grows up to 50 m high and can live beyond 1000 years[1]. In South America, there are two species of genus *Araucaria*; *Araucaria angustifolia (Bertol.) Kuntze* from Brazil and *Araucaria araucana* (Molina) K. Koch endemic to the southern region of Argentina and Chile, with a current distribution between 37°20′S and 40°20′S[2]. The tree is considered sacred for the native people "Pehuenche", who lives by collecting the seeds of the tree, called "Piñon". *A. araucana* has a cylindrical and straight trunk up to 2 m in diameter. Its appearance is characteristic of having a pyramidal crown like an umbrella, due to the lower branches are naturally pruned. The branching pattern is a regular monopodial, with 3 to 7 branches per whorl, perpendicular to the trunk, which produces straight and cylindrical logs.

The wood of *A. araucana* is light and has a yellowish-white color. Therefore, the logs were preferred in boat masts' manufacturing. Nowadays, *Araucaria* is officially protected in Chile as an endangered species. The thick bark (up to 10 cm) of the tree, gives defense against predators, but processed wood gets easily degraded by several biotic (insects, fungi) and abiotic factors. *Pleurotus ostreatus* (Jacq.) P. Kumm is the most common pathogen found on dead trees and it is involved in the decomposition of the deadwood[3]. Intriguingly, though the dead tree is entirely biodegraded, the tree's knots remain intact without decomposition in the forest. The knots popularly called *picoyo* or *chochín* are light brown in color, tough, and heavy. The knots formed by wood compression contain higher amounts of lignans than heartwood and sapwood[4]. For example, in the conifer "Norway spruce" (*Picea abies*), the average of organic extractables from knots is 120-folds higher than heartwood and 240-folds than sapwood, being lignans the major components[5, 6].

Phytochemical studies of *A. angustifolia* knots showed a variety of lignan compounds, such as pinoresinol, that gives higher resistance to biodegradation by xylophages fungus compared to the rest of the wood[7]. It is known that the heartwood and resins from *A. araucana* contain lignans, terpenoids, and resinic acids[8,9]. While knotwood has a higher composition of lignans as eudesmin[4]. Therefore, the present study aimed to determine the chemical composition of *A. araucana* knots and assess its role in the resistance against biodegradation by xylophagous fungus *Pleurotus ostreatus* (Jacq.) P. Kumm.

2. EXPERIMENTAL

2.1 Plant material

Samples of stemwood, branch and knots of *A. araucana* were collected in May 2018 from Nahuelbuta Range (Biobío Region) on the border with the Araucanía Region (37°41′43.56″S 73°07′49.34″W. Altitude of 716 m above sea level). The samples were identified by the botanist Dr. Roberto Rodríguez from the University of Concepcion. After verification, 100 g samples were chopped, dried at 40 ºC for 8 h, and milled to 1–3 mm. The samples were then stored with the code GMAA2018 in the Laboratory of Chemistry of Natural Products of the University of Concepcion.

2.2. Extraction and purification of extractives from or wood samples *A. araucana*

The wood samples of *A. araucana* were extracted in a continuous extraction system (Soxhlet) following the protocol of Willfor et al. 2006[10]. Briefly, 100 g of stemwood, branch, and knots were extracted continuously in acetone (1200 mL) by 10 h. Then the organic solvent was evaporated *in vacuo* to obtain the final extract (26.1 g). The extracts were then kept at −20°C until use.

10 grams of the total knot extract was fractionated by silica gel column chromatography (CC) using Merck silica gel 60 (25−100 µm; Aldrich, Santiago, Chile), in four fractions (AA-F1 to AA-F4) by different solvents of increased polarity from n-hexane to ethyl acetate (EtOAc). The fractioning was followed by analytical thin-layer chromatography (TLC, Merck Silica Gel 60F254 sheets, Darmstadt, Germany), for visualization UV light (254/365 nm) together with molibdophosforic acid were used. Fraction AA-F1 (1.5 g) was eluted by CC with n-hexane/EtOAc 1:1 ($^{v}/_{v}$) and further crystallized at −20 °C, giving compound 1 (1.1 g, white solid, 0.75% yield in dry wood). Fraction AA-F2 (3.2 g) was eluted with n-hexane/EtOAc 1:3 ($^{v}/_{v}$), producing the compound 2 (250 mg after crystallization, amorphous solid, 0.17% yield in dry wood). Fraction AA-F3 (3.5 g) was eluted with n-hexane/EtOAc 1:4 ($^{v}/_{v}$), producing compound 3 (8 mg, amorphous solid, 0.0054% yield in dry wood). Fraction AA-F4 (2.2 g) produced a mixture of polyphenols which were not purified.

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2.3 Identification of natural compounds from *Araucaria araucana*

2.3.1 NMR analysis

The ^{1}H NMR and ^{13}C NMR spectra were recorded in acetone-d_6 or DMSO-d_6 solution in 5 mm tubes at 25ºC on a Bruker Avance NEO 400 MHz, AVANCE NEO 500 MHz or AVANCE III 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), with the signal of TMS (for ^{1}H) or the solvent (for ^{13}C) as the internal standards. All spectra (^{1}H, ^{13}C, gs-H,H−COSY, edited HSQC, and gs-HMBC) were acquired and processed with the standard Bruker software.

2.3.2 Gas chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS analysis was performed using Agilent 7890 GC system (California, USA), equipped with an Agilent 5975 mass detector, using a column HP5-MS 30 m x 0.25mm silica and a 0.25 m film thickness. Mass spectra were obtained from the total ion current (TIC) and compared with standard mass spectra in addition to the NIST07 database (NIST 2008, National Institute of Standards and Technology). The obtained mass spectra were compared with those reported in the literature for interpretation (Yamamoto et al. 2004; Yamamoto et al. 2010).

2.3.3 Planar chromatography or High-performance Thin Layer Chromatography-Mass Spectrometry (HPTLC-MS)

HPTLC-MS was carried out on 10 x 10 cm HPTLC Silica gel 60 F254 plates. 2D chromatography was performed under the following conditions: 30 µL of *A. araucana* knotwood extract (12 mg mL^{-1}) was added to the plate and first eluted with CH_2Cl_2/EtOH (94:6 $^{v}/_{v}$) mobile phase two times. The eluate was removed, dried at 25°C, and developed with toluene/EtOAc/formic acid mixture (6:4:1 $^{v}/_{v}$). Selected bands were eluted from the plate to MS using a CAMAG TLC-MS interface assembled with an oval elution head (4.0 x 2.0 mm) using a mixture of methanol and acetonitrile (1:1 $^{v}/_{v}$) at a flow rate of 0.1 mL min^{-1} for 60 s. MS analysis was performed on Shimadzu (Kyoto, Japan) LC-MS 8030 triple quadrupole mass spectrometer with electrospray ionization (ESI) source operated with the following conditions: ESI in positive and negative modes, capillary voltage 4.5 kV, nebulizing gas (N_2) 3 L min^{-1}, drying gas (N_2) 15 L min^{-1}, desolvation line temperature 250 ºC, and block temperature 400 ºC. Mass spectra were acquired in full scan mode between m/z values of 50 and 2000. Plate background signals were subtracted for each analysis. Data were acquired and recorded by Shimadzu LabSolution software version 5.51.

2.4 Determination of total phenols

Total phenol content was determined using the Folin-Ciocalteu method. Briefly, 200 µL of extract solution (1 mg mL^{-1}) was added to 1000 µL of Folin-Ciocalteu (0.1 g L^{-1}) 2 N Merck® 10 % ($^{v}/_{v}$) reagent and the mixture was kept at room temperature for 8 min. Then 800 µL of Na_2CO_3 (75 g L^{-1}) solution was added, and the mixture was incubated at 45 °C for 15 minutes. The absorbance of the complex blue mixture was then measured at 750 nm using Bio-Tek ELx800 microplate reader against water as a blank sample. The obtained results were expressed as µg mL^{-1} gallic acid equivalent GAE (µg mL^{-1} GAE/DW). Three independent experiments (n=3) were carried for determination total phenols.

2.5 DPPH Assay

The antioxidant activities of knotwood, stemwood, and branch extracts were evaluated by DPPH radical reduction test. Briefly, the stock solutions (1000 mg mL^{-1}) were diluted to final concentrations of 100, 50, 25, and 10 µg mL^{-1} in ethanol. An aliquot of 150 µL was taken from each sample and mixed with an equal volume of DPPH solution (60 mM). The absorbance was measured at 520 nm. The results were expressed as the percentage of antioxidant activity. The % DPPH radical inhibition was calculated with the following formula:

$$DPPH\ (\%) = (Abc - Abs)/Abc \times 100$$

where Abc is the absorbance of DPPH˙ mixed with an ethanol solution, and Abs represents the absorbance of DPPH˙ mixed with samples. Three independent experiments (n=3) were carried for assay.

2.6 Decay resistance test against basidiomycetes xylophagous fungi

The resistance against wood-destroying basidiomycetes was performed with the assay code UNE-ENV 12038, with few modifications. Briefly, Pinus radiata wood or *A. araucana* stemwood blocks (50 mm x 25 mm x 15 mm), with the longitudinal faces parallel to the fiber direction, were extracted with acetone for 3 h. Then, blocks were dried at room temperature for 48 h, followed by at 103 ºC for 16 h. The woodblocks were weighed to obtain the initial dry mass (m1). 10 test woodblocks per treatment were impregnated with the following solutions separately: commercial fungicide Nipacide® P511 5% $^{w}/_{v}$, as the positive control T1; knotwood-extract of 1, 5 and 15 % $^{w}/_{v}$ T2, T3 and T4 respectively; eudesmin 0.5% $^{w}/_{v}$ T5, and blank control CT (without treatment).

Pleurotus ostreatus is a native strain collected in the Nahuelbuta mountain range. It was provided by the fungal collection of the laboratory of Natural Products, University of Concepción, code FQ1646. The fungus was cultured onto malt-agar, incubated for one week in flasks of 1 L. Then, two wood blocks were introduced into the flask with the fungus. The flasks were then filled with vermiculite until the blocks were covered entirely, and then were incubated for 16 weeks at 25 ºC in dark. Afterward, the woodblocks were removed, and the adhering mycelium was carefully removed, then they were dried at 103 ºC until reaching a constant weight (m2). The loss of biomass was calculated using the following formula:

$$\%\ Loss\ of\ biomass = (m1 - m2)/m1 \times 100.$$

Where m1 is the initial dry mass. Three independent experiments (n=3) were carried for assay.

2.7 Statistical analysis

The data were presented as means ± standard deviation (SD). The one-way analysis of variance (ANOVA) was performed to compare the differences of each treatment. Subsequently, a Tukey HSD post-hoc test was used to compare the means of each treatment. Prior to these analyses, the assumptions of Shapiro-Wilk normality and Levene variance homogeneity test were performed. Differences were statistically significant at $P< 0.05$. All statistical analyses were performed with the program Statistica™ 12.0.

3. RESULTS AND DISCUSSION

The organic compounds from different parts of *A. araucana* were remarkably higher in knotwood (26.1% $^{w}/_{w}$), compared to the branch (1.2% $^{w}/_{w}$) and stemwood (0.9% $^{w}/_{w}$) (TABLE 2). The GC-MS analysis showed that extract of *A. araucana* knotwood was rich in lignans (89.57%). In addition, it also contained terpenes and fatty acids (3.76%) and triterpenes (6.93%). The purification by chromatographic column and crystallization yielded three compounds as solids (Figure 1).

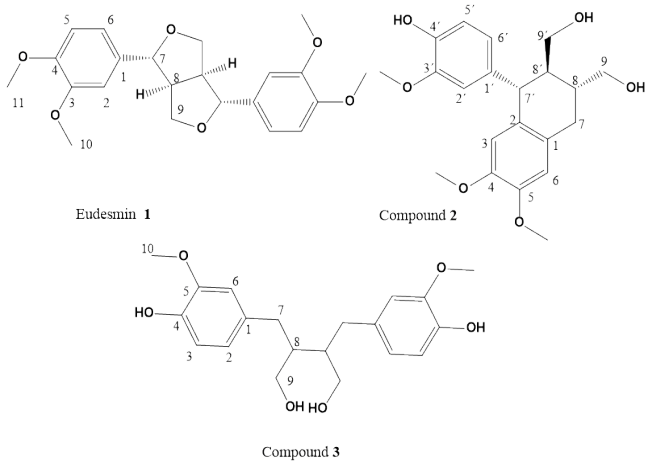

Figure 1. Structure of isolated lignans from *Araucaria araucana* knotwood.

The structure of isolated compounds was determinate by NMR analysis. The results agree with data reported in literature [14, 15]. Eudesmin. ^{1}H NMR (600 MHz,$CDCl_3$, ppm): 7.02 (d, J = 1.0 Hz, 1H, H-2), 6.92 (d, J = 8.5 Hz, 1H, H-5), 6.94 (dd, J = 2.0, 6.0 Hz, 1H, H-6), 4.73 (d, J = 4.5 Hz, 1H, H-7), 4.78 (m, 1H,

H-9), 4.28 (m, 1H, H-9), 3.92 (s, 3H, C-11), 3.90 (s, 3H, C-10), 3.12 (m, 1H, H-7). ^{13}C NMR (150 MHz, CDCl3, ppm): 149.0 (C-3), 147.9 (C-4), 134.2 (C-1), 118.8 (C-6), 111.2 (C-5), 109.7 (C-2), 86.9 (C-7), 71.4 (C-9), 55.6 (C-11), 55.5 (C-10), 54.3 (C-8). Formula: $C_{22}H_{26}O_6$.

Compound 2. ^{1}H NMR (500 MHz, DMSO-d_6, ppm): 6.70 (overlap, 1H, H-5´), 6.68 (overlap, 1H, H-6), 6.66 (d, *J* = 1.7 Hz, 1H, H-2´), 6.50 (dd, *J* = 1.7, 6.5 Hz, 1H, H-6´), 6.21 (s, 1H, H-3), 3.84 (d, *J* = 9.8 Hz, 1H, H-C7´), 3.71 (s, 3H, OCH_3/C-5), 3.69 (s, 3H, OCH_3/C-4), 3.57 (m, 1H, H-9), 3.46 (overlap, 1H, H-9), 3.46 (overlap, 1H, H-9´), 3.46 (s, 3H, OCH_3/C-3´), 3.20 (m, 1H, H-9´), 2.71 (m, 2H, H-7), 1.83 (m, 1H, H-8), 1.66 (m, 1H, H-8´). ^{13}C NMR (125 MHz, DMSO -d_6, ppm): 147.3 (C-3´), 146.8 (C-5), 146.6 (C-4´), 144.6 (C-4), 137.0 (C-1´), 132.3 (C-2), 129.3 (C-1), 121.4 (C-6´), 115.3 (C-5´), 113.3 (C-3), 113.1 (C-2´), 111.7 (C-6), 63.6 (C-9), 59.9 (C-9´), 55.7 (OCH_3/C-3´), 55.6 (OCH_3/C-4), 55.4 (OCH_3/C-5), 45.9 (C-8´), 45.9 (C-7´), 38.0 (C-8), 32.2 (C-7). Formula: $C_{21}H_{26}O_6$.

Compound 3. Secoisolariciresinol. ^{1}H NMR (600 MHz, acetone-d_6, ppm): 6.73 (s, 1H, H-6), 6.71 (d, *J* = 7.8 Hz, 1H, H-2), 6.61 (d, *J* = 7.8 Hz, 1H, H-3), 3.75 (s, 3H, H-10), 3.69 (dd, *J* = 2.3, 10.9 Hz, 1H, H-9), 3.54 (dd, *J* = 3.7, 10.9 Hz, 1H, H-9), 2.68 (m, 1H, H-7), 1.92 (m, 1H, H-8). ^{13}C NMR (150 MHz, acetone-d_6, ppm): 148.0 (C-5), 145.4 (C-4), 133.5 (C-1), 122.3 (C-3), 115.4 (C-2), 113.2 (C-6), 61.2 (C-9), 56.1 (C-10), 44.6 (C-8), 36.0 (C-7). Formula: $C_{22}H_{26}O_6$.

The phytochemical study of the knotwood extract by HPTLC-MS and GC-MS identified eleven related lignan compounds by analyzing the *m/z* signals of each spectrum and confirmed with supporting literature [11,12,13] and the fragmentation patterns correlated with the library NIST17 database (Table 1, Figure 1).

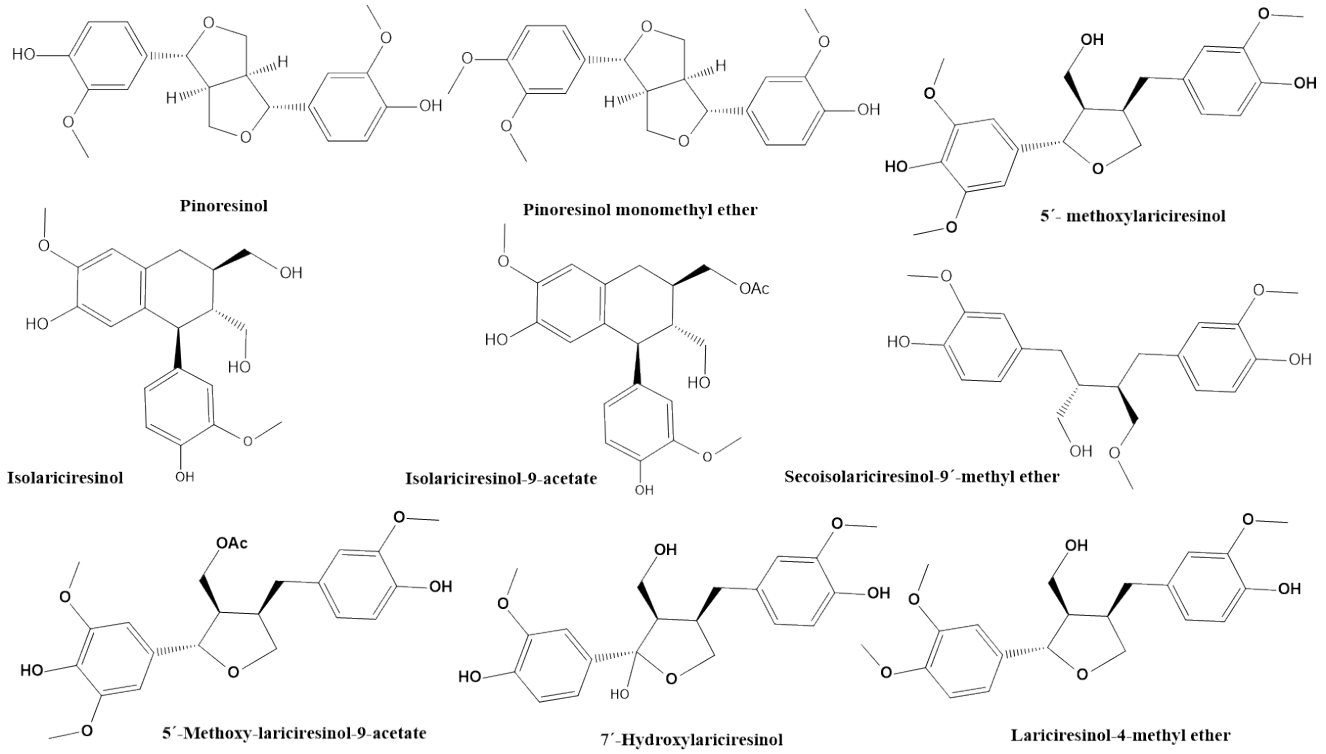

Figure 2. Structures of lignans determined by HPTLC-MS.

Table 1. Compounds identified in *Araucaria araucana* knots by HPTLC-MS.

Compounds	Formula	Mw	Rf-1D	Rf-2D
Secoisolariciresinol (3)	C20H26O6	362	0.330	0.242
Secoisolariciresinol-9'-methyl ether	C21H28O6	376	0.302	0.368
Lariciresinol-4' (or 4)-methyl ether	C21H26O6	374	0.375	0.547
5´(5 or 7´)-Methoxy-lariciresinol-9-acetate	C23H28O8	432	0.391	0.829
7'-Hydroxylariciresinol (or isomer)	C20H24O7	376	0.658	0.129
7' (or 5)-Methoxylariciresinol	C21H26O7	390	0.375	0.412
Pinoresinol	C20H22O6	358	0.565	0.776
Pinoresinol monomethyl ether	C21H24O6	372	0.569	0.881
Pinoresinol dimethyl ether (1)	C22H26O6	386	0.581	0.948
Isolariciresinol	C20H24O6	360	0.253	0.207
Isolariciresinol-9 (or 9´)-acetate	C22H26O7	402	0.385	0.141

Rf-1D: Retention factor of the first development with dichloromethane-EtOH 92:8 ($^v/_v$) mobile phase two times. Rf-2D: Retention factor of the second development with toluene/ethyl acetate/formic acid mixture 6:4:1 ($^v/_v$).

These compounds were previously reported in conifers as *Araucaria angustifolia* (Bertol.) Kuntze[7], *Picea abies* (L.) H.Karst, *Abies alba Mill*.[3] and *Fitzroya cupressoides* (Molina) I.M.Johnst[8]. Moreover, knots of *A. angustifolia*, from Brazil, have shown to contain secoisolariciresinol, pinoresinol, eudesmin, monomethyl pinoresinol, hinokiresinol, monomethyl lariciresinol, dimethyl lariciresinol, and lariciresinol[7,6], with a similar composition than the knots of *Araucaria araucana*. The knots of these both conifers have a similar biodegradation pattern, remaining in the soil for years without degradation, suggesting the protective effects of higher lignan content in these parts of the tree. Lignans could be responsible for the chemical defense in response to an eventual injury of the tree, and their content change under abiotic stress e.g., cold and snow[6,9]. The total phenols in stemwood, branch and knotwood of *A. araucana* were analyzed by the Folin-Ciocalteu method and DPPH antioxidant activity (Table 2). The yield of extracts from knotwood, branch, and stemwood of *A. araucana* were significantly different. The yield of knotwood extracts was 29- and 21.8-fold higher than that of stemwood and branch extracts, respectively. Similarly, the total polyphenol content of knotwood extract was the highest of three samples (365 µg mL^{-1} GAE/DW), followed by branch (336 µg mL^{-1} GAE/DW) and stemwood extracts of *A. araucana* (147 µg mL^{-1} GAE/DW). The DPPH activities of the extracts from *A. araucana* were in the order of knotwood > branch > stemwood, reflecting a direct correlation between the extracts and the total polyphenol content (Table 2). Extractives from *A. araucana* knotwood is 26% $^{w}/_{w}$, which compared with literature values of heartwood extracts from *Austrocedrus chilensis, Fitzroya cupressoides, Pilgerodendron uviferum* species, *A. araucana* exceeds the rest of the coniferous species[15].

Table 2. The total yield of extracts (% $^{w}/_{w}$), total phenol content (TPC) and antioxidant effect of reducing DPPH radical from stemwood, branch and knotwood of *Araucaria araucana*. Results are compared with heartwood extracts obtained from other woody conifers with higher natural durability of their wood.

	Yield of extracts	TPC (µg mL^{-1} GAE/DW)	% DPPH radical inhibition at different concentrations (µg mL^{-1})			
Extracts	% w/w		10	25	50	100
Araucaria stemwood	0.9	147.48 ± 0.18^{A}	8.88 ± 0.8^{D}	16.97 ± 1.7^{C}	36.73 ± 1.2^{B}	68.16 ± 2.0^{A}
Araucaria branch	1.2	336.60 ± 0.46^{B}	35.43 ± 0.7^{D}	50.40 ± 1.9^{C}	65.57 ± 1.5^{B}	73.35 ± 1.8^{A}
Araucaria knotwood	26.1	365.94 ± 0.32^{C}	42.51 ± 1.6^{C}	64.37 ± 2.0^{B}	66.47 ± 1.8^{B}	84.03 ± 0.6^{A}
Austrocedris chilensis stemwood [a]	6	-	79.30±0.8	83.00±0.1	86.20±1.0	84.20±1.0
Fitzroya stemwood [a]	2	-	68.20±0.7	79.30±0.4	89.20±1.1	84.10±0.3
Pilgerodendron uviferum stemwood [a]	4	-	66.80±0.4	72.00±0.5	83.80±0.5	87.00±0.8

Data are shown as Mean ± SD (n=3). Values indicated by different superscript letters differ significantly (Tukey's test). [a] Donoso et al., 2008[1].

The decay resistance test to basidiomycetes xylophagous fungi shows that after 12 weeks, the pinewood blocks evidence the progressive decay of biomass caused by *Pleurotus ostreatus*. The control blocks (CT) lost 11 ± 0.5% $^{w}/_{w}$ ($p < 0.05$), in contrast to pinewood blocks treated with different extracts (T1=Nipacide 5%; T2= Knotwood Extract 1%; T3= Knotwood Extract 5%; T4= Knotwood Extract 15%; T5= eudesmin 0.5%), which reported a lower loss of biomass within 1 ± 0.3 to 5 ± 0.7%. Tukey's test showed that there were no significant differences between T1 and T3 or T3 and T4 treatments, respectively (Figure 3).

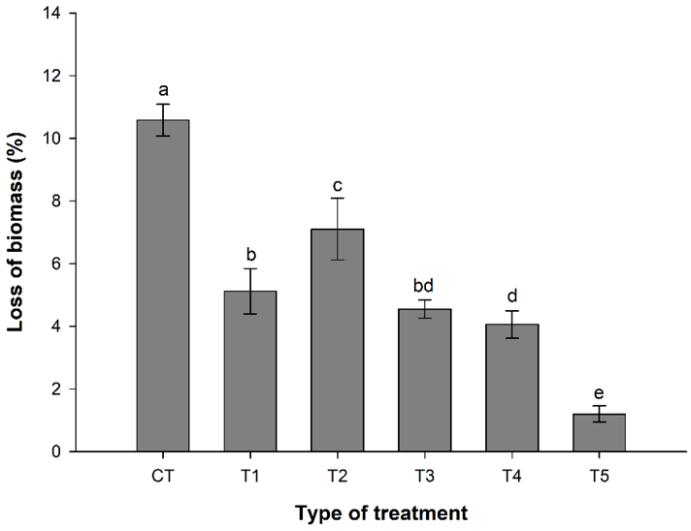

Figure 3. Percentage loss of biomass in wood (mean ± standard deviation) exposed to different treatments with white-rot fungi for 12 weeks (n=10). Values with different letters are significantly different (Tukey's test). CT= Control; T1= Nipacide; T2= 1% $^{v}/_{v}$ Knotwood Extract; T3= 5% $^{w}/_{v}$ Knotwood Extract; T4= 15% $^{w}/_{v}$ Knotwood Extract; T5= 0.5% $^{w}/_{v}$ eudesmin.

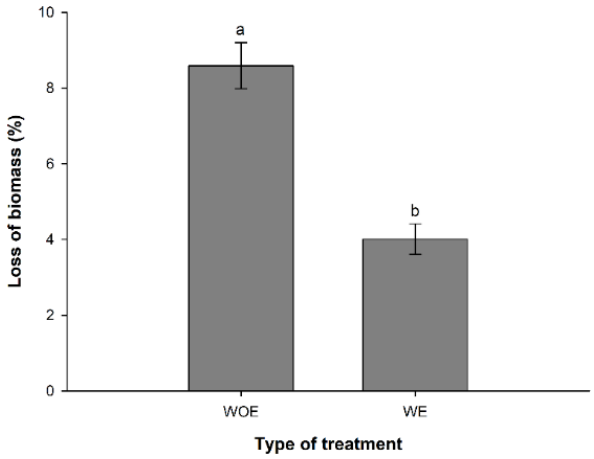

Figure 4. Percentage loss of biomass in the stemwood of *A. araucana* ± standard deviation exposed to treatments with white-rot fungi for 12 weeks (n=10). Values with different letters differ significantly (Tukey's test). WOE=Araucaria stemwood after remotion of extractables with acetone; WE=Araucaria stemwood control.

However, treatment with 0.5% eudesmin (T5) was significantly more effective in preventing the xylophagous fungus attack, even better than the commercially available product Nipacide (Figure 4). Moreover, the stemwood of *A. araucana* which was removed of organic extractables (WOR), lost a significantly higher percentage of biomass (8.59 ± 0.6%) compared to the Control (WE) These results confirmed the role of extractives in resistance to fungi biodegradation (Figure 4).

CONCLUDING REMARKS

The phenolic compounds and extractables have been reported to influence natural wood durability. In *Araucaria araucana*, the accumulation of lignans in the knots generates a chemical barrier that protects an area vulnerable to attack by pathogens, due to the constant loss of branches by self-pruning. This is an explanation of why the knotwood has a remarkable higher content of organic extractables with 26.1% $^{w}/_{w}$, compared to stemwood and branchwood with 0.9 $^{w}/_{w}$ and 1.2% $^{w}/_{w}$ respectively. Moreover, these organic components showed high antioxidant levels, which suggests that the protective activity is due to the antioxidant action of these compounds. In the knots of *A. araucana* the furofurane lignan eudesmin showed to be effective in wood protection by fungi degradation.

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