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Actividad inhibidora de lignanos presentes en *Araucaria araucana* (Molina) K. Koch, frente a hongos degradadores de madera, un modelo de estudio

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DEDICATORIA

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RESÚMEN

Araucaria araucana (Molina) K. Koch también llamada "Pehuén", es una conífera nativa de Chile, que crece hasta los 50 m en las altas montañas del sur del país. Su madera fue apreciada en el pasado y posee una moderada durabilidad natural, sin embargo, los nudos de la *A. araucana* se caracterizan por ser excepcionalmente duros y muy resistentes a la biodegradación ambiental. Estas propiedades son aprovechas por artesanos de la zona de la Araucanía para confeccionar hermosas piezas de joyería, denominando a los nudos con el popular nombre *picoyo* o *ámbar chileno.* Con el fin de explicar esta durabilidad natural, se evaluó la composición química de diferentes partes de *A. araucana*, madera de tronco, ramas y nudo, encontrando en este último 26 veces más de extraíbles que en el resto de la madera. Se identificaron 12 tipos de lignanos por HPTLC-MS. El secoisolariciresinol (45 mg $g^{-1)}$ es el lignano más abundante seguido de eudesmin (23 mg $g^{-1)}$ y otros lignanos en menor concentración. El extracto de madera de nudos y la eudesmin se utilizaron como tratamiento de impregnación en ensayos de resistencia de la madera al ataque del hongo xilófago de pudrición blanca *Pleurotus ostreatu*s, mostrando un efecto protector en concentraciones del 1% p/v del extracto, mientras que el tratamiento con eudesmin al 0,5% p/v mostró una disminución significativa del ataque del xilófago. Se determinó que la acumulación de lignanos en los nudos genera una barrera química que protege una zona vulnerable del árbol de *A. araucana* al ataque de patógenos.

ABSTRACT

Araucaria araucana (Molina) K. Koch also called "Pehuén", is a conifer native to Chile, which grows up to 50 m in the high mountains of the south of the country. Its wood was appreciated in the past and has a moderate natural durability, however, the knots of the *A. araucana* are characterized by being exceptionally hard and very resistant to environmental biodegradation. These properties are used by artisans from the Araucania region to make beautiful pieces of jewelry, calling the knots with the popular name of Picoyo or Chilean amber. In order to explain this natural durability, the chemical composition of different parts of *A. araucana*, trunk wood, branches and knots was evaluated, finding in the latter 26 times more extractable than in the rest of the wood. Twelve types of lignans were identified by HPTLC-MS. The secoisolariciresinol (45 mg g-1) is the most abundant lignan followed by eudesmin (23 mg g-1) and other lignans in lower concentration. Knots wood extract and eudesmin were used as impregnation treatment in trials of wood resistance to attack by the white rot xylophagous fungus *Pleurotus ostreatus*, showing a protective effect in concentrations of 1% w/v of the extract, while the treatment with eudesmin at 0.5% w/v showed a significant decrease in the attack of xylophagous. It was determined that the accumulation of lignans in the nodes generates a chemical barrier that protects a vulnerable zone of the *A. araucana* tree to the attack of pathogens.

CAPITULO I. Introducción General

Breve historia de las coníferas

Las gimnospermas son plantas vasculares y espermatofitas, originadas en la era carbonífera (período Pennsylvanense, 300 millones de años atrás) de gran importancia ecológica y económica. Aunque este antiguo y extenso linaje de plantas comprende actualmente un poco más de 1000 especies, son dos o tres órdenes de magnitud inferiores a las aproximadamente 300.000 especies de angiospermas existentes. Su sobrevivencia y éxito evolutivo a nuestros días se debe en parte a su capacidad de tolerar climas hostiles, suelos de bajos nutrientes y poseer una defensa química efectiva contra depredadores (Trapp y Croteau 2001; Wang y Ran 2014).

Las gimnospermas están distribuidas en todos los continentes excepto en la Antártida, de las cuales dos tercios son coníferas, un grupo que constituye más del 39% de los bosques del mundo (Figura 2). Este grupo de plantas desempeñan un papel importante en los ciclos mundiales del carbono, proporcionan importantes fuentes de madera, resinas e incluso fármacos y

alimentos (Zonneveld 2011; Wendel et al. 2012) además de ser cruciales para prevenir la erosión del suelo.

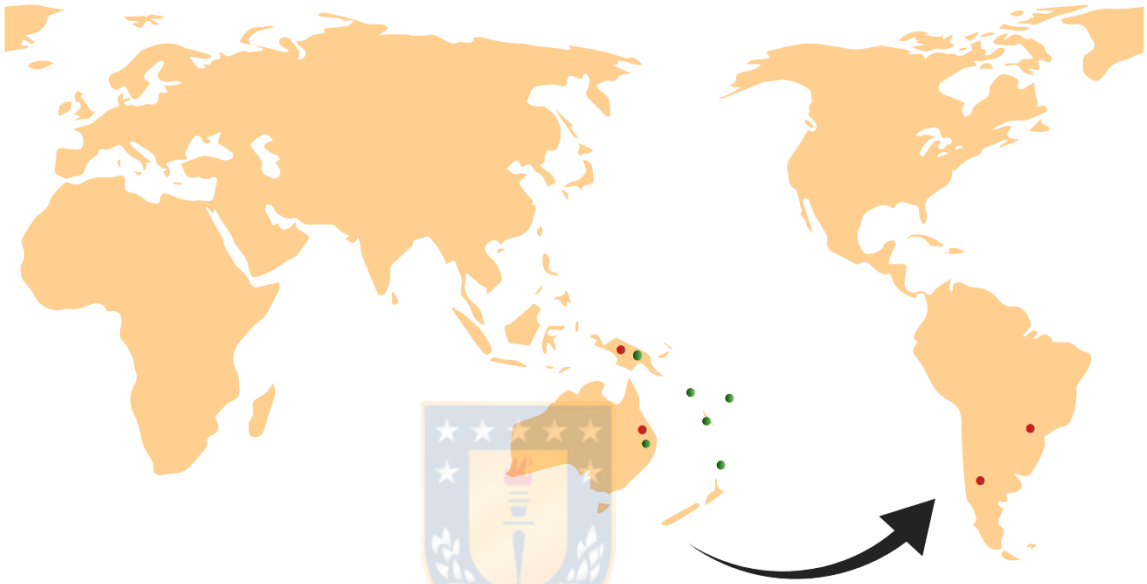

Figura 1 Distribución global de especies de Araucariaceae. En rojo especies de la sección Araucaria y en verde la sección Eutacta. La flecha indica la posible ruta de migración considerando la Antártida (Kershaw y Wagstaff 2001; Wang y Ran 2014).

La familia Araucariaceae está representada por 3 géneros (*Agathis*, *Wollemia* y *Araucaria*), y el género *Araucaria* tiene 19 especies distribuidas en el hemisferio Sur (Figura 1). Esta familia de plantas leñosas es considerada como una de las más antiguas coníferas cuyos primeros ancestros se reportan del período Carbonífero (360 millones de años atrás) alcanzando máxima diversidad y distribución en casi todo el mundo en los períodos Jurásico y

Cretácico (200-150 millones de años atrás) (Stockey 1982; Axsmith, Escapa, y Huber 2008).

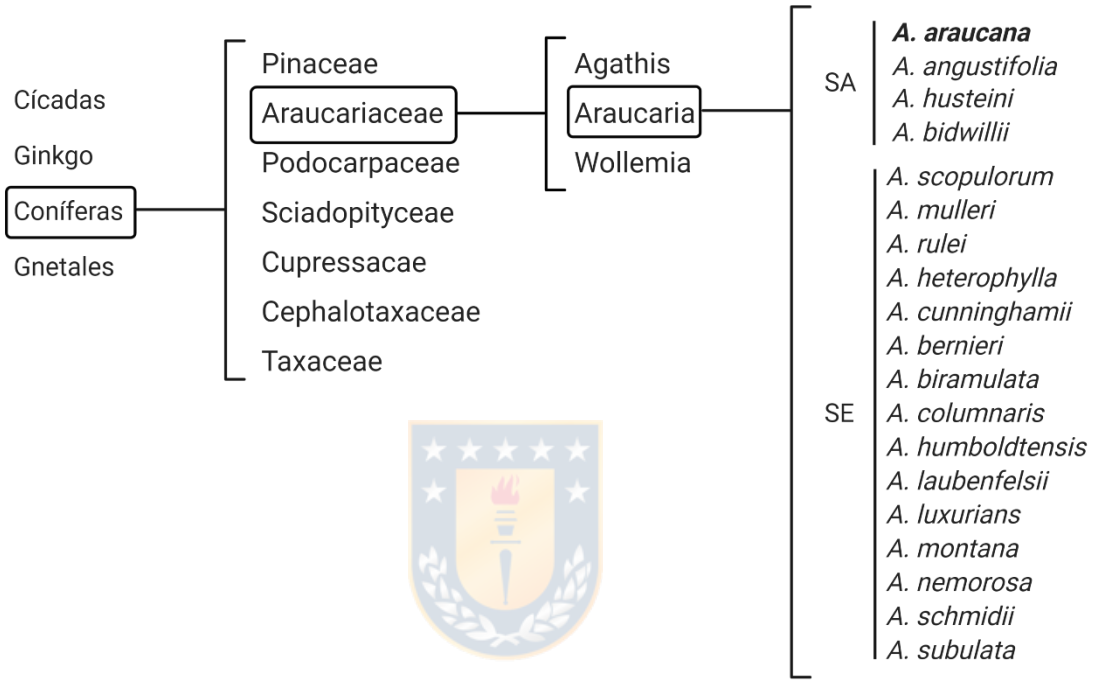

Figura 2 Filogenia de la conífera *A. araucana*. SA Sección Araucaria. SE Sección Eutacta (Kershaw y Wagstaff 2001).

Dentro del género de Araucaria, hay dos secciones, que comparten morfología y parentesco en los análisis moleculares (Stefenon, Gailing, y Finkeldey 2006):

En la sección Araucaria con 4 especies se caracteriza por tener hojas anchas, conos de más de 12 cm de diámetro y germinación de semillas hipogea.

La sección Eutacta con 15 especies caracterizada por hojas estrechas aciculares, como punzones; conos de menos de 12 cm de diámetro y germinación de semillas epigea.

Las especies de la sección Eutacta tienen una amplia distribución en los territorios de Australia, Nueva Guinea, la isla de Norfolk y Nueva Caledonia, lo que sugiere una considerable antigüedad geológica para esta sección (Figura 1). Se presume que su distribución y la gran diversidad de especies de Nueva Caledonia es el resultado de la diversificación de la población ancestral en Gondwana durante el período eoceno, cuando gran parte de la isla estaba cubierta de rocas ultramáficas (Kershaw y Wagstaff 2001). Sin embargo, hay otras pruebas geológicas que sugieren que la isla estaba bajo el nivel del mar a principios del Cenozoico, y el transporte a larga distancia desde Australia fue a través del resurgimiento, levantamiento y emplazamiento de rocas ultramáficas durante el Eoceno. El aislamiento de la sección de Araucaria en América del Sur de las regiones de Australasia, es explicada cuando estos territorios estaban unidos en Gondwana, y la migración hacia Suramérica fue a través de la Antártida probablemente a principios del Cenozoico (Kershaw y Wagstaff 2001).

Las especies del género *Araucaria,* parecen haber mantenido una preferencia por las condiciones subtropicales o mesotérmicas. Esto queda bien ilustrado por las actuales distribuciones latitudinales de las principales especies continentales de araucaria (*A. angustifolia* y *A. araucana* en el sudeste de Sudamérica; *A. cunninghamii* y *A. bidwillii* en Australia) que son notablemente similares, a pesar de un período obviamente largo de separación continental y representación en diferentes secciones del género. La precipitación media anual no es un factor significativo, ya que *A. angustifolia* requiere un mínimo de 1400 mm por año, mayor que la media de las especies australianas. Todas las especies tienen un requerimiento mínimo de alrededor de 10ºC en los meses de invierno que, quizás junto con el requerimiento de precipitaciones significativas en verano, podría explicar la extensión meridional de la especie (Wang y Ran 2014).

Solo dos especies del género *Araucaria* están presentes en Sudamérica: *A. araucana* (Molina) K. Koch y *A. angustifolia* (Bertol) Kuntze (Farjon 2010).

La especie *A. araucana*

A. araucana es una especie nativa en Chile y posee una distribución geográfica discontinua, estando presente en la Cordillera de los Andes, entre los 37º24'S y 40º03'S, mientras que en la Cordillera de la Costa tiene un rango de distribución más restringido, con solo dos pequeñas poblaciones, una ubicada entre los 37º30'S y 38º'S y otra ubicada a los 38º30' aproximadamente. Las poblaciones costeras y andinas están separadas por el valle longitudinal chileno (Ruiz et al. 2007).

Respecto al hábitat de *A. araucana*, destaca la amplia variación climática, en la cual se desarrolla la especie, con un clima templado fresco y húmedo, con nieve en invierno y atmósfera seca durante el verano, con una variación pluviométrica que oscila entre más de 4000 mm en Chile a cerca de 1100 mm en el límite este con Argentina (Veblen et al. 2005; Montaldo 1974). En la cordillera andina del sur-centro de Chile están expuestos a disturbios de escala gruesa tales como derrumbes causados por el viento, deslizamientos de tierra provocados por terremotos, actividad volcánica, avalanchas de nieve y daños relacionados con la nieve, e incendios forestales (González, Veblen, y Sibold 2010). Por otro lado, existen significativas diferencias climáticas

entre las poblaciones del rango costero y las de los Andes. Estas diferencias se relacionan principalmente, con temperatura, suelo, precipitaciones en forma de nieve y pluviometría (Donoso 1993). El sustrato en el cual crece *A. araucana* ha sido descrito como suelos arcillosos o volcánicos (Montaldo 1974), habitando lugares menos favorables de suelo rocoso, pobremente drenados, lo que es habitual en las coníferas chilenas.

Se estima que la superficie original ocupada por poblaciones de *A. araucana* eran alrededor de 500.000 hectáreas a la llegada de los colonizadores españoles, la cual se redujo drásticamente a 261.000 ha en el transcurso del siglo XX ("CONAF" 2017)debido a la intervención del hombre que utilizaban su madera para la construcción de durmientes de ferrocarril, vigas de túneles, mástiles de buque (Herrmann 2006), fabricación de muebles, puertas, ventanas y tableros. Esta explotación y talas incontroladas (algunas de ellas concesionadas por el Estado entre la década de 1940-1960) causaron que en 1976 la especie fue declarada Monumento Natural, prohibiéndose su tala. Este estatus fue revocado en el año 1987 pero restablecido debido a la presión de los grupos Pehuenches y conservacionistas en 1990 (Cabrera-Becerra 2008).

En la actualidad *A. araucana* sigue bajo esta categoría de Monumento Natural, por lo que cualquier corta o explotación que ponga en riesgo su integridad y la sustentabilidad de los bosques de araucaria está penada por la ley. Sin embargo, a pesar de su protección legal, los bosques de *A. araucana* siguen estando bajo una continua presión ya sea por pastoreo, incendios, cosecha de semillas y ocasionalmente tala ilegal (Bekessy et al., 2004) (González et al., 2006).

Esta especie de lento crecimiento que ha logrado adaptarse con éxito en hábitats climáticamente hostiles y pobres en nutrientes. Su crecimiento y menor dispersión de semillas cuando se desarrollan en conjunto con las especies de *Nothofagus*, estos rápidamente dominan el dosel. Frente a perturbaciones como incendios las especies de *A. araucana* logran colonizar y prosperar mediante brotes. Esto se observó claramente luego del primer año del incendio en la Reserva Nacional China Muerta ocurrido en marzo del 2015 (centro-sur de Chile 38°S, 71°W), cuando brotes vegetativos de *A. araucana* crecieron desde las copas quemadas y desde raíces superficiales (Figura 3), además se comprobó que las semillas mantuvieron una alta tasa de germinación (más del 60%) (Fuentes-Ramírez et al. 2019).

Figura 3 Imágenes de araucarias quemadas con brotes en a) copa y b) raíz (Fuentes-Ramírez et al. 2019).

Durabilidad natural de la madera

Según la Asociación Española de Normalización UNE-EN 350 la durabilidad natural de una madera se define como su resistencia intrínseca, es decir sin haber recibido ningún tratamiento, para resistir el ataque de un agente xilófago.

La madera de *A. araucana* fue apreciada en el pasado en numerosos usos para la construcción, la Norma Chilena Oficial NCh 789.Of 87. Maderas - Parte 1 referente a "Clasificación de maderas comerciales por su durabilidad natural" ubica a la *A. araucana* en la Categoría 4 de "Poco durable", sin tratamiento,

fijada en el terreno, y en las condiciones climáticas existentes en Chile. Según la norma la madera tiene una vida útil de 5 a 10 años máximo, por debajo de las maderas consideradas muy durables como de *Nothofagus obliqua*, *Pilgerodendron uviferum* o *Fitzroya cupressoides* cuya vida útil esperada es mayor a 20 años. Podemos entender la razón detrás de esta diferencia observando, por ejemplo, en los contrastes en las densidades de las maderas, donde la *A. araucana* posee valores inferiores a las especies con mayor durabilidad (Gayoso et al. 2013; Atencia 2003). Además, el contenido de extraíbles en la madera difiere mucho entre estas especies y, por lo tanto, poseen un rol fundamental en la protección de la madera (Donoso et al. 2008).

La protección de la madera contra el biodeterioro está estrechamente vinculada a la acumulación de sustancias extraíbles, típicamente en el duramen. El contenido de extraíbles es muy variable no sólo de un árbol a otro, sino también dentro de un mismo árbol. Estos componentes químicos no estructurales desempeñan un papel importante en la resistencia de la madera contra los organismos de descomposición de la madera (Kirker et al. 2013; González-Laredo et al. 2015). Se sabe que el duramen, parte más

coloreada u obscura, es la parte naturalmente resistente de las maderas de árboles, mientras que la albura en casi todas las especies tiene una menor durabilidad natural (Schultz y Nicholas 2002).

Extraíbles de la madera

El éxito evolutivo de las coníferas se debe en parte en los complejos mecanismos de defensa para disuadir a sus depredadores. La presencia de extraíbles como componentes constitutivos o inducibles en sus estructuras, es la principal clave de defensa química frente a depredadores (Trapp y Croteau 2001).

Los extraíbles cumplen roles importantes en la planta tales como: regulación hormonal, agentes alelopáticos, atrayentes de polinizadores, repelentes de insectos, adaptación al estrés. Se caracterizan, además, por su gran diversidad de compuestos (entre familias, géneros, especies e incluso entre variedades de la misma especie) (Azcon-Bieto 2010). Además, la producción de estos metabolitos depende directamente de la distribución geográfica, del estado de desarrollo de la planta, periodos de estrés, y del ataque de patógenos (Buchanan, Gruissem, y Jones 2002; Cates 1996; Ramawat y Merillon 2007).

Los extraíbles juegan un importante rol en la defensa y resistencia de las coníferas frente al ataque de patógenos, estos se depositan específicamente en la pared celular, en espacios intercelulares y dentro de la célula vegetal. En la madera, los metabolitos secundarios se acumulan principalmente en el duramen, en la resina que producen y su concentración depende de la especie de árbol, edad, posición dentro del árbol, ubicación geográfica, o al estrés sometido entre otros factores bióticos y abióticos.

Los extraíbles pertenecen a compuestos no estructurales de la madera, además, la producción de resinas es una las características más comunes en las plantas. Según Langenheim, la resina es una mezcla liposoluble de terpenos y/o compuestos fenólicos, que pueden ser volátiles o no-volátiles, secretados por estructuras especializadas hacia el interior o superficie de la planta (Langenheim 2003). Estos compuestos juegan un rol importante en las interacciones ecológicas (Avalos-García y Pérez-Urria Carril 2011).

La resina se puede obtener directamente del árbol a través de heridas o cortes, también se pueden obtener de la madera por métodos simples de extracción,

usando solventes (orgánicos o acuosos) que contendrán estos terpenos y otras sustancias fenólicas (Becerra et al. 2002).

Rol de los extraíbles en la protección de la madera de coníferas

Las plantas, están sujetas a la depredación por una amplia gama de herbívoros, insectos y patógenos como hongos degradadores de madera. Donde los MS específicamente terpenos y compuestos fenólicos tienen un importante papel en la defensa de las coníferas, contra el ataque de depredadores (Zhao 2011).

En el trabajo de Donoso 2008 los extraíbles fueron removidos de la madera de 3 cupresáceas chilenas e inoculados con un hongo de pudrición parda (*Laetiporus sulphureus*) la madera sin extraíbles fue más afectada en comparación de la madera que contenía sus terpenos y compuestos fenólicos, esto es muy notorio en la madera de *Pilgerodendron uviferum* (D.Don) cuyo contenido de terpenos alcanza el 74%. Chedgy 2009 demostró la facilidad con que hongos como *Coniophora puteana, Heterobasidion annosum, Phellinus sulphurascens,* y *Phellinus weirii* fueron capaces de crecer y degradar madera lixiviada de cedro rojo occidental más fácilmente que la

madera no lixiviada. Este fenómeno se correlacionó con la cantidad de extraíbles, ya que la madera lixiviada contenía un 80% menos de extractivos que la madera de cedro sin lixiviación (Chedgy, Lim, y Breuil 2009). Además se ha encontrado que lignanos como el secoisolariresinol presenta actividad antifúngica frente a *Tramentes versicolor, Ceratocystis pilifera* y el pinoresinol presenta un efecto insecticida contra *Spodoptera frugiperda* (Céspedes A et al. 2006).

Todos estos estudios relacionan el papel directo que desempeñan los extraíbles en la protección de la madera frente a xilófagos.

Compuestos terpenos en los extraíbles

Los terpenos son el grupo de metabolitos secundarios más abundantes en las plantas, existiendo alrededor de 30.000 tipos de moléculas terpénicas (Connolly y Hill 1991). La resina de las coníferas esta constituida principalmente por trementinas y colofonia en una relación 1:1, la composición de la primera es de mono y sesquiterpenos, y la de la segunda es de diterpenos y fenoles (Trapp y Croteau 2001).

Las resinas acumuladas en los tejidos de los árboles se liberan tras una lesión o por infección de un patógeno, con la finalidad de eliminar el insecto u hongo, atrapándolos en la resina, expulsándolo o repeliendo su ataque. Este proceso se llama "*pitching out*" y el resultado no es sólo eliminar a los patógenos y limpiar la zona de la herida, sino también mover las resinas a la superficie, donde se evapora la trementina para permitir que los terpenos de la resina formen una barrera física y química que sella la herida (Gijzen et al. 1993; Johnson y Croteau 1987).

Diterpenos de tipo labdano, también ha sido reportados en la madera de *A. araucana* y estudios recientes han confirmado sus efectos antifúngicos contra *Geotrichum candidum* y *Botrytis cinerea* (Hawas et al. 2015; Mendoza et al. 2015; G. Schmeda-Hirschmann et al. 2005)

Compuestos fenólicos en los extraíbles

En plantas, se han reportado aproximadamente 4.000 estructuras fenólicas que son biosintetizadas en diferentes rutas metabólicas, pero en coníferas, la mayoría provienen de la ruta del ácido shiquímico (Zhao 2011). Tal como los terpenos, los compuestos fenólicos, entre ellos taninos, flavonoides y

lignanos, tienen un importante rol en la resistencia de las coníferas al ataque de patógenos como insectos u hongos (F. Lieutier 2007; Ralph et al. 2006).

Los lignanos son derivados del fenilpropano, formados por acoplamiento estereospecífico β-β de dos radicales o más monolignoles; p-cumaril alcohol, coniferil alcohol y sinapil alcohol. Las moléculas que surgen de dos radicales monolignol que son acoplados estereospecíficamente β-O-4 o β-5 se llaman neolignanos (Umezawa 2003). Debido a sus propiedades antioxidantes, se cree que los neolignanos participan en las respuestas de defensa (Calvo-Flores et al. 2015).

Actualmente han sido identificados al menos 5 lignanos en *A. araucana* y evaluado su actividad biológica frente a bacterias, hongos e insectos.

Mecanismos de acción antifúngica de los extraíbles

El efecto antifúngico de algunas moléculas encontradas en extraíbles de madera ha sido demostrado extensamente en la literatura. Su bioactividad se ha atribuido a diversos mecanismos (Valette et al. 2017), que se detallan a continuación.

1. Actividad quelante y antioxidante

La propiedad quelante de metales de los extraíbles de madera pueden afectar drásticamente las actividades de degradación por parte de los hongos, que usan peroxidasas y lacasas dependientes del cobre para la degradación de los hongos de pudrición blanca y de hierro (mediante reacción de Fenton) en el caso de hongos de pudrición parda (Pollegioni, Tonin, y Rosini 2015).

Dentro de los compuestos que destacan acción quelante, se encuentran los troponoides, una familia de compuestos terpénicos que poseen alta actividad antifúngica en hongos de pudrición blanca y de pudrición parda, a través de la unión de hierro férrico o ferroso para formar precipitados $[Fe(trop)_3]$ o $[Fe(trop)_2]$ respectivamente. Mediante la inhibición de reducción de Fe(III) se evita el inicio de la reacción de Fenton (Diouf et al. 2002; Yen et al. 2008). Hinokitiol, uno de los troponoides más estudiados de su tipo, reduce la actividad de las lacasas, así como también otras enzimas que contienen metal como la tirosinasa y la lipoxigenasa (Saniewski, Horbowicz, y Kanlayanarat 2014). Este compuesto se encuentra en altas concentraciones en el duramen de *Austrocedrus chilensis* lo que puede explicar en parte su alta resistencia a

la biodegradación (Guillermo Schmeda-Hirschmann, Rachel Olate, y Goikoetxeaundia 2012).

Además de la actividad quelante de metales, los extractos de madera pueden eliminar las especies reactivas de oxígeno que son necesarias en el proceso enzimático u oxidativo para la descomposición de la madera. Las enzimas lignocelulolíticas son demasiado grandes para penetrar en las paredes de células de madera por lo tanto liberan radicales libres responsables del ataque de lignina local.

Los lignanos y flavonoides tienen un alto efecto antioxidante con la capacidad de eliminación de radicales (Pietarinen et al. 2006). Por ejemplo, se ha reportado que lignanos como eudesmin presentan una interesante actividad contra numerosos hongos fitopatógenos como: *Cladosporium cucumerinum*, *Diaporthe ampelina*, *Colletotrichum fragariae*, *Phomopsis obscurans* y *Fusarium oxysporum* (Cantrell et al. 2005; Cavin et al. 1998).

2. Interacción enzimática

Existen compuestos en los extraíbles que pueden afectar las enzimas involucradas en la degradación de la madera, como es el caso de medicarpin,

isoflavonoide presente en el tejido leñoso de *Dalbergia congestiflora* que es capaz de inhibir la lacasa de *Trametes versicolor* a través de 3 mecanismos de unión directa (1) al sitio T1 de la proteína, reduciendo la unión con componentes de lignina, (2) en el sitio del canal O_2 que impide la reducción de O_2 y (3) en el sitio del canal H_2O bloqueando la liberación de moléculas de agua (Martínez-Sotres et al. 2015).

En otros estudios, se ha demostrado que con la adición de vainillina se desactiva y precipita la enzima celulasa. Investigación de compuestos con estructura similar a la vainillina, se demostró que el aldehído y los grupos hidroxilo fenólicos de la vainillina son responsables del efecto inhibidor de la celulasa (Y. Li, Qi, y Wan 2014). Varios otros compuestos fenólicos inhiben la degradación de celulosa con diversas eficacias dependiendo de la presencia de grupos hidroxilo, carbonilo y metoxi en las moléculas o en su estado de oxidación (Oliva-Taravilla et al. 2016; Qin et al. 2016; Valette et al. 2017).

3. Alteración de la pared celular y membrana plasmática de hongos

Se ha reportado el efecto de la molécula *cinamaldehído*, presente en la corteza y hojas de árboles, poseen actividad antifúngica contra especies de hongos pudridores blancos (*Trametes versicolor* y *Lenzites betulina*) y parda (*Laetiporus sulphureus*) (Shreaz et al. 2016). Además este compuesto afecta la estructura de la levadura, *Saccharomyces cerevisiae*, mediante inhibición de la síntesis de componentes de la pared celular, específicamente las enzimas quitina sintasa y la β-(1,3)-glucano sintasa (Bang et al. 2000).

También la membrana plasmática de hongos puede ser alterada por la presencia de compuestos derivados del alcohol coniferilo se les ha atribuido su capacidad de formar poros dentro de la membrana plasmática, dando como resultado cambios de osmolaridad y reducción de su tamaño celular (Choi et al. 2012).

4. Alteración de la homeostasis iónica

Muchos terpenos fenólicos poseen la capacidad de desestabilizar el equilibrio iónico e inducir a la muerte a células fúngicas (Zhang, Muend, y Rao 2012). El compuesto carvacrol es capaz de inducir una rápida entrada de Ca^{2+} del

medio extracelular y también desde la vacuola, este cambio en el citosol ha mostrado ser letal en *Saccharomyces cerevisiae, Candida albicans*, *Staphylococcus aureus*, y *Pseudomonas aeruginosa* (Zhang, Muend, y Rao 2012). Similar efecto posee el compuestos cinamaldehído en ensayos contra *Phytophthora capsici* (Hu et al. 2013).

5. Efecto caotrópico

La actividad caotrópica se considera la causa principal de la inhibición metabólica y crecimiento microbiano de muchos componentes encontrados en los extraíbles (como alcoholes y fenoles) (Cray et al. 2015). Este efecto consiste en el cambio o disrupción de la red de enlaces de las moléculas de agua y en la interacción de estas con macromoléculas tales como proteínas, ADN o ARN, tendiendo a desnaturalizarlas o disolverlas. Ejemplos de estos son la vanillina y el alcohol bencílico, moléculas que pueden inhibir las enzimas especificas resultando en un desequilibrio redox celular y causando una reducción en la generación de energía (de Almeida et al. 2009).

6. Otros efectos

Algunos compuestos fenólicos, como resveratrol, han demostrado interferir con los genes de expresión y desarrollo de procesos metabólicos secundarios de formación de conidios, aflatoxina y crecimiento micelial (Wang et al. 2015).

Los nudos como importante fuente de almacenamiento de extraíbles

Es interesante que, si bien los extraíbles se acumulan en el duramen, se ha detectado mayor cantidad en los nudos. En coníferas, la concentración de extraíbles puede ser hasta 6 veces mayor en la zona de los nudos que en el resto de la madera. En algunas especies de abetos el contenido de extraíbles es hasta 100 veces mayor que en resto de la madera (Kebbi-Benkeder et al. 2016; Willfor et al. 2004).

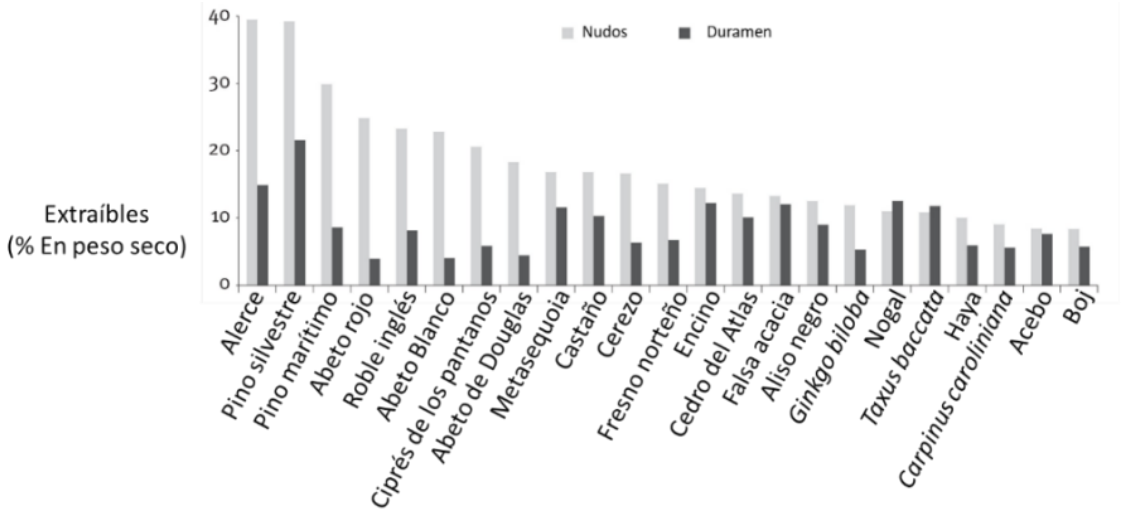

Figura 4 Comparación de extraíbles obtenidos de duramen y de nudos en diferentes especies leñosas del hemisferio norte (Kebbi-Benkeder et al. 2016).

Estudios han demostrados que los extractos de los nudos de *Pinus sylvestris* poseen mayor efecto de protección frente al ataque hongos de pudrición parda, comparados con los extractos obtenidos del duramen. Además existe una directa relación con la capacidad antioxidante de los extraíbles (Belt, Hänninen, y Rautkari 2017).

Las razones biológicas de esta alta concentración en los nudos de extraíbles no han sido del todo aclaradas, existen varias sugerencias para explicarla, pero ninguna se ha confirmado con certeza hasta la fecha. Desde un punto de vista fisiológico, es bien sabido que estos metabolitos secundarios se sintetizan en la albura y se acumulan en el duramen, en especial cuando existe

un daño en la madera, pero no hay información relacionada para los nudos (Kebbi-Benkeder et al. 2017).

Lignanos en extraíbles de la madera de *A. araucana*

Los nudos de *A. araucana* son conocidos vulgarmente en Chile como "Picoyo", un material leñoso extremadamente duro y altamente resistente a la pudrición que se ha convertido en la materia prima de numerosos artesanos quienes producen hermosas piezas de orfebrería y se ha convertido para ellos en una importante fuente de ingresos. Este material sólo puede ser obtenido de los árboles muertos de *A. araucana* que hayan sido totalmente degradados el resto su madera, quedando solo los nudos que pueden permanecer muchos años en el suelo del bosque. Popularmente se usaban los "picoyos" como combustible de leña por su alto poder calorífico, y en la medicina local mapuche las machis usaban parte del nudo para tratar molestias y dolores severos (información obtenida personalmente a través de entrevistas).

Figura 5 Nudos de *A. araucana* o "*picoyos*". A troncos de araucarias cortados en la zona de los nudos. B "picoyos" expuestos encontrados en el bosque. C Joyería creada a partir de "picoyos". D corte transversal delgado de un nudo de araucaria.

Los extraíbles de los nudos de *A. araucana* no han sido estudiados, sin embargo existen trabajos sobre la composición de los extraíbles de nudos de *A. angustifolia* donde señalan la alta presencia de lignanos como eudesmin, secoisolariciresinol y lariciresinol que aportan información para explicar la razón de su alta dureza y resistencia a la biodegradación (Anderegg y Rowe 1974; Ohashi et al. 1992; S. M. Willför, Smeds, y Holmbom 2006).

En base a los antecedentes mencionados, en el presente estudio se caracterizará el perfil químico de los extraíbles de madera de duramen y nudos de *A. araucana* y se evaluará el rol específico de sus componentes en la defensa contra hongos degradadores de madera.

HIPOTESIS 1

La diferencia de durabilidad que existe entre la madera del duramen con respecto a los nudos de *A. araucana* se debe a las diferencias cuali y cuantitativas entre los componentes de los extraíbles.

HIPOTESIS 2

La resistencia a la biodegradación por hongos xilófagos de la madera de los nudos de *A. araucana*, se debe al alto contenido de lignanos presentes en sus extraíbles.

OBJETIVO GENERAL

Determinar el rol de la concentración y composición de los extraíbles en la durabilidad de la madera de *A. araucana* frente a hongos xilófagos.

OBJETIVOS ESPECIFICOS

1. Aislar, cuantificar e identificar los componentes de los extraíbles en la madera de *A. araucana*.
2. Determinar el rol de los extraíbles y de los principales constituyentes de estos en la durabilidad natural de la madera de *A. araucana* frente a hongos xilófagos.
3. Evaluar la actividad antioxidante y quelante de los lignanos presentes en *A. araucana*.

CAPITULO II. Chemical characterization of lignans from *Araucaria Araucana* a native conifer of Chile and evaluation of their cytotoxicity and antioxidant activities

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ABSTRACT: *Araucaria araucana* is a native conifer of Chile, commonly called araucaria. The knots of araucaria are very hard wood and highly rot-resistant; they can be found in the forest decades after that the tree has died and decomposed. Here we report the phytochemical characterization of different parts of the araucaria as stemwood, branch, and knots, founding a remarkable difference in the extractable content in these parts, as well as the lignan composition, which is higher in knots than in branches or stemwood. Eudesmin was isolated and crystallized from the organic extract of knots; its structure was determinate by NMR. Moreover, secoisolariciresinol, lariciresinol and matairesinol were identified by GCMS and HPLC using standards, and quantified in stemwood, branch and knots. The results showed that secoisolariciresinol is the main lignan with 45.77 mg g^{-1}, followed by eudesmin with 22.68 mg g^{-1}, lariciresinol 4.57 mg g^{-1} and matairesinol with 1.19 mg g^{-1}. The antioxidant activity, in terms of DPPH assay, showed that knotwood extract displays the higher activity, meanwhile that eudesmin did not display activity in such assay. The cytotoxic activity against SHSY5Y neuroblastoma and P3X myeloma cell line revelated a moderate activity of extracts, while eudesmin did not show activity.

Keywords: *Araucaria araucana*, knotwood, lignan, eudesmin, DPPH activity, cytotoxic activity

INTRODUCTION

Lignans are a family of natural compounds formed by stereospecific coupling β-β` of two phenylpropane units (C_6C_3) with a wide range of rearrangements, different oxidation degree, and condensations, emerging a broad type of compounds (Vermerris y Nicholson 2008). Lignans have been studied for their potential medicinal uses as phytohormone, treatment or prevention of various types of cancer, and neuroprotective effects, among others. They have been isolated from almost all vascular plants. However, their role in nature is not fully known. It has been suggested that they are part of the defence and resistance of conifers against pathogen attacks (Kebbi-Benkeder et al. 2015; Valette et al. 2017). In conifers, the extractable give protection from predators (Trapp y Croteau 2001), and their concentration depends on the specie of tree, age, geographical location, stress, and different parts of the same tree. In fact, in hardwood as knots, extractable accumulate in higher concentration than in the heartwood, even up to 6 folds, with a concentration

of lignans than exceed 10% w/w. While, in coniferous species such as *Abies alba, Picea abies,* and *Pinus sylvestris* the concentration of lignans in knots can go up to 200-folds (Kebbi-Benkeder et al. 2016; Willfor et al. 2004). In *Pinus sylvestris* L., the extracts obtained from knots have shown a higher protective effect against brown rot fungus attack than extracts obtained from the heartwood, also pointing to a direct relationship with the antioxidant capacity and components of extractable (Belt, Hänninen, y Rautkari 2017).

In Chile, *Araucaria araucana*, commonly called araucaria, grow up over 2000 m in the centre south of the country, and their knots are commonly known as "picoyo". They are a tough wood and highly rot-resistant material that has become used by artisans who collect them from degraded *A. araucana* trees to produce beautiful pieces of silverware. The *picoyos* of araucaria can endure in the forest for many years without fungal attack, probably due to a high concentration of extractables (Anderegg y Rowe 1974; Ohashi et al. 1992; Willför, Smeds, y Holmbom 2006). For this reason, they were used as fuel for firewood with high calorific value. Knots of the Brazilian conifer *A. angustifolia* has a high presence of lignans, such as eudesmin, secoisolariciresinol and lariciresinol, but knots of araucaria has not been chemically characterized in depth. The present study reports the

chemical profile of heartwood, branches, and knots of *A. araucana* and their antioxidant and cytotoxic activity.

EXPERIMENTAL

General Information

Preparative chromatography was performed using Merck silica gel 60 (25−100 μm; Aldrich, Santiago, Chile). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60F254 sheets (Darmstadt, Germany), eluted with EtOH/dichloromethane 7% v/v and revelated with sulfuric acid EtOH 5%, then eating at 150 °C. Solvents as HPLC-grade water, acetonitrile, ethanol, acetone, formic acid were obtained from Merck (Darmstadt, Germany). Lignan standards of pinoresinol, matairesinol, lariciresinol, and secoisolariciresinol were obtained from Phytolab (Vestenbergsgreuth, Germany). Cells P3X63Ag8.653 were purchased from ATCC (ATCC® CRL-1580). Fractions were concentrated in a Büchi R100 rotavap. Solvents used in this study were distilled prior to use and dried over appropriate drying agents. NMR spectra were recorded on a

Bruker 400 MHz spectrometer, Bruker Biospin GmbH, Rheinstetten, Germany, and TMS were used as an internal standard.

Gas chromatography-Mass Spectrometry (GC-MS) analysis was performed on an Agilent 7890, California, USA, with an Agilent 5975 mass detector, using a column HP5-MS 30 m x 0.25mm silica and a 0.25 μm film thickness. Spectra were compared with standard mass spectra in addition to the NIST07 database (NIST 2008, National Institute of Standards and Technology).

Plant material and extraction

Stemwood, branch and knots of *A. araucana* were collected in May 2018 on Nahuelbuta range (Biobío Region, 37°41''43.56''S 73°07'49.34''W). Samples were identified by the botanist Dr. Roberto Rodríguez from the University of Concepcion. 100g of stemwood, branch, and knots of *A. araucana* were chopped, dried at 40 ºC for 8 h, and milled to 1-3 mm, and then they were continuously extracted on a Soxhlet. Briefly, in a 250 mL Soxhlet, 2-5 g of sample was placed in a cellulose thimble with acetone (250 mL) and refluxed for 12 h. The organic layer was filtered through a GV Durapore filter (0.22 μm pore size, 13 mm diameter, Millipore, Bedford, MA,

USA) and evaporated *in vacuo*, giving a total organic extract, which was weighted and kept frozen at -20°C until use.

Additionally, a branch of *A. araucana* was processed according to Figure 3A. Briefly, the branch of 1.2 m and around 10 cm diameter, was cut in 22 slices of 4 mm width, using an electric saw, previously cleaned with acetone. The wood powder of each slice was carefully gathered and extracted in Soxhlet, as previously described.

Quantification of lignans by HPLC

Quantification of lignans was done by HPLC (Young Lin, Anyang, South Korea) with PDA detector (PDA YL9160), using a reverse phase column Kinetex® 2.6 µm C18 100Å, 150 x 4.6 mm. The mobile phase was: A) water with 0.1 % formic acid and Phase B) acetonitrile. A gradient program was used as follows: 0-42 min 15-45% B, 42-45 min 45-100% B, 45-50 min 100% B, 50-60 min 100-15% B. The flux was constant to 0.4 mL min^{-1}, at 25 ºC and injections were of 4 µL. A calibration curve of eudesmin was measured at 280 nm. The analytical parameters were calculated from the calibration curve, with n = 6: R^2 = 0.9972, LOD = 1.11 mg L^{-1}, LOQ = 3.72 mg L^{-1}.

The lignans eudesmin, matairesinol, secoisolariciresinol, and lariciresinol were identified according to retention time and molecular absorption spectra comparing to commercially available standards. Matairesinol, secoisolariciresinol, and lariciresinol were quantified by extrapolation on the calibration curve of eudesmin, and they are expressed in terms of eudesmin content.

Antioxidant activity against DPPH radical

The antioxidant activity of the extract from the knotwood, stemwood and branch were evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazil) radical reduction test according to the methodology proposed by Hatano et al. (Hatano et al. 1989). Concentrations of 100, 50, 25, and 10 µg mL^{-1} were prepared from the total extracts in ethanol. An aliquot of 150 µL was taken from each sample and mixed with an equal volume of DPPH solution (60 mM). The resulting solution was vortexed, and the absorbance was measured against the target on Biotek® ELX800 microplate reader at a wavelength of 520 nm. The results were expressed as the percentage of antioxidant activity and compared with the TROLOX reference standard (6-hydroxy-2,5,7,8-

tetramethylcroman-2-carboxylic acid) as a control (Hatano et al. 1989). The DPPH radical reduction activity was calculated with the following formula:

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

Where Abc is the absorbance of the control (ethanol), Abs is the absorbance of the sample, lectures at 520 nm. Data is reported as IC50 DPPH radical inhibition.

TLC and bioautography with DPPH

Extracts of stemwood, branchwood and knotwood in concentrations of 12 mg mL^{-1} in acetone were seeded on a TLC and eluted using dichloromethane-ethanol 93:7 v/v. Then, the TLC plate was sprayed with a solution of 0.5 % w/v of DPPH in ethanol. After 30 min, the plate was examined to the daylight, according to the method of Dewanjee et al. (Dewanjee et al. 2015). Free-radical scavengers appear as cream/yellow spots against a purple background.

Cytotoxic activity against cancer cells

Cell culture

Murine myeloma cells (P3X) and human neuroblast cells (SH-SY5Y) were used to investigate the cytotoxicity of extract from stemwood, branchwood, knotwood and isolated compound eudesmin. SH-SY5Y cells lines were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium – Nutrient Mixture F-12) culture medium while P3X RPMI 1640, either complemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin, maintained at 37 °C, with 5% CO_2 flow, in a humidified incubator. Cells were diluted three times per week to keep them in a normal condition of growth and collected for the antiproliferative assays in the exponential phase of growth.

Cytotoxicity assay

Cell viability was investigate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric method to visualize the functionality of mitochondrial succinate dehydrogenase (correlated with cell metabolic activity) by reduction of MTT into formazan crystals, as reported by Garzoli and colleagues (Garzoli et al. 2019). Briefly, SH-SY5Y ($2x10^4$ cells/well) and P3X ($2x10^4$ cells/well) cells were seeded in 96-well plates and treated with seven 2-fold dilutions (1000, 500, 250, 125,

62.5, 31.2, and 15.6 µg mL^{-1}) with stemwood, branchwood, knotwood extracts and eudesmin in triplicate for 24h. As a negative control, cells without treatment were added. Following treating incubation time, the media was carefully removed, and MTT solution (0.5 mg mL^{-1}) was added each well and leave to react for 3 h at 37°C to allow formazan crystals formation. After this incubation time, cells were centrifuged in a plate centrifuge, the medium was removed and an organic solvent (dimethyl sulfoxide) was added to dissolve the formazan crystals completely. After 15min plates were read by a microplate reader (SunRise, TECAN, Inc, USA) at 595 nm to evaluate the number of viable cells, directly correlated with the amount of colour produced. The % of viability was calculated as follow:

$$\% \text{ viability} = (1 - (S\lambda / C\lambda)) \times 100$$

Sλ: absorption of wells treated with the stemwood, branchwood, knotwood extracts, and eudesmin;Cλ: absorption of untreated cells.

The relation between viability and sample concentration is plotted to obtain a survival curve of tumor cell lines. The effective concentration at 50% (IC50) was estimated from graphs of the dose-response curve for each concentration using GraphPad Prism software (version 8.4.2).

RESULTS AND DISCUSSION

Identification of lignans from stemwood, branchwood, knotwood of *A. araucana*

The acetone extraction of 100 g of knotwood of araucaria yield 26.1 g of total extract, which was concentrated by slow evaporation at 4°C, precipitating 1.4 g of colorless crystals identified as eudesmin, Figure 1.

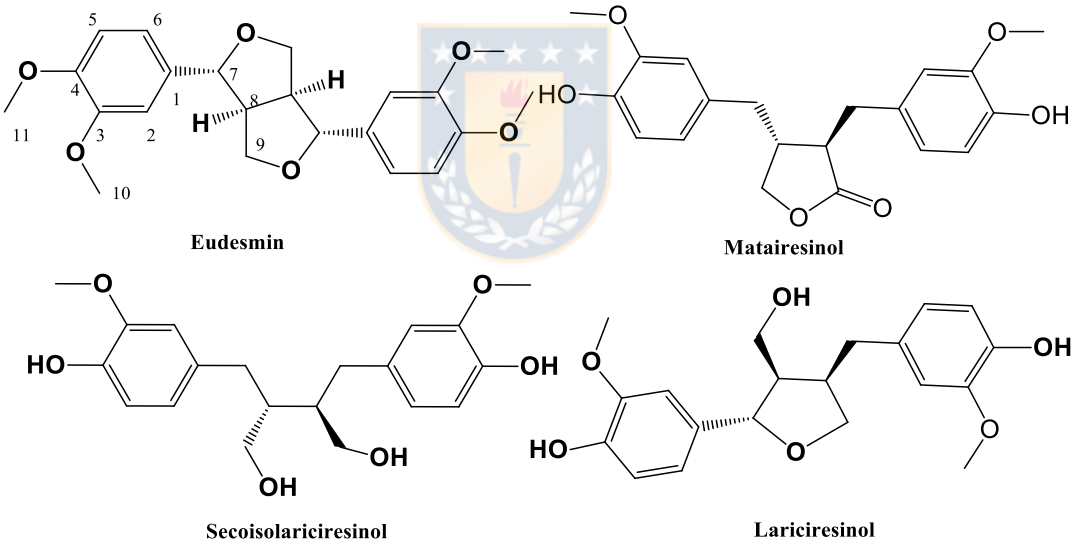


Figure 1 Structure of lignans identified form *Araucaria araucana* knotwood.

The structure of eudesmin was determinate by analysis GC-MS and NMR. The results agree with data reported in literature (Yamamoto, Otto, y Simoneit 2004; Yamamoto, Cox, y Simoneit 2010).

Eudesmin or (1R,3aS,4R,6aS)-1,4-bis(3,4-dimethoxyphenyl) hexahydrofuro [3,4-c] furan. MS m/z (rel.int.): 386 (M) + (82), 371 (3), 355 (9), 337 (2), 325 (2), 230 (2), 287 (3), 265 (2), 248 (2), 235 (5), 219 (15), 189 (12), 177 (68), 165 (100), 151 (54), 135 (15), 107 (6), 95 (8), 77 (10), 55 (5). ^{1}H NMR (400 MHz, CCl_3D) δ (ppm): 6.92 (1H, broad, H-2), 6.89 (2H, overlap, H5, H-6), 4.78 (1H, m, H-7), 4.28 (1H, m, H-9), 3.90 (1H, overlap, H-9), 3.92 (s, 3H, C-11), 3.90 (s, 3H, C-10), 3.12 (1H, m, H-8). Mass: 386 g mol^{-1}. Formula: $C_{22}H_{26}O_6$.

Quantification of extractable and lignans from stemwood, branchwood and knotwood of *A. araucana*

The acetone extraction of a representative sample of milled wood collected from three *A. araucana* tissues gave the following yields: Stemwood 0.9 %w/w, branchwood 1.2 %w/w, knotwood 26.1 %w/w. These results showed high differences in the extractable concentration in *A. araucana* tissues , suggesting that the preservation of knots of araucaria in the forest, without decomposition, could be due to a high amount of extractable in this tissue, which is 29-folds higher than in stemwood.

Four lignans were quantified by HPLC in stemwood, branchwood and knotwood of *A. araucana*, Figure 2, Table 1.

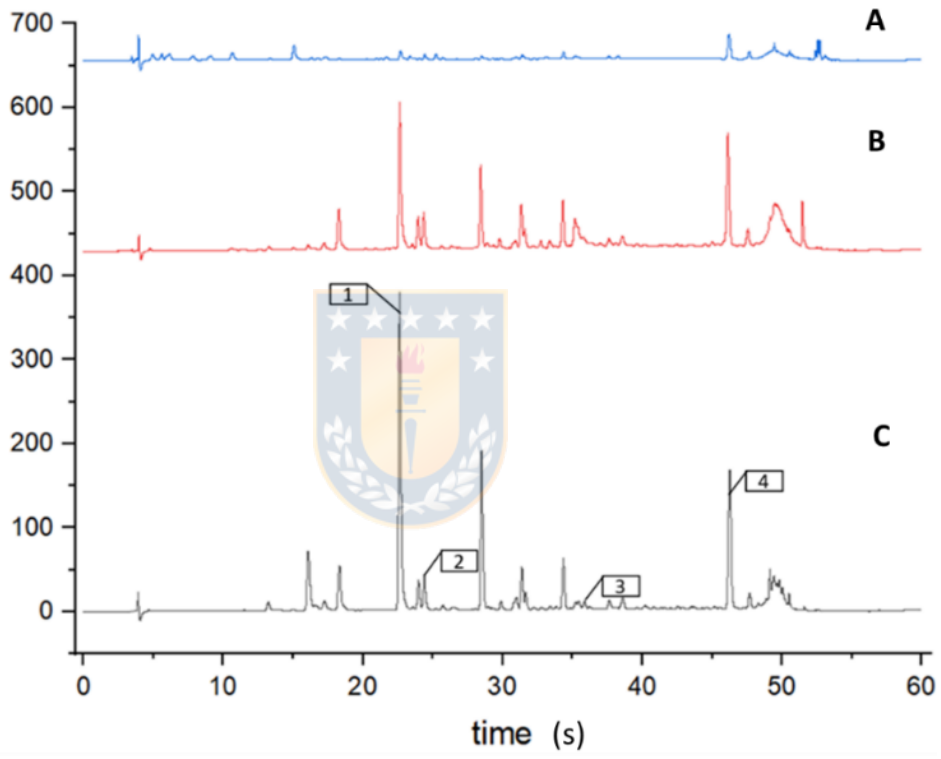


Figure 2 Representative HPLC-PDA chromatograms of total extracts at the same concentration (10mg mL^{-1}) of A) stemwood, B) branchwood and C) knotwood. The peaks of the standards of secoisolariciresinol 1, lariciresinol 2, matairesinol 3, eudesmin 4, are given in figure C.

Table 1 Concentration of four lignans in tissues of *Araucaria araucana*.

Tissue	Secoisolariciresinol mg g^{-1}	Eudesmin mg g^{-1}	Lariciresinol mg g^{-1}	Matairesinol mg g^{-1}
Stemwood	0.03	0.10	0.02	0.02
Branchwood	1.50	1.43	0.24	0.42
Knotwood	45.77	22.68	4.57	1.19

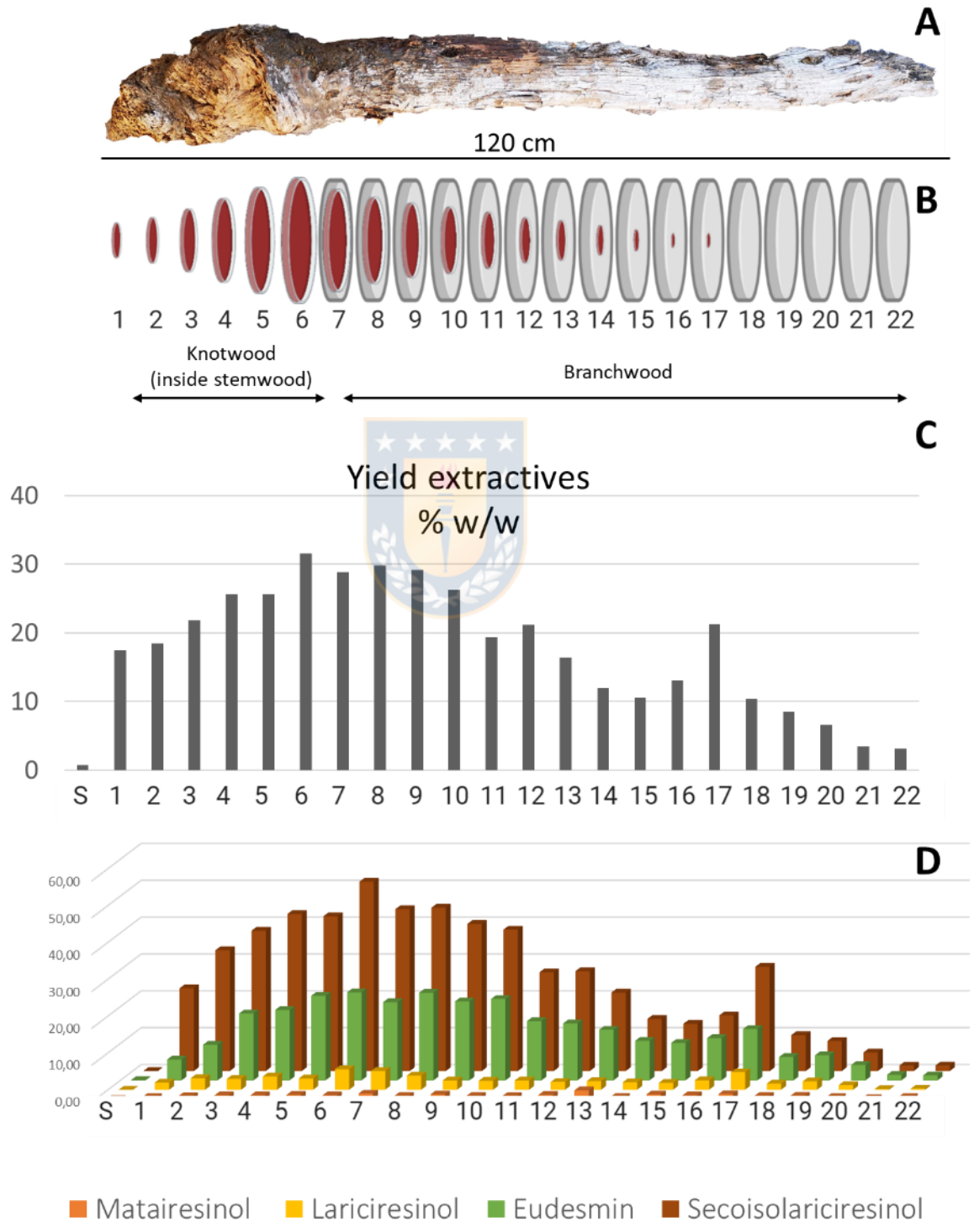
The chromatogram in Figure 2C, shows at least 12 compounds in the knotwood extract, in spite that in this study, only four of them were identified as eudesmin, matairesinol, secoisolariciresinol and lariciresinol (Figure 1), which were quantified, in terms of eudesmin. The compound secoisolariciresinol is the lignan present in higher concentration in stemwood, branchwood and knotwood of araucaria. In the knots, its concentration is remarkable, which is 30.5-folds and 1526-folds higher than in stemwood and branchwood, respectively. The second lignan in major concentration is eudesmin, followed by lariciresinol and matairesinol, showing the same pattern as secoisolariciresinol.

Quantification of extractable and lignans from different parts of a branch of *A. araucana*

The quantification of extractable and lignans were measured in all length of a branch of *A. araucana* of 1.20 m length, Figure 3A. The branch was cut in

22 slices from left (part of knot in red) to the right (branchwood in green color), as it is shown in Figure 3B. Extractable obtained from milled wood of each cut were quantified by gravimetry and summarized in Figure 3C. Results showed a higher concentration of extractable in the samples, which include the knot part (left side), than in samples of branchwood (right side). Figure 3D summarizes the concentration of four lignans; eudesmin, matairesinol, secoisolariciresinol, and lariciresinol, determined by HPLC. These results agree with the concentration of extractives showed in Table 1, where higher amounts of lignans were found in the knot.

Figure 3 Quantification of extractable and pure lignans in different parts of a branch of *A. araucana*. **A)** Branch of *A. araucana* including the knot in left side. **B)** Scheme of 22 wood samples cut from the branch of *A. araucana*. **C)** Concentration of extractable



in %w/w from each sample. **D)** Graph of quantification of eudesmin, matairesinol, secoisolariciresinol and lariciresinol in the 22 wood samples from 3B. **S** Stemwood. **3B** created with BioRender.com

The concentration of lignans varies long a branch of *A. araucana* as was shown in Figure 3D. The results of each sample are given in Table 2, Where is it shown the higher the concentration of each lignan, the close is to the knots between samples 5 to 7, and decreases to the right side of the branch moving away to the knot.

Table 2 Quantification of lignans along a branch of *A. araucana*.

Sample	Matairesinol mg g-1	Lariciresinol mg g-1	Eudesmin mg g-1	Secoisolariciresinol mg g-1
1	0.56	1.94	5.66	22.44
2	0.82	3.15	9.68	32.79
3	1.02	2.94	18.16	38.07
4	1.01	3.60	19.10	42.62
5	1.12	3.07	22.93	42.01
6	0.99	5.54	23.88	51.38
7	1.47	5.09	21.22	43.93
8	0.89	3.83	23.77	44.33
9	1.26	2.52	21.45	39.94
10	0.87	2.44	22.09	38.36
11	0.86	2.52	16.10	26.79
12	1.13	2.12	15.45	27.10
13	2.38	2.34	13.74	21.29
14	0.58	2.03	10.73	14.15
15	1.27	1.87	10.15	12.83
16	1.00	2.57	11.45	15.13
17	1.34	4.73	13.94	28.32
18	0.88	1.67	6.35	9.78
19	0.90	2.27	6.83	8.15

20	0.56	1.27	4.16	5.03
21	0.28	0.20	1.51	1.49
22	0.56	0.28	1.35	1.51

Results expressed on a dry basis.

Antioxidant and cytotoxic activity of extractable from stemwood, branchwood and knotwood of *A. araucana.*

The antioxidant capacity of extracts was visualized by bioautography, Figure 4. TLC to the left side was stained with DPPH, and yellow spots showed antioxidants compounds. According to previous results, a a higher number of polyphenols are found in knotwood, and branchwood samples than that in stemwood, which is in line with their antioxidant activity, in terms of DPPH reduction, showing an IC50 10.18, 30.49 and 72.17 µg mL^{-1} respectively (Table 3). In Figure 4, knotwood (right side), the position of each lignan was pointed out. Eudesmin is the less polar compound with higher retention factor on silica gel TLC, followed by matairesinol, lariciresinol and secoisolariciresinol. In the DPPH radical reduction test by bioautography, eudesmin showed no activity, where the band corresponding to eudesmin did not give positive coloration, as well as by spectrophotometry (Table 3). These results are consistent with the literature (Parhoodeh et al. 2011).

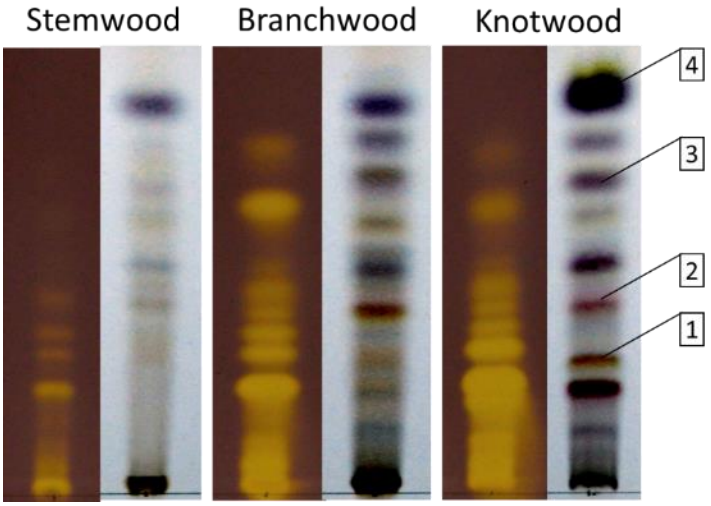


Figure 4 TLC analysis followed by bioautography DPPH activity. The plates were revelated with DPPH reagent (left) and a duplicate was revelated with sulfuric acid/ethanol (right). secoisolariciresinol 1, lariciresinol 2, matairesinol 3, eudesmin 4.

The cytotoxic activity results of the extracts and eudesmin are given in Table 3. The extracts display moderate activity against both neuroblastoma and myeloma cancer cell lines. While eudesmin did not show activity over 200 µg mL^{-1}.

Table 3 Activity of the total extract, in acetone, of knotwood, branch and stemwood of *A. araucana* in DPPH radical reduction analysis and cytotoxic assays in SHSY5Y neuroblastoma and P3X myeloma cell lines, n = 3.

Treatment	**DPPH IC50 (µg mL^{-1})**	**SH-SY5Y IC50 (µg mL^{-1})**	**P3X IC50 (µg mL^{-1})**
Knotwood	10.18	71.52	67.9
Branch	30.49	83.4	79.13

Stemwood	72.17	145.52	120.2
Eudesmin	n.a.	>200	>500
Trolox	5.58	-	-

DPPH is expressed in amount of extract needed to reduce 50% of the DPPH radical (IC50). n.a. no activity.

Possible role of *A. araucaria* wood extractable

Based on the results obtained, the difference in lignan concentration between stemwood and knotwood from *A. araucana* is notorious. This accumulation of phenolic compounds in tree knots has been studied in other gymnosperms from the northern hemisphere. Those results suggest that the presence of phenols such as lignans or stilbenes would be exerting a chemical barrier as free radical scavengers. Such properties counteract the attack of xylophagous fungi, such as brown rot, which generate free radicals to gain access to the cellulose (Kebbi-Benkeder et al. 2015).

Another interesting role of lignans from knotwoods on the soil surface is that they could be exerting an allelopathic effect, because some of these compounds, like lariciresinol and secoisolariciresinol, are highly phytotoxic.

Therefore, they would be exerting an ecological local impact that needs further study (DellaGreca, Zuppolini, y Zarrelli 2013).

Lignans are an important family of bioactive compounds with antifungal, antibacterial and insecticidal effects, which are used by conifers as a chemical defence; moreover, they have also shown pharmacological properties as anti-carcinogens, and as a food supplement because of their beneficial effects on human health (Céspedes A et al. 2006; Landete 2012; Yatkin et al. 2014). For instance, extracts of lignan-rich *Picea abies* knots are added to the winemaking process to improve its properties, enhancing its quality (Balík et al. 2017; Josef et al. 2016). The lignans secoisolariciresinol, eudesmin, lariciresinol and pinoresinol have already been reported in the heartwood of native Chilean species such as *A. araucana, Fitzroya cupressoides, and Austrocedrus chilensis* (Céspedes A et al. 2006; Calvo-Flores et al. 2015; Donoso et al. 2008). Chemical investigation on extracts of stemwood, branchwood and knotwood from araucaria was carried out. Our results, in *Araucaria araucana*, evidenced the presence of secoisolariciresinol, lariciresinol, matairesinol and eudesmin in higher concentration in the knots of araucaria, than in stemwood. The biological reasons for this high chemical concentration in knots have not been fully clarified, because physiologically,

the secondary metabolites of extractable are synthesized in the sapwood and transport to the heartwood, activity that is enhanced when the tree is damaged, but how they move and accumulate in knots is not well understood (Kebbi-Benkeder et al. 2017).

The knots of araucaria was used for a long time as wood to burn and recently as material for artisans. However, it has potential use as a source of lignans, specially eudesmin, which crystallize in organic solvents at low temperature, facilitation its purification. Extracts of stemwood, branchwood, knotwood, and all isolated compounds were evaluated for their cytotoxicity and antioxidant bioassays (Table 3). In the cytotoxic analysis of eudesmin, it did not give positive cytotoxic activity in SHSY5Y and P3X cells., In comparison to other studies in PC3 (Prostate carcinoma), MCF (Breast carcinoma), PA1 (Ovary carcinoma), DLD1 (Colon carcinoma), or M4Beu (Malignant melanoma) (Lim et al. 2007), that showed that eudesmin possesses significant antitumor effects on lung cancer A549 (IC50 18.3 uM) via to induction of mitochondria-mediated apoptosis (Jiang et al. 2017), as well as in nasopharyngeal carcinoma cells eudesmin inhibited cell viability and induced apoptosis of NPC cells (Yu et al. 2019).

CONCLUSION

A high concentration of antioxidant phenolic compounds are accumulated in *A. araucana* knotwood. We suggest that knots with a high concentration of extractable rich in lignans are part of a chemical protection mechanism. The union between the trunk and the branches supports critical mechanical loads due to the weight of the branches and environmental factors such as wind, snow, and precipitation that induce strong mechanical stress. Self-pruning of branches after death could also be the origin of the entry of pathogens and oxygen that could damage the living tissues of the stem. Consequently, the knots must be both mechanically and resistant to xylophage predation.

Regarding the cytotoxic tests, extracts of *A. araucana* showed a moderate effect and eudesmin showed no activity against the treated cell lines. Nevertheless, the cytotoxic activity is not fully understanding, it could be suggested that eudesmin is highly specific against some particular cancer cell lines. However, this deserves more in-depth studies, and it could become a potential cancer-fighting drug.

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Authors declare no conflicts of interest.

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CAPITULO III. Isolated lignans of *Araucaria araucana* provide wood protection against attack by xylophagous fungus *Pleurotus ostreatus*

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Abstract

Araucaria araucana (Molina) K. Koch also called "*Pehuén",* is a native conifer of Chile, grows up to 50 m in high mountains in the south of the country, with longevity over 1000 years. *A. araucana* is a sacred tree for the native people "Pehuenche", who live by collecting their seeds. The knots of *Araucaria* are characterized by their extreme hardness and high resistance to environmental biodegradation, with the aim to explain this capability, we evaluated the chemical composition of different parts of *A. araucana*, such as knot, stemwood, and branches, finding 12 lignans by planar chromatography coupled to mass spectrometry. Further purification of the total organic extract of knots, gave three compounds, identified by 1D and 2D NMR as eudesmin **1**, compound **2**, or ((1S,2R,3R)-1-(4-hydroxy-3-methoxyphenyl)-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene-2,3-diyl) dimethanol, reported for the first time in conifers, and compound **3** or secoisolarisiresinol. The knotwood extract and eudesmin were used as impregnation treatment in wood resistance trials to attack by the white-rot xylophagous fungus *Pleurotus ostreatus*, showing a protective effect of the extracts at concentrations of 1% wt/wt, while treatment with eudesmin at 0.5% wt/wt showed a significant decrease in the attack of xylophages.

Moreover, the total phenol content and antioxidant capacity were quantified in terms of DPPH, evidencing a higher total polyphenol content and antioxidant capabilities values in compression wood zones.

IMPORTANCE. Our study of the *Araucaria araucana* species suggests a relationship between the abundance of lignan compounds in the wood located at the base of the branches, i.e. the knots, and the high resistance of this zone of the wood to degradation by xylophagous fungi. The protective effect would be linked to the antioxidant capacity of the phenolic compounds found in high abundance and variety in the wood of the knots. The findings suggest that the species *A. araucana* has a chemical defense strategy against white-rot fungi, to reinforce its knots with a high accumulation of extractives, because this species self-prunes its branches.

Keywords: *Araucaria araucana*, lignan, eudesmin, wood resistance, white rot fungus, knotwood extractives

Introduction

In Chile, *Araucaria araucana* (Molina) K. Koch is a native conifer that grows up to 50 m high, and its longevity can exceed 1000 years. The three is considered sacred for the native people "Pehuenche" who lives by collecting their seeds 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, because when the tree grows the lower branches are automatically pruned, giving a shape like an umbrella (Figure 1 A). The branching pattern is a regular monopodial, with 3 to 7 branches per whorl, perpendicular to the trunk (Figure 1 B).

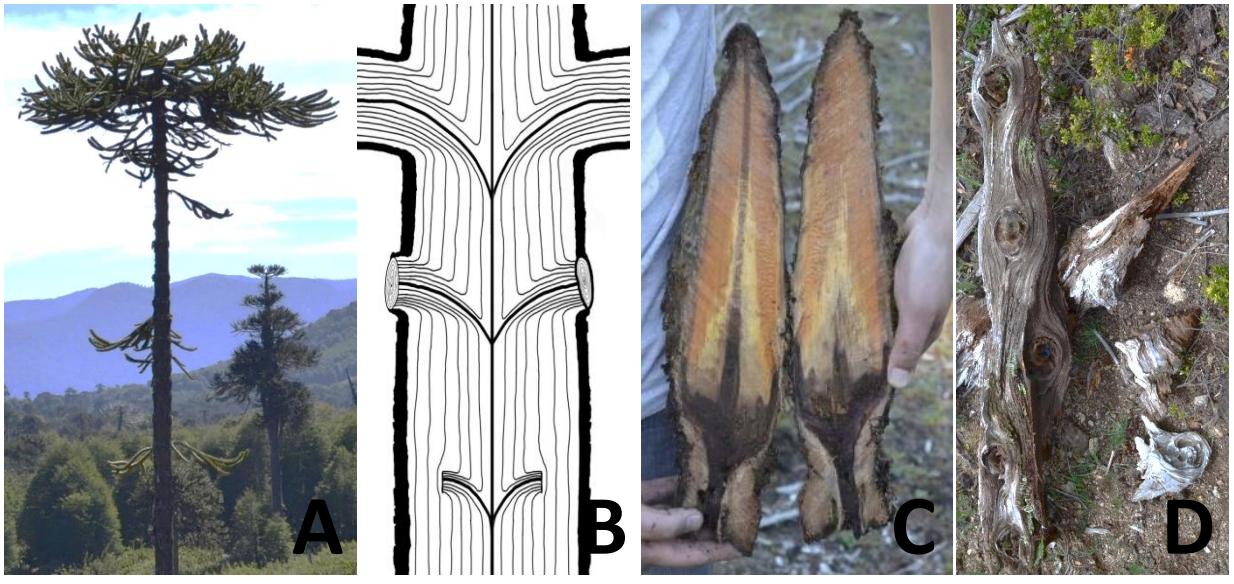

Figure 1 Photographs of *Araucaria araucana*. A) Araucaria adult tree. B) Diagram of the trunk and its knots C) Longitudinal cut of a knot. D) Araucaria knots exposed on the forest floor after the rest of the wood was degraded.

The wood of *A. araucana* has excellent quality, lightweight, easy to work, and yellowish-white, for this reason, straight and cylindrical logs were used in the manufacture of boat masts (Rodríguez et al., 1983), fortunately nowadays Araucarias is protected and is not allowed to cut it.

The main defense of Araucarias against pathogens is its thick bark (up to 10 cm) and the abundant resin that produces when they are damaged, but its wood exposed to the elements can be easily corrupt by biotic and abiotic factors. An interesting phenomenon is possible to see in forests of *A. araucana*, where the dead tree is entirely degraded, but their knots are spread on the ground, almost without decomposition (Figure 1. C and D).

The knots are popularly called *picoyo* or *chochín*, and they are characterized by their light brown color, tough and heavy, thereby, they are used by artisans to create pieces of jewelry such as earrings, pendants, or rings. They correspond to compression wood from the trees, and present higher amount of extractives than other woods such as heartwood and sapwood, for example, in the conifer "Norway spruce" (*Picea abies*), the yield of extractive from

knots is 120 folds higher than heartwood and 240 folds than sapwood, being lignans as the majority component (Holmbom et al. 2003; S. Willför et al. 2003).

In South America, there are two species of genus Araucaria, *Araucaria angustifolia* from Brazil and *Araucaria araucana* from Chile. Phytochemical studies from knots of *Araucaria angustifolia* reported high content of lignans such as pinoresinol, also with a remarkable resistance to biodegradation by xylophages in comparison with the rest of the wood (Anderegg y Rowe 1974). While, in *A. araucana* there is not information about the composition of knotwood, in spite that stemwood and resins reported lignans, terpenoids, and resinic acids (Schmeda-Hirschmann et al. 2005; Céspedes et al. 2006). With the aim to determinate the chemical composition of knots of *Araucaria araucana* and assess its role in the resistance against biodegradation, we compared the total phenolic content and antioxidants properties in extractives of stemwood, branch, and knotwood of *A. araucana*, together with the purification and elucidation of the major components of the knotwood extract, as well as the protective role in wood decomposition against white-rot fungi were evaluated.

Materials and methods

Plant material

Samples of stemwood, branch (10 cm in diameter) and knots of *A. araucana* were collected in May 2018 on Nahuelbuta Range (Biobío Region) on the border with the Araucanía Region (37°41′43.56″S 73°07′49.34″W), were identified by the botanist Dr. Roberto Rodriguez from the University of Concepcion. The samples of 100g each were chopped, dried at 40 ºC for 8 h, and milled to 1-3 mm. The samples were stored with the code GMAA2018 in the Laboratory of Chemistry of Natural Products of the University of Concepcion.

Extraction and purification of *A. araucana*

The wood samples of *A. araucana* were extracted in a continuous extraction system (Soxhlet) following the protocol of Willfor et al. 2006. Briefly, the samples of stemwood, branch, and knots (100 g of each one) were extracted continuously in acetone (1200 ml), then the organic solvent was evaporated

in vacuo, giving a total extract for: stemwood 0.9 g, branch, 1.2 g and knots 26.1 g. Total extracts were kept at -20°C until use.

A part of the total extract of knots (10 g) was further fractionated by silica gel column chromatography, giving a primary fractioning of four fractions (AA-F1 to AA-F4) by using increasing polarity of solvents from n-hexane to MeOH. Fraction AA-F1 was eluted with n-hexane/EtOAc 1:1 (vol/vol), obtaining a concentrate of eudesmin, which was further crystallized from methanol at -20 °C obtaining pure colorless crystals (536 mg, 1.4 % yield in dry wood). Fraction AA-F2 was eluted with n-hexane/EtOAc 1:3 (vol/vol), giving a mixture of lignans. Further purification gave compound **2** (8 mg, amorphous solid, 0.021 % yield in dry wood). Fraction AA-F3 was eluted with n-hexane/EtOAc 1:4 (vol/vol), giving the compound **3** (8 mg, colorless crystals, 0.021% yield in dry wood). Fraction AA-F4 showed a dark gummy residue, without compounds visualized by TLC. Pure compounds and fractions were dried *in vacuo* and frozen at -20 °C until use.

Identification of natural compounds from Araucaria araucana

Purified compounds from knots of *Araucaria araucana* were identified by 1D and 2D NMR. 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 RT on a Bruker Avance III 500 MHz or 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), with the deuterium signal of the solvent as the lock and TMS (for ^{1}H) or the solvent (for ^{13}C) as internal standard. All spectra (^{1}H, ^{13}C, gs-H,H−COSY, edited HSQC, and gs-HMBC) were acquired and processed with the standard Bruker software.

Gas chromatography-Mass Spectrometry (GC-MS) analysis was performed using gas chromatography-mass spectrometry (GC-MS) (Agilent 7890, California, USA). Equipped with an Agilent 5975 mass detector, using a column HP5-MS 30 m x 0.25mm silica and a 0.25 μm film thickness, under the following conditions: Temperature: 250 °C; Detector (mass): 280 °C; Oven: initial at 100 °C for 5 min, increasing by 8 °C min^{-1} to 250 °C and held for 15 min. The detector was configured in scan mode from 50 to 500 amu. The carrier gas flow (electronic grade helium) was 1 ml min^{-1}. 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). For interpretation of the obtained mass spectra, they were compared with those reported in the literature (Yamamoto, Cox, y Simoneit 2010; Yamamoto, Otto, y Simoneit 2004).

Planar chromatography or High-performance Thin Layer Chromatography Mass Spectrometry (HPTLC-MS) was carried out on 10 x 10 cm HPTLC Silica gel 60 F_{254} plates. 2D chromatography was carried out under the following conditions: 30 µl of *A. araucana* knotwood extract (12 mg ml^{-1}) was applied on the plate and first eluted with dichloromethane-EtOH (94:6) mobile phase two times, removed, dried at room temperature and developed with toluene - ethyl acetate - formic acid mixture (6:4:1). Selected bands were eluted from the plate to MS by means of CAMAG TLC-MS interface assembled with oval elution head (4.0 x 2.0 mm) using a mixture of methanol and acetonitrile 1:1 vol/vol at a flow rate of 0.1 ml min^{-1} for 60 seconds. MS analysis was performed in Shimadzu (Kyoto, Japan) LCMS 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.

Determination of total phenols

Total phenol content was determined using the Folin-Ciocalteu method, based on the protocol of Ragazzi and Veronese with some modifications (Ragazzi y Veronese 1973). It was added in 200 µl of extract solution (at 1 mg ml^{-1} of concentration) to 0.1 g L^{-1} with 1000 µl of Folin-Ciocalteu 2 N Merck® 10 % (vol/vol) reagent by resting the mixture at room temperature for 8 min. Then 800 µl of Na_2CO_3 75 g l^{-1} solution was added and left to incubate for 15 min at 45 °C. The complex blue mixture was then measured at an absorbance of 750 nm and the equivalent result expressed as µg ml^{-1} acid gallic equivalent GAE. Water was used as a blank.

Antioxidant activity against DPPH radical

The antioxidant activity of the extract from the knotwood, steamwood and branch were evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazil) radical reduction test according to the methodology proposed by Hatano et al. 1989. Concentrations of 100, 50, 25, and 10 µg ml^{-1} were prepared from the total extracts in ethanol. An aliquot of 150 µL was taken from each sample and mixed with an equal volume of DPPH solution (60 mM). The resulting solution was vortexed and the absorbance was measured against the target on Biotek® ELX800 microplate reader at a wavelength of 520 nm. The results were expressed as the percentage of antioxidant activity and compared with the TROLOX reference standard (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid) as a control (Hatano et al. 1989). The DPPH radical reduction activity was calculated with the following formula:

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

Where Abc is the absorbance of Control (ethanol). Abs is the absorbance of the sample, lectures at 520 nm. Data is reported as % DPPH radical inhibition.

Decay resistance test to basidiomycetes xylophagous fungi

For this assay the method of test for determining the resistance against wood-destroying basidiomycetes code UNE-ENV 12038 was used, with few modifications. Briefly, *Pinus radiata* wood blocks 50 mm x 25 mm x 15 mm with the longitudinal faces parallel to the fiber direction and humidity 12%, were extracted with acetone for 3 h to remove their extractives. Then, they were dried at room temperature for 48 h then at 103 ºC for 16 h. The wood blocks were weight getting the initial dry mass (**m1**), then 10 test wood blocks per treatment were impregnated with the following solutions: Nipacide® P511 5% (wt/vol), as positive control, stemwood-extract at 1, 5 and 15 % (wt/vol), branch-extract of 1, 5 and 15 % (wt/vol), knotwood-extract of 1, 5 and 15 % (wt/vol), eudesmin 0.5% (wt/vol), and blank control (without treatment).

Pleurotus ostreatus code FQ1646, corresponding to a native strain collected in the Nahuelbuta mountain range, provided by the fungal collection of the laboratory of Natural Products of University of Concepción, was cultured in Agar-malt culture medium, incubate 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 they incubated by 16 weeks in the dark at 25 ºC. After this incubation, the wood blocks 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 × 100

Decay resistance test to basidiomycetes xylophagous fungi by wood of *A. araucana*

This assay was following the previous method UNE-ENV 12038, replacing the blocks of *Pinus radiata* for blocks of *A. araucana* stemwood, which is more susceptible to decay. Ten blocks of *A. araucana* stemwood were continuously extracted with methanol in a soxhlet to remove their extracts completely, then it was dried at 25 ºC for 48 hours. The other ten blocks of *A. araucana* stemwood were kept as a control without treatment. Then all of them were inoculated with the fungus *P. ostreatus* code FQ1646 and incubated in the same conditions described in the previous method.

Statistical analyses

All results were presented with their means ± SD. To compare the differences of each treatment, a one-way analysis of variance (ANOVA) was performed. Subsequently, a Tukey HSD post-hoc test was performed to compare the means of each treatment. Prior to these analyzes, the assumptions of Shapiro-Wilk normality and Levene variance homogeneity test were evaluated. Differences were considered to be statistically significant when $P < 0.05$. All statistical analyses were performed with the program Statistica™ 12.0.

Results and discussions

The aim of this research was carried out by analyzing the chemical components of *A. araucana* knots and correlate their properties to the natural resistance to degradation against white-rot fungus (Figure 2).

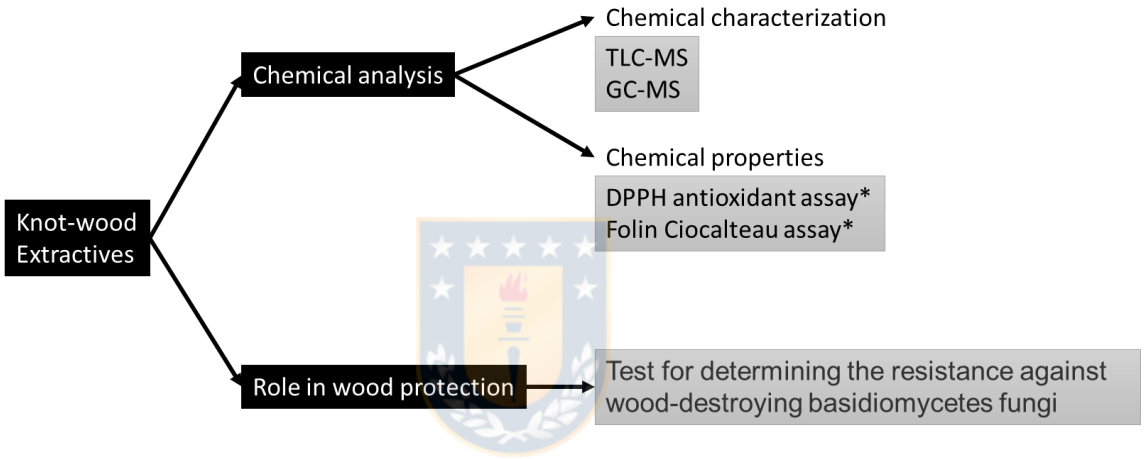

Figure 2 Steps to understand the effect of extractives in the protection of *A. araucana* wood. *Data comparison with wood extracts from other conifers and extracts from other *A. araucana* tissues.

Phytochemical composition of wood of *A. araucana*

The extraction of organic compounds with acetone of different parts of *A. araucana* gave a remarkable higher yield from the knotwood with 26.1 % (wt/wt), followed by the branch 1.2 % (wt/wt) and stemwood 0.9 % (wt/wt).

The results of GC-MS analysis indicated a chemical profile abundant in lignans in the extract of *A. araucana* knotwood. GC-MS results in knot wood showed a presence of 3.76% terpenes and fatty acids, 89.57% lignans, and 6.93% triterpenes.

The purification by silica gel column chromatography and crystallization gave three compounds as solids, among them, only compound 1 identified as eudesmin was gotten as colorless crystals, Figure 3.

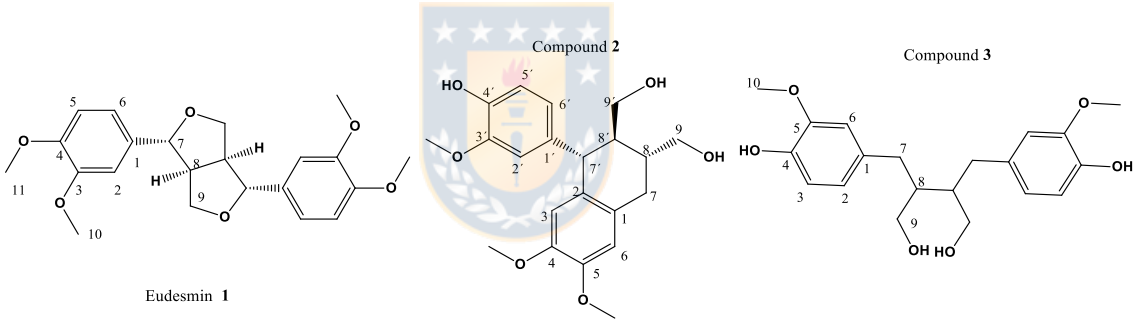

Figure 3 Structure of isolated lignans from *Araucaria araucana* knotwood.

Compound **1**, or Eudesmin or (1R,3aS,4R,6aS)-1,4-bis(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c] furan. ^{1}H NMR (600 MHz, CCl_3D) δ: 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). Mass: 386. Formula: $C_{22}H_{26}O_6$.

Compound **2**, ***Araucariol*** or ((1S,2R,3R)-1-(4-hydroxy-3-methoxyphenyl)-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene-2,3-diyl) dimethanol. ^{1}H NMR (500 MHz, DMSO) δ 6.68 (d, J = 8.0 Hz, 1H, H-5'), 6.67 (s, 1H, H-6), 6.65 (d, J = 2.0 Hz, 1H, H-2'), 6.50 (dd, J = 2.0, 8.1 Hz, 1H, H-6'), 6.21 (s, 1H, H-3), 3.84 (d, J = 9.6 Hz, 1H, H-7'), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OMe) 3.56 (dt, J = 4.4, 10.3 Hz, H-9), 3.45 (s, 3H, OCH_3), 3.44 (t, J = 2.8 Hz, 1H, H-9'), 3.42 (t, J = 2.7 Hz, 1H, H-9) 3.19 (td, J = 4.3, 10.90 Hz, 1H, H-9'), 2.72 (dd, J = 5.4, 16.0 Hz, 1H, H-7), 2.67 (dd, J = 10.4, 16.0 Hz, 1H, H-7), 1.83 (m, J = 5.1 Hz, 1H, H-8), 1.65 (m, J = 3.3 Hz, 1H, H-8').^{13}C NMR (500 MHz, DMSO) δ 147.3 (C-3'), 146.8 (C-5), 146.6 (C-4), 144.6 (C-4'), 136.9 (C-1'), 132.3 (C-2), 129,2 (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 (3'-OCH_3), 55.6 (4-OCH_3), 55.4 (5-OCH_3), 45.9 (C-8'), 45.9 (C-7'), 38.0 (C-8), 32,2 (C-7). Mass: 374,17. Formula: $C_{21}H_{26}O_6$.

Compound **3**, secoisolarisiresinol, or 2,3-bis(4-hydroxy-3-methoxybenzyl) butane-1,4-diol. ^{1}H NMR (600 MHz, Acetone) δ: 6.72 (d, J= 2.1 Hz, 1H, H-6), 6.70 (d, J= 8.1 Hz, 1H, H-3), 6.61 (dd, J= 8.2, 2.1 Hz, 1H, H-2), 3.76 (s, 3H, H-10), 3.68 (m, 1H, H-9), 3.53 (m, 1H, H-9), 2.67 (m, 2H, H-7), 1.90

(m, 1H, H-8). ^{13}C NMR (600 MHz, Acetone) δ: 147.2 (C-5), 144.5 (C-4), 132.7 (C-1), 121.5 (C-2), 114.5 (C-3), 112.4 (C-6), 60.3 (C-9), 55.2 (C-10), 43.8 (C-8), 36.2 (C-7). ESIHRMS [M+1]: 363.1803. Formula: $C_{20}H_{27}O_6$

Eudesmin has been previously identified in stemwood of *A. araucana* (Céspedes et al. 2006). Here we obtain eudesmin in a remarkable extraction yield of 1.4 g of eudesmin per 100 g of dry knotwood. Compound **2** has never isolated in conifer species, while compound **3** or secoisolarisiresinol was previously isolated from stemwood of *A. araucana* (Céspedes et al. 2006).

The phytochemical study of the knotwood extract by HPTLC-MS and GC-MS gave eleven related compounds of type lignans, which have been identified with an excellent agreement to the library NIST17 database and bibliography, Figure 4.

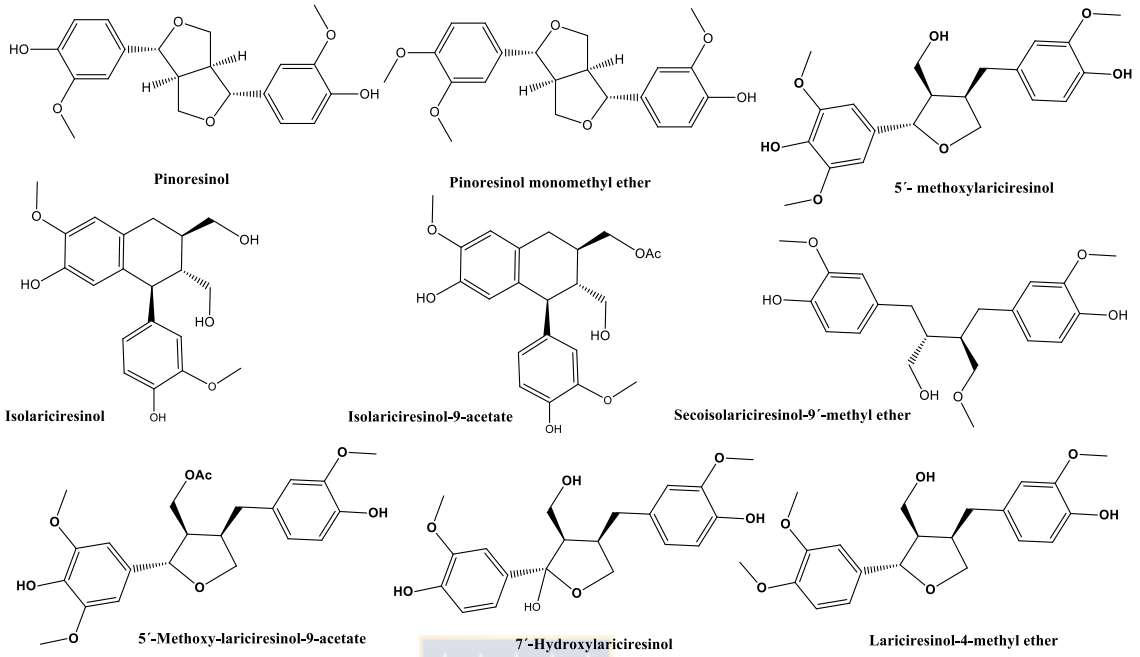

Figure 4 Some structure of compounds determinate by HPTLC-MS

Putative identification was carried out by analyzing the *m/z* signals of each spectrum iterating to the most frequent adducts in HPLC-ESI-MS and comparing it with lignan databases in the literature (Fischer et al. 2012; Horai et al. 2010; Meagher et al. 1999).

These results revealed 11 lignans (Table 4) as the main constituents in the knotwood extract of *A. araucana*. Three dibenzyl butane derived compounds: secoisolariciresinol, secoisolariciresinol-9'-methyl ether, secoisolariciresinol-4(or 9)-methyl ether-9'-acetate; Four lariciresinols

derived compounds: Lariciresinol-4' (or 4)-methyl ether, 5' (5 or 7')-Methoxy-lariciresinol-9-acetate, 7'-Hydroxylariciresinol (or isomer), 7' (or 5)-Methoxylariciresinol; Three compounds of the furofuran type pinoresinol, pinoresinol monomethyl ether, pinoresinol dimethyl ether (eudesmin); Two non-lactone naphthalene derivatives isolariciresinol and isolariciresinol-9 (or 9')-acetate.

Table 4 List of putative compounds identified in *Araucaria araucana* knots.

compound(s)	Formula	Mw	Rf-1D	Rf-2D
Secoisolariciresinol Compound 3	$C_{20}H_{26}O_6$	362	0.330	0.242
Secoisolariciresinol-9'-methyl ether	$C_{21}H_{28}O_6$	376	0.302	0.368
Lariciresinol-4' (or 4)-methyl ether	$C_{21}H_{26}O_6$	374	0.375	0.547
5´(5 or 7´)-Methoxy-lariciresinol-9-acetate	$C_{23}H_{28}O_8$	432	0.391	0.829
7'-Hydroxylariciresinol (or isomer)	$C_{20}H_{24}O_7$	376	0.658	0.129
7' (or 5)-Methoxylariciresinol	$C_{21}H_{26}O_7$	390	0.375	0.412
Pinoresinol	$C_{20}H_{22}O_6$	358	0.565	0.776
Pinoresinol monomethyl ether	$C_{21}H_{24}O_6$	372	0.569	0.881
Pinoresinol dimethyl ether (Eudesmin)	$C_{22}H_{26}O_6$	386	0.581	0.948
Isolariciresinol	$C_{20}H_{24}O_6$	360	0.253	0.207
Isolariciresinol-9 (or 9´)-acetate	$C_{22}H_{26}O_7$	402	0.385	0.141

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

Total Phenolic content and antioxidant capacity DPPH

The total phenols in samples of Stemwood, Branch and Knotwood of *A. araucana* were analyzed by the Folin-Ciocalteu method, expressed in gallic acid equivalent (GAE), results are summarized in Table 5.

Table 5 Total yield of polar extractives (% wt/wt) and total phenols obtained by the Folin-Ciocalteu method from stemwood, branch and knotwood of *Araucaria araucana*. Each value represents the average and standard deviation of 3 measurements.

Type of tissue	Stemwood	Branch	Knotwood
% (wt/wt) yield	0.9	1.2	26.1
µg ml^{-1} GAE	147.48 ± 0.18^{A}	336.60 ± 0.46^{B}	365.94 ± 0.32^{C}

*values ± SD, n=3, ** Values whit by different letters differ significantly (Tukey's test).

The extractive amount of samples of knotwood, branch, and stemwood of *A. araucana* showed a big difference. The knotwood extractive is 29 folds higher than the stemwood extractive and 21.8 folds higher than the branch extractive. The total polyphenol content in terms of GAE for the three samples showed that extracts of knotwood and branch of *A. araucana* have near values of 365 and 336 µg ml^{-1} respectively, in contrast to the extract of the stemwood of *A. araucana* which showed a phenolic content of 147 µg ml^{-1} GAE.

The DPPH radical reduction tests of samples of knotwood, branch and stemwood of *A. araucana* were compared with the DPPH values reported by Donoso-Fierro, et al. 2009 of the heartwood extract samples of species *Austrocedrus chilensis, Fitzroya cupressoides, Pilgerodendron uviferum*, where the decay wood protection for these three woods was explained by the high amount of phenols in these species, conferring by the high antioxidant activity (Table 6).

Table 6 DPPH radical reduction activity of knot, stemwood, and branch extracts.

	% Inhibition at different concentrations				
Extracts	100µg ml^{-1}	50µg ml^{-1}	25µg ml^{-1}	10µg ml^{-1}	Reference
A. chilensis	84.20±1.0	86.20±1.0	83.00±0.1	79.30±0.8	(Donoso-Fierro et al. 2009)
F. cupressoides	84.10±0.3	89.20±1.1	79.30±0.4	68.20±0.7	(Donoso-Fierro et al. 2009)
P. uviferum	87.00±0.8	83.80±0.5	72.00±0.5	66.80±0.4	(Donoso-Fierro et al. 2009)
Araucaria knotwood	$84.03±0.6^{A}$	$66.47±1.8^{B}$	$64.37±2.0^{B}$	$42.51±1.6^{C}$	
Araucaria branch	$73.35±1.8^{A}$	$65.57±1.5^{B}$	$50.40±1.9^{C}$	$35.43±0.7^{D}$	
Araucaria stemwood	$68.16±2.0^{A}$	$36.73±1.2^{B}$	$16.97±1.7^{C}$	$8.88±0.8^{D}$	
Trolox	$86.23±1.2^{A}$	$86.23±1.2^{A}$	$85.73±1.5^{A}$	$86.13±1.1^{A}$	

*values ± SD, n=3

** Values whit by different letters differ significantly (Tukey's test).

The extracts from *A. araucana* present a rank order in terms of DPPH activity of knotwood > branch > stemwood, with a direct correlation of the extractives amount and the total polyphenol content, Table 5.

Decay resistance test to basidiomycetes xylophagous fungi

Figure 5 shows that when the wood was subjected to the different types of treatments for 12 weeks, significant differences were reported in the percentage of biomass loss ($p < 0.05$). The results show that the highest biomass loss was recorded in the untreated control (CT) with 11 ± 0.5 %, indicating a more significant degradation of the xylophagous fungus. In contrast, treatments with different extractives (T1-T5) report less biomass loss, with values that fluctuated between 1 ± 0.3 and 5 ± 0.7 %. Tukey's test showed that there were no significant differences between T1-T3 and T3-T4. However, treatment with 0.5% Eudesmin (T5) was significantly more effective, preventing the attack of the xylophagous fungus (Figure 4).

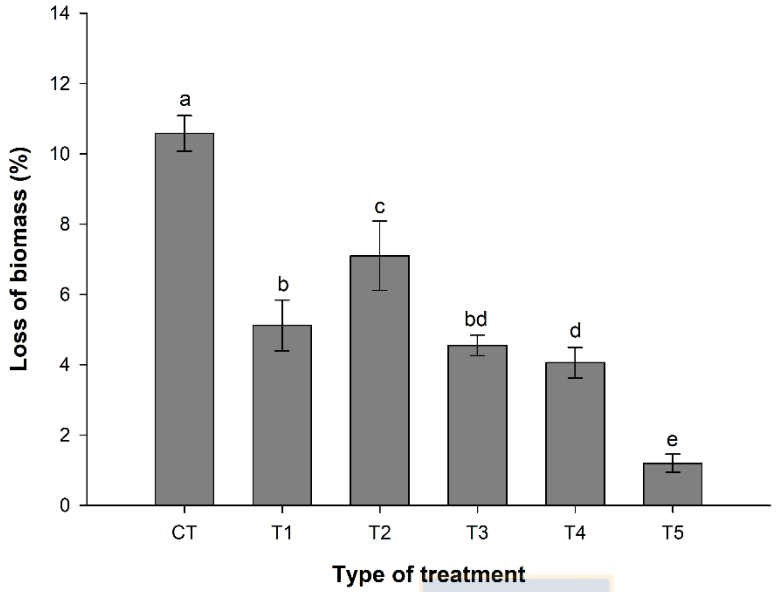

Figure 5 Percentage loss of biomass in wood ± standard deviation exposed to different treatments with white rot fungi, for 12 weeks (n=10). Values whit by different letters differs significantly (Tukey's test). CT=Control; T1=Nipacide; T2= 1% (wt/vol) Knotwood Extract; T3= 5% (wt/vol) Knotwood Extract; T4=15% (wt/vol) Knotwood Extract.; T5=0.5% (wt/vol) Eudesmin.

In the test of natural resistance of *A. araucana* (stemwood) to the biodegradation of *P. ostreatus* **Figure 6**, a higher percentage of biomass 8.59 ± 0.6 % was lost in the stemwood with removed extractives (WOR). Compared to the non-extracted araucaria wood (WE), which reported a significantly lower loss of 4.01 ± 0.4%.

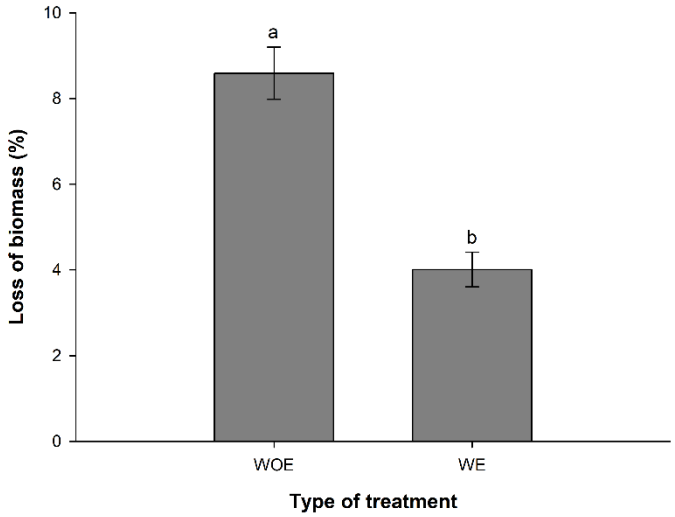

Figure 6 Percentage loss of biomass in wood of *A. araucana* ± standard deviation exposed to treatments with white-rot fungi, for 12 weeks (n=10). Values with by different letters differ significantly (Tukey's test). WOE=Araucaria wood without its natural extractives; WE=Araucaria wood with its natural extractives.

Discussion

In the present work, it also proved to be an effective technique to obtain a fast-general profile of other lignans in *A. araucana* knotwood extracts Previous work has shown that HPTLC can be a simple, rapid, and accurate method for the quantification of sesamin and sesamolin in sesame oil and its polyherbal formulations (Sukumar, Arimboor, y Arumughan 2008). This technique could be useful to study other plants that are abundant in lignans, such as knotwood from other conifers, or other tissues or matrices containing phenolic compounds.

Previous studies on *A. araucana* identified five lignans such as; secoisolariciresinol (33% of total extract), eudesmin (18.24%), lariciresinol (10.09%) pinoresinol (7.32%) and 4'-methoxy-pinoresinol (2.96%) in the extracts obtained from stemwood with MeOH (Céspedes et al. 2006). The lignans secoisolariciresinol and isolariciresinol have also been reported in the heartwood of the Chilean conifer *Fitzroya cupressoides* (Donoso et al. 2008). While knots of *A. angustifolia,* from Brazil, has shown secoisolariciresinol, pinoresinol, eudesmin, monomethyl pinoresinol, hinokiresinol, monomethyl lariciresinol, dimethyl lariciresinol, and lariciresinol (Anderegg y Rowe 1974; S. M. Willför et al. 2003). The knots of these species also have a similar pattern of remaining for years in the soil without degrading, suggesting than their lignans content gave wood protection.

In species of the north hemisphere, branches of the Norway spruce produce a higher concentration of lignans than knots, which could indicate an important role of lignans in the lignification process to reinforce the tissues of the knots as support of the branches, in the same species it has also been observed a higher production of lignans in less favorable environments with more climatic stress by cold and snow, so it would be a chemical defense in response to an eventual injury (Piispanen et al. 2008; S. Willför et al. 2003).

Our results reveal insights into the chemical defense mechanism of *A. araucana* and highlight the role of lignans in protecting against xylophage fungi. We found the highest amount of extractives, polyphenols and DPPH activity in *A. araucana* knotwood extract, than branch wood extract and lowest values were observed in *A. araucana* stemwood extracts, this results are close related with the resistance of different parts of *A. araucana* wood to xylophagous fungi attack, suggesting that the resistance of knots against *P. ostreatus* is because the high content of polyphenols. Moreover, we have seen that eudesmin gives wood protection even at 0.5% (wt/wt) more efficiently than commercially available products, and considering the high concentration of eudesmin in *A. araucana* knots, 1.4% (wt/wt), it could be an essential factor in the resistance of the knotwood to degradation in the environment. Eudesmin has reported also a high antifungal activity against some phytopathogens such as *Colletotrichum gloeosporioides*, *Phomopsis obscurans* and *Fusarium oxysporum* at a concentration between 50 and 150 µM (Cantrell et al. 2005), while eudesmin, lariciresinol and pinoresinol at 100 µg/ml suppress the growing of the white-rot fungi, *Trametes versicolor* with an inhibition effect of 33.6% respect to ketoconazole as positive control,

only overcome by secoisolariciresinol with a 45.1% inhibition at the same concentration (Céspedes et al. 2006).

Lignocellulosic fungal enzymes are unable to penetrate the walls of wood cells, therefore, they developed mechanisms of free radical release responsible for local lignin attack. Due to their chemical properties, lignans have a high chelating capacity, which is effective in combating degradation attack by xylophagous fungi, which use copper-dependent peroxidases and laccases in the case of white rot fungi and iron (Fenton reaction) in the case of brown rot fungi (Donoso-Fierro et al. 2009). In addition to metal chelating properties, lignans are also capable of reducing reactive oxygen species (e.g. hydroxyl ions, superoxide) that are necessary for the enzymatic or oxidative process for wood decomposition (Donoso-Fierro et al. 2009; Valette et al. 2017).

Hence, the role of the high presence of lignans in the knots of *A. araucana*, over branches, could be because the branches constitute a potential weakness in the tree, due to biotic and abiotic lesions. Thus, after a break, or cut of the branch and exposure of the internal tissues, the knots could act as a chemical barrier against the entry of invading organisms (bacteria, fungi, and insects).

Besides, *A. araucana* has the characteristic of naturally self-pruning of its monopodial branches, a common feature to other conifers whose knots also have a high lignan content, evidencing the important role of extractive in the protection of the tree to pathogens and oxidative damage (Kebbi-Benkeder et al. 2015) by antioxidant, chelating, and biocide properties.

Our results suggest that the high variety and amount of lignans in the extractives of *A. araucana* knots, give protection to decay by wood-degrading fungi.

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We declare no conflicts of interest.

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DISCUSIÓN GENERAL

En los análisis de extraíbles realizados en diferentes tejidos leñosos de *A. araucana*, mostraron notables diferencias cuali y cuantitivas. Respecto al contenido total de extraíbles se encontraron en los nudos 26%, rama 1.2% y tronco 0.9%. La madera del tronco presentó una menor cantidad de lignanos, seguido por la rama, los nudos fueron por lejos los más abundantes en variedad y cantidad de lignanos. Se encontró a lo largo de la rama un gradiente en la concentración de lignanos desde la punta al nudo.

Las razones de la alta concentración de extraíbles en los nudos, específicamente en el lumen de las traqueidas (Mansikkala et al. 2020), no se han aclarado totalmente. Los perfiles de lignanos, cualitativamente similares, entre rama y el nudo pueden ser explicados debido a que ambos poseen madera compresión, en el caso de la rama esta se encuentra solo en la zona basal o abaxial del tejido (X. Li et al. 2014), y en el caso del nudo, todo el tejido es madera compresión (Nisula 2018). Además esta madera presenta un alto contenido de lignina y está directamente relacionado a la alta abundancia de lignanos, porque que ambos tipos de compuestos fenólicos comparten rutas biosintéticas (Suzuki y Umezawa 2007). Fisiológicamente, estos se

sintetizan en la albura transportándose al duramen, actividad que aumenta cuando el árbol se daña, pero no se comprende bien cómo se mueven y acumulan en los nudos (Kebbi-Benkeder et al. 2015).

La acumulación en los nudos podría ser una adaptación al clima nevado y al peso causado por las ramas, así, en especies del hemisferio norte, individuos *Picea abies* producen en sus ramas una mayor concentración de lignanos en los nudos en ambientes menos favorables con más estrés climático por frío y nieve, lo que podría indicar un importante papel de los lignanos en el proceso de lignificación para reforzar los tejidos de los nudos como soporte de las ramas, por lo que además sería una defensa química en respuesta a una eventual lesión (Piispanen et al. 2008).

De este modo, tras una ruptura o corte de la rama y la exposición de los tejidos internos, los nudos podrían actuar como una barrera química contra la entrada de organismos invasores. Además, *A. araucana* tiene la característica de la autopoda natural de sus ramas monopodiales, un rasgo común a otras coníferas cuyos nudos también tienen un alto contenido de lignanos, lo que pone de manifiesto el importante papel de los extraíbles en la durabilidad natural de la madera. Además las propiedades antioxidantes, quelantes y

biocidas de los extraíbles, estos protegen al árbol frente a patógenos y a daños oxidativos (Kebbi-Benkeder et al. 2016; 2015).

Al analizar la composición de los extraíbles de la madera de araucaria se encontró que estaban constituidos en alrededor del 90% de lignanos, siendo dos los mayoritarios, secoisolariciresinol y eudesmin. Este porcentaje está de acuerdo con lo reportado por Céspedes et al. 2006.

En este estudio se encontró que la madera de los nudos *A. araucana* contienen un promedio de 45 mg g^{-1} de secoisolariciresinol. Este lignano ha sido reportado anteriormente en la madera de nudo de especies del género *Abies*, en una concentración de 25-60 mg g^{-1}, que cumpliría un rol fundamental en la protección de la madera frente a xilófagos (Ali et al. 2020; Holmbom et al. 2003).

Otro lignano detectado en alta abundancia en la madera del nudo de araucaria es eudesmin con un contenido promedio de 14 mg g^{-1}. Este compuesto se destaca del resto de los lignanos identificados, por la propiedad simétrica de su estructura, lo que explicaría su estabilidad frente a la biodegradación por hongos de pudrición blanca (Rúgolo et al. 2020 en prensa).

Respecto a estudios en los extraíbles de la madera de los nudos en otras especies del género (Anderegg y Rowe 1974), publicaron como componentes principales: secoisolaricirenol, isolariciresinol, pinoresinol, pinoresinol monometiléter, pinoresinol dietiléter (eudesmin) y hinokiresinol. En los extraíbles de *A. araucana* el único compuesto que no se encontró fue hinokiresinol.

Al evaluar el efecto protector de la madera con los extraíbles y eudesmin se encontró un interesante efecto de protección de la madera tratada con eudesmin, aislado desde lo nudos, frente al hongo *P. ostreatus*, siendo el tratamiento con mayor efectividad, superior incluso al impregnador comercial y al extracto total de madera del nudo. Esta acción antifúngica está de acuerdo con estudios anteriores en el cual eudesmin posee actividad frente a los hongos *Mucor miehei, Paecilomyces variotii, Ceratocystis pilífera y Trametes versicolor* (Céspedes et al. 2006). La razón de su actividad se podría explicar por acción directa antifúngica de dicho compuesto al carecer de grupos OH fenólicos que permiten la quelación de metales.

Algunos investigadores sugieren que podría existir un efecto de protección del eudesmin que se debería a sus propiedades hidrofóbicas, mediante la

impermeabilización superficial de la madera, impidiendo el ingreso de humedad y micelio del hongo xilófago (S. Willför et al. 2003).

En el caso de otros lignanos como secoisolariciresinol, lariciresinol que están presentes en los extraíbles de la madera de araucaria, poseen grupos OH fenólicos libres, tiene la capacidad quelante de metales y antioxidantes, lo que explicaría su efecto antifúngico mediante el secuestro de los cofactores enzimáticos necesarios para la degradación de la madera (Schultz y Nicholas 2000). Sumado a lo anterior, existe el efecto antioxidante de los lignanos para reducir las especies reactivas de oxígeno que son necesarias para el proceso enzimático u oxidativo de la descomposición de la madera (Belt, Hänninen, y Rautkari 2017; Donoso-Fierro et al. 2009).

El rol y la importancia de los extraíbles en la durabilidad natural de la madera quedó demostrado en el ensayo de susceptibilidad con las probetas de madera de pino, que fueron impregnadas con diferentes concentraciones de extraíbles obtenido de los nudos de *A. araucana*. Esta protección fue aún más efectiva que el producto comercial usado (NIPACIDE® P511) para impregnar madera, frente a la degradación por un hongo de pudrición blanca *P. ostreatus*.

CONCLUSIONES GENERALES

1. Los nudos de la madera de *A. araucana* poseen un 26 veces más extraíbles que en el tronco y rama, y sus principales componentes son fenoles de tipo lignano.
2. Los análisis cuali y cuantitativos de los extraíbles de los nudos de *A. araucana* mostraron que el secoisolariciresinol es el lignano más abundante seguido de eudesmin y otros lignanos en menor concentración.
3. Dentro de los lignanos se identificó un nuevo componente identificado que hemos denominado como *araucariol* y que corresponde a la estructura ((1S,2R,3R) – 1 - (4-hidroxi-3-metoxifenil)-6,7-dimetoxi-1,2,3,4-tetrahidronaftaleno) dimetanol.
4. Se encontró que los extraíbles juegan un rol fundamental en la durabilidad natural de la madera de *A. araucana* frente a hongos de pudrición blanca.
5. Los lignanos secoisolariciresinol y eudesmin destacan por su actividad protectora de la madera frente a hongos de pudrición blanca. Esta actividad se debe a su acción quelante y antioxidante de los lignanos.

6. La acumulación de lignanos en los nudos genera una barrera química que protege una zona vulnerable al ataque de patógenos debido a la autopoda.

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ANEXOS

Norma Chilena Oficial NCh 789.Of 87. Maderas - Parte 1: Clasificación de maderas comerciales por su durabilidad natural.

Categoría	Nombre común	Madera - Nombre científico
1. Muy durables	Roble	Nothofagus obliqua (MIRB)BL
	Ciprés de las Guaitecas	Pilgerodendron uvifera (D.DON)
	Alerce	Fitzroya cupressoides (MOL) JOHNSTON
2. Durables	Raulí	Nothofagus alpina (POEPP.ET ENDL.) OERST
	Lenga	Nothofagus pumilio (POEPP:ET ENDL:) KRASSER
	Linge	Persea linge (NEES)
3. Moderadamente durables	Canelo	Drimys winteri FORST.
	Coigüe	Nothofagus dombeyi (MIRB) BL
	Tineo	Weinmannia trichosperma CAV:
	Ulmo	Eucryphia cordifolia CAV:
4. Poco durables	Araucaria	Araucaria araucana(MOL.) C. KOCR.
	Eucalipto	Eucalyptus globulus LABILL.
	Laurel	Laurelia sempervirens (R.PAV)TUL
	Mañío hembra	Saxegothaea conspicua LINDL.
	Mañío macho	Podocarpus nubigenus LINDL.
5. No durables	Álamo	Populus alba. Populus nigra L, Populus tremuloides L.
	Olivillo	Aextoxicon punctatum (R.ET PAVON)
	Pino insigne	Pinus radiata D.DON
	Tepa	Laurelia philippiana LOOSER

Fotografías zonas de recolección de material en Cordillera de Nahuelbuta 2017

Corte transversal de árbol muerto de A. araucaria con un cuerpo fructífero de un hongo pudridor blanco *Pleurotus ostreatus*.

Diferentes estados de degradación de la madera de *A. araucana*. Tronco, corteza y rama son degradados totalmente hasta dejar solo los nudos en la superficie del bosque.

Mesa decorativa hecha con restos de *A. araucana*, el efecto estrellado de la superficie es debido a un corte transversal del tronco atravesando los nudos.

Artesanías de *picoyo* hechas de los nudos de *A. araucana*.

Publicación en colaboración con el Dr. Maximiliano Rúgolo

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Biotransformation of Araucaria araucana lignans: Solid-state fermentation with a naturally occurring Pleurotus ostreatus strain

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Biotransformation of *Araucaria araucana* lignans: Solid-state fermentation with a naturally occurring *Pleurotus ostreatus* strain

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Abbreviated title: Biotransformation of *Araucaria araucana* lignans

Abstract

The effects of a naturally occurring Patagonian strain of the white-rot fungus *Pleurotus ostreatus* on *Araucaria araucana* wood lignin was evaluated. Lignans of infected and noninfected wood shavings and the activity of fungal ligninolytic enzymes were studied. Lignans were identified using gas chromatography with a mass spectrometry detector. Only eudesmin lignan resisted biological degradation. The highest laccase activity was 0.111 ± 0.067 IU.g^{-1} dry matter substrate, which was reached after 60 days, whereas the highest manganese peroxidase (MnP) activity was 0.220 ± 0.109 IU.g^{-1} dry matter substrate, which was reached after 25 days, when the fungus was grown in a solid-state culture using an ME medium. The degradation properties of this fungal strain may be useful for not only treating resinous wastes from the regional forest industry to produce biofuels but also improving paper production. Moreover, the capacity of this white-rot fungus to grow using resinous *A. araucana* as substrate suggests the possibility of using the wood shavings or sawdust of this and other conifers as a food source to culture *P. ostreatus*, an edible mushroom.

Key words: lignans; laccase; *Pleurotus*; *Araucaria*; GC-MS

Resumen

Se evaluaron los efectos de una cepa patagónica de *Pleurotus ostreatus* sobre la composición de los lignanos de madera de *Araucaria araucana*. Se estudiaron los lignanos presentes en virutas de madera infectada y no infectadas por el hongo y se evaluaron las actividades de las enzimas ligninolíticas. Los lignanos se identificaron mediante cromatografía de gases acoplada a espectrómetro de masas (GCMS). Solamente el lignano Eudesmin resistió la degradación biológica. La mayor actividad de la enzima lacasa detectada fue de 0,111 ± 0,067 UI.g^{-1}, que se alcanzó luego de 60 días, mientras que la mayor actividad de la enzima manganeso peroxidasa (MnP) fue de 0,220 ± 0,109 UI.g^{-1} a los 25 días de cultivo. Las propiedades degradativas de esta cepa fúngica pueden ser útiles, no solamente para tratar los desechos resinosos de la industria forestal regional sino también para producir biocombustibles a partir de estos, o aplicarlos en la producción de papel. Además, la capacidad de este hongo de podredumbre blanca para crecer en un sustrato resinoso como *A. araucana* sugiere

la posibilidad de usar las virutas o aserrínes de coníferas como sustrato para cultivar un alimento de valor como *P. ostreatus*.

Palabras claves: lignanos; lacasa; *Pleurotus*; *Araucaria*; GC-MS

Introduction

Only two species of the genus *Araucaria* Juss. occur in South America: *A. araucana* (Mol.) K. Koch, present in the Patagonian Andes of Argentina and Chile, and *A. angustifolia* (Bert.) O. Kuntze, present in northeastern Argentina, Brazil, and eastern Paraguay (Veblen 1982). *A. araucana* is a coniferous tree that can reach a height of 50 m and a diameter of 2 m. The Andean populations of this species are distributed between 37°24'S and 40°03'S at 900-1700 m above sea level; another population is located in the Chilean coastal range between 37°30'S and 38°30'S at altitudes ranging from 600 to 1400 m and receiving an annual precipitation of 1500-2500 mm. They are long-lived conifers that have survived the many glaciation events that have led to the various present-day distributions of araucaria forests (Villagrán & Armesto 2005).

Araucaria araucana wood is of excellent quality, light, easy to work, and has a yellowish-white color. For these reasons, it is highly valued for construction and carpentry work, and straight and cylindrical logs are used in the manufacture of ship masts. Its main defense against pathogens is its thick bark (which reaches 10 cm thick in adults) and the abundant resin they secrete when damaged. However, when exposed to the elements, its wood can be easily damaged by biotic and abiotic factors.

In addressing the durability of the species, Eaton & Hale (1993) defined natural durability as the wood's ability to resist biological degradation. Plants defend against pathogenic attacks via structural, physical and biochemical barriers that inhibit pathogen invasion and propagation. Lignans are secondary metabolites biosynthesized via the phenylpropanoid pathway and defined by IUPAC as dimeric C_6C_3 motifs linked at carbons 8 and 8′ (Moss 2000). These and other compounds are responsible for the natural resistance of wood against the actions of insects, fungi, and bacteria. It is important to analyze the antimicrobial activity of lignans as potential inhibitors of fungal/bacterial cytotoxins and enzymes and as inhibitors of microbial growth (Rowe 1989; Castro *et al*. 1996). Cespedes *et al*. (2006) revealed that lignans found in *A. araucana* extracts show antifungal activities against white-rotting and wood-staining fungi. Lignans derivatives have high antioxidative

potency and radical scavenging capacity (Heitner *et al*. 2010; Valette *et al*. 2017). However, lignans are not involved in the formation of physical barriers, such as the plant cell wall, for protection against fungal attacks.

Pleurotus ostreatus (Jacq.) P. Kumm. (Agaricales, Basidiomycota) is an edible white-rot fungus that is capable of growing on the trunk of *A. araucana.* In addition, a few species of xylophagous fungi, such as Hyphomycetes and Basidiomycetes, have been reported growing on *Araucaria* trees (Lechner *et al*. 2002), which possess strong antifungal activity (Cespedes *et al*. 2006). The most common pattern of white-rot fungi attack consists of the simultaneous decay of all wood components. This fungus is able to secrete specific ligninolytic enzymes that cause significant phenolic degradation (Shah & Nerud 2002; Ruiz-Rodríguez *et al*. 2011; Rugolo *et al*. 2016). The enzyme manganese peroxidase (MnP), produced by white-rot fungi, is capable of oxidizing phenolic lignin units and other phenolic compounds. It has been shown that the native ferric MnP enzyme donates two electrons to H_2O_2, allowing the formation of a porphyrin cation radical (Wariishi *et al*. 1988). Other enzymes produced by the white-rot fungus include laccases, a group of oxidoreductases that catalyze the oxidation of unspecific substrates (*p*-diphenols, *o*-diphenols, aryldiamines, aminophenols, and hydroxyindols) with the concurrent reduction of dioxygen to water (Cai *et al*. 1993). Lignin peroxidase has not reported in *Pleurotus* species.

Although *P. ostreatus* is typically isolated from nonresinous species, several specimens have been found growing and producing basidiomes on *Araucaria* The above-described properties of *Pleurotus ostreatus* have stimulated interest in the degradation patterns of strains of this fungus isolated from conifers in Patagonia, with the goal of developing potential applications to treat waste produced by the regional forestry industry, which is dominated by *Pinus* exploitation. Presently, the focus is on the development of biological technologies due to their low cost and environmental advantages over existing chemical methods. The pretreatment of agricultural and forestry waste using fungi has attracted considerable interest with respect to biofuel production (Madadi & Abbas 2017). Moreover, in the process of wood pulping, a portion of the unwanted lignocellulosic material is carried over to the following stages of processing and causes problems, such as the settling of deposits,

interference with chemical processes, and reduced efficiency. The use of enzymes, such as laccases, improves efficiency and decreases production costs in the paper industry (Buchert *et al.* 2002).

The aim of the present work was to evaluate the degradation, as measured by gas chromatographic/mass spectrometric (GC-MS) analysis, of lignans in wood extractives of *A. araucana* by a naturally occurring *P. ostreatus* strain.

Material and Methods

Fungal strain and culture conditions

Pleurotus ostreatus LPSC 1254 strain (La Plata Spegazzini Collection) was used in this study. The strain was maintained in Petri dishes at 4°C on ME agar (malt extract 1.2%, glucose 1% and agar 2%).

Spawn and solid-state cultures

Spawn production was prepared in 350 mL glass flasks filled with 200 g of wet boiled (for 20 min at 90–100°C) oat grains (*Avena sativa*) and 1% (w/w) $CaCO_3$. Flasks were sterilized for 1.5 h at 121°C, cooled to 23°C, inoculated with a 1 cm diameter plug of mycelium and then incubated at 23°C in the dark for 20 d with periodic shaking.

Another set of glass flasks were filled with 10 g (dry weight) of *Araucaria araucana* wood shavings as a substrate. The samples of wood shavings were produced in our laboratory and were collected from different trees. The humidity was adjusted to 75% (w/w) with distilled water. The flasks were covered with cotton plugs to allow air circulation and autoclaved at 121°C for 2 h. After cooling, they were inoculated with 5% w/w of the spawn (colonized oat grains) and then incubated at 23°C in the dark until total substrate colonization (30–40 days) with *P. ostreatus* mycelia. Wood shavings without fungus were used as controls.

Chromatographic techniques

P. ostreatus-colonized and control *A. araucana* wood shavings (in samples of 10 g each) were harvested and extracted with maceration three times using ethyl acetate at 30°C. The crude extract was then filtered and evaporated to dryness under vacuum. The total crude extract was separated using the TLC technique (Willför *et al*. 2006) in normal phase Merck silica gel with an ethanol-dichloromethane (7:93, v/v) solution as eluent. The separated analytes were detected by spraying TLC plates with a sulfuric acid-ethanol mixture (95:5, v/v) followed by a hold at 150°C for 2min in a hot plate.

The isolation and identification of lignans were performed according to Willför *et al*. (2006) by preparative layer chromatography (PLC) silica gel 60 F_{254} plates of 2 mm thickness (Merck) using ethanol-dichloromethane (7:93, v/v) solution as eluent. Bands were injected on the plate with a micropipette containing 500 mg of extract. Visible bands under UV light were removed manually, washed with ethyl acetate, filtered and injected into GC-MS equipment for identification.

GC-MS analysis was performed by gas chromatography/mass spectrometry (GC-MS) instrument (Agilent 7890, California, USA) equipped with an Agilent 5975 mass detector and a silica HP5-MS capillary column of 30 m x 0.25 mm internal diameter and 0.25 µm film thickness under the following conditions: temperature: 250°C; detector (mass): 280°C; oven: 100°C for 5min, increased at 8°C/min to 250°C, and maintained at 250°C for 15 min. The detector was set in scan mode from 50 to 500 amu. The carrier gas (electronic grade helium) flow rate was 1 $mL.min^{-1}$. Mass spectra were obtained from the total ion current (TIC), and identification of the constituents was based on comparison of the retention times with those of reference standards, with their linear retention indices determined relative to a series of n-hydrocarbons, and on computer matching against commercial libraries (NIST05 National Institute of Standards and Technology 2008).

Identification of compounds was performed based on comparisons with mass spectra in the literature (Yamamoto *et al.* 2004; Yamamoto *et al*. 2010) and database interpretation of mass spectrometric fragmentation patterns (NIST05a).

Enzyme activities

Laccase activity (E.C.: 1.10.3.2) was measured using 0.5 mM 2,2'-azino-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) in 0.1 M sodium acetate buffer (pH 3.6) at 30°C. Oxidation of ABTS was determined by monitoring the increase in absorbance at 420 nm (ε 420 = 36 $mM^{-1}.cm^{-1}$). Manganese peroxidase activity (MnP) (E.C.:1.11.1.13) was measured using phenol red and $MnSO_4$ as substrate in 0.1 M sodium dimethyl succinate buffer (pH 4.5) (ε 610 = 22 $mM^{-1}.cm^{-1}$) at 50°C. Enzyme activity was expressed in International Units (U) standardized as the amount of enzyme required to release 1 µmol of product in 1min. In terms of production, the activity was defined as $IU.g^{-1}$ dry residue. Samples from solid-state cultures were harvested after 25, 35 and 60 days. Crude extracts were obtained by adding distilled water to the samples from each freshly harvested culture (5:1, w/w), stirring for 20min, filtering and centrifuging (modified from Vares *et al*. (1995)). The supernatants were stored at -10°C until needed.

Results and discussion

TLC analysis of noninfected *A. araucana* wood shavings (control), referred to as Aa, revealed the presence of four highly visible spots that were identified based on their retention factor (Rf) values as eudesmin (Rf = 0.91), pinoresinol monomethyl ether (Rf = 0.82), lariciresinol (Rf = 0.47), and secoisolariciresinol (Rf = 0.32). Two bands with Rf values of 0.54 and 0.24 were not identified but corresponded to derivatives of lariciresinol (having molecular weights of 374 and 360). A similar pattern was observed in infected wood, referred to as Aa Inf, but the spots were less intense than those of the controls (Fig. 1).

The GC-MS chromatogram of the total extract of *A. araucana* wood shavings (Aa) is shown in Fig. 2, and that of the total extract of *A. araucana* wood shavings infected with *P. ostreatus* (Aa Inf) is shown in Fig. 3. Extracted ion chromatograms at m/z 151 were obtained for better detection of lignans. The identified lignans and derivatives and their molecular weights, molecular formulas, abundance percentages (relative peak area %) and retention times (min) in Aa and Aa Inf are listed in Table 1. The chemical structures of the detected lignans are shown in Fig. 4.

In Aa, the major components were eudesmin (28%), secoisolariciresinol-4-methyl ether-9'-acetate (25.8%), and lariciresinol (21.5%). The minority component corresponded to pinoresinol monomethyl ether (5.72%). Other components accounting for less than 2 % included shonanin (1.95%) and isovanillin (1.34%). In contrast, in Aa Inf, the number of lignans and derivatives was lower; they included isovanillin (26.2%), eudesmin (19.41%), and (E)-conipheryl alcohol (3.66%). The minority components (< 2%) were shonanin (1.67%) and secoisolariciresinol-4-methyl ether-9´-acetate (1.61%). However, three lignans detected in Aa, secoisolariciresinol, pinoresinol monomethyl ether, and lariciresinol, were not detected in the Aa Inf sample. The GC-MS analysis revealed the presence of 6 lignans, including the dibenzylbutanediol-type lignans secoisolariciresinol and secoisolariciresinol-4-methyl ether-9'-acetate, the tetrahydrofuran-type lignans lariciresinol and shonanin, and the furofuran-type lignans pinoresinol monomethyl ether and eudesmin. All of these lignans have also been reported in *Araucaria angustifolia* resin extracts (Yamamoto *et al.* 2004).

Additionally, some differences were observed in the relative abundances of compounds between Aa and Aa Inf that can be attributed to the fungus. In particular, the presence of eudesmin decreased from 28% in Aa to 19.41% in Aa Inf, whereas the amount of isovanillin increased from 1.34% in Aa to 26.62% to Aa Inf. The presence of (E)-coniferyl alcohol and isovanillin in the Aa Inf sample indicate wood degradation. Both compounds are not only precursors and abundant components in lignin polymer but also products of ligninolytic degradation. In fact, these products result from the activities of enzymes such as laccases (Su *et al*. 2018), MnP and aryl-alcohol oxidases, a small group of enzymes detected in *P. ostreatus* that are likely involved in lignan degradation (Lupo *et al*. 2009).

The white-rot-infected *A. araucana* wood contained mainly eudesmin, which was less reactive/more recalcitrant than the other lignans. This result could have been due to the absence of hydroxyl groups and the presence of β–β (α-O-γ) linkages in eudesmin. Moreover, the aryl-OCH_3 groups in these lignans might increase the stability of this molecule under fungal degradation. These phenomena likely explain why the LPSC 1254 strain of *P. ostreatus* was not able to easily degrade eudesmin. However, it is unclear how this compound is integrated into the lignin structure (potentially masked) and whether the mycelium lacks the enzymatic reactions that favor the

degradation of this compound. Eudesmin has been reported to have high antifungal activity against some phytopathogens, such as *Colletotrichum gloeosporioides*, *Phomopsis obscurans* and *Fusarium oxysporum*, at concentrations between 50 and 150 μM (Cantrell *et al.* 2005). In another study (Martínez *et al.* 2005), GC-MS analysis of *Eucalyptus globulus* hardwood degraded by the white-rot fungus *Ceriporiopsis subvermispora* showed a decrease in lignin peaks and an increase in carbohydrate peaks relative to fungus-treated wood but no difference in the total degradation of lignin-derived compounds.

Lignans have high chelating capacity, a feature that is especially effective in combating the degradation by xylophagous fungi that use peroxidases, Cu-dependent laccases, and iron-promoted Fenton reactions. In addition to having metal chelating properties, lignans are capable of reducing the levels of reactive oxygen species, which are necessary for the enzymatic or oxidative processes that lead to wood decomposition (Donoso-Fierro *et al*. 2009).

The laccase and MnP activities in solid culture are shown in Table 2. Enzymatic activity appeared first as high laccase activity, and the maximum level of enzymatic activity was observed on day 25 (0.148 ± 0.023 IU.g^{-1} dry matter substrate). Thereafter, the level of activity decreased rapidly, although low levels of laccase activity continued to be detected throughout the incubation period. MnP was first detected in the extracts after 25 days of fermentation. The level of activity increased throughout the experiment, reaching its maximum level on day 60 (0.220 ± 0.010 IU.g^{-1} dry matter substrate).

Elisashvili *et al.* (2006) reported that the presence of a lignocellulosic substrate is necessary for MnP production by *Pleurotus dryinus* (Pers.) P. Kumm. When *P. ostreatus* was cultured in wheat straw and tree leaves of *Fagus sylvatica*, it showed laccase activity values of approximately 75–4.00 and 1.75–3.50 IU.g^{-1} dry matter substrate, respectively, using ABTS, and MnP activities of 1.75–3.75 and 0.03–1.48 IU.g^{-1} dry matter substrate, respectively (Elisashvili *et al*. 2008). In a study of grapevine sawdust, Stajic *et al*. (2004) reported a laccase activity of 3.49 IU.g^{-1} dry matter substrate using syringaldazine for laccase detection.

The white-rot fungi *Pleurotus* spp. possess the ability to degrade the lignin polymers of wood tissues. Solid-state fermentation is an efficient method to study the biotransformation of *P. ostreatus* substrates and simultaneously produce hydrolytic and ligninolytic enzymes when fermenting lignocellulosic materials (Ozcirak Ergun & Ozturk Urek 2017). Our main aim in this study was to investigate the biotransformation of *A. araucana* extractives by the white-rot fungus *P. ostreatus* under solid-state fermentation conditions.

Conclusion

Lignans can be efficiently oxidized by *P. ostreatus* enzymes. Among the lignans detected in *A. araucana* wood in this study, only the lignan eudesmin showed little degradation. Of the lignans analyzed in this study, eudesmin seems to be the most recalcitrant to lignocellulolytic action by *P. ostreatus*. Nevertheless, all of the lignans seemed to be influenced; no unreacted lignan molecules in Aa Inf were detected in the GC-MS analysis. In addition, our study suggests that this white-rot strain can degrade other phenolic groups present in *A. araucana* lignans. This is the first study to culture *P. ostreatus* on *A. araucana* as a substrate. Future research should explore the ability of native strains of *P. ostreatus* to grow on, degrade and develop basidiomes on substrates generated from conifer debris from the regional forest industry, which is dominated by the use of *Pinus* species.

To determine the *in vitro* and *in vivo* roles of various lignans, further assays should be undertaken with purified molecules and mixtures to determine their independent and interactive effects.

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Conflict of interest

We declare that the authors have no conflicts of interest in connection with this manuscript.

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Figure captions

Figure 1 TLC analysis of white-rot-infected *A. araucana* wood shavings showing bands of ethyl acetate soluble compounds. Bands of infected samples are less intense than those of noninfected samples. **Aa Inf**: infected wood shavings, **Aa:** noninfected wood shaving. Reference bands correspond to eudesmin (Eu), pinoresinol monomethyl ether (Pi), secoisolariciresinol (Se), lariciresinol (La), and derivatives of lariciresinol (L1 and L2) are shown.

Figure 2 GC-MS chromatogram of the total extracts from healthy *Araucaria araucana* wood shavings

Figure 3 GC-MS chromatogram of the total extracts from *Araucaria araucana* wood infected with the white-rot fungus *Pleurotus ostreatus*

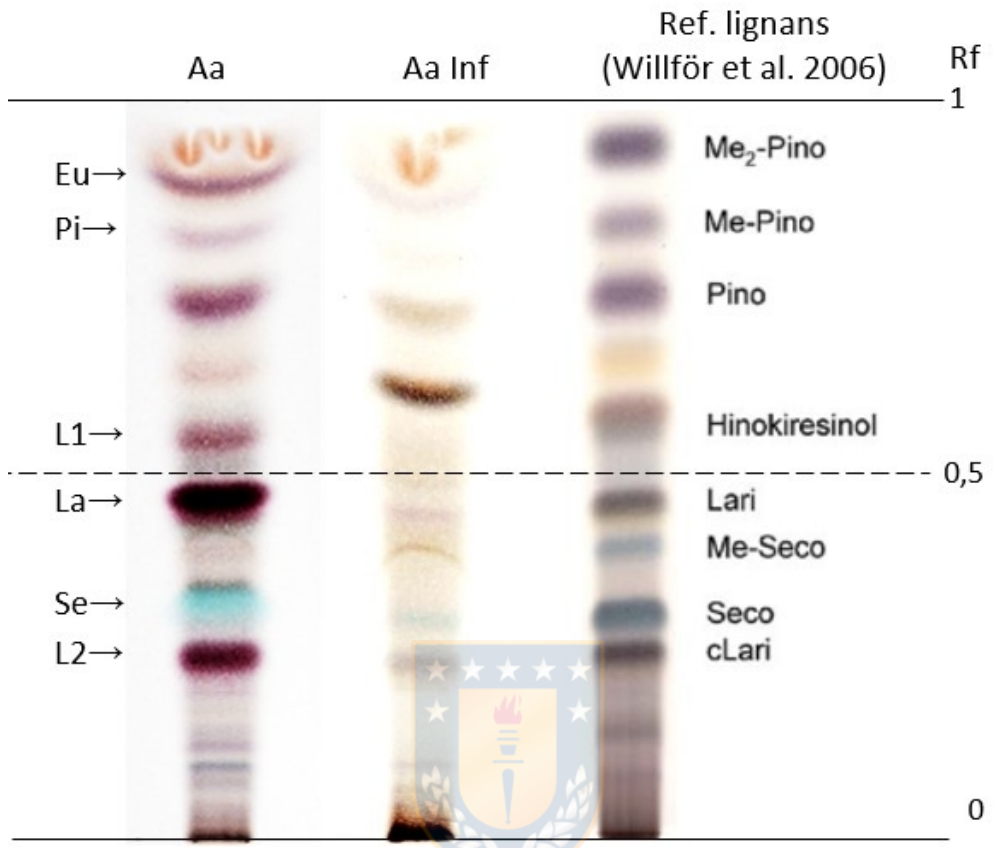
Figure 4 Chemical structures of lignan and other phenolic compounds. Se4: secoisolariciresinol-4-methyl ether-9'-acetate, Se: secoisolariciresinol, Eu: eudesmin, Pi: pinoresinol monomethyl ether, La: lariciresinol, Shy: shonanin, Co (E)-conipheryl alcohol, Is: isovanillin

Table 1. Lignans and derivatives detected in *A. araucana* wood shavings infected or uninfected with the white-rot fungus *Pleurotus ostreatus*

	Compound	Molecular formula	Molecular weight	Retention time (min)	Percentage of total in healthy wood	Percentage of total in infected wood
Is	isovanillin	$C_8H_8O_3$	152	8.32	1.34%	26.62%
Co	(E)-conipheryl alcohol	$C_{10}H_{12}O_3$	180	11.81	-	3.66%
Sh	shonanin	$C_{20}H_{24}O_5$	344	22.92	1.95%	1.67%
Se4	secoisolariciresinol-4-methyl ether-9′-acetate	$C_{23}H_{30}O_7$	418	24.21	25.83%	1.61%
Se	secoisolariciresinol	$C_{20}H_{26}O_6$	362	26.92	13.01%	-
Pi	pinoresinol monomethyl ether	$C_{21}H_{24}O_6$	372	27.17	5.72%	-
Eu	eudesmin	$C_{22}H_{26}O_6$	386	27.432	28.00%	19.41%
La	lariciresinol	$C_{20}H_{24}O_6$	360	27.910	21.51%	-
	Not identified				2.60%	47.03%
	Total				100%	100%

Table 2. Activities of laccase and manganese peroxidase (IU.g^{-1} dry matter) of *Pleurotus ostreatus* strain 1254 growing on *Araucaria araucana* wood shavings

Number of culture days	Enzyme activity (IU.g^{-1} dry matter)	
	Laccase	MnP
25	0.148 ± 0.023	0.030 ± 0.006
35	0.087 ± 0.006	0.054 ± 0.006
60	0.069 ± 0.015	0.220 ± 0.010



TLC analysis of white-rot-infected A. araucana wood shavings showing bands of ethyl acetate soluble compounds. Bands of infected samples are less intense than those of noninfected samples. Aa Inf: infected wood shavings, Aa: noninfected wood shaving. Reference bands correspond to eudesmin (Eu), pinoresinol monomethyl ether (Pi), secoisolariciresinol (Se), lariciresinol (La), and derivatives of lariciresinol (L1 and L2) are shown.

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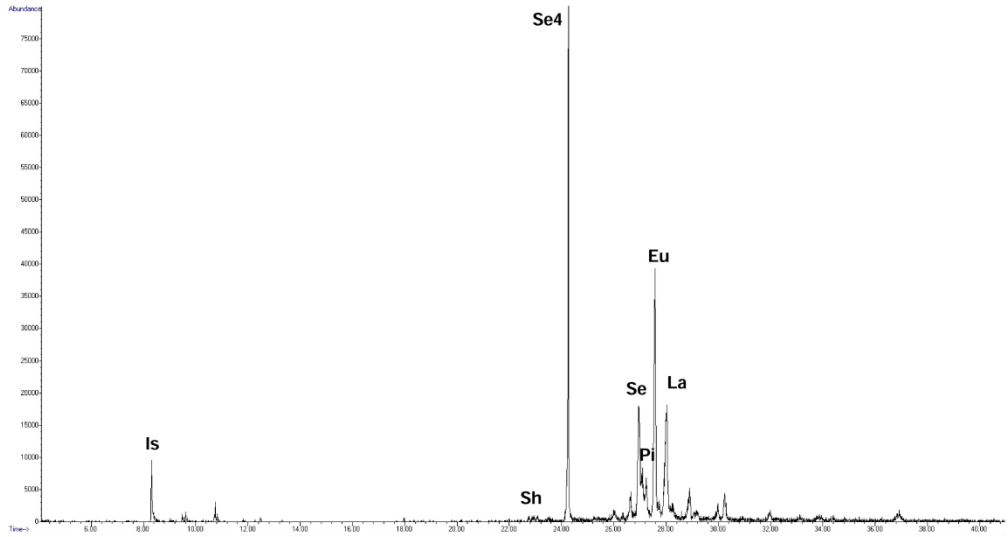
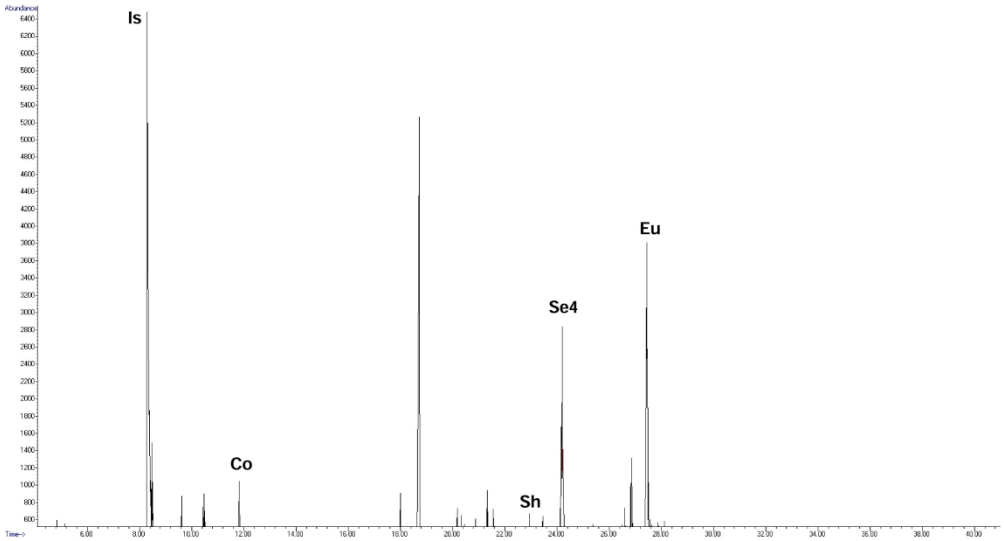


GC-MS chromatogram of the total extracts from healthy Araucaria araucana wood shavings

330x175mm (300 x 300 DPI)



GC-MS chromatogram of the total extracts from Araucaria araucana wood infected with the white-rot fungus Pleurotus ostreatus

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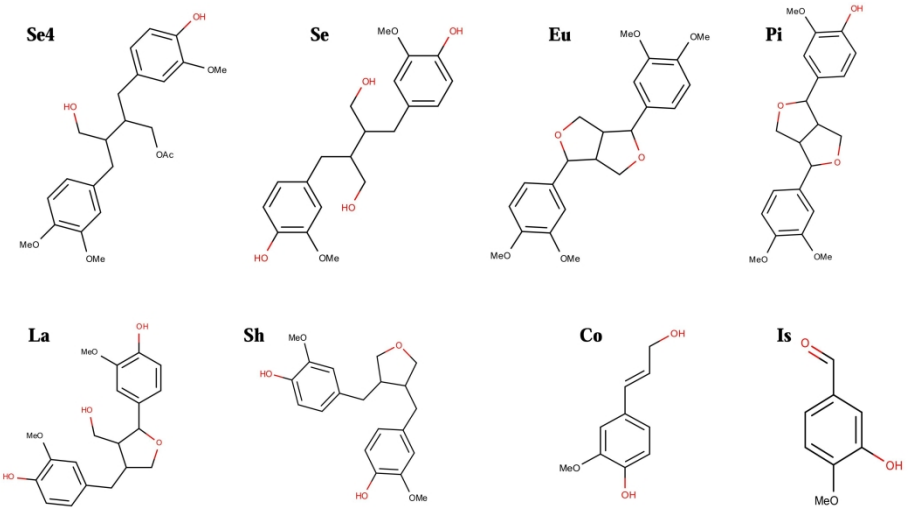

Chemical structures of lignan and other phenolic compounds. Se4: secoisolariciresinol-4-methyl ether-9'-acetate, Se: secoisolariciresinol, Eu: eudesmin, Pi: pinoresinol monomethyl ether, La: lariciresinol, Shy: shonanin, Co (E)-conipheryl alcohol, Is: isovanillin

338x190mm (300 x 300 DPI)