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ESTUDIO SOBRE EL COMPORTAMIENTO MORFOFISIOLÓGICO Y FITOQUÍMICO DE HOJAS DE *Aristotelia chilensis* (MOL.) STUNTZ BAJO DISTINTAS CONDICIONES DE AMBIENTE NATURAL E *IN VITRO*

Tesis presentada a la Facultad de Ciencias Forestales de la Universidad de Concepción para optar al grado académico de Doctor en Ciencias Forestales

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DEDICATORIA

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RESUMEN

Aristotelia chilensis (Mol.) Stuntz es una especie antioxidante perenne endémica de Chile. Crece en áreas abiertas o bajo el dosel de los árboles, y sus hojas emergen a principios de la primavera y el verano. El objetivo de este estudio fue analizar el comportamiento morfofisiológico y fitoquímico de hojas de *A. chilensis* con diferente edad ontogenética bajo diferentes condiciones de estacionalidad natural y crecimiento *In vitro*. Se realizó un estudio fisiológico sobre la respuesta del PSII en hojas adultas que representaban diferentes edades: hojas jóvenes, del tercio superior de la rama (hojas apicales, AP), y hojas completamente expandidas, del tercio inferior de la rama correspondiente a las hojas basales (BS). Tanto las BS como las AP de las estaciones estudiadas (invierno, primavera y verano) no mostraron estrés, lo que se refleja en los valores óptimos de Fv/Fm. El comportamiento del apagamiento no fotoquímico (NPQ) no se vio influido por el tipo de hojas y la estación del año. Los resultados sugirieron que un aumento en la intensidad de la luz (primavera) afectó positivamente a la actividad antioxidante y al contenido de fenoles totales, lo que se correlacionó con mayores valores de la tasa transportadora de electrones (ETR) y el apagamiento fotoquímico (qP). Los principales resultados de anatomía, las AP mostraron adaptaciones morfológicas, ya que las áreas de los espacios intercelulares y del parénquima en empalizada fueron mayores que en las BS. Para explicar las diferencias en la composición fenólica de las hojas adultas y de las hojas generadas *In vitro*, se evaluó la composición química del material vegetal. Se identificaron y cuantificaron un total de 16 compuestos fenólicos en las hojas originadas *In vitro* y 20 en los extractos de las hojas *ex vitro* de *A. chilensis* mediante HPLC-DAD-ESI (Ion Trap)-MS^n y otros equipos cromatográficos. Los grupos de familias identificados son los derivados del ácido gálico, el ácido cafeoilquínico, los derivados del ácido elágico, los elagitaninos y derivados de flavonoides. Además, se observa mediante las pruebas histoquímicas la acumulación de algunos metabolitos generales como polifenoles, terpenoides y alcaloides en las hojas adultas y hojas obtenidas *In vitro* de *A. chilensis*.

ABSTRACT

Aristotelia chilensis (Mol.) Stuntz is an evergreen antioxidant species endemic to Chile. It grows in open areas or under tree canopy, and its leaves emerge in early spring and summer. The aim of this study was to analyse the morpho-physiological and phytochemical behaviour of leaves of *A. chilensis* with different ontogenetic age under different conditions of natural seasonality and *In vitro* growth. A physiological study was carried out on the response of PSII to adult leaves represented different ages: young leaves, from the upper third of the branch (apical leaves, AP), and fully expanded leaves, from the lower third of the branch corresponding to basal leaves (BS). Both BS and AP from the studied seasons (winter, spring, summer) did not show stress, reflected in optimum Fv/Fm values. The behaviour of non-photochemical quenching (NPQ) was not influenced by the kind of leaves and season of the year. The results suggested that an increase in light intensity (spring) positively affected the antioxidant activity and total phenol content, which correlated with higher of electron transport rate (ETR) and photochemical quenching (qP) values. In the main anatomy results, AP showed morphological adaptations as area intercellular spaces and parenchyma palisade areas were larger than in the basal leaves. To explain differences in the phenolic composition of adult leaves and leaves generated *In vitro*, the chemical composition vegetative material was evaluated. A total of 16 phenolic compounds were identified and quantified in leaves originating *In vitro* and 20 in leaf extracts *ex vitro* of *A. chilensis* by HPLC-DAD-ESI (Ion Trap)-MS^n and other chromatographic equipment. The identified family groups are gallic acid derivatives, caffeoylquinic acid, ellagic acid derivatives, ellagitannins and flavonoids derivatives. In addition, histochemical tests show the accumulation of some general metabolites such as polyphenols, terpenoids and alkaloids in adult leaves and leaves obtained *In vitro* from *A. chilensis.*

INTRODUCCIÓN GENERAL

Las plantas constituyen una fuente importante de obtención de compuestos biológicamente activos, debido a la riqueza y complejidad de las estructuras químicas que se producen en el metabolismo secundario, incrementando su producción cuando la planta se encuentra en condiciones de estrés (Scossa & Fernie, 2020). Debido a esto, existen plantas silvestres que son sobreexplotadas ya que no existe una normativa ni organismos que regulen su recolección. Esto eventualmente, podría conducir a la extinción de un patrimonio genético y su biodiversidad. En esta situación se encuentra, *Aristotelia chilensis* (Mol.) Stuntz, ya que debido a la abundancia de compuestos bioactivos que contiene, existe un interés comercial creciente en su explotación (Misle et al., 2011). En este sentido, es fundamental evaluar su comportamiento fisiológico y fitoquímico bajo distintas condiciones en su hábitat natural, así como, encontrar una fuente alternativa de obtención de su biomasa vegetal mediante su multiplicación vegetativa por cultivo *In vitro*.

Las plantas, bajo determinadas condiciones, en su interacción con el ambiente, son capaces de producir más de 100.000 compuestos que se denominan metabolitos secundarios, que normalmente, no son esenciales para el crecimiento y desarrollo de las plantas y están implicados en los mecanismos de supervivencia de las mismas (Dai & Mumper, 2010). Dentro del metabolismo primario, la fotosíntesis es la principal fuente productora de energía mediante un proceso que se inicia con la absorción de la luz, por medio de los pigmentos fotosintéticos (clorofilas a, b y carotenoides) ubicados en el complejo antena en la membrana de los tilacoides, que puede seguir tres rutas diferentes: la disipación de la energía fotoquímica, la disipación térmica (o no fotoquímica) y la emisión de fluorescencia (Murchie & Lawson, 2013). La fotosíntesis es fundamental para

la producción de azúcares que son necesarios para la respiración celular, y la obtención de energía y esqueletos carbonados para el metabolismo secundario (Figura 1). Del mismo modo, muchas de las moléculas carbonadas generadas en el metabolismo primario son esenciales para la formación de compuestos en el metabolismo secundario, que intervienen en las respuestas de la planta frente a factores externos (Dai & Mumper, 2010).

Existe una gran variedad de metabolitos secundarios cuya biosíntesis pertenece a grupos taxonómicos concretos. Los precursores de estos compuestos proceden de rutas metabólicas básicas del metabolismo primario como el ciclo de Krebs, la glucólisis o la asimilación del nitrógeno. Así, los metabolitos secundarios se clasifican en tres grupos de acuerdo con su origen biosintético y estructura química, en compuestos nitrogenados o productos nitrogenados secundarios, compuestos fenólicos y terpenos o isoprenoides. Dentro del metabolismo secundario, la producción de compuestos fenólicos y compuestos nitrogenados se realiza por la ruta del ácido shikímico, y en menor medida, por la ruta del ácido malónico (Taiz et al., 2018).

La ruta del ácido shikímico es la principal vía de producción de compuestos aromáticos (Figura 2). Los precursores para la formación de aminoácidos aromáticos son la eritrosa 4-fosfato (E4P) proveniente de la ruta de las pentosas fosfato y fosfoenolpiruvato (PEP) que se forma en la glucólisis. Estos dos compuestos se condensan para formar deshidrocinato cíclico acompañado de la liberación de ambos grupos fosfatos, seguido de la eliminación del agua y la reducción del grupo carbonilo, se forma shikimato. Después de la protección del grupo 3-hidroxilo mediante la fosforilación catalizada por la enzima shikimato sintasa, el grupo 5-hidroxilo de shikimato reacciona con PEP para sintetizar enolpiruvilshikimato-3-fosfato (EPSP). Luego, la eliminación del grupo fosfato, da origen a corismato, que es el precursor común de la síntesis de los tres amino ácidos aromáticos: fenilalanina, tirosina y triptofano. La síntesis del triptofano se realiza mediante la vía del antranilato, y para la síntesis de fenilalanina (Phe) y tirosina (Tyr) a través de la vía del arogenato (Barros & Dixon, 2020).

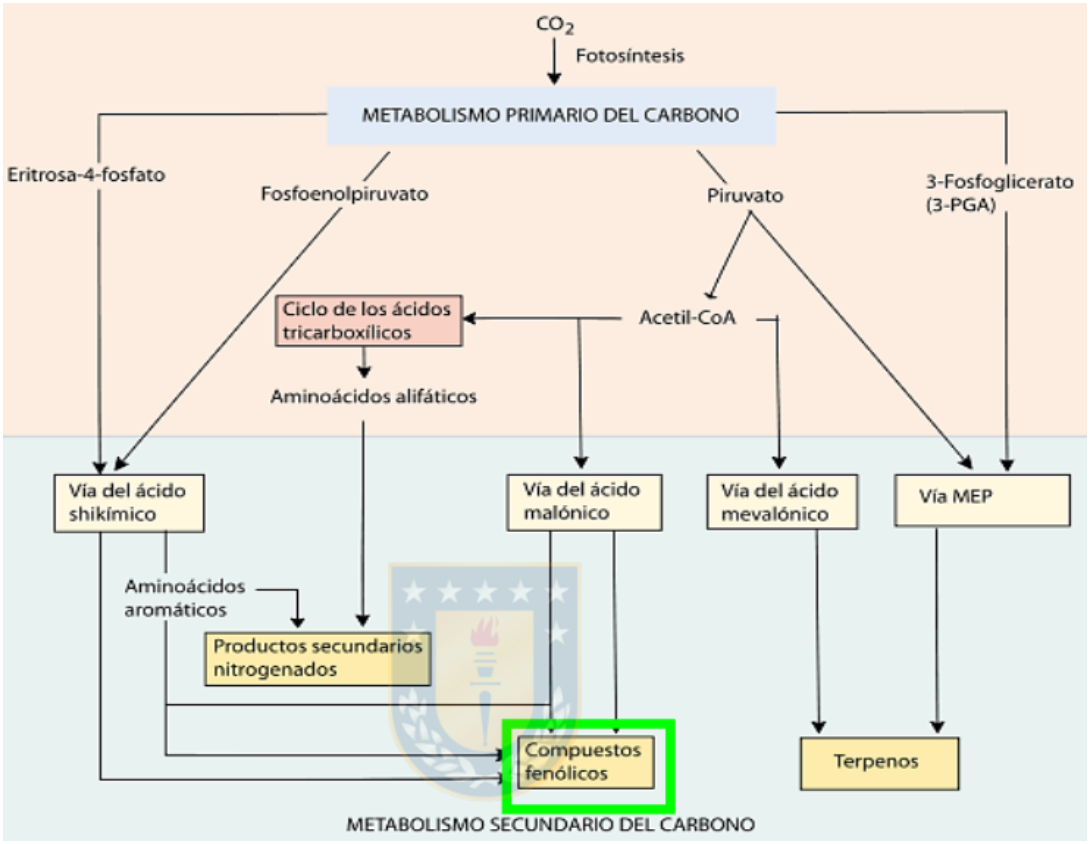

Figura 1. Metabolismo primario del carbono y su relación con la biosíntesis de metabolitos secundarios. Extraído de Taiz et al., (2018).

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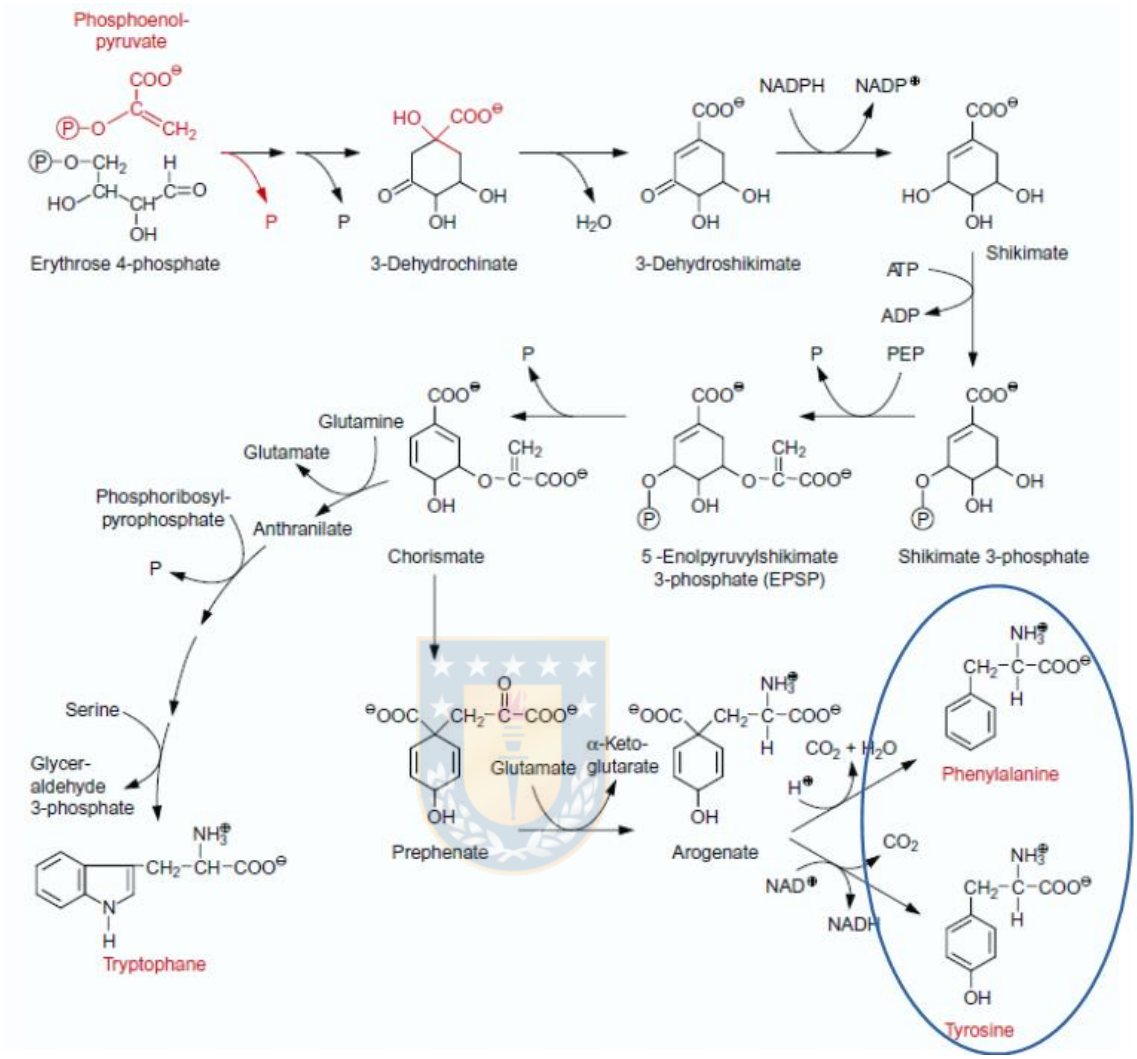


Figura 2. Ruta de síntesis de amino ácidos aromáticos (triptófano, fenilalanina y tirosina). Extraído de Heldt & Piechulla (2011).

En la vía del arogenato, la síntesis de prefenato se realiza por el reordenamiento de la cadena lateral que pasa a la posición 1 del anillo aromático, seguido por la transaminación del grupo ceto para la formación de arogenato. El arogenato es el producto principal para la biosíntesis de fenilalanina y tirosina, que posteriormente, darán origen a los fenilpropanoides, precursores de los compuestos fenólicos. Por lo tanto, la ruta del ácido shikímico no está restringida solamente a la formación de aminoácidos para la síntesis de proteínas, sino que además proporciona los precursores para la síntesis de una variedad de

compuestos formados por la planta que son parte de los metabolitos secundarios (Heldt & Piechulla, 2011).

La ruta de la fenilalanina (Figura 3), se puede dirigir bien hacia la biosíntesis de proteínas o bien, hacia la biosíntesis de compuestos fenólicos según las necesidades fisiológicas de la planta. Los compuestos fenólicos están formados por un anillo aromático tipo fenol con un grupo hidroxilo. Representan aproximadamente el 40% de carbono orgánico de las plantas y derivan del esqueleto de fenilpropanoide (estructuras C6-C3). Estos se clasifican en fenoles simples, cumarinas, ligninas, lignanos, suberina y cutina. Adicionalmente, a estos fenoles simples se adicionan grupos malonil-CoA dando lugar a los estilbenos y las chalconas. Los compuestos fenólicos en general, son utilizados como componentes estructurales de las paredes celulares, y también se sintetizan en respuestas de defensa de la planta; muchos de ellos son los responsables del color de las flores y frutos, aromas en los órganos de las plantas, actuando también como antioxidantes, entre otras funciones, que son esenciales para la supervivencia de plantas. Algunos compuestos fenilpropanoides como las cumarinas y lignanos tienen una función de defensa en la planta. Las ligninas son componentes estructurales de las paredes celulares, mientras que la suberina y la cutina forman parte de las capas impermeables de las células. Los estilbenos tienen funciones antifúngicas, y los flavonoides y taninos protegen a la planta contra los herbívoros (Buchanan et al., 2015; Taiz et al., 2018).

Durante la síntesis de los fenilpropanoides, actúa la enzima fenilalanina amonio liasa (PAL) sobre el precursor inicial, fenilalanina, para formar el ácido *trans*-cinámico. Esta reacción de síntesis del ácido cinámico, libera una molécula de NH_3 que probablemente se vuelve a fijar como ion amonio por la reacción de glutamina sintetasa. Esto determina la participación de una ruta perteneciente al metabolismo primario de la planta para el desarrollo de ciertos metabolitos secundarios. El proceso natural de la adquisición de nitrógeno por los organismos requiere de tres vías: la luz, las reacciones fotoquímicas y la fijación biológica del nitrógeno (Buchanan et al., 2015).

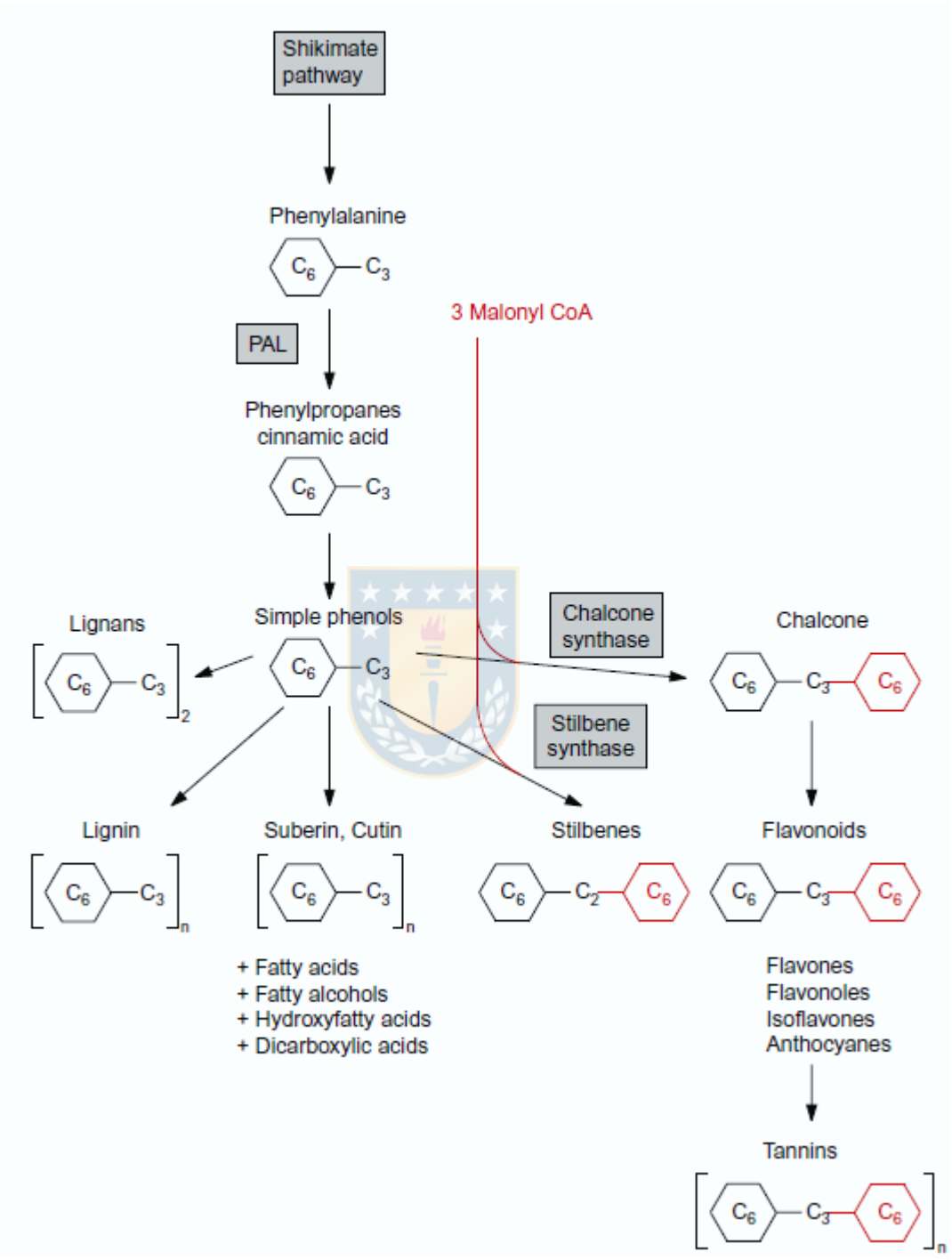


Figura 3. Descripción general de las rutas biosintéticas de compuestos fenólicos en plantas a partir de fenilalanina. Extraído de Heldt & Piechulla (2011).

La fijación biológica del nitrógeno por la planta está relacionada con el ciclo del nitrógeno, donde se fija el ion amonio (NH_4^+) o nitrato (NO_3^-), que pasan a una serie de formas orgánicas e inorgánicas hasta fijar la molécula de nitrógeno. La asimilación del ion amonio se realiza mediante el ciclo glutamina sintetasa-glutamato sintasa (GS-GOGAT). La conversión de fenilalanina a ácido cinámico para la posterior síntesis de compuestos fenólicos contribuye a una acumulación del ion amonio, el cual es tóxico a altas concentraciones. Para afrontar esta acumulación, el NH_4^+ entra al ciclo GS-GOGAT para producir glutamato suficiente, y así asegurar el flujo a través de la formación de arogenato que da origen a la Phe y Tyr, (Buchanan et al., 2015; Taiz et al., 2018).

Como se nombra anteriormente, la PAL cataliza la desaminación de la fenilalanina, formando un doble enlace de carbono-carbono en la cadena lateral, produciendo el ácido *trans*-cinámico (Figura 4). Posteriormente, ocurren una serie de adiciones de grupos hidroxilo y otros sustituyentes para formar los distintos fenilpropanoides. PAL es una de las enzimas más estudiadas dentro de las rutas de biosíntesis de los metabolitos secundarios.

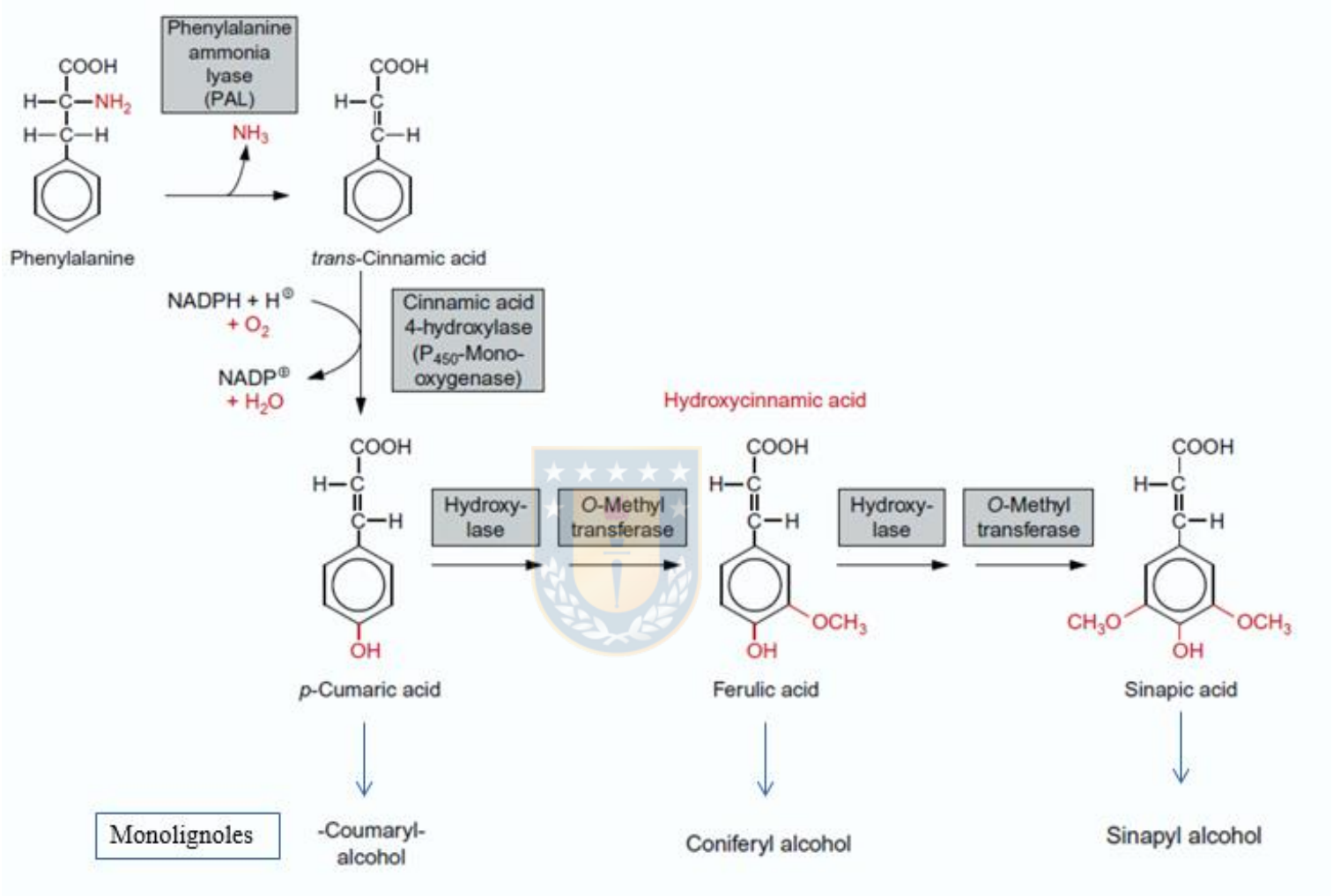


Figura 4. Síntesis de ácidos hidroxicinámicos y derivados, a partir de fenilalanina. Extraído de Heldt & Piechulla (2011).

La introducción del grupo hidroxilo en el anillo fenólico del ácido *trans*-cinámico ocurre gracias a la reacción catalizada por una monooxigenasa. Esta reacción está determinada por la enzima cinamato 4 hidroxilasa (C4H), que utiliza el citocromo P_{450} como sitio de unión del O_2 (Heldt & Piechulla, 2011). La enzima C4H se caracteriza por la presencia de un grupo hemo que absorbe luz a una longitud de onda de 450 nm. La reacción global está representada de acuerdo con:

$$NADPH + H^+ + R\text{-}CH_3 + O_2 \rightarrow NADP^+ + R\text{-}CH_2\text{-}OH + H_2O$$

Se trata de una reacción de monooxigenación en la que sólo uno de los átomos de oxígeno es incorporado en la molécula del substrato, mientras que el otro es reducido hasta agua. Las reacciones subsiguientes en la vía fenilpropanoide conducen a la adición de otros grupos –OH y de sustituyentes metoxilo (-OCH_3), dando origen a los ácidos ferúlico y sinápico, que forman el grupo de los ácidos hidroxicinámicos. A partir de estos tres derivados diversifican las ligninas, lignanos, suberina y cutina (Heldt & Piechulla, 2011).

Los componentes básicos para la síntesis de ligninas son los alcoholes *p*-cumarílico, sinapílico y coniferílico, que constituyen los precursores de los monolignoles. La síntesis de los monolignoles requiere de la reducción de los grupos carboxilos de los ácidos correspondientes a alcoholes (Heldt & Piechulla, 2011). Por otro lado, los lignanos se sintetizan por la dimerización de los monolignoles, o algunas veces, por la condensación de los anillos aromáticos. El mecanismo de síntesis de los lignanos no está del todo claro. Se conoce que el lignano pinorresinol es un constituyente de la resina y que el lignano malognol inhibe el crecimiento de bacterias y hongos (Heldt & Piechulla, 2011).

En cambio, las ligninas, forman parte de la estructura de las paredes celulares vegetales. En conjunto con la celulosa, otorgan la rigidez a los tejidos, posibilitando el crecimiento en altura y la conducción de agua a través de los elementos anatómicos de conducción, las traqueidas (en Gimnospermas) y los elementos de los vasos leñosos (en Angiospermas)

que conforman el xilema. Otra función importante, es proporcionar a la planta resistencia frente al ataque mecánico dado por los herbívoros y enzimáticos dado por bacterias y hongos (Ringuelet & Viña, 2013). Se han realizado estudios de los intermediarios para la síntesis de ligninas en tomate, *Arabidopsis thaliana* y tabaco (Mouradov & Spangenberg, 2014). En la especie modelo *A. thaliana* el silenciamiento del gen que expresa las enzimas HQT (hidroxicinamoyl-CoA quinato hidroxicinamoyl), dan lugar a la represión de la síntesis de ligninas y la redirección del flujo metabólico al incremento en la actividad de flavonoides (Mouradov & Spangenberg, 2014).

La síntesis de suberina y cutina está relacionada con la formación de compuestos fenilpropanoides asociados a ácidos grasos y/o alcoholes de cadena larga. La cutina es una molécula formada por varios ácidos grasos unidos mediante un grupo éster, donde se distinguen por su cadena lateral dado por grupos époxidos o hidroxilo. La cutícula que recubre las paredes celulares externas de las células de la epidermis vegetal está formada principalmente por cutina, sustancivina que le otorga a esta estructura propiedades de impermeabilidad al agua y a los gases. La suberina es un constituyente importante de las paredes celulares más externas de los órganos vegetales subterráneos como lo es la banda de Caspary que se encuentra en la endodermis de las raíces. Se asocia también a las células del súber de la peridermis, tejido constituyente de la corteza exterior de tallos y raíces que experimentan crecimiento secundario, en las plantas leñosas (Heldt & Piechulla, 2011; Ringuelet & Viña, 2013).

Otro grupo importante de los fenilpropanoides son los derivados del ácido benzoico. La evidencia actual de esta ruta no es del todo clara, pero se sugiere que el ácido benzoico es sintetizado a partir de la fenilalanina o directamente a partir de corismato (Figura 2). Derivados del ácido benzoico, como el ácido salicílico y vainillina proceden de esta ruta fenilpropanoide. La vainillina es la sustancia aromática de la vainilla, y en cambio, el ácido acetilsalicílico que proviene de la corteza de *Salix alba*, su derivado acetilado (ácido

acetilsalicílico) se conoce con el nombre comercial de aspirina, y se utiliza contra la fiebre, dolores de cabeza y otras dolencias (Heldt & Piechulla, 2011).

Para la síntesis de flavonoides y estilbenos, procedentes de la ruta de la fenilalanina, se requiere un segundo anillo aromático derivado de residuos de acetato (Figura 5). Posiblemente el grupo más grande de los compuestos fenólicos es el de los flavonoides (Mouradov & Spangenberg, 2014).

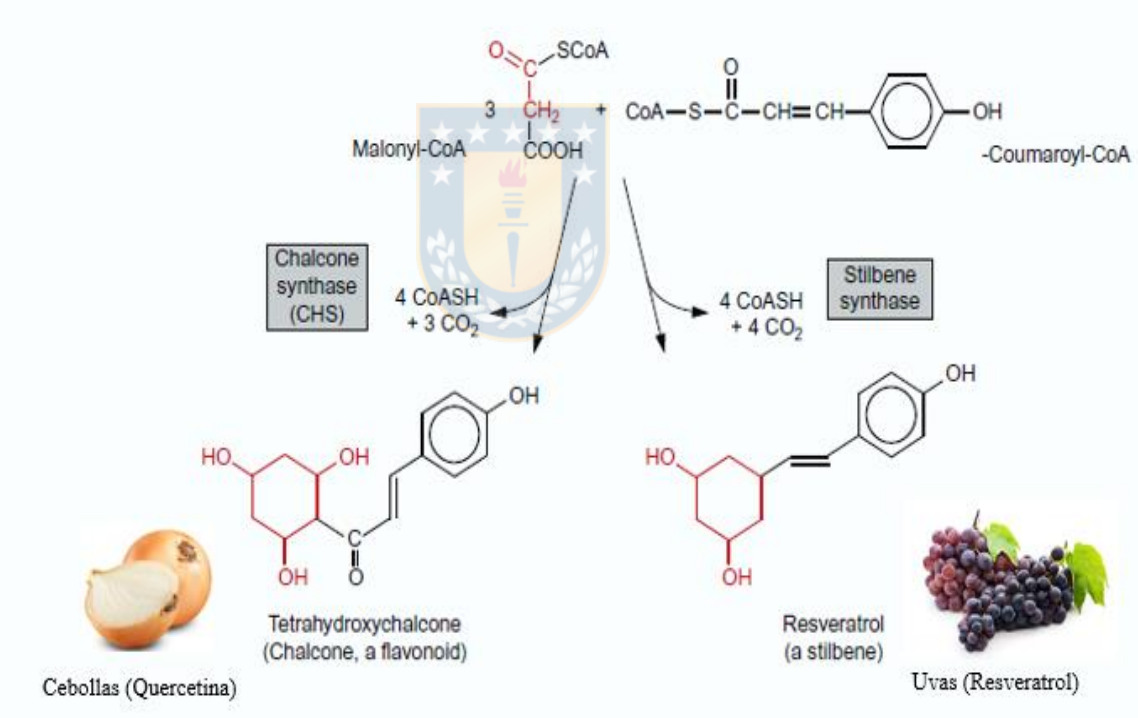


Figura 5. Síntesis chalconas y estilbenos. Extraído de Heldt & Piechulla (2011).

El precursor para la síntesis de los flavonoides es la chalcona, sintetizada por la chalcona sintasa (CHS) a partir de *p*-cumaril-CoA y tres moléculas de malonil-CoA. Esta reacción irreversible libera tres moléculas de CO_2 y cuatro moléculas de coenzima A (CoA).

Diversos estudios han descrito que la síntesis de CHS, al igual que la síntesis de PAL, está sujeta a múltiples controles de expresión génica por factores internos y externos (Heldt & Piechulla, 2011).

Por otra parte, para la síntesis de estilbenos, actúa la enzima estilbeno sintasa (STS) utilizando *p*-cumaril-CoA y tres moléculas de malonil-CoA. A diferencia de la síntesis de chalconas, la reacción libera cuatro moléculas de CO_2 y cuatro moléculas de CoA. El resveratrol, es uno de los estilbenos más sencillos, que se encuentran en las plantas pertenecientes a la familia de las Vitáceas como la vid. Las uvas y los productos derivados de las mismas, constituyen la fuente principal de estilbenos disponibles en la naturaleza para la dieta humana. El resveratrol es también una fitoalexina a la que se le atribuye propiedades antioxidantes y antifúngicas, lo que hace interesante la síntesis de estos compuestos mediante el uso de herramientas biotecnológicas (Belchi-Navarro et al., 2013; Heldt & Piechulla, 2011),

Una vez sintetizada la chalcona, comienza la biosíntesis de flavonoides y sus derivados. La biosíntesis de los flavonoides es catalizada por la enzima chalcona isomerasa (Figura 6). En primera instancia, se sintetiza la flavona que es el precursor de la síntesis de flavonas, flavonoles, antocianidinas e isoflavonas (Heldt & Piechulla, 2011).

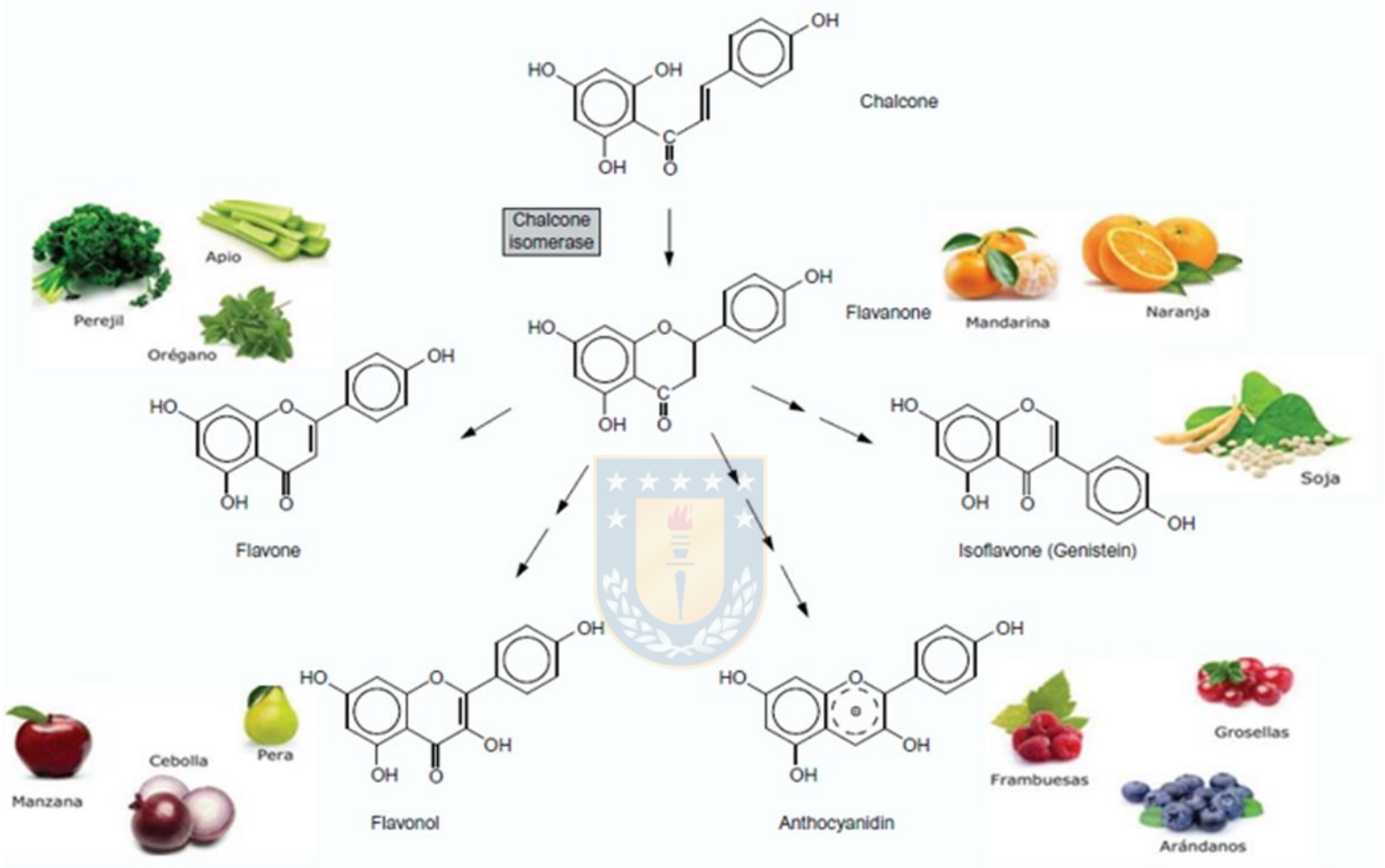
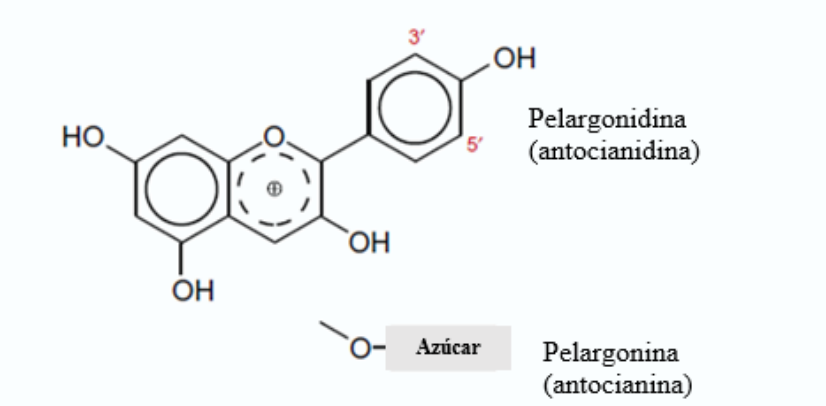


Figura 6. Chalcona como precursor para la síntesis de flavonoides. Extraído de Heldt & Piechulla (2011).

Los flavonoides se caracterizan por ser protectores contra herbívoros, actuando como fitoalexinas y como canal de comunicación para la interacción de las plantas con simbiontes. Las flavonas y flavonoles tienen una absorción máxima en la región ultravioleta, actuando como pigmentos protectores contra los rayos UV. Se ha demostrado que la biosíntesis de flavonoides aumenta, al tener hojas expuestas a luz UV, para evitar el daño en la planta por los rayos UV. También, se ha descrito que muchos flavonoides son antioxidantes y actúan como captadores de radicales para especies reactivas de oxígeno, evitando así la peroxidación de los lípidos de las membranas. Otra característica asociada a los flavonoides, especialmente las antocianidinas junto con las flavonas y flavonoles es que son los facilitadores de los pigmentos y co-pigmentos, contribuyendo a la coloración de órganos vegetales. Además, de otorgar ciertos aromas y sabores característicos de algunas especies vegetales, actuando como atrayentes de polinizadores y dispersores de frutos y semillas (Heldt & Piechulla, 2011; Ringuelet & Viña, 2013).



Antocianidina	Sustituyente	Color
Pelargonidina	-	Naranja-rojo
Cianidina	3′-OH	Rojo
Peonidina	3′-OCH_3	Rosa
Delfinidina	3′-OH, 5′-OH	Púrpura azulado
Petunidina	3′OCH_3, 5′-OH	Púrpura
Malvidina	3′.OCH_3, 5′-OCH_3	Púrpura rojizo

Figura 7. Ejemplos de antocianidinas. Extraído de Heldt & Piechulla (2011).

Las antocianinas son glucósidos de antocianidinas en las que el componente de azúcar, que consiste en una o más hexosas, generalmente está vinculado al grupo -OH del anillo aromático. Las antocianinas son pigmentos hidrosolubles que se localizan en las vacuolas de las células vegetales y que otorgan el color rojo, púrpura o azul a las hojas, flores y frutos. Un ejemplo de antocianidina es la pelargonidina que es un pigmento floral presente en la naturaleza asociado a un glucósido, llamado pelargonina (Figura 7).

En cuanto a la biosíntesis de los taninos, se lleva a cabo mediante la vía fenilpropanoide, a partir del cual se forman los polímeros fenólicos. Los taninos se han utilizado en la industria para convertir la piel de los animales en cuero, dando la característica de resistencia al agua, calor y ataque de microorganismos. Los grupos fenólicos asociados a los taninos, le dan las características de tener un sabor astringente y muchas veces desagradable. De esta manera, cuando el animal come las hojas que contienen estos compuestos, hace que sean menos digeribles y tenga un efecto negativo a nivel del tracto digestivo del herbívoro. Por esta razón, los taninos son muy efectivos en la protección de las hojas contra los herbívoros (Heldt & Piechulla, 2011).

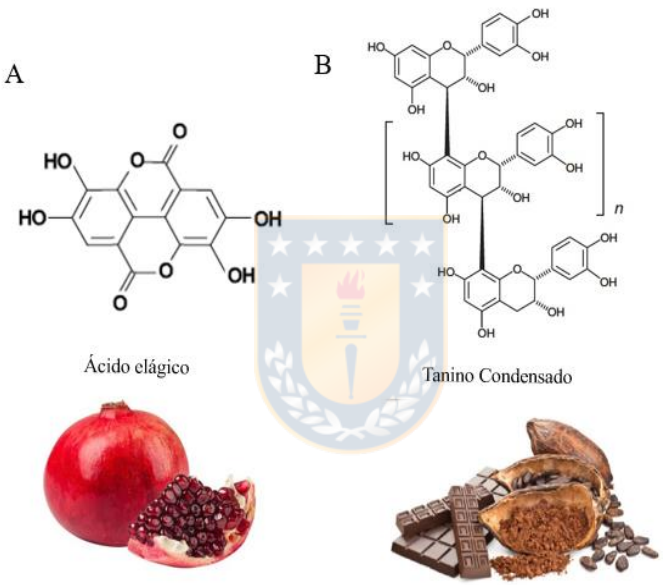


Figura 8. Ejemplo de taninos hidrolizados (A) y taninos condensados (B). Extraído de Heldt & Piechulla (2011).

Los taninos, se clasifican en general en dos grandes grupos dependiendo de su vía de síntesis (Heldt & Piechulla, 2011; Ringuelet & Viña, 2013):

a) Taninos hidrolizables: se corresponde a moléculas de ácido gálico o su dímero, el ácido elágico, asociado a glucósidos (Figura 8). Dentro de los azúcares, generalmente está presente

la glucosa. El ácido gálico se sintetiza a partir de shikimato. El ácido elágico se encuentra presente en nueces y frutas, en especial en las granadas y frambuesas. Del mismo modo, están presentes en muchos frutos rojos (fresas, arándanos, moras) y otros frutos secos (nueces pacanas y castañas) y también en kiwis y uvas.

b) Taninos condensados: se corresponde a polímeros de flavonoides y por lo tanto son productos del metabolismo de los fenilpropanoides. La vía de síntesis de los diferentes taninos condensados se desconoce del todo. Se conoce con el nombre de proantocianidinas, los que derivan de la reacción de oxidación que ocurre durante el calentamiento de estos polifenoles en soluciones alcohólicas ácidas, produciendo antocianinas, pigmentos de diferentes colores comprendidos entre el rojo y el violeta, a los que ya se ha hecho referencia con anterioridad (Figura 9). Las proantocianidinas contienen entre dos y hasta cincuenta unidades de flavonoides. Las proantocianidinas son polímeros de flavanoles (catequina y/o epicatequina), y se distribuyen en la naturaleza en las frutas rojas, frutos secos, vino tinto, piel de la uva, té, café y cacao.

La actividad biológica de los taninos se relaciona con su capacidad de unirse a las proteínas y combinarse con enzimas. Desde el punto de vista farmacológico, se ha estudiado cómo esta interacción afecta a los procesos patológicos. Además, los taninos actúan como inhibidores del crecimiento de bacterias, hongos y levaduras, debido a su capacidad para inactivar a las enzimas y proteínas de estos microorganismos. Las investigaciones dedicadas a la búsqueda de agentes antivirales también han revelado que los taninos resultarían útiles al respecto, especialmente los taninos hidrolizables, mostrando una mayor actividad los de menor peso molecular (Ringuelet & Viña, 2013).

Los taninos también son los responsables de las propiedades de astringencia de ciertos órganos vegetales. En los frutos, los taninos se acumulan en su estado inmaduro, donde actúan como mecanismo de disuasión para herbívoros y posibilitan cierta protección para que le fruto alcance su madurez. Incluso, esta protección para alcanzar el fruto un estado maduro, permite obtener semillas viables, favoreciendo la multiplicación de la especie (Ringuelet & Viña, 2013).

Si bien, se conoce la vía de síntesis de cada uno de los compuestos fenólicos, las actividades biológicas de estos compuestos, así como su acción a nivel *In vitro* e *in vivo* son objeto de investigación.

En general, los compuestos fenólicos presentan varias funciones biológicas entre las que destacan su actividad antioxidante, anticancerígena (Dai & Mumper, 2010) y antifúngica (Stohs & Hartman, 2015), entre otras. En algunas especies vegetales, principalmente en leñosas, la producción de estos compuestos se incrementa cuando se encuentran bajo condiciones ambientales adversas.

Aristotelia chilensis: productor de compuestos antioxidantes

Aristotelia chilensis (Mol.) Stuntz, es una especie nativa que pertenece a la familia *Eleocarpaceae* y se distribuye en Chile desde la Región de Coquimbo hasta la Región de Aysén, incluyendo el Archipiélago de Juan Fernández y en los bosques subantárticos de Argentina. *A. chilensis*, se conoce con el nombre común de maqui y se considera una especie forestal no maderera que, en época estival da origen a una baya comestible a la que se le atribuyen actividades antioxidantes (Salinas & Caballé, 2020).

A. chilensis es una especie perenne que se desarrolla preferentemente en suelos húmedos del valle central, de las cordilleras de la Costa y de los Andes, desde el nivel del mar hasta los 2.500 metros de altitud. Crece tanto en áreas abiertas como debajo del dosel de los bosques. Es una especie dioica, con flores femeninas y masculinas amarillo pálido, de 5-6 mm de diámetro, donde las flores femeninas dan origen al fruto comestible de maqui. Esta baya negra brillante posee de dos a cuatro semillas en su interior de 3 mm de largo y 2 mm de ancho (Figura 9). Sus hojas emergen en dos periodos del año, uno a principios de primavera y otras, en menor cantidad, en verano (Salinas & Caballé, 2020). Las hojas son fotosintéticamente activas tanto en los días de temperatura moderada de otoño e invierno como durante la primavera temprana (Damascos & Prado, 2001). En primavera, la formación

de hojas nuevas requiere de sumideros activos y de energía para su crecimiento y mantenimiento de la maquinaria fotosintética y posteriormente, para la floración ya que la formación y crecimiento de las estructuras reproductivas de la planta son procesos con alta demanda de energía (Damascos & Prado, 2001). En otros estudios realizados en especies siempreverdes, se encontró que la conservación de hojas de invierno hasta la primavera no estaba asociada a la translocación de nutrientes foliares antes de la formación de hojas nuevas, pero si al mantenimiento de un balance positivo de carbono en periodos menos favorables (Mendoza et al., 2014).

Figura 9. Variación de coloración en frutos de *A. chilensis*, durante periodo de maduración.

Con respecto a los estudios asociados a las semillas de maqui, Rodríguez Beraud & Tampe Pérez, (2017) estudiaron el comportamiento en la germinación de las semillas de maqui *In vitro* en diferentes tratamientos contra la dormancia que presenta esta especie. En general, las citoquininas y las giberelinas son reguladores del crecimiento que se utilizan para provocar la salida de la dormición en las semillas, y el inhibidor de este fenómeno es el ácido abscísico (ABA) (Taiz et al., 2018). Así, para comprobar el efecto del ABA en la dormancia de las semillas de maqui, Rodríguez Beraud & Tampe Pérez, (2017) realizaron tratamientos con un inhibidor de ABA, la fluridona (Flu), cuya acción impide la biosíntesis de carotenoides que son precursores de ABA; y tratamientos con las sustancias promotoras de la salida de la dormición, bencilaminopurina (BAP) como citoquinina y ácido giberélico. Estos autores concluyeron que BAP (100μM) es la citoquinina que mejor promueve la ruptura de la dormancia en las semillas de maqui, alcanzando valores de un 85% de germinación a los 21 días.

Repetto-Giavelli et al., (2007) describieron en esta especie una plasticidad fenotípica, que contempla rasgos asociados a la ganancia de carbono y economía del agua, que le permite sobrevivir tanto en un hábitat con poca luz y mucha agua, como el bosque natural, y en uno con mucha luz y poca agua, como los fragmentos de bosque. Esta plasticidad, apoya la hipótesis que en un futuro próximo el maqui, sería una de las especies dominantes de los fragmentos de bosque (Bustamante et al., 2005).

Dado los cambios ambientales, por condiciones de estrés, las plantas podrían combinar respuestas del tipo morfológicas, bioquímicas y fisiológicas relacionadas con su distribución geográfica. Dentro de las respuestas morfológicas, la anatomía foliar de las hojas es una variable importante, donde hojas expuestas a la luz tienden a desarrollar un parénquima de mayor grosor y una mayor cantidad de capas de parénquima esponjoso que una hoja bajo sombra, lo que incide directamente en el proceso fotosintético (Repetto-Giavelli et al., 2007).

En ambientes donde las temperaturas son elevadas y predominan los vientos secos y combinados con una alta radiación, se puede generar daño fotooxidativo del aparato fotosintético (Steiner, 2020). De acuerdo con Moreno (2016), la especie *A. chilensis* mantiene un mecanismo de ajuste bioquímico y funcional en las diferentes altitudes

estudiadas. Los resultados manifiestan que a medida que aumenta la altitud, existe una mayor cantidad de carbohidratos solubles que actúan como osmoprotectores; estos resultados están asociados aumento en carbohidratos están asociado a una disminución de la tasa transportadora de electrones (ETR) y en un menor contenido de clorofilas y carotenoides. Sin embargo, el contenido de prolina, como indicador de estrés, no se ve alterado en ninguno de los puntos estudiados. Moreno, (2016), concluye que la planta es capaz de desarrollarse en cada condición medioambiental, sin manifestar síntomas de estrés, lo que se corrobora con la medida de los valores de la eficiencia cuántica del fotosistema II (PSII).

Actividades biológicas de los compuestos orgánicos asociadas al fruto *A. chilensis*

Se ha reportado que *A. chilensis* posee uno de los frutos más saludables, debido a su elevado contenido de polifenoles y su alta actividad biológica (Gironés-Vilaplana et al., 2014). Estudios realizados por Fuentealba et al., (2012) concluyeron que el consumo frecuente del fruto de maqui y de sus derivados, ayudan a reducir el riesgo de padecer enfermedades neurodegenerativas como la enfermedad de Alzheimer (EA). Además, en los últimos años ha cobrado importancia como producto nutracéutico, suministrándose en formato comercial (pastillas, cápsula, polvo, infusiones, entre otros).

Céspedes et al., (2008) han descrito el efecto cardioprotector del fruto de *A. chilensis*, debido a su participación como productos de degradación de grasa. Específicamente, estos autores describen que los fenoles presentes en las bayas inhiben la peroxidación lipídica en sistemas celulares *In vitro* y en animales de experimentación, previniendo así las lesiones cardíacas. Otra propiedad descrita por Céspedes et al., (2010), es la actividad antiinflamatoria, debido a sus componentes antioxidantes: ácidos fenólicos, antocianinas y flavonoides (Tabla 1), aisladas bajo la técnica de cromatografía líquida de alta eficacia con arreglo de diodos (HPLC-DAD). Estos componentes actúan en actividades inhibitorias sobre la producción de las especies reactivas de oxígeno (ERO) que influyen en procesos fisiológicos y patológicos (Céspedes et al., 2010).

Adicionalmente, se han evaluado las diferencias en el contenido de antocianinas totales, fenoles totales y actividad antioxidante de los frutos de maqui de diferentes zonas geográficas. Este estudio realizado por Fredes et al., (2014) concluyó que existe una variabilidad de concentraciones de fenoles totales y su actividad antioxidante, en los frutos de maqui dependiendo del lugar de recolección. De acuerdo con lo anterior, otros autores citados por Fredes et al., (2014), describen que muchos factores ambientales influyen en la biosíntesis de flavonoides en plantas, incluyendo la luz, temperatura, altitud, tipo de suelo, agua, estado nutricional, interacciones microbianas, patogénesis, heridas, defoliación, y reguladores de crecimiento.

Tabla 1. Antocianinas aisladas desde frutos de *A. chilensis*. Contenido en mg/100g de masa seca. Expresadas en mg equivalentes de delfinidina-3-glucósido (HPLC-DAD y UV utilizando λ = 254, 280, 365 and 520 nm). Extraído de Céspedes et al., (2010).

Nombre compuesto	**Contenido**	**%**
Delfinidina-3-sambubiosido-5-glucósido	101,05	35,10
Delfinidina-3,5-diglucósido	49,80	17,30
Cianidina-3-sambubiosido-5-glucósido	20,73	7,20
Cianidina-3,5-diglucósido	18,71	6,50
Delfinidina-3-sambubiosido	30,51	10,60
Delfinidina-3-glucósido	32,53	11,30
Cianidina-3-sambubiosido	17,37	6,03
Cianidina-3-glucósido	17,20	5,97
Total Antocianinas	**287,9**	

Estudios clínicos realizados por diferentes autores (Garcia-Diaz et al., 2019; Girones-Vilaplana et al., 2014) han sugerido que el consumo de alimentos ricos en polifenoles, pueden atenuar el estrés oxidativo y el estado inflamatorio crónico asociado a enfermedades no transmisibles. Davinelli et al., (2015) realizaron una evaluación con un extracto estandarizado de frutos de maqui (Delphinol®) rico en antocianinas. Este estudio fue desarrollado en 42 pacientes (45-65 años) que consumían este extracto encapsulado 3 veces

al día durante 4 semanas. Las observaciones realizadas sugerían que una dieta rica en este extracto podría mejorar el estado de estrés oxidativo en adultos sanos, adultos con sobrepeso y fumadores (Davinelli et al., 2015).

También, se han realizado estudios asociados a enfermedades inflamatorias crónicas. En el caso de la obesidad asociada a la insulina resistente, Reyes-Farias et al., (2016) evaluó el efecto antiinflamatorio en ensayos *In vitro* con tejido adiposo de ratón, preadipocitos 3T3-L1 y monoadipocitos RAW264.7. Todos ellos se trataron con extractos por separado de frutos de *A. chilensis, Berberis microphylla* y *Vaccinium corymbosum.* Los resultados sugirieron que los frutos de *A. chilensis* y *B. microphylla* bloqueaban el estrés oxidativo, inflamatorio e insulino resistente a nivel *In vitro.* Cabe destacar, que el maqui fue el único que presentó un incremento en la actividad MMP-2 (metaloproteinasas), capaces de regular la acumulación de tejido adiposo blanco. Este último, es el principal responsable de regular la obesidad, los estados inflamatorios crónicos y el estrés oxidativo (Reyes-Farias et al., 2016). Sandoval et al., (2019) realizaron un estudio relacionado con la resistencia a la insulina y desarrollo de tejido adiposo blanco subcutáneo en ratón, en el que concluyeron que la ingesta de liofilizado de las bayas de maqui en ratones obesos, mejoraba la respuesta de la insulina, disminuyendo el peso y mejorando la expresión de genes asociados a la oxidación de ácidos grasos, formación de grasa parda y la termogénesis del tejido adiposo blanco subcutáneo. Al mismo tiempo, Tenci et al., (2019) realizaron un estudio para evaluar las propiedades antiinflamatorias del maqui como tratamiento local para enfermedades inflamatorias intestinales (EII). Para ello, los autores desarrollaron un diseño con una mezcla de polímeros para aplicar el extracto de frutos de maqui y evaluar su efecto en el colon. Los tres polímeros que utilizaron se caracterizaban por tener un efecto sinérgico al combinarse con el extracto de maqui al 0,5% p/p, y aumentaban la permanencia del extracto sobre la mucosa, formando una capa protectora de gel. La mezcla de polímeros con el extracto de maqui se evaluó en líneas celulares de fibroblastos humanos y Caco-2 (adenocarcinoma colorrectal humano) y concluyeron que el diseño de la mezcla de polímeros con el extracto de maqui no tiene un efecto citotóxico en los test *In vitro* de las líneas celulares nombradas y sus propiedades antioxidantes tampoco se veían afectadas, resultando una alternativa para tratamiento contra las EII. Zhou et al., (2019) desarrollaron otro estudio asociado al uso de extractos de fruto de

maqui en tratamientos contra las EII, específicamente contra la colitis ulcerosa en ratones C57BL/6 y concluyeron que el extracto de maqui tenía acción terapéutica con efecto antiinflamatorio y mejoraba la regulación de la microbiota intestinal.

En otro estudio realizado por Céspedes et al., (2017a) se utilizaron fracciones del fruto del maqui para analizar la actividad de dos enzimas inflamatorias: óxido nítrico sintasa inducible (iNOS) y ciclooxigenasa-2 (COX-2) que son las responsables de catalizar la síntesis de óxido nítrico (NO) y prostaglandina E2 (PGE2), respectivamente. La importancia de este estudio radica en que el exceso de estas dos enzimas puede provocar septicemia, shock séptico y síndrome de respuesta inflamatoria sistémica. El estudio se realizó con diferentes fracciones del fruto del maqui en líneas celulares del macrófago murino RAW 264.7 que es rico en ácidos grasos poliinsaturados. Estos ácidos grasos poliinsaturados pueden sufrir peroxidación lipídica, y en presencia de lipopolisacáridos (LPS) activados producen neuroinflamación y posterior síntesis de NO. Dentro de las fracciones analizadas por estos autores, destacan la presencia de quercetina, ácido gálico, luteolina y miricetina como potentes antiinflamatorios, debido a su elevada actividad antioxidante reflejada en los ensayos de SOD, ABTS, TBARS, ORAC, FRAP y DPPH. Estos resultados sugerían que los antocianos y flavonoides presentes en las fracciones del fruto (extractos de metanol y metanol-acetona) suprimían la producción de NO inducida por LPS, a través de la regulación de las proteínas inflamatorias iNOS y COX-2. Por lo tanto, los flavonoides y antocianos presentes en el fruto del maqui pueden actuar como agentes antagonistas para mejorar los efectos del estrés oxidativo, en respuesta a un proceso inflamatorio (Céspedes et al., 2017a). De modo complementario, otros autores han señalado que el fruto del maqui presenta propiedades anti-hemolíticas (Rubilar et al., 2011) y antidiabéticas (Rojo et al., 2012), en estudios realizados en condiciones *In vitro*.

Recientemente, Di Lorenzo et al., (2019) analizaron los efectos antidepresivos y la actividad antioxidante de un extracto de fruto de maqui. Para dilucidar el efecto del extracto como antidepresivo, se utilizó un modelo murino de tejido cerebral de ratón afectado con depresión posterior al accidente cerebrovascular (PSD). Posteriormente, se evaluó *in vivo,* con pruebas físicas, la restauración del comportamiento normal en el modelo de ratón, después del uso de extracto de maqui. Además, en el tejido cerebral del ratón, también se evaluó la actividad

antioxidante de las enzimas y los productos asociados a la peroxidación lipídica. En dicho estudio se identificaron, por primera vez, nuevos compuestos asociados a los frutos que se correspondían a malvidina-glucósido, ácido γ- aminobutírico (GABA), colina y el alcaloide trigonelina. Estos autores concluyeron que los frutos de maqui podrían ser útiles para reforzar la terapia farmacológica de la PSD mediante la regulación del estrés oxidativo (Di Lorenzo et al., 2019).

Además, Chen et al., (2020) identificó 18 compuestos de la baya de maqui con la técnica de Ultra-HPLC, y demostró el efecto fotoprotector del extracto hidroetanólico de maqui frente a los rayos UV-B *In vitro* e *in vivo*. Adicionalmente, dentro de los compuestos aislados, el ácido gálico, el ácido protocatecúico, el granatín B y el ácido elágico son excelentes contra el fotodaño (Chen et al., 2020).

Por otra parte, Ojeda et al., (2011) evaluaron el efecto de un concentrado de jugo de maqui sobre la expresión de la enzima COX-2 con respecto a las vías de señalización y viabilidad en células de cáncer de colon de rata, concluyendo que el jugo concentrado de maqui provocaba un efecto anticancerígeno y antiinflamatorio.

En relación al consumo del jugo del fruto de maqui como bebida beneficiosa para la salud humana, Gironés-Vilaplana et al., (2015) realizaron un estudio del licor anisado de maqui como preservante natural y lo compararon con pacharán, que es un preservante tradicional obtenido de la maceración de bayas de *Prunus spinosa* L., y aceite esencial de *Pimpinella anisum* L. o *Illicium verum* H. Los resultados de este estudio mostraron que el licor anisado de maqui tenia, además de un color atractivo, una alta actividad antioxidante debido a la presencia de antocianinas y de 35 compuestos volátiles aromáticos (Gironés-Vilaplana et al., 2015). Además, Gironés-Vilaplana et al., (2016) también analizaron preparados de bebidas isotónicas de frutos de maqui liofilizados y jugo de limón, con la finalidad de dilucidar el efecto de la temperatura durante el proceso de esterilización y conservación de las bebidas. Estos autores concluyeron que en la bebida isotónica de maqui se mantuvo la actividad antioxidante, independientemente de las temperaturas de los tratamientos térmicos, y a una temperatura de conservación de 7ºC (Gironés-Vilaplana et al., 2016). Al mismo tiempo, se

han realizado diversos estudios del efecto de la temperatura y técnicas de secado en la obtención de extractos de fruto de maqui y su influencia en la conservación de las propiedades antioxidantes (Bastías-Montes et al., 2019; Garrido Makinistian et al., 2019; López de Dicastillo et al., 2019; Quispe-Fuentes et al., 2017, 2020). Concluyendo que la temperatura de secado influye en las propiedades antioxidantes, antocianinas totales, fibra dietética, actividad antiinflamatoria y antidiabética. Además, López de Dicastillo et al., (2016) describieron el uso de extracto de maqui en la elaboración de material de envasado a base de polímeros biodegradables. Resultando un efecto antimicrobiano y antioxidante de los envases realizados con un polímero de metilcelulosa en combinación de una cantidad de glutaraldehído.

Actividades biológicas asociadas a las hojas de *A. chilensis*

Investigaciones referentes al uso de las hojas de *A. chilensis*, en el contexto de su uso como antioxidante y/o antiinflamatorio, se han identificado alcaloides y compuestos fenólicos. Los alcaloides aislados e identificados en las hojas de maqui fueron aristotelina (ARI), aristotelona, aristona, aristotelinona, aristotelinina, protopina, serratolina, hobartinol, 8-oxo-9-dehidro-hobartine, hobartine, 8-dehidro-9-oxo-makomakina, 8-oxo-9-dehidro-makomakina y makonina (Muñoz et al., 2011; Turchetti & Paz, 2019).

Vidal et al., (2013) describieron que las hojas de maqui contenían una proporción mayoritaria de ácidos fenólicos (54,36%), flavonoides (42,10%) y estilbenos (3,55%), cuando se realizaba un extracto de hojas maceradas en etanol y agua 40% v/v (Tabla 2). En este estudio, el extracto de hojas fue microencapsulado con una emulsión de agua y aceite, para determinar las propiedades antioxidantes de los compuestos bioactivos, antes y después del proceso de microencapsulación. Estos autores concluyeron que la actividad antioxidante del extracto de hojas disminuye desde un valor de 99.66% a un 30-35% una vez microencapsulado.

Tabla 2. Compuestos fenólicos de extractos de hojas de *A. chilensis* determinados en HPLC-DAD. Extraído de Vidal et al., (2013).

Componentes fenólicos	**Compuestos fenólicos**	**µM**	**%**
Ácido fenólico			
	Hidroxibenzoico: ácido gálico	399.18	47.55
	Hidroxicinámico: ácido cumárico	57.14	6.81
Flavonoides			
Flavonoles	Quercetina	10.92	1.30
	Isoquercetina	2.91	0.35
	Mirecetina	18.95	2.26
	Rutina	15.05	1.79
Antocianinas	Pelargonidina	121.29	14.45
	Peonidina	1.71	0.20
Flavanoles	Catequina	182.59	21.75
Estilbenos	Resveratrol	29.79	3.55

Por otra parte, Muñoz et al., (2011) realizaron un estudio de la actividad antiinflamatoria, analgésica y antioxidante en diferentes fracciones de extractos de hoja de maqui, utilizando diferentes tipos de solventes: HE (*n*-hexano), DCM (diclorometano), ME (metanol), INFU (infusión, agua), ALK-MIX (DCM+ME). En todos ellos se identificaron alcaloides y otros compuestos fenólicos, siendo la fracción más enriquecida en alcaloides la correspondiente a ALK-MIX (ARI, aristona, serratolina, horbanitol), seguido de INFU (ARI, protopina). En relación con otros compuestos, la fracción DCM presentaba un elevado contenido de ácido ursolico, friedelina, quercetina 5,3´-dimetil éter), seguido de ME (quercetina 3-O-β-glucosidasa, kaempferol) y de INFU (ácido ferúlico, ácido cafeíco). Además, la actividad antiinflamatoria y analgésica de las diferentes fracciones se realizó utilizando un modelo de ratón (CF-1). Los resultados sugerían que el mayor efecto contra la inflamación inducida con 13-acetato-12-O-tetradecanoilforbol, se obtuvo en los extractos de DCM y ME (63,9% y 66% respectivamente). En otro ensayo que consiste en inducir la inflamación con ácido

araquidónico para confirmar la actividad antiinflamatoria se observó la mejor respuesta antiinflamatoria en los extractos INFU (56,2%). El efecto analgésico se analizó con tres diferentes ensayos utilizando como control la acción del ibuprofeno. En el test de "writhing", que se utiliza para evaluar agentes analgésicos o antiinflamatorios, donde se obtuvo el mejor resultado con los extractos de DCM y HE, ambos con valores de efecto analgésico de 89,2%. En el test "tail flick", es una prueba con ratones que se realiza para evaluar la sensibilidad al dolor en respuesta de un estímulo térmico, donde el mejor resultado se obtuvo en la fracción ALK-MIX con un 58,2%, superando a la acción del control. En el test "formalin", que es una prueba modelo válido y confiable para evaluar la actividad analgésica, donde se obtuvo el mejor resultado en la fracción ME (74,1%), que resultó ser similar al control. Por último, para confirmar la actividad antioxidante se realizaron dos ensayos: la inhibición de la xantina oxidasa y DPPH. En el primer ensayo, el mejor resultado se obtuvo en los extractos INFU y ME (52,9% y 62,7%). En el ensayo con DPPH para evaluar la actividad antioxidante, el resultado más significativo se obtuvo en los extractos ME (9,7 µg/ml). Como conclusión, se demostró que las hojas de maqui poseían una potente actividad antiinflamatoria, efecto analgésico y actividad antioxidante (Muñoz et al., 2011).

Céspedes et al., (2017b) realizaron los primeros estudios en los que se utilizaron los extractos de hojas de maqui como tratamiento para la EA. En el caso de las enfermedades neurodegenerativas y en el proceso de envejecimiento, hay una pérdida de la neurotransmisión de las fibras colinérgicas y un aumento de la producción de ERO. Los pacientes con la EA tienen una menor producción cerebral de acetilcolina (ACh), debido a alteraciones en la expresión y actividad de la acetilcolinesterasa (AChE) y butirilcolinesterasa (BChE). El uso de inhibidores de AChE se ha utilizado como tratamiento clínico para mantener de los niveles de ACh y mejorar la función colinérgica. Además, otra enzima involucrada en las enfermedades neurodegenerativas es la tirosinasa que cataliza la degradación de la tirosina hacia el precursor de melanina. Un mal funcionamiento de tirosinasa implica repercusiones en la formación de neuromelanina y problemas en la absorción de radicales libres a nivel cerebral (Céspedes et al., 2017b; Orta-Salazar et al., 2014). Céspedes et al., (2017b), realizaron un estudio con diferentes fracciones de extractos de hojas de maqui (compuestos fenólicos y alcaloideos) para evaluar la inhibición de AChE,

BChE y tirosinasa *In vitro*. Para evaluar la actividad inhibitoria de tirosinasa, se realizó un ensayo con la enzima tirosinasa de hongos (EC 1.14.18.1). La mejor respuesta de inhibición fue en la fracción etil/acetato (F3). Estos resultados se explican por la presencia de quercetina, miricetina y apigenina en esta fracción, datos que se correlacionan de forma positiva con la actividad antioxidante. Asimismo, se evaluó la inhibición de colinesterasa (AChE y BChE) realizando ensayos colorimétricos. Los mejores resultados de la inhibición de AChE y BChE, se obtuvieron en la F3 y en la fracción de alcaloides (F5) por lo que se concluyó que las hojas de *A. chilensis* se podrían utilizar para inhibir colinesterasa y tirosinasa (Céspedes et al., 2017b).

Adicionalmente, se ha estudiado el uso de alcaloides aislados de hojas de maqui para contrarrestar enfermedades neurológicas, dadas por el mal funcionamiento de los receptores de acetilcolina de tipo nicotínico (nAChR). Arias et al., (2019) estudiaron la actividad farmacológica de la interacción de ARI, aristoquinolina y aristona, con dominios extracelulares de modelos humanos (h) $\alpha 3\beta 4$, $\alpha 4\beta 2$ y nAChR $\alpha 7$, por mediciones de afluencia de Ca^{2+}. En conclusión, la mejor actividad inhibitoria e interacción molecular es dado en la combinación de $\alpha 3\beta 4$ y ARI, debido a la alta selectividad del alcaloide y potencial uso como inhibidor no competitivo.

Recientemente, Romero et al., (2019) propusieron el uso de ARI, aislada de la parte vegetativa de *A. chilensis*, para el tratamiento de enfermedades vasculares como la hipertensión. Para ello, evaluaron los mecanismos relacionados con la relajación/contracción de los anillos aórticos aislados de ratas con problemas de hipertensión. Los resultados obtenidos sugerían que ARI aislada de hojas de maqui poseía propiedades vasodilatadoras ya que se observó una respuesta de relajación de los anillos aórticos sometidos a tratamiento con ARI, cuando previamente se había realizado un tratamiento de contracción con KCl y fenilefrina. Otro resultado importante es que la vasodilatación inducida por ARI puede ser modulada por los canales de potasio. Estos autores concluyeron que ARI se podría utilizar como prototipo de droga para mejorar la actividad farmacológica debido a sus propiedades vasodilatadoras (Romero et al., 2019), expandiendo así el uso de sus actividades biológicas antes descritas.

Por otra parte, González-Villagra et al., (2019) estudiaron la implicación de ABA en la biosíntesis de compuestos fenólicos, principalmente de antocianinas, en plantas de maqui sometidas a estrés por sequía. Anteriormente, Finkelstein, (2013) describió que el ABA regulaba los mecanismos fisiológicos y bioquímicos para la tolerancia de las plantas al estrés hídrico, y otros autores Bucchetti et al., (2011) describieron que el ABA podría tener una función relevante en la acumulación de compuestos fenólicos, específicamente de antocianinas. El estudio de González-Villagra et al., (2019) se realizó con dos tipos de plantas, unas totalmente irrigadas (Di) y otras con estrés por sequía (Ni). En ellas, se realizaron diferentes tratamientos, unas fueron tratadas con Flu (un inhibidor de ABA) y otras sin Flu, y/o con la adición de ABA a las 24 horas, y los resultados obtenidos se compararon con las plantas control, Di y Ni. De esta manera, se determinó la influencia del ABA en hojas adultas y jóvenes y el contenido de compuestos fenólicos (quercetina, ácido ferúlico, ácido cumárico, y rutina), concluyendo que el ABA estaba involucrado en la regulación de la biosíntesis de antocianinas en plantas de *A. chilensis* bajo estrés por sequía (González-Villagra et al., 2019). Además, los resultados describían que el contenido de antocianinas totales en hojas adultas procedía mayoritariamente de la presencia de los compuestos delfinidina, petunidina, malvidina y cianidina. En efecto, los niveles de delfinidina disminuyeron en plantas Ni tratadas con Flu y este efecto se revertía al añadir ABA. Además, las concentraciones de petunidina y malvidina se incrementaron en las plantas estresadas al añadir ABA y los niveles de cianidina fueron más elevados en las hojas adultas control, en plantas Ni y en las plantas Ni en presencia de Flu y posterior adición de ABA (González-Villagra et al., 2019). Los resultados obtenidos por otros autores sugerían que el estrés por sequía inducía un aumento de la concentración de antocianinas debido a la sobre expresión de los genes que codifican para la dihidroflavonol 4-reductasa (DFR), UDP-glucosa-flavonoide 3-O-glucosil transferasa (UFGT) y para factores de transcripción MYB A1 y MYB 5A (Borsani et al., 2010; González-Villagra et al., 2019). De esta manera se comprobó la existencia de una correlación positiva entre los estudios descritos anteriormente con los resultados obtenidos en este estudio ya que se observó un aumento del transcrito UFGT y del contenido de antocianinas totales en las plantas estresadas tratadas con Flu y ABA (González-Villagra et al., 2019).

Cultivo de plantas *In vitro:* factores implicados

A diferencia de los cultivos agronómicos tradicionales, el cultivo *In vitro* de plantas se define como "el cultivo sobre un medio nutritivo, en condiciones estériles, a partir de parte y/o células de las plantas madres, semillas o embriones de plantas superiores", y generalmente, no responden al patrón normal de desarrollo de una planta cultivada en el campo (Hussain et al., 2012). En particular, esto refleja la capacidad de las células vegetales de regenerar un organismo completo bajo las condiciones de cultivo *In vitro*, gracias a que las células vegetales son capaces de expresar la totalidad del potencial genético de una célula madre (totipotencialidad) (Hussain et al., 2012).

El control y regulación de las condiciones de cultivo se lleva a cabo mediante la manipulación de los factores físicos como son la temperatura, luz, humedad relativa, así como el tamaño y forma de los recipientes de cultivo, volumen de medio de cultivo, aireación y agitación de los cultivos (Hussain et al., 2012; Phillips & Garda, 2019). La temperatura es un factor determinante que influye en la mayoría de los procesos fisiológicos y oscila entre 22 y 28 ºC, 16 horas de luz/8 horas de oscuridad. La intensidad lumínica de las cámaras de crecimiento varía entre los 50 a 100 μmol $m^{-2}s^{-1}$ de radiación fotosintéticamente activa. Esta intensidad lumínica ha sido considerada baja por lo que es un factor limitante para la fotosíntesis. En general, las plantas cultivadas *In vitro* crecen en porcentajes elevados de humedad. Sin embargo, una humedad relativa excesiva conduce a una malformación en la anatomía del tejido, afectando su desarrollo morfológico. Además, el uso de envases que promuevan el intercambio gaseoso que favorezca la acumulación del CO_2 necesario para la fotosíntesis (Phillips & Garda, 2019).

Por otra parte, los factores químicos, concretamente el pH del medio de cultivo que, oscila entre 5 y 6, y normalmente, disminuye durante la asimilación de las sales de amonio y aumenta con la asimilación de los nitratos, y el propio medio de cultivo que aporta los nutrientes minerales (macro- y microelementos). También es importante realizar un aporte orgánico, que contiene una fuente de carbono (generalmente sacarosa), vitaminas, una fuente de nitrógeno orgánico como el hidrolizado de caseína, y hormonas vegetales (Narayani &

Srivastava, 2017), por lo que la adecuación de la composición del medio de cultivo para el desarrollo de plantas *In vitro* es fundamental.

Los carbohidratos añadidos al medio como azúcares tienen varias funciones esenciales ya que son los sustratos para la respiración. Estos carbohidratos juegan un papel en la biosíntesis de muchos compuestos, sirven de elementos básicos para la elaboración de las biomoléculas y participan en el desarrollo de las plantas. (Phillips & Garda, 2019).

Por otro lado, las plantas *In vitro* presentan diferencias a nivel anatómico y funcional con respecto a las plantas crecidas *ex vitro*. Barupal et al., (2018), describieron que las diferencias a nivel de organización del tejido fotosintético en hojas *In vitro* eran causadas por los cambios en el medio ambiente. De hecho, a nivel anatómico se ha descrito que las plantas *In vitro* presentan carencia de las ceras cuticulares en las hojas (Sánchez & Suárez, 2011), escaso desarrollo de la cutícula (Sánchez & Suárez, 2011) y una alta densidad de estomas no operativos (Apóstolo et al., 2005).

Las perspectivas y uso de los métodos de propagación *In vitro* de plantas, tejidos u órganos y células vegetales, se han centrado hasta ahora en la micropropagación acelerada de material vegetal seleccionado o transformado, la propagación de variedades libres de virus, el mantenimiento de bancos de germoplasma, la obtención de plantas resistentes o tolerantes a estrés abiótico y a enfermedades y plagas, la obtención de plantas resistentes a herbicidas, insecticidas, fungicidas y nematicidas, la obtención de plantas que produzcan frutos con mayor valor nutricional, la producción de metabolitos secundarios y la biotransformación de compuestos orgánicos (Hussain et al., 2012).

Por otra parte, para la introducción de cualquier especie vegetal en cultivo *In vitro*, es necesario superar la contaminación por los microorganismos del ambiente en el que conviven las plantas. En el caso de las especies leñosas, es fundamental superar la etapa de desinfección inicial con el fin de reducir la mayor cantidad de contaminantes que poseen. A pesar de los grandes avances en la biotecnología forestal, el cultivo *In vitro* de leñosas sigue siendo muy difícil y a menudo se limita a la utilización de explantes juveniles (Bonga et al., 2010).

Uso del cultivo *In vitro* en *A. chilensis*

En cuanto a técnicas de micropropagación de *A. chilensis*, principalmente el cultivo *In vitro*, aborda la formación de callos a partir de hojas (Céspedes et al., 1995) y estudios de cuantificación de *trans*-resveratrol en callos originados a partir de hojas adultas (Albornoz, 2012). Otro estudio realizado por Sadino-Riquelme (2015) donde desarrolló un protocolo de inducción de callos *In vitro* de maqui para la obtención de antocianinas. Sadino-Riquelme (2015) realizó una evaluación de diferentes combinaciones de medios de cultivo basal rico en sales MS (Murashige & Skoog, 1962) y medio con menor concentración de nitratos B5 (Gamborg, et al., 1968), hormonas de crecimiento, y factores físicos luz/oscuridad para la obtención de callos de maqui a partir de cotiledón, hojas o embrión. Concluyendo que los callos obtenidos desde cotiledones, realizados en medios de cultivo semisólido MS, hormona 2,4-D y en presencia de luz; sugieren una mejor alternativa para la producción de antocianinas (Sadino-Riquelme, 2015).

Por otra parte, Navarrete (2019) realizó un trabajo de micropropagación de brotes de maqui en sistemas de inmersión temporal SETIS™ Biorreactor, de la casa comercial Duchefa Biochemie B.V. En este estudio, los brotes de maqui propagados corresponden a clones de la variedad "Luna Nueva", que presentan características del árbol de producción frutal de alto rendimiento y buena calidad de la fruta. Esta selección de la variedad con fines de producción de frutales se realiza en base a otro estudio descrito por Brauch et al., (2017) quienes describen a esta variedad con un alto contenido de delfinidinas. Navarrete (2019) utiliza de medio de cultivo basal MS para el establecimiento en medio semisólido y posteriormente medio líquido Woody Plant Medium (WPM), descrito por Lyold & McCown (1981) que tiene una baja concentración de sales. En conclusión, Navarrete, (2019) desarrolla un protocolo para la multiplicación brotes *In vitro* de maqui variedad "Luna Nueva", utilizando la tecnología de sistemas de inmersión temporal.

El problema de establecer cultivos del tipo leñosos, es la aparición de necrosis en el tejido vegetal y en el medio de cultivo que provoca una disminución en el rendimiento y posterior muerte del material (Azofeifa, 2009). Para combatir este problema en el cultivo *In vitro* de

A. chilensis, se determina la composición del medio de cultivo pobre en sales, la adición de polivinilpolipirrolidona (PVPP) que es una poliamida utilizada en la prevención de oscurecimiento de tejidos vegetales, y una disminución en la fuente de carbono (Azofeifa, 2009).

En particular, la extracción de metabolitos secundarios desde plantas superiores es poco usual debido a la dificultad en la cantidad de masa vegetal a tratar. La producción biotecnológica de metabolitos secundarios utilizando cultivo *In vitro* de plantas es una salida factible (Narayani & Srivastava, 2017).

Uso de metabolómica para dilucidar los compuestos activos

El metabolismo refleja todos los cambios bioquímicos de la planta, y la actividad metabólica es la característica esencial y material básico de la vida (Chen et al., 2015). Para la mejor compresión de la biosíntesis de metabolitos, es importante abarcar los mecanismos de biosíntesis a nivel genético, proteómico y metabolómico (Figura 10). Desde un punto de vista general, se cree que la información fluye desde ADN (ácido desoxirribonucleico) a los ARNm (ácidos ribonucleicos mensajeros), luego a las proteínas y luego en metabolitos catalizados por enzimas proteicas; donde finalmente, estos productos convergen e interactúan para producir una variedad de fenotipos biológicos diferentes (Chen et al., 2015). La genómica ha logrado dilucidar los mecanismos de las funciones putativas de los genes, mediante el análisis comprensivo de la función del ADN en varias especies, pero no es lo suficientemente específico para describir los roles biológicos y funcionales. En cambio, la proteómica se ha vuelto una herramienta para el estudio sistemático de las proteínas, que son los que regulan el sistema biológico (Chen et al., 2015).

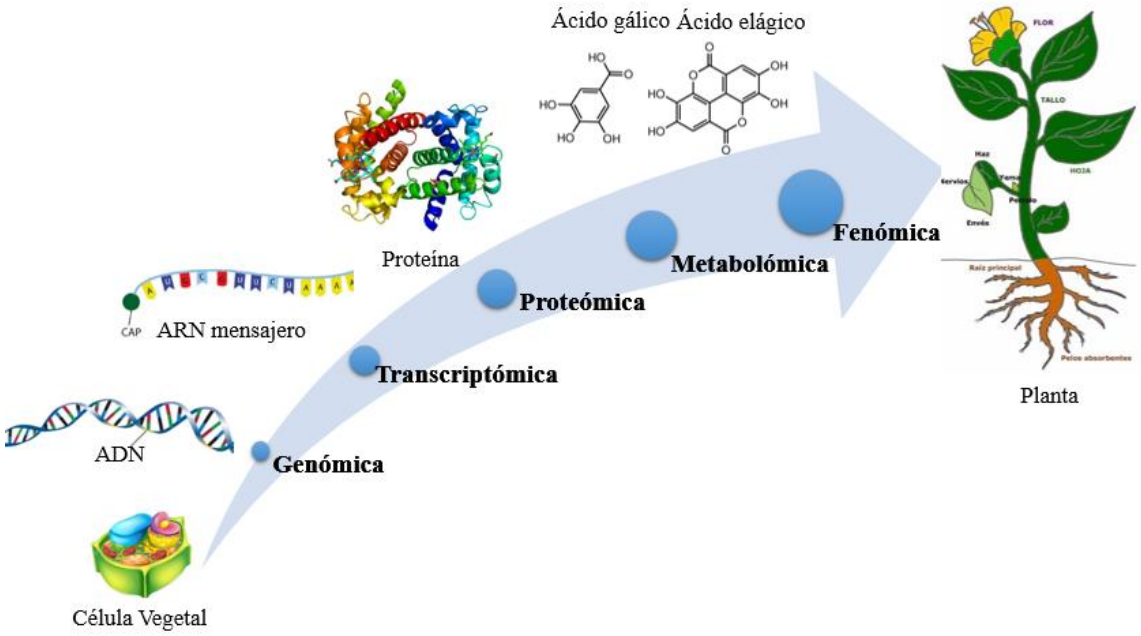


Figura 10. Mecanismos de regulación de los sistemas biológicos en plantas.

En los últimos años el desarrollo de la metabolómica ha tomado gran importancia, debido a su alto impacto en trabajos científicos, ya que, permite obtener resultados concisos de cuáles son los metabolitos simples o complejos presentes en una muestra, tejido o fluido. Considerada como la más reciente de las grandes ómicas, la metabolómica permite la descripción, identificación y cuantificación, desde una perspectiva holística de todos los metabolitos presentes en la muestra, considerados como los productos finales de la expresión génica (Barallobre-Barreiro et al., 2013).

La síntesis de metabolitos a partir de enzimas proteicas, son dependientes de la especificidad del sustrato, tal especificidad podría explicar los diferentes metabolitos en las plantas (Fiehn, 2002). Se desconoce del todo, la proporción de metabolitos que realizan funciones biológicas en plantas. Esto es debido a que muchos de los metabolitos sintetizados son pertenecientes a familia de una planta o son especie especifica de una planta. La importancia de conocer los

diferentes metabolitos radica en identificar cuáles de ellos al menos cumplen funciones biológicas importantes en las plantas o cuales son característicos de cada especie, que las ayudan a sobrevivir en nichos ecológicos variables (Chen et al., 2015).

La metabolómica de las plantas es una de las partes importantes de la investigación, debido a que es un importante módulo de la biología de sistemas y es lo más cercano al fenotipo. En términos del desarrollo actual de tecnologías, no existe ningún método analítico específico que sea capaz de detectar todos los metabolitos, lo que hace que la investigación sobre metabolómica de las plantas sea más desafiante (Barallobre-Barreiro et al., 2013; X. Chen et al., 2015).

El uso de técnicas analíticas de alta resolución como la cromatografía líquida de alta eficacia (HPLC) o la cromatografía líquida de ultra alto rendimiento (UPLC), la resonancia magnética nuclear (NMR), espectrometría de masas (MS), han ido en avance con el fin de comprender la composición de las muestras (Narayani & Srivastava, 2017). La metabolómica dirigida permite la acotación en la búsqueda de metabolitos de interés.

Por ejemplo, Tortosa, (2018) utilizó de tecnologías "ómicas" para monitorizar el estado biológico de plantas de *Brassica oleracea* sometidas a un estrés biótico por *Xamthomonas campestris* pv. *campestris* (Pammel) Dowson (Xcc), considerando los cambios que se producen durante la interacción planta-patógeno. Los cultivos comerciales de *B. oleracea* son susceptibles a la enfermedad de podredumbre negra causada por Xcc. Es por ello, que las plantas de brásicas han desarrollado diferentes mecanismos moleculares para eliminar o frenar el avance del patógeno. Debido a la complejidad de dicha interacción, utiliza la transcriptómica, proteómica y metabolómica para comprender el mecanismo de acción de la planta frente al estrés y diseñar un sistema de respuesta. Para el desarrollo de la investigación de Tortosa, (2018) se utilizaron hojas plantas de brócoli inoculadas con Xcc y control, tomando muestras en los días posteriores de la infección (1, 2, 3, 6 y 12 días). En el análisis transcriptómico, se observó que la mayoría de los transcritos a los 12 días. En el análisis de proteómica, se combinaron técnicas de marcajes de péptidos y cromatografía liquida, siendo importante destacar que proteínas relacionadas con procesos metabólicos esenciales de la

planta se identificaron a los 3 días, en cambio, a los 12 días se reactivaron proteínas relacionadas con procesos del metabolismo primario y producción de EROs. Por último, en el análisis metabolómico, se llevó a cabo en cromatografía líquida acoplado al detector QTOF (cuadrupolo-tiempo de vuelo), identificando que post-infección se afecta la síntesis de la familia de cumarinas y compuestos lipídicos. En conclusión, se identificaron una serie de cambios del brócoli en todos los niveles moleculares, siendo considerable en estadios más avanzados de la infección (Tortosa, 2018).

Por lo tanto, en vistas del potencial que tiene la especie *A. chilensis*, descrita como antioxidante, es que se desarrolla esta tesis doctoral. Por ende, estudios de *A. chilensis* que consideren la integración de la energía lumínica en condiciones naturales e *In vitro*, relacionado con la producción de compuestos fenólicos con actividad antioxidante y que pudieran ser reflejadas en la anatomía e histoquímica foliar de *A. chilensis*, son requeridos. Es por ello por lo que se ha propuesto desarrollar la siguiente investigación que podría dar información relevante en la producción de fenoles específicos (metabolómica) en hojas de diferente edad ontogénica en forma natural como también en microtallos originados bajo condiciones *In vitro*.

Problemática por estudiar

Son numerosos los estudios que existe acerca de las propiedades farmacéuticas, nutracéuticas y biológicas atribuidas al fruto de maqui en diferentes estados de su desarrollo y en especial durante la secuencia de maduración. Sin embargo, sus hojas si bien existen reporte de sus propiedades, son pocos los estudios realizados en éstas especificándose propiedades muy similares a las del fruto. La edad de un tejido determina tasas metabólica y productividad por lo que en los estudios realizados en esta especie no se ha tomado en cuenta esta problemática. En la presente tesis se plantean las siguientes hipótesis.

HIPÓTESIS

Hojas de *Aristotelia chilensis* (Mol.) Stuntz con diferente edad fisiológica, aumentan su producción de compuestos fenólicos con capacidad antioxidante.

OBJETIVO GENERAL

Analizar el comportamiento morfofisiológico y fitoquímico en hojas de *Aristotelia chilensis* (Mol.) Stuntz con diferente edad fisiológica bajo diferentes condiciones de estacionalidad natural y de crecimiento *In vitro*.

OBJETIVOS ESPECÍFICOS

1.- Evaluar el comportamiento fotosintético en hojas basales y apicales de *A. chilensis* bajo condiciones de estacionalidad de ambiente natural y condiciones de crecimiento *In vitro*.

2.- Evaluar la variación del contenido de compuestos fenólicos y su actividad antioxidante en hojas basales y apicales de *A. chilensis* bajo diferentes condiciones de estacionalidad de ambiente natural y condiciones de crecimiento *In vitro*.

3.- Estudiar las variables en la anatomía en hojas basales y apicales de *A. chilensis* bajo diferentes condiciones de ambiente natural y condiciones de crecimiento *In vitro*.

GLOSARIO

ABA	Ácido abscísico
ABTS	2,2'-azino-bis(3-etilbenzotiazolin-6-sulfónico)
ACh	Acetilcolina
ARI	Aristotelina
CoA	Coenzima A
COX-2	Ciclooxigenasa-2
DPPH	2,2-Difenil-1-picrilhidrazil
EA	Enfermedad de Alzheimer
EII	Enfermedades inflamatorias intestinales
ERO	Especies reactivas de oxígeno
FRAP	Poder antioxidante reductor del hierro
Flu	Fluridona
HPLC-DAD	Cromatografía líquida de alta eficacia con arreglo de diodos
NO	Óxido nítrico
ORAC	Capacidad de absorción de radicales de oxígeno
PAL	Enzima fenilalanina amonio liasa
PEP	Fosfoenolpiruvato
Phe	Fenilalanina
SOD	Superóxido dismutasa
TBARS	Sustancias reactivas al ácido tiobarbitúrico
Tyr	Tirosina

CAPÍTULO I

Seasonal changes in photosynthesis, phenolic content, antioxidant activity and anatomy of apical and basal leaves of *Aristotelia chilensis* (Mol.) Stuntz

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Abstract

Aristotelia chilensis (Mol.) Stuntz is an evergreen antioxidant species endemic to Chile. It grows in open areas or under tree canopy, and its leaves emerge in early spring and summer. The objective of this study was to determine annual station influence (winter, spring and summer) on photosynthetic activity, total phenol content (TPC), antioxidant activity and anatomy of apical and basal leaves of *A. chilensis*. Photosynthesis performance was determined by measuring electron transport rate (ETR), the quantum efficiency of photosystem II (F_v/F_m), photochemical quenching (qP) and non-photochemical quenching (NPQ) with a fluorimeter. Leaf extracts were analysed to determine total phenol content (TPC) and antioxidant activity using DPPH and ABTS methods. The maximum ETR and qP were recorded in spring and summer when the photosynthetically active radiation (PAR) at midday was higher (1901 and 1968 μmol m^{-2} s^{-1}, respectively). F_v/F_m had typical physiological values

in both types of leaves (about 0.8 in all the seasons). The behaviour of NPQ was not influenced by the kind of leaves and season of the year. In concordance, the basal spring leaves had higher TPC values (42.8 mg GAE g^{-1} dm). In contrast, the highest values of antioxidant activity were recorded in basal winter leaves followed by basal spring leaves. The results suggested that an increase in light intensity (spring) positively affected the antioxidant activity and TPC, which correlated with higher ETR and qP values. In the main anatomy results, apical leaves showed morphological adaptations as area intercellular spaces and parenchyma palisade areas were larger than in the basal leaves.
Additional key words: A. chilensis, chlorophyll fluorescence, phenolic compounds.

Abbreviations: ABTS - 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonate; DPPH - 1,1-diphenyl-2-picrylhydrazyl; ETR- electron transport rate; F_0 - minimal fluorescence; F_m - maximal fluorescence; F_v - variable fluorescence; F_v/F_m - maximal photochemical efficiency of PSII; NPQ - non-photochemical quenching; PAR – photosynthetically active radiation; PSII - photosystem II; qP- photochemical quenching; ROS - reactive oxygen species; TPC- total phenol content; ΦPSII - efficiency of photosystem II.

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Introduction

Some plant species, mainly woody type, grow under adverse environmental conditions just like the *Aristotelia chilensis* (Mol.) Stuntz, a dioic evergreen tree which is commonly known

as *"maqui"* and native to Chile. This plant develops preferably in humid and drained soils of the central valley, in the slopes of both mountain ranges, streams and margins of forests, from near sea level to 2500 m altitude (Zúñiga *et al.* 2017). *A. chilensis* grows up to 4-5 m high, it has a soft and smooth bark, and abundant, thin and flexible ramifications. Leaves are simple, oval-lanceolate form with dentate edges and range from 4 to 9 cm in size. The leaves veins are marked with a long reddish petiole and stems are characterized by an intense red colour. Flowering occurs in the beginning of spring and the fruits are harvested once a year, from December to February (Turchetti and Paz 2019). In addition, *A. chilensis* plants tolerate drought periods of less than one month. This species appears in succession as a colonizer of newly burned or exploited soils, forming dense and monospecific groups known as "*macales"* with the function of reducing erosion and generating the conditions for establishing other species forming secondary shrubs (Salinas and Caballé 2020).

Variations in environmental factors such as temperature, light radiation, water availability, among others, can cause stress and therefore, changes in plant metabolism. In fact, the photosynthetic rate decreases due to an alteration in the electron transport mechanism and CO_2 assimilation, which is finally reflected in a decrease in carbohydrate production (Mathur *et al.* 2018). Plants, in their interaction with the environment, produce a high number of secondary metabolites, which are normally not essential for their primary metabolism. The synthesis of these compounds is enhanced under stress conditions and many of them have biological activities which are beneficial to human health, and are used as biologically active compounds (Scossa and Fernie 2020). The phenolic compounds are useful in the prevention of arteriosclerosis, cancer, diabetes, neurodegenerative diseases and arthritis (Gonçalves and Romano 2017).

A. chilensis fruits have been studied intensively (Masoodi *et al.* 2019) and they are used for pharmacological (Céspedes *et al.* 2017, Ortiz *et al.* 2021) and nutraceuticals purposes (Fredes *et al.* 2018, Agulló *et al.* 2021). Their fruits are bright black edible berries, with a high level of anthocyanin, thus, they have up to four times more antioxidants properties than other berries (Fredes *et al.* 2014, 2018, Fuentes *et al.* 2019). However, the national and international demand for these fruits is growing and thus, affects its genetic heritage and biodiversity. Faced with the drawbacks of the unregulated collection of fruits, the proposal

to use leaves as a source of phenolic compounds arises, constituting an alternative that can be sustainable, permanent and that does not affect the state of conservation of the species.

A. chilensis leaves emerge along the branch in two periods of the year, one more abundant in early spring, and another, in summer. As evergreen species, *A. chilensis* plants retain their leaves during winter and they remain photosynthetically active, both in the days of moderate temperature in autumn and winter, and during the early spring (Moya *et al.* 2019). However, there are limited studies on the behaviour of phenolic compounds in leaves in relation to their morpho-phenological and physiological development in a seasonal period.

The present work relates the age of the leaves of *A. chilensis* (Mol.) Stuntz to the photosynthetic capacity and accumulation of phenolic compounds as well as their antioxidant capacity under different natural growth conditions.

Materials and methods

This research was conducted between August 2017 and January 2018, the period in which the leaves of *A. chilensis* were collected, at the Universidad de Concepción, Biobío Region, Chile (36° 50'02.6 "S, 73° 01'54.3" W). The influence of climatic conditions on annual seasons in which this study was carried out is shown in Table 1 Suppl. (data from Dirección Meteorológica de Chile, 2018). In addition, the photosynthetically active radiation (PAR) measurements obtained at midday in the study seasons are presented in Table 1.

Table 1. Climatic conditions of Región del Biobío, Concepción, Chile, where *A. chilensis* is growing. Each parameter corresponds to the monthly average for the three seasons of the year.

Season	**Winter (August)**	**Spring (November)**	**Summer (January)**
Minimum temperature [°C]	0	8	9
Maximum temperature [°C]	14	22	29
Average temperature [°C]	9	14	17
Average rainfall [mm]	586.73	849.72	1.45
Humidity [%]	85	81	75
Average wind speed [km h^{-1}]	13.7	13.6	14.8
Maximum wind speed [km h^{-1}]	85.2	50	50
PAR at midday [µmol photons m^{-2} s^{-1}]	435.98 ± 10.4	1901.7 ± 3.6	1968.7 ± 7.2

*Source: Dirección Meteorológica de Chile, 2018.

Adult male *A. chilensis* plants growing in a natural environment and reaching a uniform height of 3 m were used. Six plants were selected within the university campus. The study was carried out with fully expanded leaves, where adult and young leaves were distinguished. According to González-Villagra *et al.* (2018) two different positions represented different ages: young leaves, from the upper third of the branch (apical leaves), and fully expanded leaves, from the lower third of the branch corresponding to basal leaves.

Light response curves: The chlorophyll fluorescence was evaluated through light responses. The branch obtained from the complete plant was previously put in the dark for 30 min and then exposed to different light intensities of 12.13, 25.62, 51.39, 86.64, 241.50, 479.08, 878.08 and 1280.83 μmol photons m^{-2} s^{-1}. Fluorescent signals were measured with a pulse amplitude fluorimeter (FMS 2, Hansatech Instrument, U.K). Following the terminology of Murchie and Lawson (2013), the minimum value for chlorophyll fluorescence (F_o) in the dark-adapted state was determined by applying a weak pulse of modulated light, and the maximum fluorescence (F_m) was induced by a short pulse (0.8 s) of saturating light. The fluorescence signals were followed until they reached a steady state (F_s). To determine the maximal fluorescence in light (F_m'), various pulses of saturating light were applied. The minimal value for chlorophyll fluorescence (F_o') in the light-adapted state was determined by turning off the actinic light, and immediately applying a 2 s far-red pulse.

The maximum photochemical efficiency of photosystem II (ΦPSII), F_v/F_m (variable fluorescence/maximal fluorescence) was calculated considering $F_v = F_m - F_o$. Once data from fluorimeter were obtained, the efficiency of photosystem II ($\Phi PSII = (F_m' - F_s)/F_m'$), and the electron transport rate ($ETR = 0.8 \times \Phi PSII \times PAR \times 0.5$) were calculated. The constants of equation are factor 0.8 in which the average value of the absorbance for the green leaves, and the factor 0.5 assumes that the efficiency of both photosystems is equal, and that the radiation is distributed equally among them (Yang *et al.* 2020). In addition, photochemical quenching ($qP = (F_m' - F_s)/(F_m - F_o')$) and non-photochemical quenching ($NPQ = (F_m - F_m')/F_m'$) were calculated (Yang *et al.* 2020).

In the different seasons of the year (winter, spring and summer), branches of *A. chilensis* were collected, and apical and basal leaves were identified. The samples were collected at midday where the light intensity is higher. At this time, the photosynthetically

active radiation (PAR) was measured, and the light condition of the exposed environment of the study plants was established based on the criteria described by Zhen and Bugbee (2020). The PAR data is shown in Table 1.

Preparation of *A. chilensis* samples for anatomical studies: For anatomical studies, the tissue was selected from the central portion of the both types of leaves of the study, cut quickly and fixed in 37 % formalin, acetic acid and 70 % ethanol (FAA_{70}). The samples were dehydrated through serial solutions in ethanol and *n*-butyl acetate, and embedded in paraplast. The cuts were made with a Jung Biocut 2035 microtome and stuck in glass sheets with Hatsup and Bissmut adhesives. After removing the paraplast by dipping in butyl acetate and washing with ethanol, the samples were coloured with safranin and Astra blue. Subsequently, they were re-dehydrated in a series of dilutions of ethanol and finally in butyl acetate. Glass sheets were visualized in a Leica ICC50 HP optical microscope. Apical and basal leaves were selected from the three seasons of the year. Anatomical analysis was performed on all the plant materials using a Leica ICC50 HP optical microscope according to nomenclature proposed by dos Santos Isaias *et al.* (2011). The measurement of morphological characteristics was done using AxioVision SE64 Rel. 4.9.1 Software (Zeiss, Germany) with contrasting the scale to determine the area of adaxial epidermis, palisade parenchyma, spongy parenchyma, abaxial epidermis and intercellular space.

Preparation of *A. chilensis* samples for chemical study: Fresh basal and apical leaves (40 g) were dried at 37 °C for two days. Dried leaves were crushed to obtain powder and then, maceration was performed by exhaustion in methanol-HCl 0.1 %. The total extract was concentrated in a rotavaporat 37 °C and lyophilized for 24 h. The extraction yield was defined as the amount of extract (mg) recovered by leaf dry mass (mg), for each sample.

Determination of total phenolic content (TPC): The total phenolic content (TPC) in each extract/sample was determined using TPC method described by Sembiring *et al.* (2018), with slight modifications. The dried extract/sample was dissolved in distilled water to a concentration of 200 µg ml^{-1}. The calibration curve was established using gallic acid (10 to 200 µg ml^{-1}). The reaction mixture contained: distilled water (400 µl), sample or gallic acid

solution for the standard curve (20 µl), Folin-Ciocalteu reagent (40 µl) and 15 % sodium carbonate (200 µl). The reaction mixture was incubated at room temperature for 60 min in darkness, with intermittent shaking to aid colour development. Absorbance was measured at 750 nm using UV-Vis spectrophotometer (BioTeK ELx800, Winooski, USA). TPC was expressed in mg of gallic acid equivalents per gram of dry mass (GAE mg g^{-1} dm).

Determination of the antioxidant activity

*DPPH radical scavenging assay***:** The free radical scavenging activity of *A. chilensis* leaf extracts and standard solution Trolox (±-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were analysed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method as reported by Masek *et al.* (2020). The assay mixture contained 270 µl of 0.06 mmol l^{-1} DPPH radical solution, prepared in methanol, and 30 µl of Trolox at in different concentrations (10 to 200 µg ml^{-1}) or *A. chilensis* leaf extracts. The reaction mixtures were quickly mixed and incubated in darkness at 37 °C for 20 min. The decrease in absorbance of each sample was measured at 515 nm using UV/Vis spectrophotometer. Trolox, a well-known antioxidant, was used as positive control, while DPPH radical solution with 1 ml methanol was taken as blank. All determinations were performed in triplicate (n = 3).

*ABTS radical scavenging assay***:** For ABTS assay, the procedure followed was that described by Masek *et al.* (2020) with some modifications. The stock solutions included 3.5 mmol l^{-1} ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonate) and 1.22 mmol l^{-1} potassium persulfate. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 16 h at room temperature in darkness. After the $ABTS^{*+}$ radical was formed, the solution was then diluted by mixing 1 ml $ABTS^{*+}$ solution with 14 ml distilled water to obtain an absorbance value of 0.70 ± 0.01 units at 750 nm. $ABTS^{*+}$ solution was freshly prepared for each assay. The reaction (60 min, in dark conditions) contained $ABTS^{*+}$ radical (180 µl) and 20 µl of the samples at different concentrations. For standard Trolox and samples, dilutions of 10 to 200 µg ml^{-1} were prepared. All determinations were performed in triplicate (n = 3). The antioxidant activity for both methodologies was expressed as percentage of inhibition, which corresponds to the

amount of radical (DPPH and ABTS) neutralized by the extract at a certain concentration, as described in the following equation

$$\text{Inhibition (\%)} = ((A_c - A_s)/A_c) \times 100$$

Where, A_c is the absorbance of the control and A_s the absorbance of the samples. The antioxidant activity was expressed as IC_{50}, which was defined as the final concentration (µg extract ml^{-1}) of the tested sample required for the inhibition of radical by 50 % (Rubilar *et al.* 2011, de Menezes *et al.* 2021).

Statistical Analysis: For the light response curves, eight samples for six plants were used for each type of leaf in relation to the season of the year studied. For the determination of TPC, six plant samples were used for each type of leaf in relation to the season of the year studied. For the foliar anatomy measurements, five samples with fifteen repetitions were used. The analyses were carried out with the AxioVision LE 4.8.2.0 software. The assays for the determination of antioxidant activity were carried out in triplicate. The data obtained were analysed statistically using analysis of variance (ANOVA), and the differences between the means were determined through the Tukey test ($P \leq 0.05$). Statistical analyses were performed using the InfoStat/L software (FCAUNC, Argentina) and the graphic representations were made using SigmaPlot software version 10.0 (SPSS; Chicago, IL, USA).

Results

Effect of seasonal conditions on fluorescence parameters of both basal and apical leaves: According to the chlorophyll fluorescence data for *A. chilensis*, it was observed that they were affected by seasons of the year. Certainly, the season of the year had a significant effect on ETR (Table 3 Suppl.). On the other hand, quantum efficiency of the PSII (F_v/F_m) was affected by the leaf type but not by the environmental conditions or their interactions. However, qP and NPQ were significantly affected by the leaf type and the season, and an interaction between both variables (season and leaf type) was observed (Table 3 Suppl.).

Table 2. Effect of the season of the year on fluorescence parameters in basal and apical leaves of *A. chilensis*.

Parameters	Winter	Spring	Summer	*F*
Basal leaf				
ETR	64.02 ± 6.47a	129.71 ± 13.69b	108.83 ± 4.83b	13.38**
qP	0.25 ± 0.03b	0.47 ± 0.04a	0.42 ± 0.02a	13.81**
F_v/F_m	0.83 ± 0.01a	0.84 ± 0.005a	0.84 ± 0.01a	0.97ns
NPQ	3.42 ± 0.11a	3.79 ± 0.15a	3.84 ± 0.22a	1.85ns
Apical leaf				
ETR	85.75 ± 5.25a	123.08 ± 12.58b	122.19 ± 3.64b	6.84*
qP	0.35 ± 0.02b	0.48 ± 0.04a	0.51 ± 0.01a	13.35**
F_v/F_m	0.83 ± 0.004a	0.82 ± 0.01a	0.82 ± 0.004a	1.16ns
NPQ	3.85 ± 0.14a	3.89 ± 0.14a	4.27 ± 0.07a	3.44ns

Data represent the mean ± SE from eight samples of each season of the year. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from ANOVA for the different season of the year and type of leaf.

**F* values were significant at 95% level of probability.

***F* values were significant at 99% level of probability.

****F* values were significant at 99.9% v of probability.

Non-significant values are indicated by “ns”.

Table 3. Relationship between type of leaf (A) and season of the year (B) as well as interaction of both (AXB) on fluorescence parameters in *A. chilensis*.

Source of variation	ETR	qp	Fv/Fm	NPQ
Type of leaf (A)	1.76ns	8.01*	7.19*	7.08*
Season (B)	19.22**	25.07**	0.27ns	4.06*
AXB	13.4**	19.38**	2.58ns	5.06*

F-values from two-way ANOVA for ETR, qP, Fv/Fm and NPQ. *F*-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability. Non-significant values are indicated by “ns”.

Light response showed that the maximum values of ETR were obtained in the seasons of the year with greater luminosity (spring and summer) in both types of leaves (basal and apical) as shown in Table 2. In fact, the minimum values were observed in winter, regardless of the type of leaf, showing a strong correlation of ETR/PAR during this season, as described for the last parameter in the Materials and Methods section. The parameter, F_v/F_m, remained with values of about 0.80 in all the seasons of the year analysed, without significant differences among them. In the same way, F_v/F_m values showed no significant differences between basal and apical leaves (Tables 2 and 3 Suppl.).

Photochemical quenching (qP) is the ratio of excitation energy trapped by open reaction centres that has been used for electron transport (Yang *et al.* 2020). It was observed that the highest qP values were recorded in spring and summer, in both types of leaves, and they all showed significant differences with respect to the data obtained during the winter (Table 2). However, there were no significant differences in qP values in both apical and basal leaves during winter, where the lowest values of qP were obtained.

Non-photochemical quenching (NPQ) indicates the influence of non-photochemical processes on the fluorescence emission of chlorophyll from darkness to light state (Yang *et al.* 2020). The type of leaf used and the seasons of the year did not influence the behaviour of NPQ, although a trend of increase in the NPQ values with the increase in the light intensity during spring and summer, especially in apical leaves was observed (Tables 2 and 3 Suppl.).

Foliar Anatomy: Fig. 1 Suppl. shows cross sections of basal (Fig. 1A) and apical (Fig. 1B) fully expanded leaves. Important anatomical differences were observed mainly in the distribution of palisade and spongy parenchyma of both types of leaves. In apical leaves (Fig. 1B), the area of parenchyma palisade was larger than in the basal leaves. Also, the basal leaves had smaller intercellular spaces than the apical leaves. However, no significant changes were recorded in the epidermis in both types of leaves (Table 4).

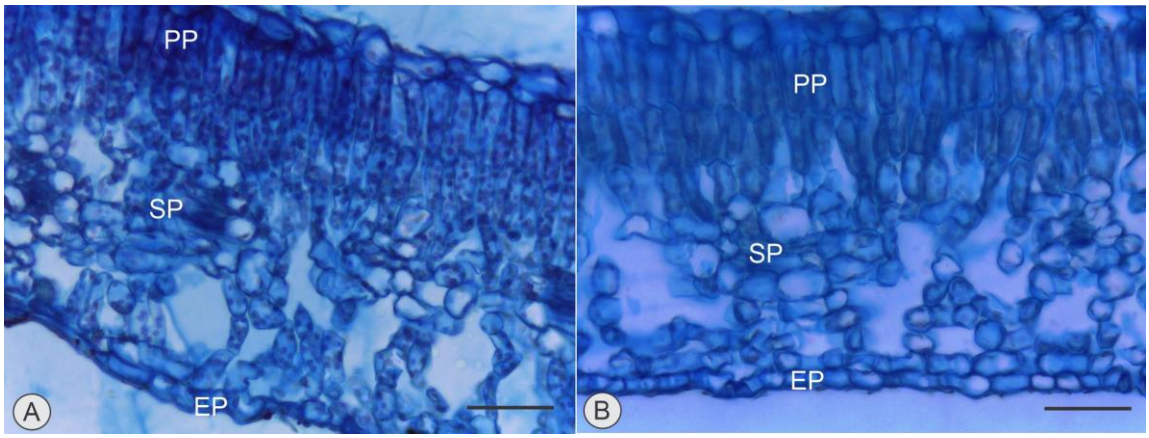

Fig. 1 Cross section of basal (A) and apical (B) leaves of *A. chilensis*. Black bars: 50 µm. EP: epidermis. PP: palisade parenchyma. SP: spongy parenchyma.

Table 4. Measurements of areas (µm2) in cross sections of basal and apical leaves of *A. chilensis*.

	Area [µm^2]		
	Basal leaf	**Apical leaf**	***F***
Adaxial epidermis	243.27 ± 8.24a	257.25 ± 7.51a	1.57ns
Palisade parenchyma	266.78 ± 7.16a	336.98 ± 5.33b	61.81**
Spongy parenchyma	203.50 ± 7.97a	214.33 ± 4.91a	2.52ns
Abaxial epidermis	135.29 ± 5.28a	140.85 ± 3.38a	0.79ns
Intercellular space	888.71 ± 23.26a	1165.04 ± 31.42b	49.97**

Data represent the mean ± SE of75 measurements. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from one-way ANOVA for the different types of leaf.

Yields of the extraction of phenolic compounds: Fig. 2 Suppl. shows the yield of the extraction process of phenolics, which was obtained from basal and apical leaves of *A. chilensis,* in the different seasons of the year (mg extract mg $^{-1}$ dm). When comparing the yields, both apical and basal leaves in winter were lower than in spring and summer. Basal and apical leaves in spring and summer, respectively, showed the highest yield levels.

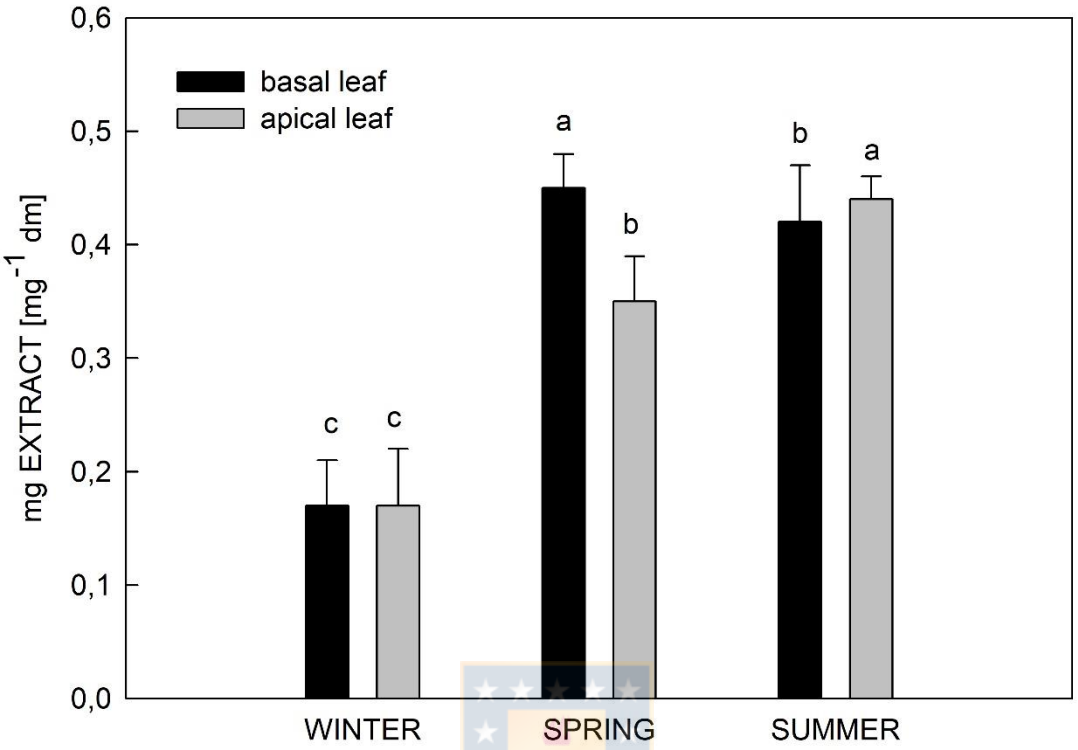

Fig. 2 Yield of extracts of basal and apical leaves of *A. chilensis* to determine phenolic content in three seasons of the year (mg extract mg $^{-1}$dm). Data represent the mean ± SE of three samples from each season of the year. Different letters in the same row.

Determination of Total Phenol Content: TPC values showed significant differences in both basal and apical leaves, and in the different seasons (Fig. 3). The highest values were recorded for extracts obtained from basal leaves in spring, followed by those of apical leaves in winter and summer. The lowest value of TPC was recorded in extracts of basal leaves in winter and in those obtained from apical leaves in spring. According to TPC data from leaves of *A. chilensis*, they were not affected by the type of leaf and interaction between both factors was observed (Table 5 Suppl.).

Table 5. Relationship between type of leaf (A) and season of the year (B), as well as the interaction of both (AXB), expressed as F-values from two-way ANOVA, for TPC and antioxidant activity (ABTS and DPPH assays) in *A. chilensis*.

Source of variation	TPC	ABTS	DPPH
Type of leaf (A)	0.65ns	25.15**	11.04*
Season (B)	1.39*	9.15*	3.97*
AXB	1.14ns	14.48*	6.32*

F-values from two-way ANOVA for TPC, ABTS and DDPH. *F*-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability. Non-significant values are indicated by "ns".

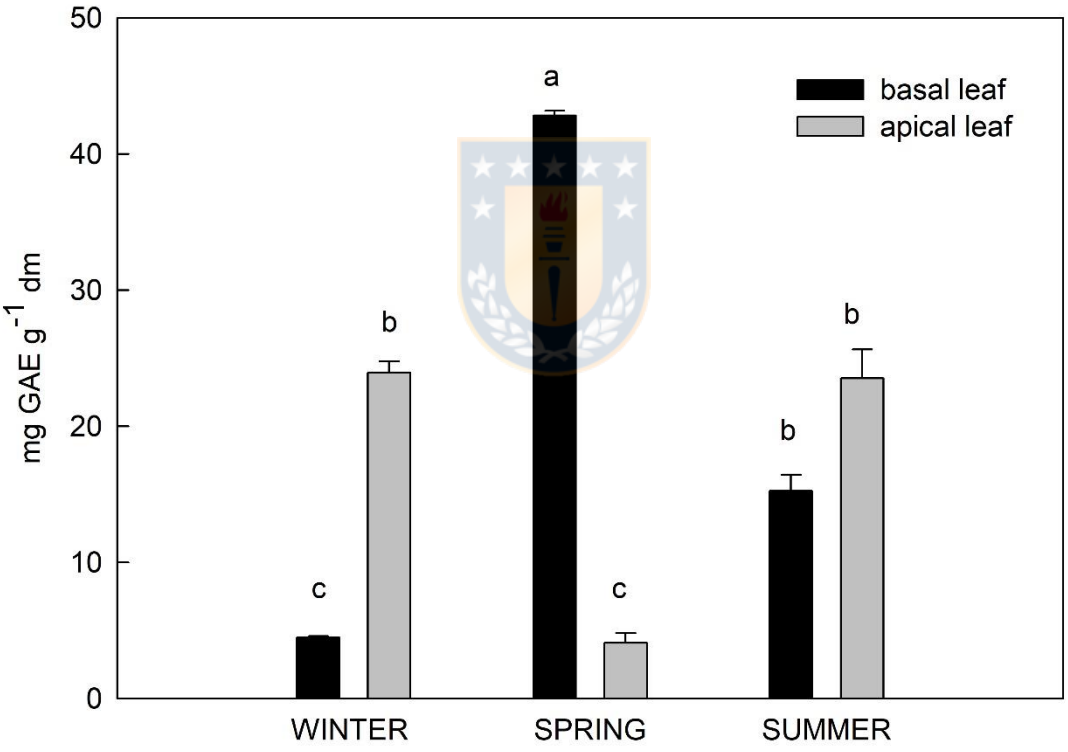

Fig. 3 TPC (expressed as mg GAE g^{-1} dm) in basal and apical leaves of *A. chilensis* in the different seasons of the year. Data represent the mean ± SE of six samples from each season of the year. Different letters indicate significant differences according to Tukey´s test (one-way ANOVA, $P \leq 0.05$).

Determination of the Antioxidant Activity: The *F*-values from two-way ANOVA for the antioxidant activities obtained by both methodologies (ABTS and DPPH), were influenced by the season of the year, the type of leaf and the interaction between both factors as shown in Table 5 Suppl. In addition, the antioxidant activity for ABTS assay expressed as a function of IC_{50} is shown in Table 6. Data described in this Table strongly support significant differences in extracts of basal leaves sampled in winter, as compared to the extracts obtained in the other two seasons, as well as significant differences between both types of leaves as seen from their *F* values. The best result of this ABTS antioxidant activity was found in basal winter leaves, which corresponds to the lower value of IC_{50}, followed by spring and summer basal leaves. Thelowest antioxidant capacity which corresponds to the higher value of IC_{50} was found in apical spring leaves by ABTS assay.

Table 6. Antioxidant activity (ABTS and DPPH assays) expressed as a function of the IC50 obtained for each type of leaf and season of the year (µg extract ml-1).

Parameters	Winter	Spring	Summer	*F*
Basal leaf				
ABTS	45.05 ± 0.30a	49.97 ± 2.24b	49.91 ± 0.97b	11.88*
DPPH	34.68 ± 0.58a	35.92 ± 0.90ab	38.08 ± 1.65b	6.93*
Apical leaf				
ABTS	51.81 ± 4.59a	69.02 ± 4.21b	56.05 ± 4.21a	12.81*
DPPH	59.49 ± 1.87c	47.37 ± 0.63b	33.89 ± 1.53a	236.03***

Data represent the mean ± SE of three samples from each season of the year. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from one-way ANOVA for the different types of leaf.

On the other hand, IC_{50} values obtained from DPPH assay are shown in Table 6. Data obtained from this assay were significantly different in both types of leaves as confirmed by their *F* values. Although, the differences in antioxidant activity were small and significant, basal leaves during winter had a higher antioxidant capacity than those in spring and summer. Regarding apical leaves, the extracts in all the seasons presented values statistically different,

being lower in summer, which means a higher antioxidant capacity, and higher levels in both winter and spring, which is related to a lower antioxidant capacity.

Discussion

Weather conditions that characterize the different seasons of the year affect both the morphology and physiology of the plant, and therefore, modify the growth and development of its organs, including the leaves. Consequently, the objective of this work was to analyse the effect of seasons on *A. chilensis* leaves. As maqui leaves emerge in two periods of the year, one more abundant in spring, and another, in summer, these two seasons were selected for this study. In addition, a third season, winter, was also selected because *A. chilensis* plants retain their leaves during this period, and thus, they remain photosynthetically active. Another factor analysed was the level of growth of *A. chilensis* leaves, in both apical and basal leaves, because these leaves have different morphological and physiological characteristics. The apical or juvenile leaves are tissues that are not yet mature, while the basal leaves are more mature and have a thicker cuticle, with fewer intercellular spaces.

In fact, Damascos and Prado (2001a) indicated that adult leaves of *A. chilensis* in winter, remained photosynthetically active during the spring and 15 days before the senescence, and when subjected to a low photonic flow density (150 mmol m^{-2} s^{-1}), showed higher average values of photosynthesis and efficient use of water was expressed in area leaf than new leaves. Being an evergreen tree, its leaves emerge in two seasons of the year, spring and summer. These authors indicated that, the new leaves in the spring constitute sinks of mass and energy. However, the formation and growth of the reproductive structures of the plant are processes with high energy demand. In other studies conducted on evergreen species, it was found that the conservation of leaves from winter to spring was not associated with the translocation of foliar nutrients before the formation of new leaves but to maintain a positive carbon balance in less favourable periods (Mendoza *et al.* 2014).

Murchie and Lawson (2013) carried out a review on how fluorescence parameters can be used to evaluate changes in photosystem II photochemistry, linear electron flux and CO_2 assimilation *in vivo*, and described the theoretical basis for the use of specific fluorescence parameters. In relation to these parameters, apical leaves showed lower F_v/F_m values than basal leaves during spring and summer, although, the data obtained was

constantly within the normal physiological values (about 0.8) throughout the year, indicating no environmental stress. This agrees with the results of the current study (Table 2) and thus, the decrease of F_v/F_m values cannot be interpreted as a photo inhibition of the photosynthetic apparatus. This could be due to two reasons: either *A. chilensis* is tolerant to high light conditions (Moya *et al.* 2019), or this plant is not under stress, making it to achieve the optimum physiological performance in any season of the year (Molina-Montenegro *et al.* 2012). Other studies confirmed that this species has a great phenotypic plasticity in traits associated with carbon gain and water economy, which makes it to survive both under habitats with low light and water availability (continuous forest), and with high light conditions and water scarcity (Moya *et al.* 2019).

In the present study, ETR and qP values were well-correlated and appeared to be dependent on light intensity. However, in *A. chilensis* basal leaves, both ETR and qP values were slightly higher in spring and summer. Acosta-Motos *et al.* (2015a) studied the effect of high light intensity on chlorophyll fluorescence parameters in apical and basal leaves of myrtle plants. Under high light irradiation, basal leaves of myrtle plants showed higher values for qP, F_v/F_m and NPQ than apical leaves (Acosta-Motos *et al.* 2015a). These results partially agree with those of the present study, because F_v/F_m values of *A. chilensis* leaves were influenced by leaf type, with higher F_v/F_m values in basal leaves than apical leaves under high light conditions (spring and summer). This result can be due to a down-regulation mechanism of PSII in high light conditions in apical leaves, which were more exposed to sunlight than basal leaves. A similar response in F_v/F_m values has been observed in pea leaves subjected to high light irradiation, that is, the response is dependent on the exposure time to this high light intensity (Hernández *et al.* 2004). On the other hand, higher NPQ means that much of the light energy absorbed is dissipated by the protective mechanisms. However, in the results of the current study, NPQ behaved differently depending on the leaf type, but no significant change was recorded for NPQ values during the different seasons. These stable NPQ values suggested the capacity of *A. chilensis* leaves to use the excess light energy in photosynthesis process. In contrast, in basal myrtle leaves, high NPQ values were observed during periods of high light intensity, which could facilitate the safe removal of excess light energy and minimize the generation of ROS (Acosta-Motos *et al.* 2015a).

Variations in leaf size within each plant species depend on the chronological and physiological ages of the developing plant and their architectural model. The architectural model is an inherent growth strategy that defines the manner in which the plant expands its form in response to morphological features (Barthélémy and Caraglio 2007). In *A. chilensis*, leaves have an accepted general morphology where the basal leaves are the largest and young leaves (apical leaves) are smaller. Furthermore, in this study, some differences in foliar anatomy were found between apical and basal leaves. These differences can be reflected in F_v/F_m values, in which basal leaves are higher during spring and summer. Interestingly, apical leaves had a higher percentage of area occupied by palisade parenchyma, an adaption that could favour the photosynthetic process. Nevertheless, these results indicated that the photosynthetic activity of apical leaves was lower that of basal leaves. The photosynthetic activity could be explained thus the basal leaves are older leaves and they played the role of carbon resource through their photosynthetic activity increase (Damascos and Prado 2001b). Another interesting difference is the greater intercellular spaces observed in the apical leaves in relation to basal leaves. This anatomical modification in leaves can improve the CO_2 diffusion, and facilitate its entry into the chloroplast, especially under stress conditions. A similar modification was previously described in myrtle and Eugenia plants under salinity conditions (Acosta-Motos *et al.* 2015a,b). These authors observed a decrease in the percentage of spongy parenchyma, and an increased percentage of intercellular spaces under NaCl-stress. They concluded that these anatomical changes may serve to facilitate CO_2 diffusion in a situation of reduced stomatal aperture (Acosta-Motos *et al.* 2015a,b). In contrast to this study, the apical leaves of *A. chilensis* were not under stress condition. The evidence suggests increase of parenchyma palisade and intercellular spaces of the apical leaves are an adaptation.

A. chilensis is characterized by high phenol content as compared to other berries, and the nutritional and pharmacological effects are attributed to them. The TPC observed in this study were lower than those reported by Vidal *et al.* (2013) for *A. chilensis* leaves collected in Región del Biobío. These authors carried out the extraction in ethanol-water 50 % v/v, and obtained TPC values of 40 ± 0.57 mmol l^{-1} GAE, which is equivalent to 136 mg of phenols g^{-1} dm values, and higher when compared with samples of apical leaves of spring. A similar response was reported by Rubilar *et al.* (2011) where TPC of *A. chilensis* leaves is higher

than those of this study (69.0 ± 0.9 mg GAE g^{-1} dm). These results could indicate that the extraction method used influenced the TPC quantification. The plants are constantly exposed to multifactorial environmental changes that are unfavourable for growth and development. However, phenolic compounds like flavonoids play a significant role in UV protection. Several studies confirmed that excess light or UV radiation changes the flavonoids composition of plant leaves in response to photodestruction and photoprotection. (Naikoo *et al.* 2019).

In addition, phenolic compounds exhibit a wide range of biological effects. Some of them are powerful free radical scavengers (and so, have antioxidant activity). Depending on the extraction procedure, the antioxidant activity can vary, being in some cases, higher in leaves than in fruits (IC_{50} values, leaves: 8.0 ± 0.1 mg extract l^{-1}, fruit: 399.8 ± 17.5 mg extract l^{-1}) (Rubilar *et al.* 2011). In the case of the present study, the antioxidant activity recorded in leaves was similar to that observed by Rubilar *et al.* (2011) in stems (IC_{50} value: 43.1 ± 1.7 mg extract l^{-1}). González-Villagra *et al.* (2018) determined that fully expanded *A. chilensis* leaves subjected to drought stress, had higher antioxidant activity and major capacity to synthesize ABA regulating stomatal closure, than young leaves in the same condition. In addition, young leaves showed highest TPC and total anthocyanin levels but lower antioxidant activity in the 10^{th} day of drought stress treatment. The authors suggest that the fully expanded leaves of stressed *A. chilensis* plants have a strong antioxidant mechanism to tolerate drought stress.

The mechanism by which an antioxidant compound interacts with a radical molecule depends on the structural conformation of the antioxidant (Mishra *et al.* 2012). Granato *et al.* (2018) indicated that no single antioxidant activity assay will reflect the total antioxidant capacity. The antioxidant activity methods have particularities depending on mechanisms of action, types of radical, pH, time of exposition and temperature. One of the tests used in this study was the DPPH assay, which is simply due to its stable nitrogen radical, but has problems with many antioxidants by reacting with different kinetics or not reacting at all (Mishra *et al.* 2012). It was also indicated that the DPPH assay is pH-dependent, and the final result could be influenced by the deprotonation of the phenolic group (Mishra *et al.* 2012, Tirzitis and Bartosz 2010). The other method used in this study was the ABTS assay, which consists of an oxidation reaction of the coloured cation $ABTS^{*+}$.The ABTS assay can be applied to

lipophilic and hydrophilic compounds (Huyut *et al.* 2017). Thus, the results of the current study determined in the DPPH assay, an increase in antioxidant activity in summer in both types of leaves, coinciding with the greater luminosity, without the same trend in ABTS assay.

On the another hand, Harnly (2017) described how the measurement of *In vitro* antioxidant activity and total phenolic content using the Folin-Ciocalteu reagent are not suitable. In this regard, chromatography techniques used to identify and quantify phenolic compounds in foods, beverages and herbal extracts have sufficient accuracy or precision. It is also necessary to highlight what the author indicates about the results of the method *a* is (usually) not comparable with the data of method *b* or even between laboratories (Harnly 2017). Therefore, as indicated by Granato *et al.* (2018), it is evident that "antioxidant activity" involves complex interactions. However, screening spectrophotometric methods to characterize the samples give an idea of total phenolic content in the matrix. The relationship between TPC and antioxidant activity has been reported in several studies (Rawat *et al.* 2011). However, Kabra *et al.* (2019) indicated that in the extracts of *Myrica esculenta* leaves, the relationship between phenolic compounds and antioxidant activity could be influenced by phytochemical composition of each extract and the use of two different mechanisms of radical scavenging (DPPH and ABTS assays). Similarly, Zhang *et al.* (2018) indicated in their DPPH and ABTS assays, different values for nineteen citrus genotypes belonging to *Citrus reticulata* Blanco, in their peels, juice, pulp and seeds. In addition, in this study, it is important to highlight the differences of TPC content and the relation with light in the basal spring leaves. The phenolic content may be influenced by the quantity and quality of this active substance, type of plant habitat, season of the year and conditions in which plants are grown (Stanković *et al.* 2017). In this context, the light in the spring season could be favourable for the synthesis of these active compounds in *A. chilensis* leaves and their relationship with the increased photosynthesis to leads to an increase in secondary metabolism. In summary, the TPC and antioxidant activity variation between apical and basal *A. chilensis* leaves in the same season might be due to the different phytochemical composition.

As a general conclusion, in this study, it was determined that the *A. chilensis* basal leaves showed a better photosynthetic performance as indicated by higher F_v/F_m values, which correlates with higher total phenol content in high light conditions. In both types of

leaves, the increase in light intensity was accompanied by an increase in NPQ values, reflecting a safe mechanism to dissipate excess light energy. In addition, apical leaves display some morphological adaptions, such as increase in intercellular spaces to facilitate the entry of CO_2 inside the chloroplasts, as a mechanism to protect the photosynthetic process. Finally, in order to take advantage of the research, it is necessary to know in the future, the performance of the activities of the different antioxidant enzymes and compare these antioxidants mechanisms in both types of leaves.

References

Acosta-Motos, J.R., Diaz-Vivancos, P., Álvarez, S., Fernández-García, N., Sanchez-Blanco, M.J., Hernández, J. A.: Physiological and biochemical mechanisms of the ornamental *Eugenia myrtifolia* L. plants for coping with NaCl stress and recovery. - Planta **242(4)**: 829–846, 2015.

Acosta-Motos, J.R., Diaz-Vivancos, P., Álvarez, S., Fernández-García, N., Sánchez-Blanco, M.J., Hernández, J. A.: NaCl-induced physiological and biochemical adaptative mechanisms in the ornamental *Myrtus communis* L. plants. - J. Plant Physiol. **183**: 41–51, 2015.

Agulló, V., García-Viguera, C., Domínguez-Perles, R..: Beverages based on second quality citrus fruits and maqui berry, a source of bioactive (poly)phenols: sorting out urine metabolites upon a longitudinal study vicente. - Nutrients **13**: 312, 2021. https://doi.org/ 10.3390/nu13020312.

Barthélémy, D., Caraglio, Y.: Plant architecture: A dynamic, multilevel and comprehensive approach to plant form, structure and ontogeny. - Ann. Bot. **99(3)**: 375–407, 2007.

Céspedes, C.L., Pavón, N., Domínguez, M., Alarcón, J., Balbontín, C., Kubo, I., El-Hafidi, M., Ávila, J.G.: The chilean superfruit black-berry *Aristotelia chilensis* (Elaeocarpaceae), maqui as mediator in inflammation-associated disorders. - Food Chem. Toxicol. **108**: 438–450, 2017.

Damascos, M.A., Prado, C.H.B.A.: Defoliación en la especie invierno-verde *Aristotelia chilensis* y su efecto sobre el crecimiento inicial de hojas y ramas. - Bosque **22(1)**: 45–50, 2001a. https://doi.org/10.4206/bosque.2001.v22n1-05.

Damascos, M.A., Prado, C.H.B.A.: Leaf phenology and its associated traits in the wintergreen species *Aristotelia chilensis* (Mol.) Stuntz (Elaeocarpaceae). - Rev. Chil. Hist. Nat. **74(4)**: 805–815, 2001b.

de Menezes, B.B., Frescura, L.M., Duarte, R., Villetti, M.A., da Rosa, M.B.: A critical examination of the DPPH method: Mistakes and inconsistencies in stoichiometry and IC50 determination by UV–Vis spectroscopy. - Anal. Chim. Acta. **1157**: 338398, 2021.

dos Santos Isaias, R.M., de Oliveira Coelho, D., da Silva Carneiro, R.G.: Role of *Euphalerus ostreoides* (hemiptera: Psylloidea) in manipulating leaflet ontogenesis of *Lonchocarpus muehlbergianus* (Fabaceae). - Botany **89(9)**: 581–592, 2011.

Fredes, C., Montenegro, G., Zoffoli, J.P., Santander, F., Robert, P.: Comparación de los contenidos de fenoles totales, antocianos totales y la actividad antioxidante de frutos ricos en polifenoles que crecen en Chile. - Cienc. e Investig. Agrar. **41(1)**: 49–59, 2014. https://doi.org/10.4067/S0718-16202014000100005.

Fredes, C., Osorio, M.J., Parada, J., Robert, P.: Stability and bioaccessibility of anthocyanins from maqui

(*Aristotelia chilensis* [Mol.] Stuntz) juice microparticles.- LWT - Food Sci. Technol. **91**: 549–556, 2018.

Fuentes, L., Figueroa, C.R., Valdenegro, M., Vinet, R.: Patagonian berries: healthy potential and the path to becoming functional foods. - Foods **8(8)**: 2019.

Gonçalves, S., Romano, A.: Inhibitory properties of phenolic compounds against enzymes linked with human diseases. - Phenolic Compounds - Biological Activity 2017.

González-Villagra, J., Rodrigues-Salvador, A., Nunes-Nesi, A., Cohen, J.D., Reyes-Díaz, M.M.: Age-related mechanism and its relationship with secondary metabolism and abscisic acid in *Aristotelia chilensis* plants subjected to drought stress. - Plant Physiol. Biochem. 1**24(December 2017)**: 136–145, 2018.

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L.D., Hidalgo, F.J., Miyashita, K., Camp, J.V., Alasalvar, C., Ismail, A. B., Elmore, S., Birch, G. G., Charalampopoulos, D., Astley, S. B., Pegg, R., Zhou, P., Finglas, P.: Antioxidant activity, total phenolics and flavonoids contents: should we ban *In vitro* screening methods?. - Food Chem. **264(April)**: 471–475, 2018.

Harnly, J.: Antioxidant methods. - J. Food Compos. Anal. **64**: 145–146, 2017.

Hernández, J.A., Escobar, C., Creissen, G., Mullineaux, P.M.: Role of hydrogen peroxide and the redox state of ascorbate in the induction of antioxidant enzymes in pea leaves under excess light stress. - Funct. Plant Biol. **31(4)**: 359–368, 2004.

Huyut, Z., Beydemir, Ş., Gülçin, I.: Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. - Biochem. Res. Int. 2017.

Kabra, A., Sharma, R., Hano, C., Kabra, R., Martins, N., Baghel, U.S.: Phytochemical composition, antioxidant, and antimicrobial attributes of different solvent extracts from *Myrica esculenta* Buch.-Ham. ex. D. Don Leaves Atul. - Biomolecules**. 9(8)**:357, 2019. https://doi.org/10.3390/biom9080357

Masek, A., Latos-Brozio, M., Kałuzna-Czaplinska, J., Rosiak, A., Chrzescijanska, E.: Antioxidant Properties of Green Coffee Extract. - Forests. **11(5)**:557, 2020.

Masoodi, H., Villaño, D., Zafrilla, P.: A comprehensive review on fruit: *Aristotelia chilensis* (maqui) for modern health: towards a better understanding. - Food Funct. **10(6):** 3057–3067, 2019.

Mathur, S., Jain, L., Jajoo., A.: Photosynthetic efficiency in sun and shade plants. Photosynthetica **56(1)**: 354–365, 2018.

Mendoza, C.A., Turrión, M.B., Aceñolaza, P.G., Gallardo, J.F., Pando, V.: Retranslocación de nutrientes en especies dominantes de bosques del espinal Mesopotámico (Argentina). - Bosque **35(2)**: 185–193, 2014. https://doi.org/10.4067/S0717-92002014000200006.

Mishra, K., Ojha, H., Chaudhury, N.K.: Estimation of antiradical properties of antioxidants using DPPH- assay: a critical review and results. - Food Chem. **130(4)**: 1036–1043, 2012.

Molina-Montenegro, M.A., Torres-Díaz, C., Carrasco-Urra, F., González-Silvestre, L.A., Gianoli, E.: Plasticidad fenotípica en dos poblaciones antárticas de *Colobanthus quitensis* (Caryophyllaceae) bajo un escenario simulado de cambio global. - Gayana - Bot. **69(1)**: 152–160, 2012. https://doi.org/10.4067/S0717-66432012000100015.

Moya, M., González, B., Doll, U., Yuri, J.A., Vogel, H.: Different covers affect growth and development of three maqui clones (*Aristotelia chilensis* [Molina] Stuntz). - J. Berry Res. **9(3)**: 449–458, 2019.

Murchie, E.H., Lawson, T.: Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. - J. Exp. Bot. **64(13)**: 3983–3998, 2013.

Naikoo, M.I., Dar, M.I., Raghib, F., Jaleel, H., Ahmad, B., Raina, A., Ahmad Khan, F., Naushin, F.: Role and

regulation of plants phenolics in abiotic stress tolerance: an overview. - Plant Signal. Mol.: 157–168, 2019. https://doi.org/10.1016/B978-0-12-816451-8.00009-5.

Ortiz, T., Argüelles-Arias, F., Begines, B., García-Montes, J.M., Pereira, A., Victoriano, M., Vázquez-Román, V., Pérez Bernal, J.L., Callejón, R.M., De-Miguel, M., Alcudia, A.: Native chilean berries preservation and *In vitro* studies of a polyphenol highly antioxidant extract from maqui as a potential agent against inflammatory diseases. - Antioxidants **10**: 843, 2021.https://doi.org/10.3390/antiox10060843.

Rawat, S., Jugran, A., Giri, L., Bhatt, I.D.,Rawal. R.S.: Assessment of antioxidant properties in fruits of *Myrica esculenta*: A popular wild edible species in indian himalayan region. - Evidence-based Complement. Altern. Med. **2011**, 512787, 2011. https://doi.org/10.1093/ecam/neq055.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., Shene, C.: Extracts of maqui (*Aristotelia chilensis*) and murta (*Ugni molinae* Turcz.): sources of antioxidant compounds and α-glucosidase/α-amylase inhibitors. - J. Agric. Food Chem. **59(5)**: 1630–1637, 2011.

Salinas, J., Cabellé, G.: Maqui, el fruto silvestre de mayor importancia en Chile. - Instituto Forestal, Chile. 248, 2020. https://bibliotecadigital.infor.cl/handle/20.500.12220/30380.

Scossa, F., Fernie, A.R.: The evolution of metabolism: how to test evolutionary hypotheses at the genomic level. - Comput. Struct. Biotechnol. J. **18**: 482–500, 2020.

Sembiring, E.N., Elya, B., Sauriasari, R.: Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of *Caesalpinia bonduc* (L.) Roxb. - Pharmacogn. J. **10(1)**: 123–127, 2018.

Stanković, M., Ćurčić, S., Zlatić, N., Bojović, B.: Ecological variability of the phenolic compounds of *Olea europaea* L. leaves from natural habitats and cultivated conditions. - Biotechnol. Biotechnol. Equip. **31(3)**: 499–504, 2017.

Tirzitis, G., Bartosz, G.: Determination of antiradical and antioxidant activity: basic principles and new insights. - Acta Biochim. Pol. **57(2)**: 139–142, 2010.

Turchetti, G., Paz, C. : *Aristotelia chilensis* (Mol.) Stuntz: a natural source of bioactive compounds. - Curr. Tradit. Med. **5(1)**: 66–74, 2019.

Vidal, J.L., Avello, L.M., Loyola, C.C., Campos, P.J., Aqueveque, M.P., Dungan, R.S., Galotto, L.M., Guarda, M.A.: Microencapsulation of maqui (*Aristotelia chilensis* [Molina] Stuntz) leaf extracts to preserve and control antioxidant properties. - Chil. J. Agric. Res. **73(1)**: 17–23, 2013.

Yang, Y.J., Zhang, S.B., Wang, J.H., Huang, W.: The decline in photosynthetic rate upon transfer from high to low light is linked to the slow kinetics of chloroplast ATP synthase in *Bletilla striata*. - Photosynth. Res. **144(1)**: 13–21, 2020.

Zhang, H., Yang, Y.F., Zhou, Z.Q.: Phenolic and flavonoid contents of mandarin (*Citrus reticulata* Blanco) fruit tissues and their antioxidant capacity as evaluated by DPPH and ABTS methods. - J. Integr. Agric. **17(1)**: 256–263, 2018.

Zhen, S., Bugbee, B.: Far-red photons have equivalent efficiency to traditional photosynthetic photons: implications for redefining photosynthetically active radiation. Plant Cell Environ. (January), 1259–1272, 2020.

Zúñiga, G.E., Tapia, A., Arenas, A., Contreras, R.A., Zúñiga-Libano, G.: Phytochemistry and biological properties of *Aristotelia chilensis* a chilean blackberry: a review. - Phytochem. Rev. **16(5)**: 1081–1094, 2017.

CAPÍTULO II

Comparative study of metabolomic profile and antioxidant content of adult and *In vitro* leaves of *Aristotelia chilensis*

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Abstract

This work aimed to identify the bioactive compounds present in adult maqui (*Aristotelia chilensis*) leaves from different stages of development and seasons of the year, and compare them with leaves obtained from maqui plants grown *In vitro*.

The quantitative and qualitative analysis showed the presence of different phenolic compounds in both apical and basal adult, and *In vitro* maqui leaves. The compounds identified were classified into the groups of galloyl and caffeoyl quinic acids, ellagitannins, ellagic acid derivatives and flavonoid derivatives. In the quantitative analysis, the total phenolics contents in the *In vitro* samples with respect to *ex vitro* samples. The highest levels of flavonoids derivatives were found in adult leaves in spring and *In vitro* leaves.

The analyses for HPLC/MS indicated the extract from spring basal leaves (BS) included quercetin, catechin, kaempferol, and 3-caffeoyl quinic acids compounds while *In vitro* leaves extract not present quercetin. Determination of lipophilic compounds was performance by GC/MS. The samples of *In vitro* leaves showed a high presence of α-tocopherol and β-sitosterol. In contrast, the samples of adult leaves presented a hight level of linolenic and linoleic acids.

1. Introduction

Aristotelia chilensis is a perennial non-wood forest species belonging to the Elaeocarpaceae family. It is an endemic specie of the sub-Antarctic forests of both Argentina and Chile. In the last one, its cultivation extends from the Region of Coquimbo to the Aysén Region, including the Juan Fernández Islands (Zúñiga et al., 2017). *A. chilensis*, known as maqui, has been recognized for its beneficial effects for human health due to its antioxidant (Girones-Vilaplana et al., 2016), anti-tumoral(Mena et al., 2021; Ojeda & Maria A.; Hancke, 2011), cardioprotective (Céspedes et al., 2008), anti-inflammatory (Céspedes, Pavón, et al., 2017; Ortiz et al., 2021), anti-hemolytic (Rubilar et al., 2011), and anti-diabetics properties (Rojo et al., 2012), as well as for its antiplatelet effect (Rodriguez et al., 2021). These biological activities are mainly attributed to its small fruits containing high levels of polyphenols, particularly a wide variety of phenolic compounds like phenolic acids, anthocyanins, pro-anthocyanidins and alkaloids (Masoodi et al., 2019).

For this reason, most of the studies carried out in maqui have been focused on using fruit extracts since they exhibit pharmacological activities of high relevance, mainly associated with the content of anthocyanins (Masoodi et al., 2019; Zhu et al., 2021). The anthocyanin

profile in maqui fruit, which is abundant in delphinidin derivatives, has been characterized by different authors (Concha-Meyer et al., 2021; Rodriguez et al., 2021). These compounds are relevant since they are absorbed and metabolized in humans, circulating as sulphated and glucuronidated forms in the blood, which are accumulated in target tissues, excreted in the urine, and can be transported across the blood-brain barrier (Felgines C, 2005; Talavéra, 2005).

It is important to highlight that the content of flavonoids and other phenolic compounds depends on the intrinsic factors that affect the plant like the expression of specific genes, as well as the different geographical areas where maqui plants are cultivated (Fredes et al., 2014). Many environmental factors such as light, temperature, altitude, soil type, water, nutritional status, microbial interactions, pathogenesis, wounds, defoliation, growth regulators, seasonality, as well as the different agricultural techniques used, can influence the biosynthesis of these compounds in maqui plants. (Fredes et al., 2014).

On the other hand, given the beneficial health properties of these compounds, there is a growing interest in the knowledge of the metabolic profile of extracts of maqui fruits and leaves, and the high demand for these bioactive compounds has promoted the search for new alternative sources for the production of these compounds. Turchetti & Paz (2019) described the presence of polyphenols and indole alkaloids like aristoteline, aristotelinine, aristotelone, aristone, aristotelinone, aristoquinoline, and makonine and hobartine and their derivatives, amongst others, in maqui leave extracts. Muñoz et al., (2011) carried out a phytochemical characterization of leaves extracts, and detected anti-inflammatory, analgesic, and antioxidant activities in them. In addition, Céspedes et al., (2017) carried out the first studies on the use of maqui leave extracts to treat Alzheimer's disease. Nevertheless, few studies have considered the effects of seasonality and the state of development of maqui leaves to understand in which seasonal they have high levels of these specific bioactive phenolic compounds.

Besides, the growing market demand for these bioactive compounds can lead to over-harvesting of the different organs (mainly fruits and leaves) from endemic forest species as is the case of maqui plants, which would make it an endangered species. In this way, when a scarce distribution in nature limits the supply of a bioactive compound, plant biotechnology can provide an alternative and sustainable system for its production. Phytochemical-

producing green factories based on the use of the plant *In vitro* cultures have several advantages over plants grown in the field: a higher production of bioactive compounds with a lower use of natural sources, the obtention of uncontaminated plant material since *In vitro* cultures are maintained in a sterile environment. Moreover, agricultural systems are not required, freeing up land for food production, and drastically reducing the use of the water (Narayani & Srivastava, 2017).

For these reasons, plant *In vitro* cultures can be considered a suitable and cutting-edge system for the sustainable production of phytochemicals that preserve their natural sources.

In this study, a comparative analysis of the bioactive compounds presents in maqui leaves collected from the field at different stages of development and different seasons of the year, and the bioactive compounds identified in maqui leaves from plants grown *In vitro* was carried out.

2. Materials and Methods

2.1 Plant material

Maqui leaves were collected between August-December 2017 at the University of Concepción, Biobío Region, Chile. The samples were collected in the winter and spring seasons. Adult maqui plants of the male sex reaching a height of 3 m were used. In them, two types of samples were collected: leaves of the upper third of the branch called apical (AP) and leaves of the lower third of the branch called basal (BS) (Crisóstomo-Ayala et al., 2021).

2.1.2 In vitro plant cultures

In vitro plant cultures of maqui were initiated using vegetative nodal segments as initial explants. This plant material was obtained from mother plants collected in November 2018. The vegetative segments were disinfected with ethanol 70% (1 min), and then, with a hypochlorite solution (0.5%) containing detergent (10 min). After the disinfection time, the segments were washed 4 times with sterile distilled water. After that, they were placed in glass tubes with 20 mL of culture medium. The culture medium contained Murashige & Skoog (1962) basal mineral medium at the halfway point of its concentration, vitamins, sucrose (20 g L^{-1}), polyvinylpolypyrrolidone (0.5 g L^{-1}), kinetin (0.5 mg L^{-1}), naphthalene

acetic acid (0.05 mg L^{-1}). Then, the pH was adjusted to 6.0 and phytagel (2.7 g L^{-1}) was added. Shoots were developed from these nodal segments, and they were subcultured every 25 days. *In vitro* plants were grown at a 16-h photoperiod (photosynthetic photon flux density (PPFD) of 40-60 µmol photons m^{-2} $s^{-1)}$, and a temperature of 25/20 °C, day/night, respectively.

2.1.3 Preparation of the samples from in vivo and In vitro maqui leaves

For phytochemical analysis, samples from adult maqui plants constituted by fresh AP and BS leaves (40 g) were dried at 37 °C for two days. Dried leaves were crushed to get a fine powder and then, maceration was performed by exhaustion in methanol-HCl 0.1%. The total extract was concentrated at 37 °C and lyophilized for 24 hours. In addition, for preparing samples from *In vitro* plant material, leaves from *In vitro* maqui plants were collected (2 g) and lyophilized for extract preparation. The lyophilized material was macerated with methanol-HCl (0.1%) for 24 hours, and then was centrifuged and the supernatant collected. It was dried at 37 °C and frozen at -20 °C until use.

2.2 Determination of polyphenolic compounds

The identification and quantification of polyphenolic compounds in the samples of maqui leaves was carried out using HPLC-DAD-ESI-MS^n and HPLC-MS.

2.2.1 HPLC-DAD-ESI (Ion Trap)-MS^n analysis

Chromatographic separations of different components of the extracts were carried out in a Mediterranea sea C18 column (150 x 4.60 mm, 3 µm particle size, Teknokroma, Spain) at room temperature, as described Ferreres et al., (2015) with minor modifications. Elution was performed with a mobile phase of two solvents, water-formic acid (1%) (A) and acetonitrile (B), starting with 10% B and using a gradient to obtain 25% B at 30 min and 70% B at 35 min at a flow rate of 0.8 mL min^{-1} and an injection volume of 20 µL. The system was HPLC (Agilent model 1200) coupled to a DAD and a mass spectrometer with ion trap technology (Bruker, model Amazon, Ultra High-Speed ion trap).

Chromatograms were recorded at 280 and 350 nm, and the MS system was operated in negative ion mode using the MS/MS Fragmentation Amplitude 1.00 V (mass fragmentation energy ramp), mass range *m/z* 100-1200. For quantitative analysis, external standard calibration curves for ellagic acid (assay ≥95%), rutin (assay ≥95%), chlorogenic acid (assay ≥99%), and gallic acid (assay ≥99%), all purchased from Sigma-Aldrich, (Germany) were used. The results were expressed in µg g^{-1} DW (dry weight).

2.2.2 HPLC-MS

BS leaves collected in spring and *In vitro* leaves were analysed by an HPLC-MS system (Agilent Series 1200, Agilent Technologies, USA), as described by Sánchez-Pujante et al., (2020). Separation was performed at room temperature, on a C18 column (4.6 mm x 250 mm, 5 µm). The mobile phase consisted of solvent A (formic acid 0.5%) and solvent B (acetonitrile-formic acid 0.1%), using a gradient described as follows: 0 min, 2% solvent B; 10 min, 20% solvent B; 36-37 min, 100% solvent B; 37.5 min, 2% solvent B; and 40 min, 2% solvent B. The flow rate was 0.8 mL min^{-1}, and the injection volume was 20 µL. Mass spectral analysis was carried out using a TOF/Q-TOF MS (Agilent Series 6220, Agilent Technologies, USA) equipped with an ESI operating in negative ion mode. The operation parameters were: capillary, fragmentor, and octopole RF voltages were 2500V, 180V, and 250V, respectively; nebulizer pressure, 60 psi; drying gas flow, 12 L/min; and drying gas temperature 350ºC. Mass range was 50-1200 m/z and scan rate were 1.9 spectra/sec. External standard calibration curves for catechin (assay ≥99%), 3-0-caffeoylquinic acid (assay ≥98%), quercetin (assay ≥95%), and kaempferol (assay ≥97%), all purchased from Sigma-Aldrich, (Germany) were used.

2.3 Determination of lipophilic compounds GC/MS

Samples were analysed by GC/MS as described by Sabater-Jara et al., (2010). The identification of metabolites was based on the mass spectra (EI, 70 eV) obtained from a gas chromatograph (Agilent Technologies 6890 Network GS System) equipped with a mass selective detector (Agilent Technologies 5973). A 30 m×0.25 mm capillary column (Agilent 19091 S-433HP-5MS) was used for GC/MS analysis. The GC oven temperature was

programmed from 60 to 310 ˚C for the analysis of the metabolites. A constant flow rate of 0.1 mL min^{-1} was set using helium as carrier gas. Th injection volume was 1 µL. Data was obtained in scan mode using electron impact ionization.

The identification of maqui metabolites was conducted by comparing the experimental mass spectra with the National Institute Standard and Technology (NIST) spectral library. Likewise, the metabolites were identified and quantified by comparing with respective retention times and mass spectra from external standards. The range of each standard curve was 0.1 – 10 µg mL^{-1}.

2.4 Total flavonoid content

Total flavonoid content (TFC) was measured by the aluminium chloride assay described by Ahmed et al., (2015) with some modifications. All samples were analysed in triplicates. An aliquot of 0.2 mL of extracts or standard rutin solution (0.02-0.4 mg L^{-1}) was added to 0,8 mL methanol (50%). Then, 60 µL aqueous sodium nitrite solution (0.5 M) was added followed by 60 µL aluminium chloride solution (0.3 M). After 5 min, 0,4 mL sodium hydroxide solution (1 M) was added. The absorbance was measured at 506 nm on a UV visible spectrophotometer against a blank. TFC was expressed as rutin equivalents RE (mg mL^{-1}).

3. Results and Discussion

3.1 Qualitative analysis of the maqui leaves

Many phenolic compounds were detected in the methanolic extracts of *A. chilensis* leaves. Figure 1 shows the HPLC-UV chromatogram (280, 350 nm) of the leaves obtained by *In vitro* culture. Figure 2 shows the HPLC-UV chromatogram (280, 350 nm) of the spring BS leaves, the rest of chromatograms of the *ex vitro* leaves are similar to the exposed. The compounds identified are described in the following item.

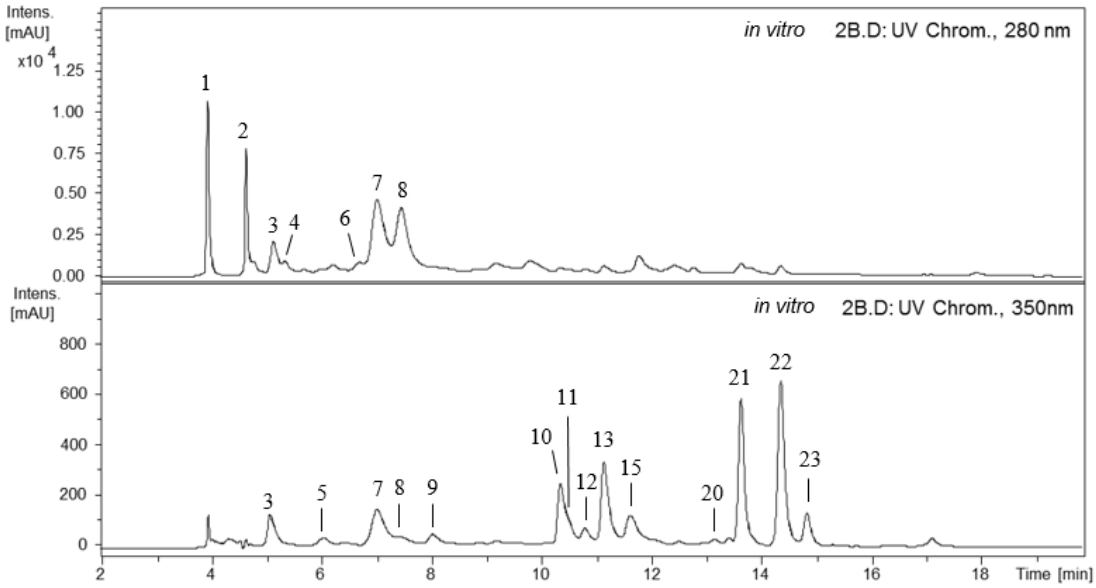


Figure 1. HPLC-DAD-ESI (Ion Trap)-MSn (280, 350 nm) phenolic profile of methanolic extract from *In vitro* leaves of *A. chilensis*. Identity of compounds is shown in Tables 1 and 2.

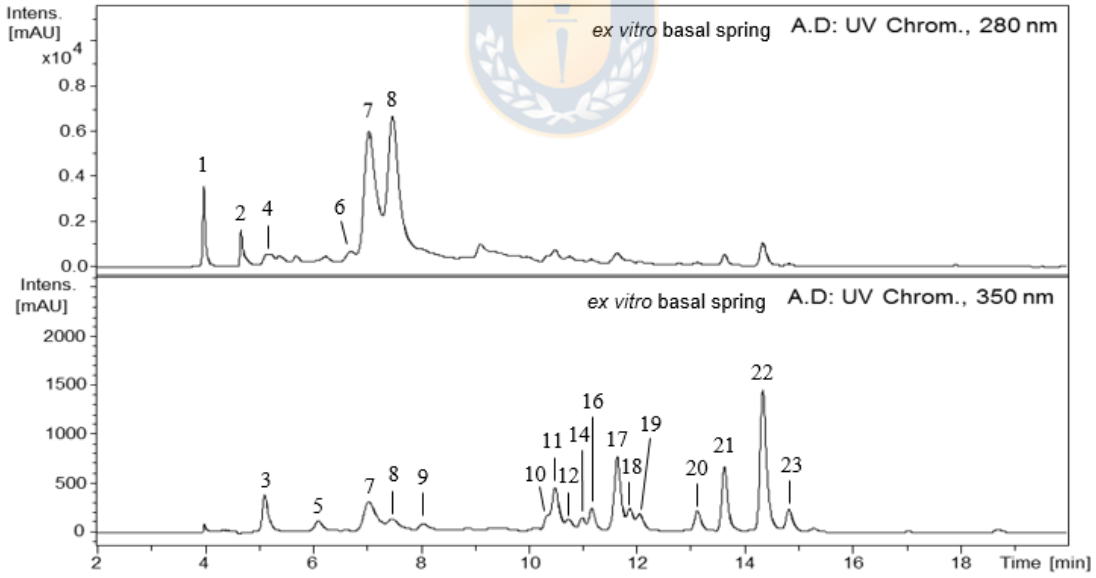


Figure 2. HPLC-DAD-ESI (Ion Trap)-MSn (280, 350 nm) phenolic profile of methanolic extract from *ex vitro* basal spring leaves of *A. chilensis*. Identity of compounds is shown in Tables 1 and 2.

Galloyl acid derivatives

The compounds **1**, **2** and **4** with Rt at 3.9, 4.6 and 5.3 min, respectively showed identical UV spectra (276 nm), and deprotonated molecular ions were at *m/z* 331, 343 and 495, respectively. In the MS fragmentation of **1,** the loss of a fragment at 162 amu (hexosyl radical) was observed leading to an ion at *m/z* 169 (deprotonated molecular ion of gallic acid). Therefore, this compound was identified as galloyl-hexoside (**1**) (Ferreres et al., 2012). In the MS fragmentations of **2,** a loss of 152 amu (radical galloyl) was detected to produce an ion at *m/z* 191 (deprotonated molecular ion of quinic acid), and so, this compound was identified as galloyl quinic acid (**2**). The MS^2 of **4** provided a deprotonated molecular ion higher than 2 (152 amu or increase with regard 2) and showed an additional loss of 152 to obtain 343 (deprotonated molecular ion of 2). This compound provided a similar fragmentation pattern ($MS^3[495 \rightarrow 343]^-$) than **2** therefore, it was identified as di-galloyl quinic acid (**4**).

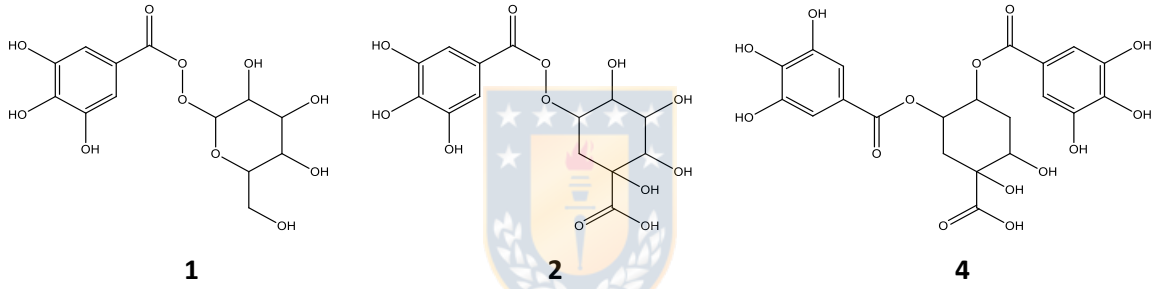
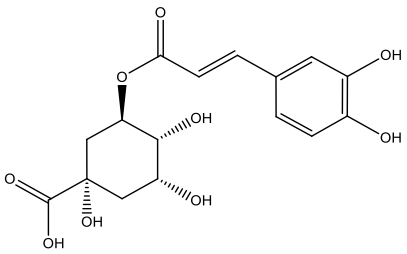


Figure 3. Structure of the main galloyl acid derivatives identified in *A. chilensis* leaves extracts.

Caffeoyl quinic acids

The compounds **3** and **5** (Rt 5.1 and 6.1 min, respectively) showed the same deprotonated molecular ions at *m/z* 353 y UV spectra (299sh, 324 nm) typical of cinnamoyl quinic acid structures. According to their MS fragmentations and their relative abundances (MS^2(**3**): 191 (100%), 179 (50%); MS^2(**5**): 179 (65%), 173 (100%)), and in accordance with Clifford et al., (2003), it can be concluded that these compounds were 3-caffeoyl quinic acid (**3**) and 4-caffeoyl quinic acid (**5**) (Andrade et al., 2019; Petreska et al., 2011).



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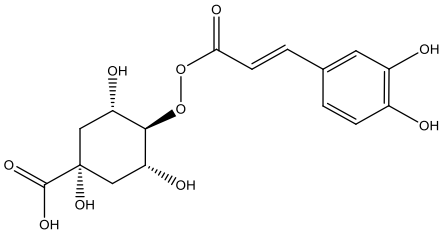

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Figure 4. Structure of the main caffeoyl quinic acids identified in *A. chilensis* leaves extracts.

Ellagitannins

The peaks **6**, **7**, **8** with Rt at 6.7, 7.0 and 7.4 min, respectively presented the same UV spectra (278 nm) with a MS fragmentation of the deprotonated ion corresponding to ellagic acid (301amu) (Mena et al., 2013). Therefore, they were unknown polymeric structures composed of galloyl and hexahydroxydiphenoyl (HHDP) esterified with glucose. The peak **7** with $[M-H]^-$ at *m/z* 951 and MS^2: 933, 463, 301 could be coincident with granatin B (galloyl-HHDP-DHHDP-hexoside) (Mena et al., 2012); Peaks **6** and **8** showed the same mass and fragmentation pattern ($[M-H]^-$ at *m/z*: 1109, MS^2: 1049, 973, 935, 301), and their structures have not been identified.

6 NOT IDENTIFIED

8 NOT IDENTIFIED

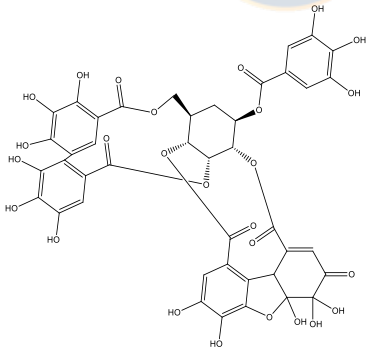

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Figure 5. Structure of the main ellagitannins identified in *A. chilensis* leaves extracts.

Ellagic acid derivatives

The compounds **9**, **10**, **12**, **13** and **15** showed UV spectra typical of ellagic acid derivatives (UV: 252, 305sh, 345sh, 364 nm) and their MS fragmentations, after the losses of 162 amu (**9**), 132 amu (**10** and **12**) and 146 amu (**13** and **15**), a base peak of ellagic acid was detected (301 amu) (Table 1) (Ferreres et al., 2013). Therefore, these compounds were identified as ellagic acid-hexoside (**9**), ellagic acid-pentoside isomers (**10** and **12**) and as ellagic acid-rhamnoside isomers (**13** and **15**)(Mena et al., 2013).

Table 1. *Rt*, Molecular Formula, $[M-H]^-$ and MS^2 $[M-H]^-$ data for the ellagic acid derivatives detected in methanolic extracts obtained from *A. chilensis* leaves. [1]

Compounds[2]		***R*t (min)**	**$[M-H]^-$, *m/z***	**$MS^2[M-H]^-$, *m/z***		
				-132	-146	-162
9	Ellag ac-Hex	8.0	463			301
10	Ellag ac-Pt	10.3	433	301		
12	Ellag ac-Pt	10.7	433	301		
13	Ellag ac-Rhmn	11.0	447		301	
15	Ellag ac-Rhmn	11.6	447		301	

[1] Main fragments observed.

[2] Ellag ac: ellagic acid; Pt: pentoside; Rhmn: rhamnoside; Hex: hexoside.

Flavonoid derivatives

The peaks **11**, **14**, **16-23** showed UV spectra characteristics of flavonoids (Table 2). Particularly, the compounds **14**, **16**, **17**, **18** and **20** had UV spectra of quercetin substituted at the 3-*O* position (Table 2). After the MS fragmentation, a base peak at *m/z* 301 (deprotonated quercetin) was observed. The deprotonated molecular ion of **14** and **16** at *m/z* 609 indicated that they were rhamno-hexosyl quercetin isomers, and the absence of other ions at the MS fragmentation spectra linked to the interglycosyl linkage suggests a bond 1→6 (Medina et al., 2017). Therefore, both compounds should be quercetin-3-*O*-(6-rhamnosyl) hexosides isomers, labelled by elution order by reverse phase interaction as quercetin-3-*O*-(6-rhamnosyl) galactoside (**14**) and quercetin-3-*O*-(6-rhamnosyl) glucoside (**16**) (Abu-Reidah et al., 2017). Compounds **17**, **18** and **20** showed a deprotonated molecular ion of

monoglycosides. The peaks **17** and **18** should be quercetin-3-*O*-hexosides isomers which would agree with quercetin-3-*O*-galactoside (**17**) and quercetin-3-*O*-glucoside (**18**) (Gil-Izquierdo & Mellenthin, 2001), and quercetin-3-*O*-pentoside (**20**) (Table 2). The peak **11** presented a deprotonated molecular ion at *m/z* 615 with a loss of 152 amu after the MS fragmentation event (galloyl radical), and other ion at *m/z* 301 (deprotonated quercetin) which indicated that this compound was quercetin-3-*O*-(galloyl) hexoside. Its UV spectrum provided a maximum at 268 nm due to the overlaying of quercetin and gallic acid UV spectra (Ferreres et al., 2012). The compound **19** had a monoglycoside mass and a UV spectrum of luteolin derivative. Its fragmentation showed a base peak at *m/z* 285 (deprotonated luteolin) confirming luteolin-7-*O*-hexoside (Ferreres et al., 2018; Ferreres et al., 2014). In the MS fragmentation of **21** and **22**, an ion at *m/z* 285 as base peak from their deprotonated aglycones was observed (tetrahydroxyflavone). Their UV spectra (Table 2) did not correspond neither with that luteolin (5,7,3´,4´-tetrahydroxyflavone) nor that kaempferol (3,5,7,4´-tetrahydroxyflavone). In fact, these UV spectra (maximum absorption peaks at 268 y 336 nm) were more similar to apigenin (5,7,4´-trihydroxyflavone), and the additional hydroxyl was not bond to the ring A since the maximal absorption would be between 270-275 nm instead of 268 then, it could be placed at the ring B 2´-position (5,7,2´,4´-tetrahydroxyflavone). Therefore, considering the comments above with regard to the glycosylated fraction of the compounds **14/16** and **17/18**, **21** and **22**, they could be labelled as 5,7,2´,4´-tetrahydroxyflavone-*O*-(6-rhamnosyl) hexoside and 5,7,2´,4´-tetrahydroxyflavone-*O*-hexoside, respectively. The peak **23** showed a UV spectrum like that of luteolin, and its deprotonated molecular ion at *m/z* 659 with MS fragment ion at *m/z* 329 as base peak (deprotonated trihydroxy-dimethoxyflavone) has not been properly identified.

Table 2. Rt, Molecular Formula, $[M-H]^-$ and $MS^2[M-H]^-$ data for flavonoids derivatives detected in methanolic extracts obtained from *A. chilensis* leaves [1]

Compounds[2]		*R*t (min)	UV (nm)	$[M-H]^-$, *m/z*	$MS^2[M-H]^-$, *m/z*	
					-152	$[Aglc-H]^-$
11	Qct-3-(Gall)Hex	10.5	268, 288sh, 352	615	463(100)	301(25)
14	Qct-3-(Rhmn)Hex	11.0	255, 266sh, 295sh, 354	609		301(100)
16	Qct-3-(Rhmn)Hex	11.2	256, 266sh, 298sh, 355	609		301(100)
17	Qct-3-Hex	11.6	256, 266sh, 298sh, 354	463		301(100)
18	Qct-3-Hex	11.9	256, 266sh, 298sh, 355	463		301(100)
19	Lut-7-Hex	12.0	256, 266sh, 348	447		285(100)
20	Qct-3-Pt	13.1	256, 266sh, 298sh, 355	433		301(100)
21	tetOHFlv-(Rhmn)Hex	13.6	248sh, 268, 290sh, 336	593		285(100)
22	tetOHFlv-Hex	14.3	248sh, 268, 290sh, 336	447		285(100)
23	triOH-diOMeFlv-der	14.8	252, 266sh, 298sh, 346	659		329(100)

[1] Main fragments observed.

[2] Aglc: aglycon; Gall: gallic acid; Qct: quercetin; Lut: luteolin; tetOHFlv: 5,7,2´,4´-tetrahydroxyflavone; triOH-diOMeFlv-der: trihydroxy-dimethoxyflavone-derivative; Pt: pentoside; Rhmn: rhamnosid; Hex: hexoside.

Table 3. Polyphenolic compounds quantified in methanolic extracts obtained from *A. chilensis* leaves (µg g-1 DW). BS W: Basal winter leaves, AP W: Apical winter leaves, BS S: Basal spring leaves, AP S: Apical spring leaves, and IVITRO: *In vitro* leaves.

Sample	Galloyl acid derivatives	Caffeoyl quinic acids	Ellagitannins	Ellagic acid derivatives	Flavonoid derivatives	Total polyphenols
AP S	777.77 ± 15.65	89.67 ± 0.70	3306.80 ± 33.88	27.99 ± 0.33	894.69 ± 2.67	5096.92 ± 37.42
BS S	1004.52 ± 10.28	87.83 ± 1.30	3355.70 ± 27.61	36.04 ± 1.45	928.35 ± 4.96	5412.43 ± 29.94
AP W	400.96 ± 5.71	21.58 ± 0.14	1055.90 ± 7.28	8.13 ± 0.10	379.24 ± 2.18	1865.81 ± 9.51
BS W	362.33 ± 9.20	6.90 ± 0.09	915.33 ± 13.98	5.33 ± 0.05	230.77 ± 2.04	1520.66 ± 16.86
IVITRO	4587.73 ± 545.87	836.01 ± 23.13	4541.77 ± 40.05	160.32 ± 24.77	485.61 ± 11.04	9775.43 ± 137.484

3.2 Qualitative differences of phenolics from In vitro and ex vitro samples

The screening of phenolic compounds from methanolic extracts obtained from *In vitro* (samples: 1 and 2) and *ex vitro* (samples: spring apical, winter apical, spring basal, winter basal) by HPLC–DAD–ESI/MS^n showed a HPLC-UV chromatogram (280 and 350 nm) (Figs 1 and 2) where the peaks detected were derivatives of galloyl acids (**1**, **2** and **4**), caffeoyl quinic acids (**3** and **5**), ellagitannins (**6**-**8**), ellagic acids (**9**, **10**, **12**, **13** and **15**) and flavonoid-derivatives (**11**, **14**, **16**-**23**) according to their UV spectra and MS data.

In vitro and *ex vitro* samples showed similar chromatographic profiles for some compounds, but significant differences were detected. The *In vitro* samples showed five ellagic acid derivatives, while in the *ex vitro* samples, **13** and **15** were not found. Also, flavonoids **14** and **16**-**19** detected in *In vitro* samples were not found in *ex vitro* samples. Besides, differences in the content were found between *In vitro* and *ex vitro* samples, and among the samples belonging at each group.

According to the results in HPLC-DAD-ESI-MS^n, the expression of phenolics compounds was more related to the type of leaf than to the season of the year. It is well-known that phenols are a distinctive feature of *A. chilensis* and some of its nutritional and pharmacological effects can be attributed to their presence in the plant (Arias et al., 2019; Turchetti & Paz, 2019).

The first description of phenolic compounds in maqui leaves was carried out by Muñoz et al., (2011), who developed a study with fractions of maqui leaf extracts, using different solvents: HE (n-hexane), DCM (dichloromethane), ME (methanol), INFU (infusion, water), ALK-MIX (DCM + ME) to determinate anti-inflammatory, analgesic and antioxidant activities. The phenolic compounds mainly identified for these authors with HPLC-ESI-MS were present in the ME and INFU fractions. Their study includes quercetin 5,3′-dimethyl ether, quercetin 3-*O*-β-D-glucoside and kaempferol detected in the ME fraction; and caffeic acid and ferulic acid in the INFU fraction. Additionally, the ursolic acid, friedelin and quercetin 5,3′-dimethyl ether was identified in the DCM fraction.

Furthermore, Vidal et al., (2013) developed a study for the microencapsulation of maqui leaf extracts, and identified the presence of phenolics acids (54.36%), flavonoids (42.10%) and stilbenes (3.55%). The authors identified and quantified gallic acid (47.55%) followed by catechin (21.75%) and pelargonidin (14.45%) as the major compounds found in the maqui leaf extracts. The phenolic acids identified by the authors include gallic acid and coumaric acid, however, these compounds were not identified in this study.

Similarly, the identification of luteonin matched with the results described in more recent studies carried out in maqui leaves by Céspedes, et al., (2017). Also, they have identified in different fractions of leaves extracts phenolic compounds like quercetin, myricetin, rhamnetin, quercitrin, rutin, apigenin, luteonin, *p*-coumaric acid and benzoic acid. The fractions consisted in successive macerations, in the first place, with water and methanol (6:4) followed by using organic solvents, from the most polar to the least polar solvents. The results described by Céspedes, et al., (2017) were similar to those obtained from the qualitative analysis carried out in the present study.

In addition, González-Villagra et al., (2019) described quantitative differences in the content of rutin, coumaric acid, ferulic acid, quercetin in young and adult plants under drought stress. Their assays consisted in evaluating the level of total anthocyanins and ABA regulation in plant response to the application of ABA exogenous and an inhibitor of ABA (Fluridone). The HPLC-DAD analyses carried out in maqui leaves showed the main presence of rutin, independent to the drought stress or ABA and Fluridone application. Compared with this study, coumaric and ferulic acids were not found in their analysed samples, although another two compounds were identified in all their samples.

3.3 Quantitative analysis of the maqui leaves

The family of compounds identified in different extracts of the present study as well as their concentrations are listed in Table 3 and detailed of the 23 compounds quantified are shown in Table S1. It is important to highlight that total phenolic content in the *In vitro* samples was higher than those found in the *ex vitro* samples. Particularly in the family of

galloyl acid derivatives, caffeoyl quinic acid, ellagic acid derivatives and ellagitannins. Moreover, basal spring leaves exhibited a slightly higher level of galloyl acids derivatives, caffeoyl quinic acids and ellagitannins with respect to apical spring leaves, while in basal winter leaves contained the lower concentrations of these compounds.

In this study, the gallic and chlorogenic acids were used as standard to quantify the family of galloyl quinic and caffeoyl quinic acids derivatives, respectively. Rivera-Tovar et al.,(2021) analysed maqui leaves performing extractions in methanol and aqueous acetone, and quantified gallic acid (0.64 ± 0.02 mg g^{-1}DW) and chlorogenic acid (1.44 ± 0.02 mg g^{-1}DW). In the present study, the gallic acid levels were higher in the spring leaves and *In vitro* leaves, and also, the level of chlorogenic acid was higher in the *In vitro* leaves than that obtained by Rivera-Tovar et al., (Rivera-Tovar et al., 2021).

Table S1. Detailed quantified profile of the methanolic extracts obtained from *A. chilensis* leaves (µg g^{-1} DW). BS W: Basal winter leaves, AP W: Apical winter leaves, BS S: Basal spring leaves, AP S: Apical spring leaves, and IVITRO: *In vitro* leaves.

	Compounds	**AP S**	**BS S**	**AP W**	**BS W**	**IVITRO**
Peak	Galloyl acids derivatives					
1	Galloyl-hexoside	250.23 ± 10.35	400.36 ± 8.21	145.39 ± 4.40	68.16 ± 6.12	2135.17 ± 144.39
2	Galloyl quinic acid	309.80 ± 9.95	265.36 ± 5.21	119.93 ± 2.07	83.09 ± 2.36	1571.63 ± 148.94
4	Di-galloyl quinic acid	217.74 ± 6.25	338.80 ± 3.32	135.64 ± 3.00	211.09 ± 6.46	880.93 ± 99.31
	Caffeoyl quinic acids					
3	3- caffeoyl quinic acid	64.89 ± 0.57	58.32 ± 1.29	15.36 ± 0.14	5.04 ± 0.05	791.28 ± 20.87
5	4-caffeoyl quinic acid	24.78 ± 0.40	29.51 ± 0.15	6.21 ± 0.02	1.86 ± 0.08	44.73 ± 0.95
	Ellagitannins					
6	Ellagitannin	125.02 ± 5.06	148.91 ± 1.15	20.06 ± 0.23	19.19 ± 0.34	273.34 ± 24.98
7	Granatin B	1644.52 ± 33.88	1338.17 ± 18.10	563.66 ± 5.93	465.13 ± 13.07	1824.00 ± 65.61
8	Ellagitannin	1537.27 ± 14.89	1868.62 ± 20.83	472.17 ± 4.22	431.01 ± 4.96	2444.44 ± 74.18
	Ellagic acid derivatives					

9	Ellagic acid-Hexoside	14.05 ± 0.31	11.29 ± 1.42	2.82 ± 0.01	2.10 ± 0.01	18.61 ± 0.91
10	Ellagic acid-Pentoside		11.69 ± 0.28			42.96 ± 3.78
12	Ellagic acid -Pentoside	13.94 ± 0.12	13.06 ± 0.11	5.32 ± 0.10	3.23 ± 0.05	20.22 ± 3.31
14	Ellagic acid-Rhamnoside					52.15 ± 5.86
17	Ellagic acid					26.39 ± 5.54
	Flavonoid derivatives					
11	Quercetin-3-(gallic acid)Hexoside	117.72 ± 0.59	135.29 ± 0.57	34.00 ± 0.69	20.13 ± 0.30	
13	Quercetin-3-(2-Rhamnoside)Hexoside	37.11 ± 0.66	36.73 ± 0.19	19.83 ± 0.18	10.36 ± 0.28	
15	Quercetin-3-(6-Rhamnoside)Hexoside	77.90 ± 0.43	68.39 ± 0.26	39.93 ± 0.60	22.71 ± 0.38	
16	Quercetin-3-Hexoside	152.18 ± 1.11	165.64 ± 2.26	59.70 ± 0.69	26.52 ± 0.57	
18	Quercetin-3-Hexoside	44.71 ± 1.24	47.14 ± 1.09	22.02 ± 0.34	10.59 ± 0.18	
19	Quercetin-3-Rhamnoside	45.45 ± 0.78	40.83 ± 0.54	7.53 ± 0.37	7.67 ± 0.17	
20	Quercetin-3-Pentoside	42.54 ± 0.43	43.51 ± 0.16	9.46 ± 0.06	2.14 ± 0.34	
21	Tetrahydroxyflavone-(6-Rhamnoside)Hexoside	143.82 ± 1.10	130.57 ± 1.04	64.45 ± 0.40	49.85 ± 1.02	224.85 ± 12.55

22	Tetrahydroxyflavone-Hexoside	194.94 ± 1.06	225.90 ± 4.03	112.68 ± 1.72	73.48 ± 1.52	220.75 ± 23.91
23	Trihydroxy-dimethoxyflavone	38.31 ± 0.48	34.35 ± 0.51	9.62 ± 0.09	7.32 ± 0.09	40.01 ± 3.33

On the other hand, many phytochemical studies of the composition of the maqui berries have been carried out (Masoodi et al., 2019). More recently, Sandoval et al., (2019) described and quantified using UPLC-DAD the presence of delphinidin-3-*O*-sambubioside-5-*O*-glucoside (19.645 ± 0.788 mg g^{-1}DW), delphinidin-3-*O*-sambubioside (17.770 ± 1.178 mg g^{-1}DW), cyanidin-3-*O*-sambubioside-5-*O*-glucoside (2.447 ± 0.063 mg g^{-1}DW), cyanidin-3-*O*-glucoside (2.148 ± 0.158 mg g^{-1}DW) and cyanidin-3-*O*-sambubioside (2.642 ± 0.201 mg g^{-1}DW). In addition to UHPLC techniques applied to maqui berries, in a recent study carried out by Chen et al., (Chen et al., 2020) used UHPLC-Q Exactive orbitrap-HRMS to identify 18 compounds and demonstrated the photoprotective effect to the hydroethanolic maqui berry extract against to UV-B induced *In vitro* and *in vivo*. They verified the protective effect on photodamage of gallic ellagic and protocatechuic acids, and granatin B. Also, quercetin derivatives and others delphinidin derivates have been proved to provide skin photoprotection due to their potent antioxidant activities. Moreover, another study carried out recently by Rodríguez et al., (2021) described in unripe maqui fruits, the presence of granatin B, kaempferol, quercetin, delphinidin, and cyanidin glucosides and others compounds. It is necessary to highlight that granatin B which have present in fruits, has not been described before in maqui leaves, until the present study. The granatin B is an ellagitannin compound which usually contains the pomegranate fruits and its functions are associated with anti-inflammatory effects (Mena et al., 2012). Gironés-Vilaplana et al., (2014) quantified granatin B (0.53 ± 0.11 mg $100g^{-1}$DW) and ellagic acid hexoside (2.01 ± 0.15 mg $100g^{-1}$DW) in maqui fruit extracts carried out with methanol and water (70:30 v/v). The content of granatin B described by these authors was lower than in the present study carried out in maqui leaves. However, the content of ellagic acid hexoside described by Gironés-Vilaplana et al., (2014) was higher than that found in maqui leaves (Table S1). Moreover, Genskowsky et al., (2016) identified and quantified phenolic compounds like ellagic acids (0.94 ± 0.01 mg g^{-1}DW) in maqui berry that were higher than that found in the *In vitro* leaves of the present study (Table S1). In addition, these authors also quantified rutin (0.20 ± 0.01 mg g^{-1}DW), obtaining similar levels that that found in our basal winter leaves (Table 3). Finally, the

presence in maqui leaves of the tetrahidroxyflavone derivatives have not been previously identified in any study carried out on this plant species.

According to HPLC-MS identification, *A. chilensis* leaves showed the presence of quercetin, catechin, kaempferol and 3-caffeoyl quinic acid. As regards to sample quantification, the compounds 3-caffeoylquinic acid and catechin showed the highest concentrations followed by kaempferol. The quercetin was only detected in *ex vitro* maqui leaves (Table 4). These results agreed with several authors who reported the presence of quercetin, catechin and kaempferol in maqui leaves (Céspedes, Balbontín, et al., 2017; González-Villagra et al., 2019; Muñoz et al., 2011; Vidal et al., 2013). The values of quercetin (7040 μM) and catechin (6669.24 μM) obtained in the *ex vitro* samples were higher than those obtained by Vidal et al.,(2013) (quercetin (10.92 μM) and catechin (182.59 μM)). The presence of caffeolyl quinic acid was very similar to those reported by Gironés-Vilaplana et al., (2014, 2012), who mentioned that caffeoyl quinic acid derivatives were present in fruits. In addition, Rivera-Tovar et al., (2021) realized that the successive extractions of maqui leaves in methanol and aqueous acetone contained higher values than those found in this study as compared data from HPLC-MS analyses. The authors used UPLC-MS for the identification and quantification of compounds like quercetin (1.35 ± 0.03 mg g-1 DW), catechin (2.25 ± 0.03 mg g-1 DW), kaempferol (0.90 ± 0.01 mg g-1 DW), and 3-caffeoyl quinic acid (1.44 ± 0.02 mg g-1 DW).

Table 4. HPLC-MS data of identified and quantified compounds from leaves of *A. chilensis* (μg g^{-1} DW).

Leaf Type	**Quercetin**	**Catechin**	**Kaempferol**	**3-caffeloyl quinic acid**
In vitro		35.44	6.69	25.93
BS Spring	145.73	132.59	25.93	253.77

GC-MS is one of the most reliable biophysical methods for its specificity and repeatability, was utilized for the phytochemical profiling of *A. chilensis* leaves. We

observed the production of β-sitosterol, α-tocopherol (Figure 6), and linoleic and linolenic acid (Figure 7). The compounds were quantified using external commercial patterns. The mass spectra and the structure of β-sitosterol, α-tocopherol, and linoleic and linolenic acid, were found in the supplementary material, respectively (Figure S1, S2). The highest values of β-sitosterol, α-tocopherol are obtained in *In vitro* leaves, followed by spring BS leaves, for both compounds. Furthermore, the lowest values obtained for β-sitosterol and α-tocopherol were in both types of winter leaves (Figure 6). In contrast, the higher values for linoleic acid and linolenic acid were obtained in *ex vitro* leaves than in *In vitro* leaves of *A. chilensis* (Figure 7). It is important to highlight that α-Tocopherol, linoleic acid and linolenic acid were not described and quantified in maqui leaves before. The level of α-tocopherol was described by Quispe-Fuentes et al., (2020) in maqui fruit (31.7 ± 0.5 µg g-1 DW) compared with the maqui leaves this value was low. Also, the authors described the presence of palmitic acid (9.79 ± 0.16 g $100g^{-1}$ of the sample), linoleic acid (44.63 ± 0.25 g $100g^{-1}$ of the sample), and linolenic acid (2.24 ± 0.23 g $100g^{-1}$ of the sample) in maqui fruit. The first quantification of phytosterol was described by Muñoz & Ramos, (2016). They identified a main phytosterol it was β-sitosterol, followed by campesterol, sitostanol and campestanol. They described the value of β-sitosterol was 4.3 ± 1.0 µg/g DW. The α-tocopherol and β-sitosterol metabolites are produced by plants at low concentrations and their production increased in samples of *In vitro* leaves (Figure 6). In this study, the highest values of linoleic acid and linolenic acid were found on BS maqui leaves (Figure 7).

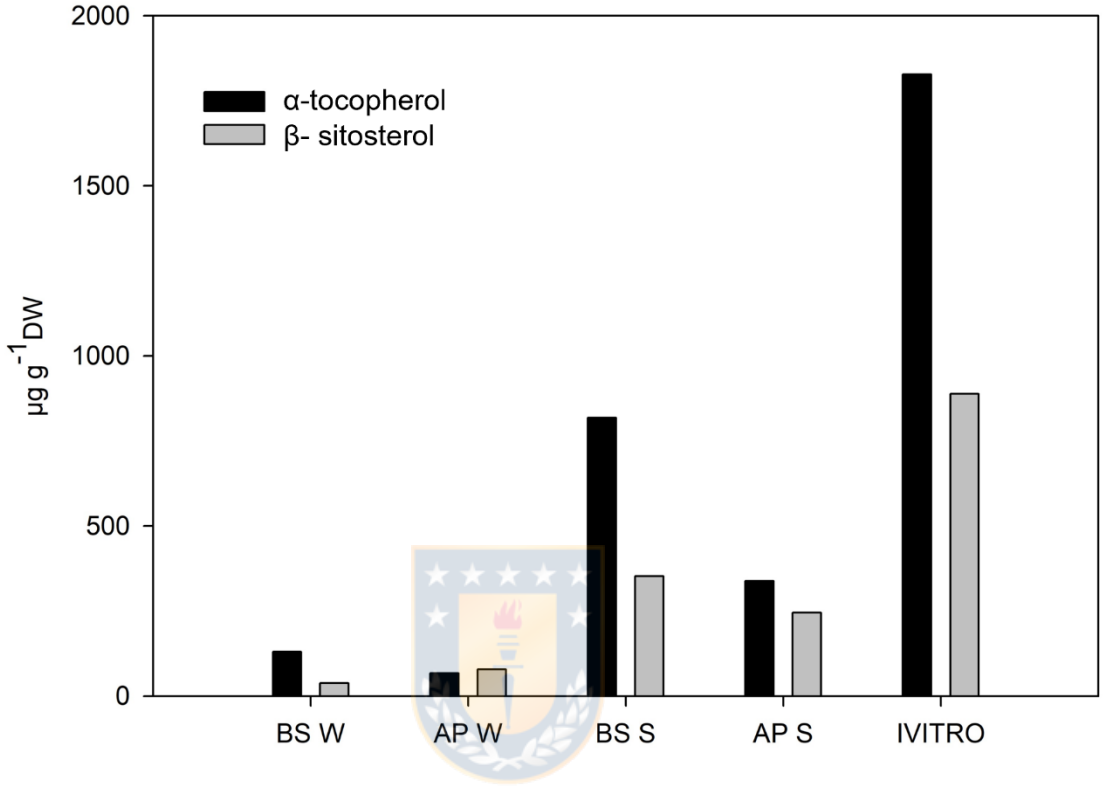

Figure 6. Yield (µg g^{-1} DW) of α-tocopherol and β-sitosterol from *A. chilensis* leaves. BS W: Basal winter leaves, AP W: Apical winter leaves, BS S: Basal spring leaves, AP S: Apical spring leaves, and IVITRO: *In vitro* leaves.

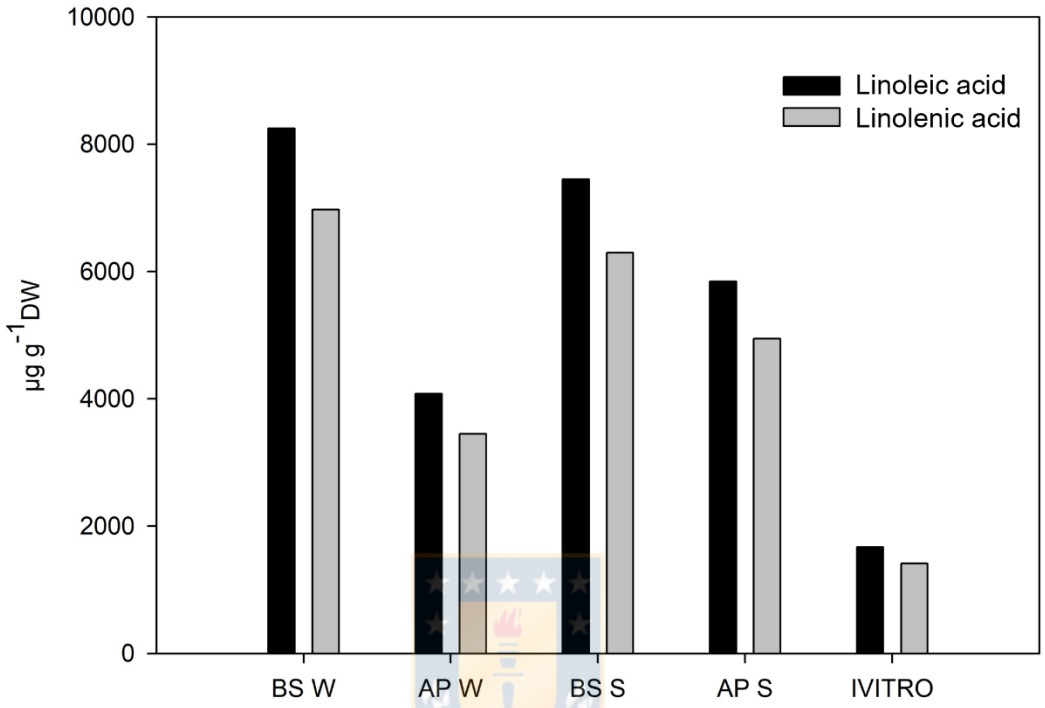

Figure 7. Yield (µg g^{-1} DW) of Linoleic acid and Linolenic acid from *A. chilensis* leaves. BS W: Basal winter leaves, AP W: Apical winter leaves, BS S: Basal spring leaves, AP S: Apical spring leaves, and IVITRO: *In vitro* leaves

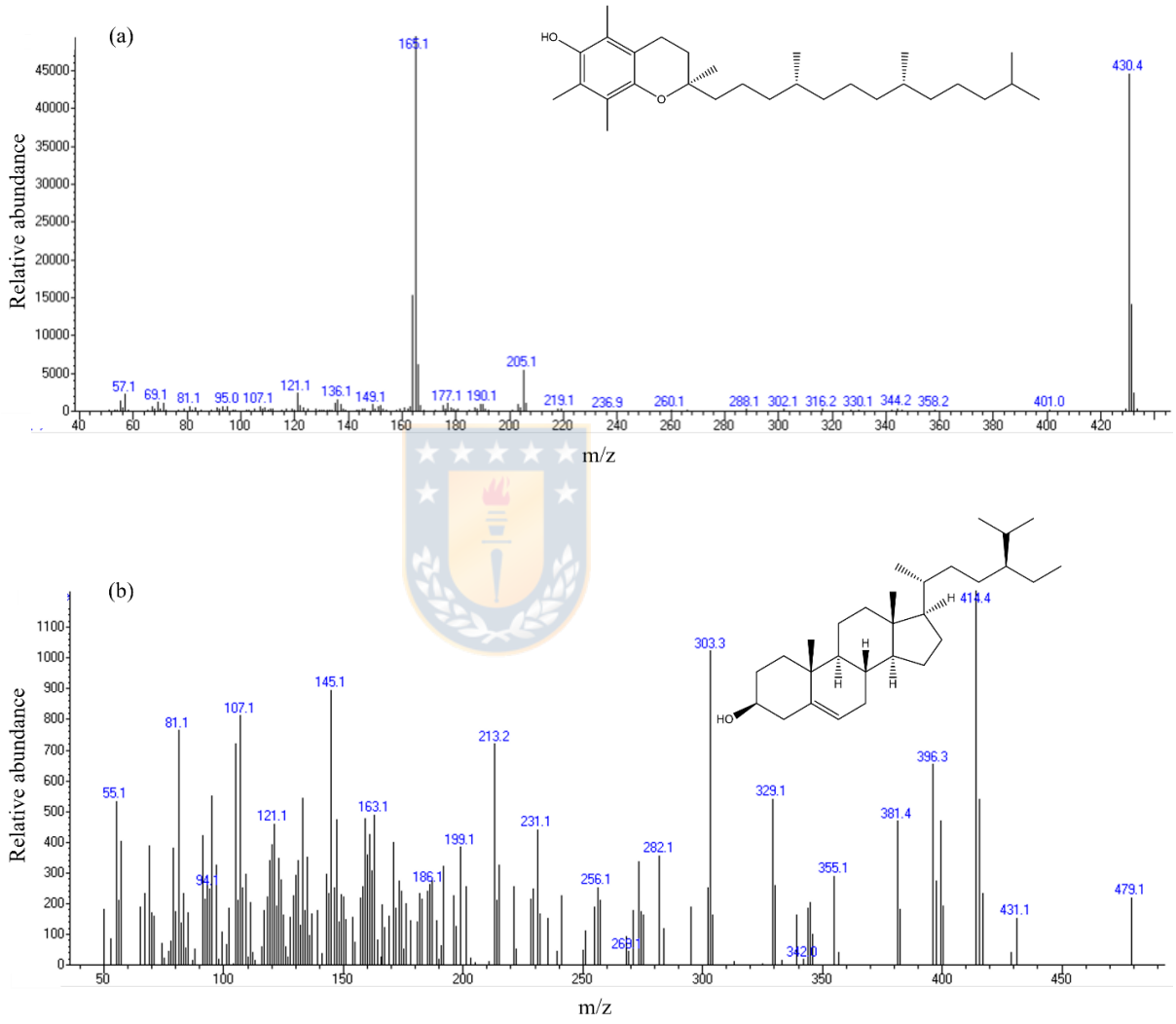

Figure S1. Mass spectra and chemical structure of α-tocopherol (a) and β-sitosterol (b).

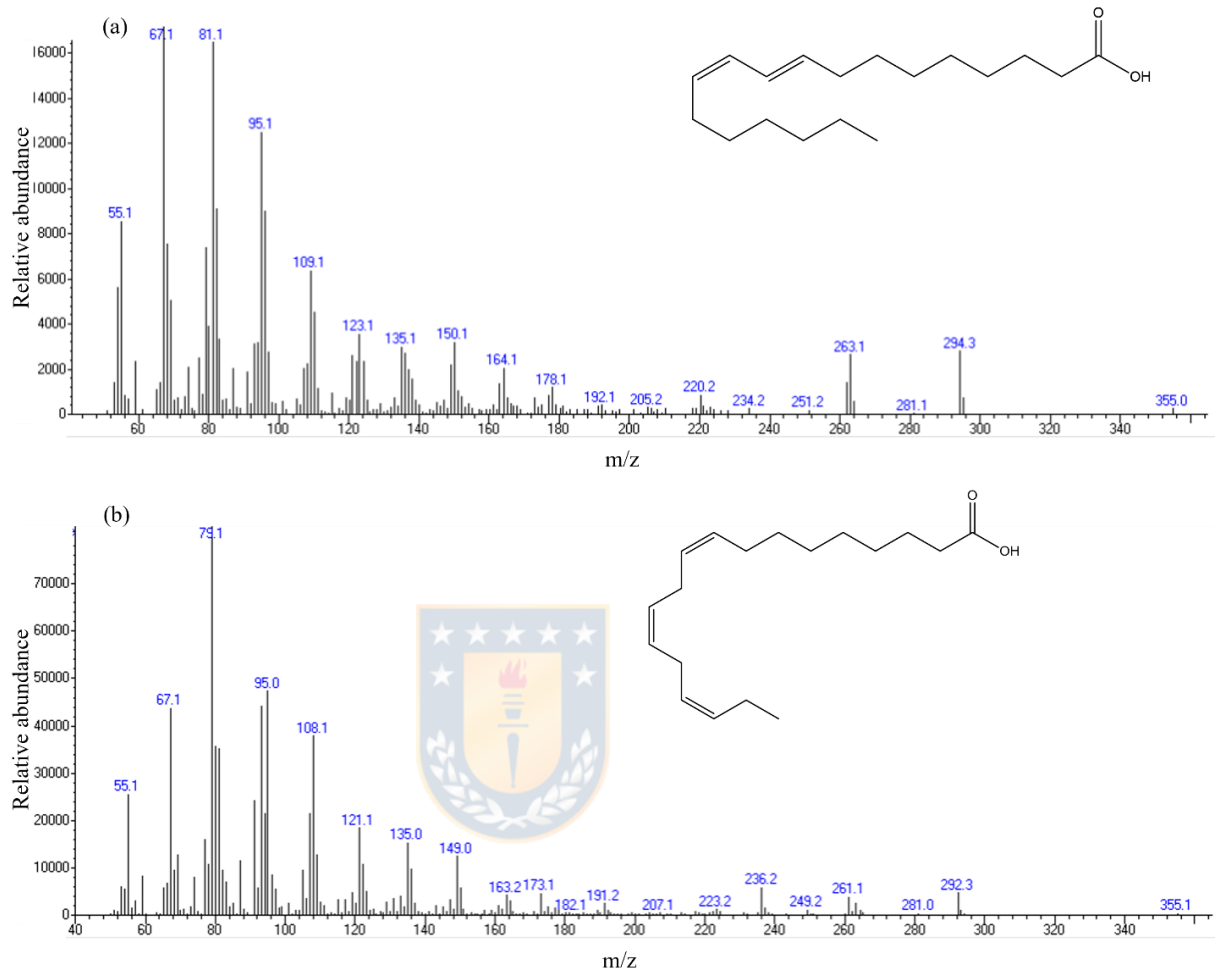

Figure S2. Mass spectra and chemical structure of Linoleic acid (a) and Linolenic acid (b).

The TFC of the different extracts of *A. chilensis* was shown in Table 5. BS winter extract had the highest TFC followed by those of apical leaves in spring and by of BS in spring, no significant differences were observed between them. The lowest value of TFC was detected in extracts of *In vitro* leaves.

Table 5. Total Flavonoids content (mg mL^{-1}) in *ex vitro* and *In vitro* leaves of *A. chilensis*. Median ± E.E. (one-way ANOVA, Tukey, $p \leq 0.05$, n=4).

Types of Leaves		TFC (mg mL^{-1})
1	BS Winter	0.086 ± 0.004a
2	AP Winter	0.074 ± 0.001c
3	BS Spring	0.081 ± 0.005bc
4	AP Spring	0.083 ± 0.002b
5	*In vitro*	0.061 ± 0.002c

Plants of maqui possess high amounts flavonoids and potent antioxidant activity leading to various defensive and disease-fighting properties (Masoodi et al., 2019). Phenolic compounds are plants secondary metabolites considered fundamental plant constituents due to the presence of one or more hydroxyl groups on their aromatic ring. The results of TFC are similarly to described by Vidal et al., (2013) to those obtained in *In vitro* leaves (0.061 ± 0.01 mg mL^{-1}). The assay was realized with autumn leaves and not described difference state of development of leaf. Probably the difference of the extract method is the major influence on the content of flavonoids. The amount of flavonoid content of the methanolic extract was approximately more than higher than hydroethanolic extracts proposed by the author.

In summary, the analyses for HPLC/MS indicated the extract from BS spring leaves included quercetin, catechin, kaempferol, and 3-caffeoyl quinic acids compounds while *In vitro* leaves extract not present quercetin. Determination of lipophilic compounds was performance by GC/MS. The samples of *In vitro* leaves showed a high presence of α-

tocopherol and β-sitosterol. In contrast, the samples of adult leaves presented a hight level of linolenic and linoleic acids. The HPLC-DAD-ESI (Ion Trap)-MS^n analysis showed the presence of different phenolic compounds in both apical and basal adult (20 compounds), and *In vitro* maqui leaves (16 compounds). The compounds identified were classified into the groups of galloyl and caffeoyl quinic acids, ellagitannins, ellagic acid derivatives and flavonoid derivatives.

Finally, the results indicated the *In vitro A. chilensis* leaves could be considered how an antioxidants and lipophilic compounds resources. This study is initial for the future investigation of the *In vitro* culture of *A. chilensis* leaves with the approach to provide secondary metabolites.

Supplementary Materials: Table S1: Detailed quantified profile of the methanolic extracts obtained from *A. chilensis* leaves (µg g-1 DW). BS W: Basal winter leaves, AP W: Apical winter leaves, BS S: Basal spring leaves, AP S: Apical spring leaves, and IVITRO: *In vitro* leaves. Figure S1. Mass spectra and chemical structure of α-tocopherol (a) and β-sitosterol (b). Figure S2. Mass spectra and chemical structure of Linoleic acid (a) and Linolenic acid (b).

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References

Abu-Reidah, I. M., Gil-Izquierdo, A., Medina, S., & Ferreres, F. (2017). Phenolic composition profiling of different edible parts and by-products of date palm (Phoenix dactylifera L.) by using HPLC-DAD-ESI/MS(n). *Food Res Int*, *100*(Pt 3), 494-500. https://doi.org/10.1016/j.foodres.2016.10.018

Ahmed, D., Khan, M. M., & Saeed, R. (2015). Comparative Analysis of Phenolics, Flavonoids, and Antioxidant and Antibacterial Potential of Methanolic, Hexanic and Aqueous Extracts from Adiantum caudatum Leaves. *Antioxidants (Basel)*, *4*(2), 394-409. https://doi.org/10.3390/antiox4020394

Andrade, C., Ferreres, F., Gomes, N. G. M., Duangsrisai, S., Srisombat, N., Vajrodaya, S., Pereira, D. M., Gil-Izquierdo, A., Andrade, P. B., & Valentao, P. (2019). Phenolic Profiling and Biological Potential of Ficus curtipes Corner Leaves and Stem Bark: 5-Lipoxygenase Inhibition and Interference with NO Levels in LPS-Stimulated RAW 264.7 Macrophages. *Biomolecules*, *9*(9). https://doi.org/10.3390/biom9090400

Arias, H. R., Ortells, M. O., Feuerbach, D., Burgos, V., & Paz, C. (2019). Alkaloids Purified from *Aristotelia chilensis* Inhibit the Human alpha3beta4 Nicotinic Acetylcholine Receptor with Higher Potencies Compared with the Human alpha4beta2 and alpha7 Subtypes. *J Nat Prod*, *82*(7), 1953-1960. https://doi.org/10.1021/acs.jnatprod.9b00314

Céspedes, C. L., Balbontín, C., Ávila, J. G., Domínguez, M., Alarcón, J., Paz, C., Burgos, V., Ortiz, L., Penaloza-Castro, I., Seigler, D. S., & Kubo, I. (2017). Inhibition on cholinesterase and tyrosinase by alkaloids and phenolics from *Aristotelia chilensis* leaves. *Food Chem Toxicol*, *109*(Pt 2), 984-995. https://doi.org/10.1016/j.fct.2017.05.009

Céspedes, C. L., El-Hafidi, M., Pavon, N., & Alarcon, J. (2008). Antioxidant and cardioprotective activities of phenolic extracts from fruits of Chilean blackberry *Aristotelia chilensis* (Elaeocarpaceae), Maqui. *Food Chemistry*, *107*(2), 820-829. https://doi.org/10.1016/j.foodchem.2007.08.092

Céspedes, C. L., Pavón, N., Domínguez, M., Alarcón, J., Balbontín, C., Kubo, I., El-Hafidi, M., & Ávila, J. G. (2017). The chilean superfruit black-berry *Aristotelia chilensis* (Elaeocarpaceae), Maqui as mediator in inflammation-associated disorders. *Food Chem Toxicol*, *108*(Pt B), 438-450. https://doi.org/10.1016/j.fct.2016.12.036

Chen, L., Zhou, G., Meng, X. S., Fu, H. Y., Mo, Q. G., & Wang, Y. W. (2020). Photoprotection of maqui berry against ultraviolet B-induced photodamage *In vitro* and in vivo. *Food Funct*, *11*(3), 2749-2762. https://doi.org/10.1039/c9fo01902b

Clifford MN, J. K., Knight S, Kuhnert N. (2003). Hierarchical scheme for LC-MSn identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry*, *7;51(10)*, 2900-2911. https://doi.org/10.1021/jf026187q

Concha-Meyer, A. A., Sepúlveda, G., Pérez-Díaz, R., & Torres, C. A. (2021). Effect of preservation processing on quality attributes and phenolic profile of maqui (*Aristotelia chilensis* mol. Stuntz) fruit. *Lwt*, *149*. https://doi.org/10.1016/j.lwt.2021.111920

Crisóstomo-Ayala, K., Hernández de la Torre, M., Pedreño, M.A., Hernández, J.A., Pérez, C., Bustos, E., Sánchez-Olate, M., & Ríos, D. (2021). Seasonal changes in photosynthesis, phenolic content, antioxidant activity and anatomy of apical and basal leaves of *Aristotelia chilensis* (Mol.) Stuntz. *Biologia Plantarum.* art.no. 6728: Manuscript accepted

Felgines C, T. S., Texier O, Gil-Izquierdo A, Lamaison JL, Remesy C. (2005). Blackberry Anthocyanins Are Mainly Recovered from Urine as Methylated and Glucuronidated Conjugates in Humans. *Journal of Agricultural and Food Chemistry*, *Oct 5;53(20)*, 7721-7727. https://doi.org/10.1021/jf051092k.

Ferreres, F., Gil-Izquierdo, A., Vinholes, J., Silva, S. T., Valentao, P., & Andrade, P. B. (2012). Bauhinia forficata Link authenticity using flavonoids profile: relation with their biological properties. *Food Chem*, *134*(2), 894-904. https://doi.org/10.1016/j.foodchem.2012.02.201

Ferreres, F., Gomes, N. G. M., Valentao, P., Pereira, D. M., Gil-Izquierdo, A., Araujo, L., Silva, T. C., & Andrade, P. B. (2018). Leaves and stem bark from Allophylus africanus P. Beauv.: An approach to anti-inflammatory properties and characterization of their flavonoid profile. *Food Chem Toxicol*, *118*, 430-438. https://doi.org/10.1016/j.fct.2018.05.045

Ferreres, F., Grosso, C., Gil-Izquierdo, A., Fernandes, A., Valentão, P., & Andrade, P. B. (2015). Comparing the phenolic profile of Pilocarpus pennatifolius Lem. by HPLC–DAD–ESI/MS n with respect to authentication and enzyme inhibition potential. *Industrial Crops and Products*, *77*, 391-401. https://doi.org/10.1016/j.indcrop.2015.09.006

Ferreres, F., Grosso, C., Gil-Izquierdo, A., Valentao, P., & Andrade, P. B. (2013). Ellagic acid and derivatives from Cochlospermum angolensis Welw. Extracts: HPLC-DAD-ESI/MS(n) profiling, quantification and *In vitro* anti-depressant, anti-cholinesterase and anti-oxidant activities. *Phytochem Anal*, *24*(6), 534-540. https://doi.org/10.1002/pca.2429

Ferreres, F., Oliveira, A. P., Gil-Izquierdo, A., Valentao, P., & Andrade, P. B. (2014). Piper betle leaves: profiling phenolic compounds by HPLC/DAD-ESI/MS(n) and anti-cholinesterase activity. *Phytochem Anal*, *25*(5), 453-460. https://doi.org/10.1002/pca.2515

Fredes, C., Yousef, G. G., Robert, P., Grace, M. H., Lila, M. A., Gomez, M., Gebauer, M., & Montenegro, G. (2014). Anthocyanin profiling of wild maqui berries (*Aristotelia chilensis* [Mol.] Stuntz) from different geographical regions in Chile. *J Sci Food Agric*, *94*(13), 2639-2648. https://doi.org/10.1002/jsfa.6602

Genskowsky, E., Puente, L. A., Perez-Alvarez, J. A., Fernandez-Lopez, J., Munoz, L. A., & Viuda-Martos, M. (2016). Determination of polyphenolic profile, antioxidant activity and antibacterial properties of maqui [*Aristotelia chilensis* (Molina) Stuntz] a Chilean blackberry. *J Sci Food Agric*, *96*(12), 4235-4242. https://doi.org/10.1002/jsfa.7628

Gil-Izquierdo, A. a., & Mellenthin, A. (2001). Identification and quantitation of flavonols in rowanberry (Sorbus aucuparia L.) juice. *European Food Research and Technology*, *213*(1), 12-17. https://doi.org/10.1007/s002170100328

Gironés-Vilaplana, A., Baenas, N., Villaño, D., Speisky, H., García-Viguera, C., & Moreno, D. A. (2014). Evaluation of Latin-American fruits rich in phytochemicals with biological effects. *Journal of Functional Foods*, *7*, 599-608. https://doi.org/10.1016/j.jff.2013.12.025

Girones-Vilaplana, A., Huertas, J. P., Moreno, D. A., Periago, P. M., & Garcia-Viguera, C. (2016). Quality and microbial safety evaluation of new isotonic beverages upon thermal treatments. *Food Chem*, *194*, 455-462. https://doi.org/10.1016/j.foodchem.2015.08.011

Girones-Vilaplana, A., Valentao, P., Moreno, D. A., Ferreres, F., Garcia-Viguera, C., & Andrade, P. B. (2012). New beverages of lemon juice enriched with the exotic berries maqui, acai, and blackthorn: bioactive components and *In vitro* biological properties. *J Agric Food Chem*, *60*(26), 6571-6580. https://doi.org/10.1021/jf300873k

González-Villagra, J., Cohen, J. D., & Reyes-Diaz, M. M. (2019). Abscisic acid is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress. *Physiol Plant*, *165*(4), 855-866. https://doi.org/10.1111/ppl.12789

Masoodi, H., Villano, D., & Zafrilla, P. (2019). A comprehensive review on fruit *Aristotelia chilensis* (Maqui) for modern health: towards a better understanding. *Food Funct*, *10*(6), 3057-3067. https://doi.org/10.1039/c8fo02429d

Medina, S., Collado-Gonzalez, J., Ferreres, F., Londono-Londono, J., Jimenez-Cartagena, C., Guy, A., Durand, T., Galano, J. M., & Gil-Izquierdo, A. (2017). Quantification of phytoprostanes - bioactive oxylipins - and phenolic compounds of Passiflora edulis Sims shell using UHPLC-QqQ-MS/MS and LC-IT-DAD-MS/MS. *Food Chem*, *229*, 1-8. https://doi.org/10.1016/j.foodchem.2017.02.049

Mena, J., Elgueta, E., Espinola-Gonzales, F., Cardenas, H., & Orihuela, P. A. (2021). Hydroethanolic Extracts of the *Aristotelia chilensis* (Maqui) Berry Reduces Cellular Viability and Invasiveness in the Endometrial Cancer Cell Line Ishikawa. *Integr Cancer Ther*, *20*, 15347354211007560. https://doi.org/10.1177/15347354211007560

Mena, P., Calani, L., Dall'Asta, C., Galaverna, G., Garcia-Viguera, C., Bruni, R., Crozier, A., & Del Rio, D. (2012). Rapid and comprehensive evaluation of (poly)phenolic compounds in pomegranate (Punica granatum L.) juice by UHPLC-MSn. *Molecules*, *17*(12), 14821-14840. https://doi.org/10.3390/molecules171214821

Mena, P., Vegara, S., Marti, N., Garcia-Viguera, C., Saura, D., & Valero, M. (2013). Changes on indigenous microbiota, colour, bioactive compounds and antioxidant activity of pasteurised pomegranate juice. *Food Chem*, *141*(3), 2122-2129. https://doi.org/10.1016/j.foodchem.2013.04.118

Muñoz, O., Christen, P., Cretton, S., Backhouse, N., Torres, V., Correa, O., Costa, E., Miranda, H., & Delporte, C. (2011). Chemical study and anti-inflammatory, analgesic and antioxidant activities of the leaves of *Aristotelia chilensis* (Mol.) Stuntz, Elaeocarpaceae. *J Pharm Pharmacol*, *63*(6), 849-859. https://doi.org/10.1111/j.2042-7158.2011.01280.x

Muñoz, O., & Ramos, F. (2016). Quantitative analysis of phytosterols in *Aristotelia chilensis* (Maqui) leaves. *International Food Research Journal*, *23(2)*, 822-826.

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tohaoco Tissue Cultures. *Physiologia Plantarum*, *15*.

Narayani, M., & Srivastava, S. (2017). Elicitation: a stimulation of stress in *In vitro* plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochemistry Reviews*, *16*(6), 1227-1252. https://doi.org/10.1007/s11101-017-9534-0

Ojeda, J. J., Evelyn; Molina, Luis; Parada, Fabiana; Burgos, Rafael A.; Hidalgo,, & Maria A.; Hancke, J. L. (2011). <11-Effects of *Aristotelia chilensis* berry juice on cyclooxygenase 2 expression,.pdf>. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, vol. 10, núm. 6, noviembre, 2011, pp. 543-552 Universidad de Santiago de Chile Santiago, Chile*.

Ortiz, T., Arguelles-Arias, F., Begines, B., Garcia-Montes, J. M., Pereira, A., Victoriano, M., Vazquez-Roman, V., Perez Bernal, J. L., Callejon, R. M., De-Miguel, M., & Alcudia, A. (2021). Native Chilean Berries Preservation and *In vitro* Studies of a Polyphenol Highly Antioxidant Extract from Maqui as a Potential Agent against Inflammatory Diseases. *Antioxidants (Basel)*, *10*(6). https://doi.org/10.3390/antiox10060843

Petreska, J., Stefova, M., Ferreres, F., Moreno, D. A., Tomás-Barberán, F. A., Stefkov, G., Kulevanova, S., & Gil-Izquierdo, A. (2011). Potential bioactive phenolics of Macedonian Sideritis species used for medicinal "Mountain Tea". *Food Chemistry*, *125*(1), 13-20. https://doi.org/10.1016/j.foodchem.2010.08.019

Quispe-Fuentes, I., Vega-Galvez, A., Aranda, M., Poblete, J., Pasten, A., Bilbao-Sainz, C., Wood, D., McHugh, T., & Delporte, C. (2020). Effects of drying processes on composition, microstructure and health aspects from maqui berries. *J Food Sci Technol*, *57*(6), 2241-2250. https://doi.org/10.1007/s13197-020-04260-5

Rivera-Tovar, P. R., Torres, M. D., Camilo, C., Mariotti-Celis, M. S., Dominguez, H., & Perez-Correa, J. R. (2021). Multi-response optimal hot pressurized liquid recovery of extractable polyphenols from leaves of maqui (*Aristotelia chilensis* [Mol.] Stuntz). *Food Chem*, *357*, 129729. https://doi.org/10.1016/j.foodchem.2021.129729

Rodriguez, L., Trostchansky, A., Wood, I., Mastrogiovanni, M., Vogel, H., Gonzalez, B., Marostica Junior, M., Fuentes, E., & Palomo, I. (2021). Antiplatelet activity and chemical analysis of leaf and fruit extracts from *Aristotelia chilensis*. *PLoS One*, *16*(4), e0250852. https://doi.org/10.1371/journal.pone.0250852

Rojo, L. E., Ribnicky, D., Logendra, S., Poulev, A., Rojas-Silva, P., Kuhn, P., Dorn, R., Grace, M. H., Lila, M. A., & Raskin, I. (2012). *In vitro* and in Vivo Anti-Diabetic Effects of Anthocyanins from Maqui

Berry (*Aristotelia chilensis*). *Food Chem*, *131*(2), 387-396. https://doi.org/10.1016/j.foodchem.2011.08.066

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., & Shene, C. (2011). Extracts of Maqui (*Aristotelia chilensis*) and Murta (Ugni molinae Turcz.): sources of antioxidant compounds and alpha-Glucosidase/alpha-Amylase inhibitors. *J Agric Food Chem*, *59*(5), 1630-1637. https://doi.org/10.1021/jf103461k

Sabater-Jara, A. B., Almagro, L., Belchi-Navarro, S., Ferrer, M. A., Barcelo, A. R., & Pedreno, M. A. (2010). Induction of sesquiterpenes, phytoesterols and extracellular pathogenesis-related proteins in elicited cell cultures of Capsicum annuum. *J Plant Physiol*, *167*(15), 1273-1281. https://doi.org/10.1016/j.jplph.2010.04.015

Sánchez-Pujante, P. J., Gionfriddo, M., Sabater-Jara, A. B., Almagro, L., Pedreño, M. A., & Díaz-Vivancos, P. (2020). Enhanced bioactive compound production in broccoli cells due to coronatine and methyl jasmonate is linked to antioxidative metabolism. *J Plant Physiol*, *248*, 153136. https://doi.org/10.1016/j.jplph.2020.153136

Sandoval, V., Femenias, A., Martinez-Garza, U., Sanz-Lamora, H., Castagnini, J. M., Quifer-Rada, P., Lamuela-Raventos, R. M., Marrero, P. F., Haro, D., & Relat, J. (2019). Lyophilized Maqui (*Aristotelia chilensis*) Berry Induces Browning in the Subcutaneous White Adipose Tissue and Ameliorates the Insulin Resistance in High Fat Diet-Induced Obese Mice. *Antioxidants (Basel)*, *8*(9). https://doi.org/10.3390/antiox8090360

Talavéra, S. F., Catherine; Texier, Odile; Besson, Catherine; Gil-Izquierdo, Angel; Lamaison, Jean-Louis and Rémésy, Christian. (2005). Anthocyanin Metabolism in Rats and Their Distribution to Digestive Area, Kidney, and Brain. *Journal of Agricultural and Food Chemistry 53 (10)*, 3902-3908. https://doi.org/10.1021/jf050145v

Turchetti, G., & Paz, C. (2019). *Aristotelia chilensis* (Mol.) Stuntz: A Natural Source of Bioactive Compounds. *Current Traditional Medicine*, *5*(1), 66-74. https://doi.org/10.2174/2215083804666181002095249

Vidal J, L., Avello L, Marcia, Loyola C, Cristina, Campos P, Jorge, Aqueveque M, Pedro, R. Dungan, Stephanie, Galotto L, Maria, & Guarda M, Abel. (2013). Microencapsulation of maqui (*Aristotelia chilensis* [Molina] Stuntz) leaf extracts to preserve and control antioxidant properties. *Chilean journal of agricultural research*, *73(1)*, 17-23.

Zhu, S., Aspera-Werz, R. H., Chen, T., Weng, W., Braun, B., Histing, T., & Nussler, A. K. (2021). Maqui berry extract prevents cigarette smoke induced oxidative stress in human osteoblasts *In vitro*. *EXCLI J*, *20*, 281-296. https://doi.org/10.17179/excli2020-3244

Zúñiga, G. E., Tapia, A., Arenas, A., Contreras, R. A., & Zúñiga-Libano, G. (2017). Phytochemistry and biological properties of *Aristotelia chilensis* a Chilean blackberry: a review. *Phytochemistry Reviews*, *16*(5), 1081-1094. https://doi.org/10.1007/s11101-017-9533-1

CAPÍTULO III

Histochemical and anatomy characterization of *ex vitro* and *In vitro* *Aristotelia chilensis* leaves

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Abstract

Studies on native antioxidant plants reinforce initiatives to preserve the environments where these species grow naturally. *Aristotelia chilensis* is dioic evergreen tree native of Chile that grows preferably in humid and drained soils of the central valley, slopes of mountain ranges, streams and forests´ margins. This study aimed to analyse and describe the anatomy of adult and *In vitro A. chilensis* leaves and evaluate primary and secondary metabolites through histochemical techniques. Samples of leaves were fixed, dehydrated,

embedded in the support material, and sectioned for mounting histological slides for the anatomical description of leaf tissues. Some leaf samples were processed as fresh material. In the cross-section of adult leaves, uniseriate epidermis cells are covered by a thin and slight cuticle on both surfaces. Whereas on the *In vitro* leaves, not presented cuticle. The mesophyll of adult leaves is formed by two or three layers of palisade parenchyma and up to five strata of spongy parenchyma. In the transverse section of *In vitro* leaves, presented round-shaped cells form the palisade tissue and spongy parenchyma. Histochemical analyses presented total phenols, alkaloids and flavonoids that coincide with the phytochemistry of the species in both *ex vitro* and *In vitro* leaves. This study contributed to expand the information about Chilean species with antioxidant properties through histochemical analysis. The use of plant material to obtain antioxidants is a viable alternative in both *In vitro* and *ex vitro* material.

1. Introduction

Elaeocarpaceae family comprises about 12 genera and 550 species, widely distributed in tropical and warm-temperature southern regions. Elaeocarpaceae species? has a very varied vegetative morphology, suffrutescent subshrubs, sometimes ericoid, and few shrubs; most species are small to large trees (Coode, 2004). The genera of *Aristotelia* is represented by five species distributed in the South Pacific´s temperate zones, found in Chile, Argentina, New Zealand, Australia and Tasmania Island (Turchetti & Paz, 2019).

Aristotelia chilensis a dioic evergreen tree native of Chile that grows preferably in humid and drained soils of the central valley, slopes of mountain ranges, streams, and forests´ margins. *A. chilensis* is commonly known as “maqui” and grows up to 4-5 meters higher (Salinas & Caballé, 2020). These plants have a soft and smooth bark and plentiful thin and flexible ramifications. Its leaves are simple, oval-lanceolate forms with dentate edges and sizes from 4 to 9 cm. The petiole and stems are characterized by reddish color. In the

summer season, its female flowers give rise to an edible purple berry. When ripe, the fruit reaches a diameter of 0.5 cm and is harvested from December to February once a year. Its female flowers give an edible berry in the summer season (Turchetti & Paz, 2019).

In general, the phytochemistry of *A. chilensis* is conducted in fruits because it is most used by the nutritional health and employed for various medicinal purposes. The fruit of *A. chilensis* stands out for its importance in the pharmaceutical and food industries. Maqui berries have a high concentration of polyphenols type anthocyanin in glycosylated form, which give the fruit an antioxidant effect and induces potent neuroprotection (Fuentealba et al., 2012). Nevertheless, leaves´ phytochemistry has increased due to phenols and alkaloids, which confer antioxidant, anti-inflammatory, and analgesic properties (Turchetti & Paz, 2019; Zúñiga et al., 2017). Besides, Céspedes et al., (Céspedes et al., 2017) described the use of leaves extracts of *A. chilensis* rich in alkaloids and phenols like neuroprotective potential. In summary, the phytochemistry study of leaves is crucial to know the potential properties of these permanent vegetative sources.

In general, plants that grow in *ex vitro* environments have more development of photosynthetic apparatus, giving rise to a more remarkable synthesis of carbohydrates. This increased synthesis of carbon elements enhances secondary metabolites, which provide the plants with unique characteristics. Furthermore, under biotic or abiotic stress conditions, plants respond with an increase in the production of secondary metabolites, some of which are beneficial for human health (Scossa & Fernie, 2020). Another productive of vegetative material using biotechnological techniques are the *In vitro* culture. The *In vitro* plants grow in a sterile and nutritional environment where they obtain vegetative material less than traditional culture. However, *In vitro* culture has a difference in the level of organization to photosynthetic layer (Barupal et al., 2018), less presence of cuticle waxes, and no operative stomata due to the environment of culture (Sáez et al., 2012). Despite these differences, modern biotechnological techniques have been used *In vitro* culture as a tool for obtaining secondary metabolites with

pharmacological and industrial uses (Nandagopal et al., 2018). Therefore, it is necessary to differentiate two types of *A. chilensis* leaves for the study: *In vitro* and *ex vitro* leaves.

Although phytochemical studies it more sensitive for the detection of chemical compounds. The use of histochemical tests through the reagent's colorants could determine the site where metabolite is produced (de Souza et al., 2018).

Therefore, this study focuses on the description and evaluation of the structure of *A. chilensis* in two different types of leaves. Thus, this study aimed to analyse and describe the anatomy of adult and *In vitro A. chilensis* leaves and evaluate primary and secondary metabolites through histochemical techniques.

2. Materials and methods

The study was carried out at the Laboratorio de Cultivo de Tejidos Vegetales, Centro de Biotecnología Universidad de Concepción, Chile and Anatomy Laboratory, Departamento de Botânica, Instituto de Ciências Biológicas, Universidad Federal de Minas Gerais, Brasil.

The *ex vitro* samples from mother plants were collected in the spring season in October 2019, at the Universidad de Concepción, Región del Biobío, Chile (36° 50'02.6 "S, 73° 01'54.3" W). Adult male *A. chilensis* plants were used, which grow in a natural environment and reach a uniform height of 3 m. The study is carried out with fully expanded leaves (Crisóstomo-Ayala et al., 2021).

2.2 *In vitro* plant cultures

In vitro plant cultures of *A. chilensis* were initiated using vegetative nodal segments as initial explants. This plant material was obtained from mother plants collected in

November 2018 (same mother plants from *ex vitro* material). The vegetative segments were disinfected with a hypochlorite solution (0.5%) containing detergent (10 min) and then, with ethanol 70% (1 min), followed by four washes with sterile distilled water. The vegetative segments were placed in glass tubes with 20 mL of culture medium. The composition of the culture medium was Murashige and Skoog, (1962) basal mineral medium with vitamins at the halfway point of its concentration, sucrose (20 g L^{-1}), polyvinylpolypyrrolidone (0.5 g L^{-1}), kinetin (0.5 mg L^{-1}), naphthalene acetic acid (0.05 mg L^{-1}). After that, the pH was adjusted to 6.0, and the phytagel was added (2.7 g L^{-1}). Shoots developed from these nodal segments were subcultures every 25 days. *In vitro* plants were grown at a 16-h photoperiod, photosynthetic photon flux density (PPFD) of 40-60 µmol photons m^{-2} s^{-1}, and temperature of 25/20°C.

2.3 Preparation of the samples from *ex vitro* and *In vitro* plant material

The tissue was selected from the central portion of the leaf for anatomical studies. It was cut quickly, and fixed in 37% formalin, acetic acid, and 70% ethanol (FAA_{70}). Samples were collected from adult *A. chilensis* fresh and *In vitro A. chilensis* leaves. Then, the samples were dehydrated through serial solutions in ethanol and *n*-butyl acetate and embedded in Paraplast® (Kuster & Vale, 2016). The cuts were made with a Jung Biocut 2035 microtome and stuck in glass sheets with bismuth adhesive. After removing the Paraplast® by dipping in butyl acetate and washing with ethanol, samples were coloured with safranin and Astra blue. Subsequently, they were re-dehydrated in a series of dilutions of ethanol and finally in butyl acetate.

For histochemical analysis, samples from adult *A. chilensis* fresh leaves and *In vitro A. chilensis* leaves were collected. The plants material was divided into three groups (Ferreira et al., 2017), T1: fresh material, T2: the samples were fixed for 24 hours in Karnovsky´s solution containing 2.5% glutaraldehyde, 4.5% formaldehyde in phosphate buffer (0.1 M, pH 7.2) (Karnovsky, 1964) and subjected to ethanol 70 %, T3: the samples were embedded

in polyethylene glycol (PEG) 6000. Simultaneously, the control tests were performed with fresh material for *In vitro* and adult *A. chilensis* leaves.

In the T1 and T2 treatment, the handmade sections were cut with razor blades. The collected samples hand-sectioned were submitted to histochemical reactions in a Petri dish.

In the T3 treatment, the embedded process was carried out at 60ºC with 25% PEG 6000 successive increments of PEG to form blocks with the samples. Once the water evaporates (24-48 hours), the polymer is added until reaching pure PEG or 90% PEG. The blocks were kept in the freezer at -20°C before sectioning in the microtome. During the observations in the microtome, the blocks were moisturized using a wet paint-brush before sectioning. The blocks of samples were cut at 25-35 µm thick. The samples were immediately immersed in warm water in a Petri dish (35-50°C).

In both studies, in the reagent itself or jelly glycerine, the slides were mounted. The sections were photographed in a Leica ICC50 HP optical microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Histochemical tests were performed on T1, T2 and T3 obtained from fresh material as described above using the following reagents for metabolites (primary and secondary): Lugol (T1 for *In vitro* leaves, T2 for adult leaves, Jensen 1962) for detection of starch, bromophenol blue 0.1% (T1 for *In vitro* leaves, T3 for adult leaves, Mazia et al., 1953) for proteins and Sudan red B (T1,Brundrett et al., 1991) for total lipids compounds, Wiesner´s test for lignin (T1, Johansen 1940), Dragendorff reagent for alkaloids (T1, Furr and Mahlberg, 1981), NADI reagent (α-naphthol and N,N-dimethyl-p-phenylenediamine) for terpenoids (T1, David and Carde, 1964). Phenolic compounds were detected using

formalin (T3, Johansen 1940). In particular, flavonoids were detected using caffeine and sodium benzoate fixation, followed by treatment with DMACA (4-dimethylamino cinnamaldehyde) reagent (Feucht et al., 1986).

3. Results and Discussion

The anatomy results of adult and *In vitro* leaves of *A. chilensis* are visualized in Figure 1. The leaves have a dorsiventral mesophyll, found mainly in dicotyledons. It is characterized by a upper epidermis with no stomata and large intercellular spaces on the adaxial side (Homem et al., 2020; Crisóstomo-Ayala et al., 2021). In the cross section of *A. chilensis* adult leaves, uniseriate epidermis cells are covered by a thin and slight cuticle on both surfaces (Fig.1b). On the *In vitro* leaves of *A. chilensis*, only distinct a uniseriate epidermis cell on both surfaces (Fig.1d).

The first layer has round shaped cells in the adaxial side (adult leaves) and the other layers have cells elongated anticlinal. The mesophyll of adult leaves presents by two or three layers of palisade parenchyma and up to five strata of spongy parenchyma (Fig.1b). In the transverse section of *In vitro* leaves, the round shaped cells form the palisade parenchyma. The abaxial side of the *In vitro* leaves present by round shaped cells that correspond to the spongy parenchyma (Fig.1d).

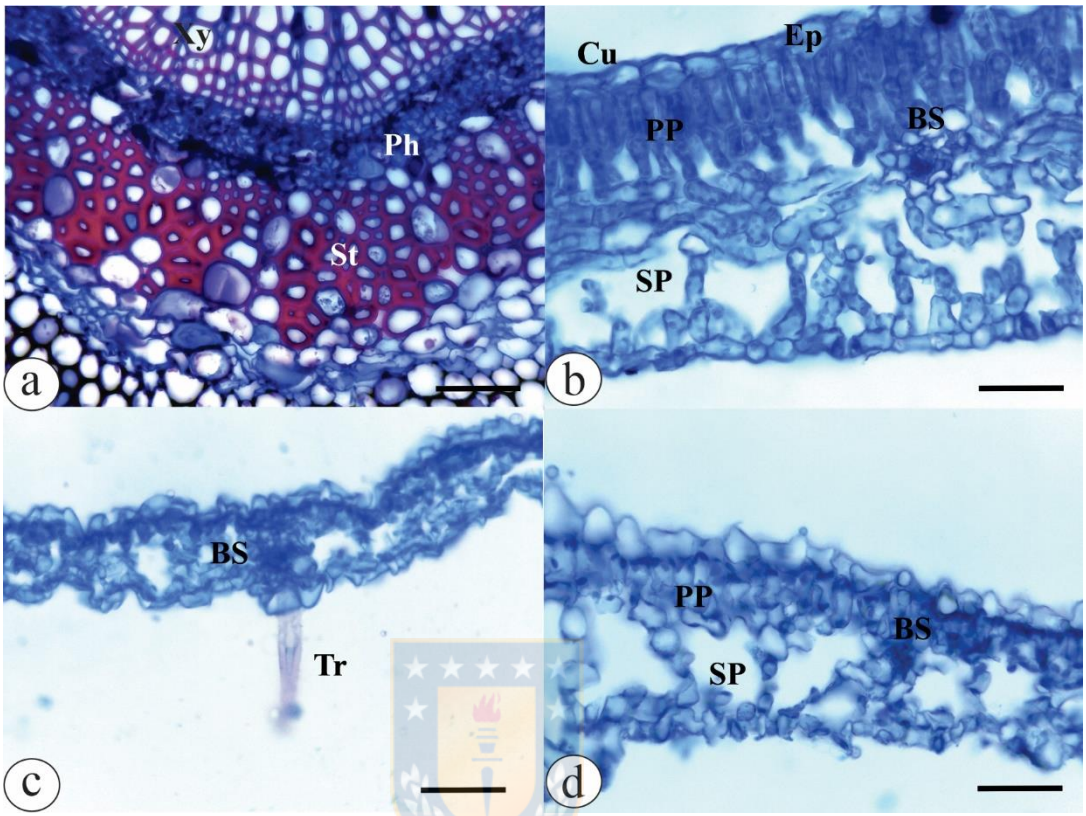

Figure 1. Cross section of *A. chilensis* adult (a, b) and *In vitro* (c, d) leaves. (a) Central vascular bundle of *ex vitro* leaves; (b) *Ex vitro* leaf mesophyll section; (c) Vascular bundle of *In vitro* leaves; (d) *In vitro* leaf mesophyll section. Abbreviations: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem; Ph, phloem; BS, bundle sheath; Cu, cuticle; Tr, trichome. Bars = 50 µm.

Adult and *In vitro* leaves of *A. chilensis* have collateral vascular bundles arranged in a single series throughout the cross-section leaf. Vascular bundles of *A. chilensis* are embedded in the middle of the mesophyll (Fig.1a, c). The midrib of the adult leaves has a biconvex contour and a more prominent face abaxial. Nevertheless, the midrib of *In vitro* leaves has a biconvex contour, but the epidermis cells continued on the abaxial side (Fig.1a, c).

Primary metabolites were identified location with histochemical techniques in the *A. chilensis* leaves. Secondary metabolites of *A. chilensis* are distributed in some specific section or almost in the plant´s whole tissues. Chemical compounds were not visualized in the negative control (Fig.2a, b; 3a). Figure 2-4 which was the cross-section of *A. chilensis* adult leaves with histochemical test results. The histochemical test results on *In vitro* leaves of *A. chilensis* are showed in Figures 3-5.

Starch grains were positive in the epidermis cells (Fig.3c). For the *In vitro* leaves, the starch grains were distributed in the mesophyll cells (Fig.3b). For the identification of lipophilic substances (lipids), it was used Sudan B reagent. The red color was observed in the cuticle and epidermis cells in *A. chilensis* leaves (Fig.2d, e; 3c). In Figure 2d, cuticle red is observed color and distinct the first layer of epidermis cells. The presence of proteins was characterized to a bright red color was developed with bromophenol blue. Proteins were localized at all mesophyll cells in *A. chilensis* leaves (Fig. 2f; 3d).

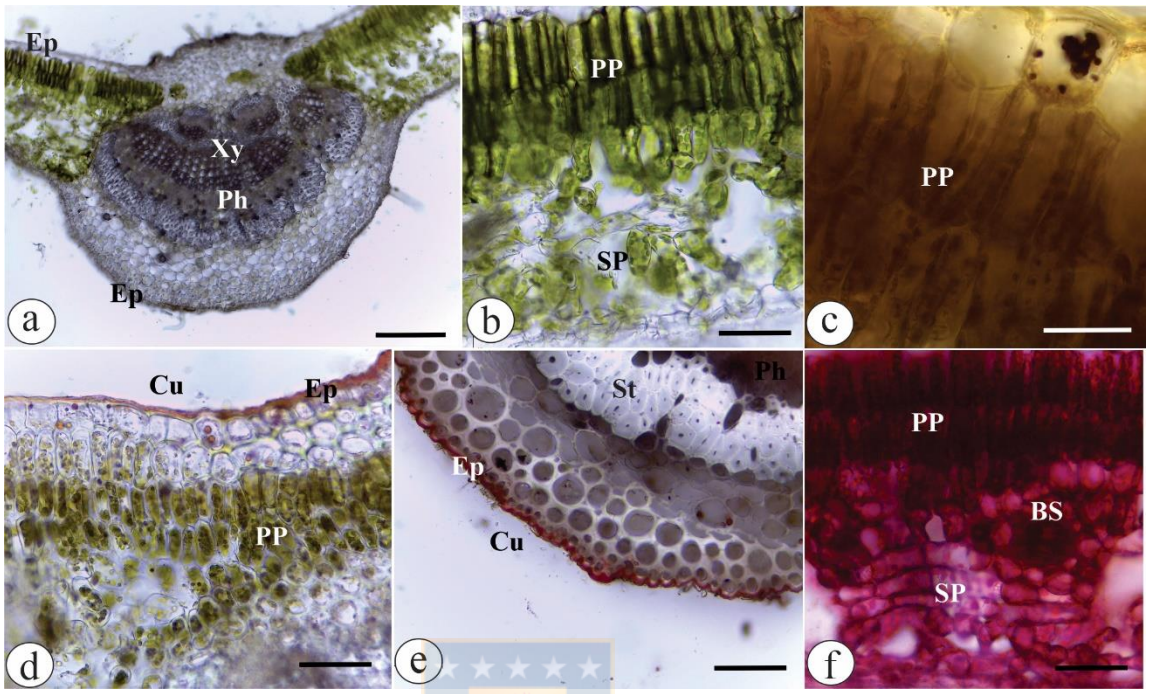

Figure 2. Histolocalization of plant primary metabolites in *A. chilensis* adult leaves. (a, b) Control test; (c) starch; (d, e) lipids; (f) proteins. Abbreviations: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; St, Sclerenchymal tissue; Xy, xylem; Ph, phloem; BS, bundle sheath; Cu, cuticle. Bars: b, d, e, f, g = 50 µm, a = 200 µm, c = 25 µm.

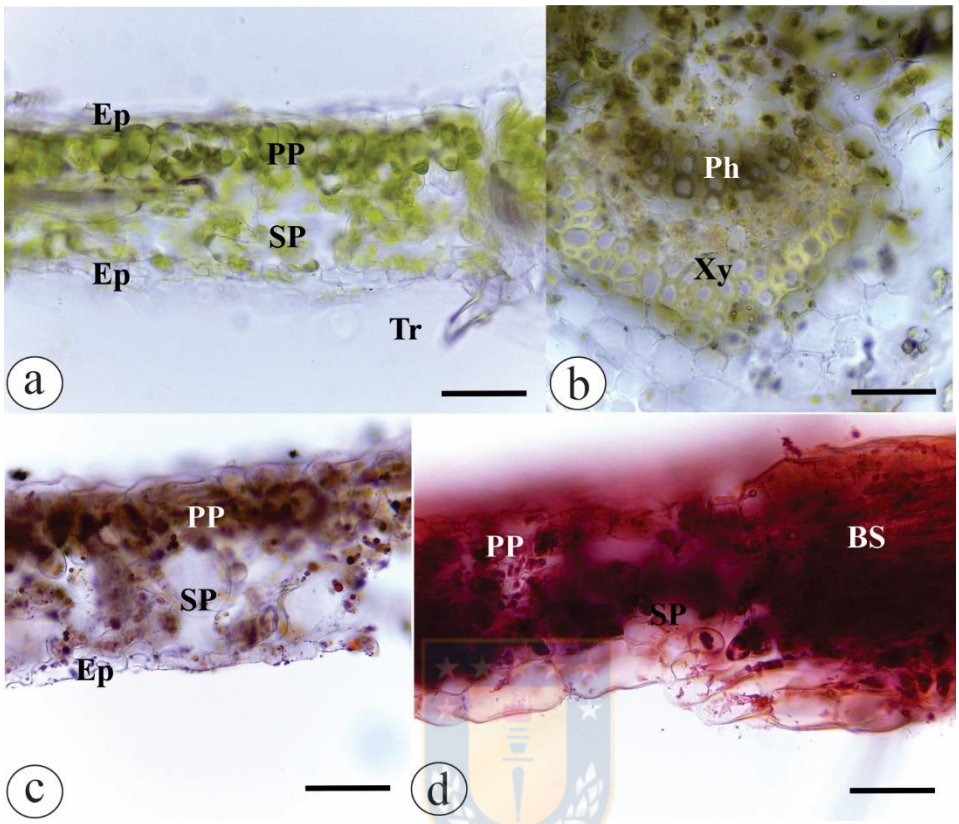

Figure 3. Histolocalization of plant primary metabolites in *A. chilensis In vitro* leaves. (a) Control test; (b) starch; (c) lipids; (d) proteins. Abbreviations: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem; Ph, phloem; BS, bundle sheath; Tr, trichome. Bars = 50 µm.

The presence of lignin was localized at the vascular bundle in the adult and *In vitro* leaves (Fig.4a, b; 5a, b). Moreover, the lignin was found in the fibber cells in the trichome (Fig.5b).

The Dragendorff reagent showed total alkaloids in the bundle sheath, palisade parenchyma and spongy parenchyma in the adult and *In vitro A. chilensis* leaves (Fig. 4c, 5c). It has been described leaves of *A. chilensis* are a source of polycyclic indole alkaloids like aristoteline, aristotelinine, aristotelone, aristone, makonine, aristotelinine, 8-oxo-9-dehidrohorbatine, 8-oxo-9-dehidromakomakine, horbatine, aristoquinoline, serratoline,

makomakine and sorreline (Turchetti & Paz, 2019). Additionally, the use of alkaloids isolated from maqui leaves has been studied to counteract neurological diseases. Arias et al., (2019) have identified aristoteline as a potent alkaloid against neurological diseases caused by the malfunction of the receptors responsible for neurotransmission.

Total phenols were detected by fixation in ferrous sulphate and formalin before PEG was embedded. The reaction resulted to precipitate the polyphenols and the sample was stored black of brown (Ferreira et al., 2017). The result showed that the polyphenols stored in enlarge to mesophyll cells were found in both the *ex vitro* and *In vitro* leaves of *A. chilensis* (Fig. 4d, e; 5d, e).

Flavonoids in *A. chilensis* adult leaves could be found in the epidermis and vascular bundle (Fig. 4f). Moreover, to confirm the positive to flavonoids result use caffeine fixation and DMACA reagent (Fig. 4g). In contrast, it was not identified flavonoids on *In vitro* leaves (Fig.5f, g). Despite the results, the presence of phenolic compounds is consistent with the species´ literature (Turchetti & Paz, 2019; Zúñiga et al., 2017). The major phenolic acids identified were gallic acid and coumaric acid, followed by flavonoids such as quercetin glucosides, myricetin, luteolin, pelargonidin, peonidin, diosmetin, apigenin, rhamnentin, catechin, and catechin. In addition, it identified the presence of aromatic compounds such as ρ-hydroxybenzoic acid, resveratrol, anthraquinone, and scopoletin. Moreover, the study realized by González-Villagra et al., (2019) identified the anthocyanins´ presence in *A. chilensis* leaves like delphinidin, cyanidin, petunidin, and malvidin. The compounds to the glycosylated form of delphinidin and cyanidin have been previously reported in edible berries of *A. chilensis* (Sandoval et al., 2019).

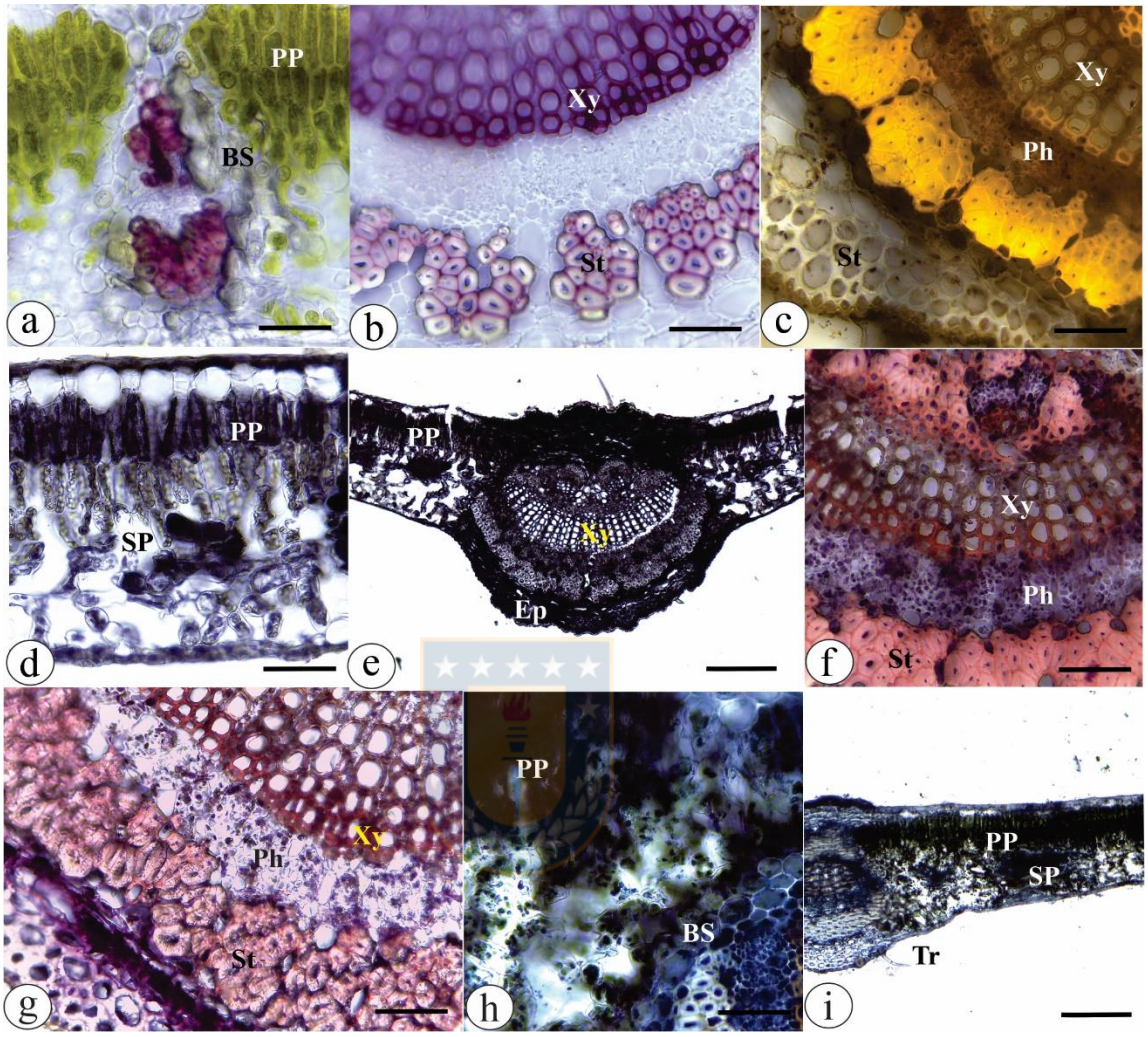

Figure 4. Histolocalization of plant secondary metabolites in *A. chilensis* adult leaves. (a, b) Lignins; (c) alkaloids; (d, e) phenolic compounds; (f, g) flavonoids; (h, i) terpenoids. Abbreviations: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; St, Sclerenchymal tissue; Xy, xylem; Ph, phloem; BS, bundle sheath; Cu, cuticle; Tr, trichome. Bars: a, b, c, d, f, g, h= 50 µm, e, i= 200 µm.

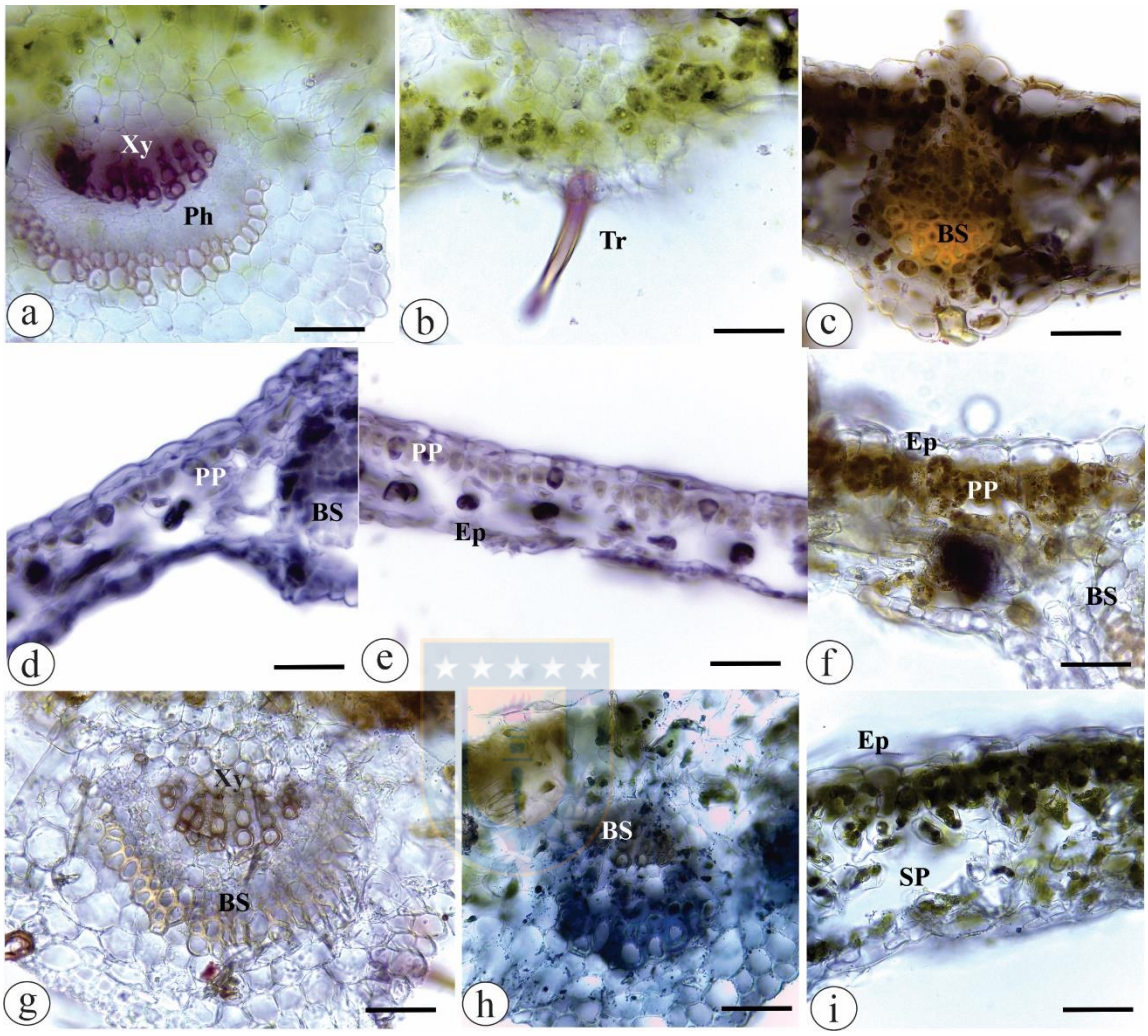

Figure 5. Histolocalization of plant secondary metabolites in *A. chilensis In vitro* leaves. (a, b) Lignins; (c) alkaloids; (d, e) phenolic compounds; (f, g) flavonoids; (h, i) terpenoids. Abbreviations: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem; Ph, phloem; BS, bundle sheath; Cu, cuticle; Tr, trichome. Bars = 50 µm.

The histochemical test showed that terpenoids gave a blue colour when stained with NADI reagent. Terpenoids were distributed to mesophyll cells on adult and *In vitro A. chilensis* leaves (Fig. 4h, i; 5h, i). The presence of terpenoids was lower in palisade parenchyma and spongy parenchyma of *In vitro* leaves (Fig 5M). Studies have reported terpenoid compounds with *A. chilensis* leaves by GC-MS and maceration of leaves in the n-hexane fraction (Céspedes et al., 2017; Muñoz & Ramos, 2016). The study realized by Muñoz and Ramos, (2016) stands out β-sitosterol presence followed by campesterol, sitostanol and campestanol in negligible concentrations. Céspedes et al., (2017) indicated in n-hexane fraction of the aerial parts of *A. chilensis* the presence of β-sitosterol and friedelane.

In summary, through the histochemical test, it was possible to identify primary and secondary metabolites in two different stages of development of *A. chilensis* leaves.

4. Conclusions

Identifying the histochemical compounds of the *A. chilensis* leaves contributes to expanding the in-formation about Chilean species with antioxidant properties. The used of *In vitro* culture techniques would be an efficiency method for accumulation of antioxidants compounds in *A. chilensis* leaves. The histochemical test used demonstrated the accumulation of some metabolites such as polyphenols and alkaloids in both types *A. chilensis* study leaves.

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References

Arias, H. R., Ortells, M. O., Feuerbach, D., Burgos, V., and Paz, C. (2019). Alkaloids purified from *Aristotelia chilensis* Inhibit the Human α3β4 Nicotinic Acetylcholine Receptor with Higher Potencies Compared with the Human α4β2 and α7 Subtypes. *Journal of Natural Products*, *82*(7), 1953–1960. https://doi.org/10.1021/acs.jnatprod.9b00314

Barupal, M., Kataria, V., and Shekhawat, N. S. (2018). *In vitro* growth profile and comparative leaf anatomy of the C3–C4 intermediate plant *Mollugo nudicaulis* Lam. *In vitro Cellular and Developmental Biology - Plant*, *54*(6), 689–700. https://doi.org/10.1007/s11627-018-9945-7

Benedetti, S. (2012). Monografía de maqui *Aristotelia chilensis* (Mol.) Stuntzs (Issue August). Programa de Investigación de Productos Forestales no Madereros, Instituto Forestal, Chile. http://www.redagricola.com/cl/maqui-un-negocio-millonario-que-apuesta-por-masificarse/

Brundrett, M. C., Kendrick, B., and Peterson, C. A. (1991). Efficient lipid staining in plant material with sudan red 7b or fluoral yellow 088 in polyethylene glycol-glycerol. *Biotechnic and Histochemistry*, *66*(3), 111–116. https://doi.org/10.3109/10520299109110562

Céspedes, C. L., Balbontin, C., Avila, J. G., Dominguez, M., Alarcon, J., Paz, C., Burgos, V., Ortiz, L., Peñaloza-Castro, I., Seigler, D. S., and Kubo, I. (2017). Inhibition on cholinesterase and tyrosinase by alkaloids and phenolics from *Aristotelia chilensis* leaves. *Food and Chemical Toxicology*, *109*, 984–995. https://doi.org/10.1016/j.fct.2017.05.009

Coode, M. J. E. (2004). Elaeocarpaceae 135 *Elaeocarpaceae*. *2*.

Crisóstomo-Ayala, K., Hernández de la Torre, M., Pedreño, M.A., Hernández, J.A., Pérez, C., Bustos, E., Sánchez-Olate, M., & Ríos, D. (2021). Seasonal changes in photosynthesis, phenolic content, antioxidant activity and anatomy of apical and basal leaves of *Aristotelia chilensis* (Mol.) Stuntz. *Biologia Plantarum.* art.no. 6728: Manuscript accepted

David, R., Carde, J.P., (1964). Coloration différentielle dês inclusions lipidique et ter-peniques dês pseudophylles du Pin maritime au moyen du reactif Nadi. C. R.Acad. Sci. 258, 1338–1340.

de Souza, D. M. F., Sá, R. D., Araújo, E. L., and Randau, K. P. (2018). Anatomical, phytochemical and histochemical study of *Solidago chilensis* Meyen. *Anais Da Academia Brasileira de Ciencias*, *90*(2), 2107–2120. https://doi.org/10.1590/0001-3765201720160280

Ferreira, B. G., Falcioni, R., Guedes, L. M., Avritzer, S. C., Antunes, W. C., Souza, L. A., and Isaias, R. M.

S. (2017). Preventing false negatives for histochemical detection of phenolics and lignins in PEG-embedded plant tissues. *Journal of Histochemistry and Cytochemistry*, *65*(2), 105–116. https://doi.org/10.1369/0022155416677035

Feucht, W., Schmid, P. P. S., and Christ, E. (1986). Distribution of flavanols in meristematic and mature tissues of *Prunus avium* shoots. *Journal of Plant Physiology*, *125*(1–2), 1–8. https://doi.org/10.1016/S0176-1617(86)80237-1

Fuentealba, J., Dibarrart, A., Sáez-Orellana, F., Fuentes-Fuentes, M. C., Oyanedel, C. N., Guzmán, J., Pérez, C., Becerra, J., and Aguayo, L. G. (2012). Synaptic silencing and plasma membrane dyshomeostasis induced by amyloid-β peptide are prevented by *Aristotelia chilensis* enriched extract. *Journal of Alzheimer's Disease*, *31*(4), 879–889. https://doi.org/10.3233/JAD-2012-120229

Furr, M., and Mahlberg, P. G. (1981). Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. *Journal of Natural Products*, *44*(2), 153–159. https://doi.org/10.1021/np50014a002

González-Villagra, J., Cohen, J. D., and Reyes-Díaz, M. M. (2019). Abscisic acid is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress. *Physiologia Plantarum*, *165*(4), 855–866. https://doi.org/10.1111/ppl.12789

Homem, I. C. M., Bobek, V. B., Szabo, E. M., Budel, J. M., Raman, V., Oliveira, V. B., and Miguel, O. G. (2020). Anatomy and histochemistry of leaf and stem of brazilian endemic species *Mollinedia clavigera* Tul. *Brazilian Archives of Biology and Technology*, *63*, 10–17. https://doi.org/10.1590/1678-4324-2020180717

Jensen, W. A. (1962). Botanical Histochemistry: Principles and Practice. W. H. Freemanand Co, San Francisco.

Johansen, D.A., 1940. Plant Microtechnique. McGraw-Hill, New York.

Karnovsky. (1985). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal Cell Biology*, *27*(November), 137–138.

Kuster, V. C., and Vale, F. H. A. (2016). Leaf histochemistry analysis of four medicinal species from Cerrado. *Revista Brasileira de Farmacognosia*, *26*(6), 673–678. https://doi.org/10.1016/j.bjp.2016.05.015

Mazia, D., Brewer, P.A., Alfert, M., 1953. The cytochemistry staining and measure-ment of protein with mercuric bromophenol blue. Biol. Bull. 104, 57–67.

Muñoz, O., and Ramos, F. (2016). Quantitative analysis of phytosterols in *Aristotelia chilensis* (maqui) leaves using GC/MS. *International Food Research Journal*, *23*(2), 822–826.

Murashige, T., and Skoog, F. (1962). Murashige1962Revised.Pdf. *Physiologia Plantarum*, *15*, 474–497.

Nandagopal, K., Halder, M., Dash, B., Nayak, S., and Jha, S. (2017). Biotechnological approaches for production of anti-cancerous compounds resveratrol, podophyllotoxin and zerumbone. *Current Medicinal Chemistry*, *25*(36), 4693–4717. https://doi.org/10.2174/0929867324666170404145656

Sáez, P. L., Bravo, L. A., Sáez, K. L., Sánchez-Olate, M., Latsague, M. I., and Ríos, D. G. (2012). Photosynthetic and leaf anatomical characteristics of C*astanea sativa*: A comparison between *In vitro* and nursery plants. *Biologia Plantarum*, *56*(1), 15–24. https://doi.org/10.1007/s10535-012-0010-9

Sandoval, V., Femenias, A., Martínez-Garza, Ú., Sanz-Lamora, H., Castagnini, J. M., Quifer-Rada, P., Lamuela-Raventós, R. M., Marrero, P. F., Haro, D., and Relat, J. (2019). Lyophilized maqui (*Aristotelia chilensis*) berry induces browning in the subcutaneous white adipose tissue and ameliorates the insulin resistance in high fat diet-induced obese mice. *Antioxidants*, *8*(9). https://doi.org/10.3390/antiox8090360

Scossa, F., and Fernie, A. R. (2020). The evolution of metabolism: How to test evolutionary hypotheses at the genomic level. *Computational and Structural Biotechnology Journal*, *18*, 482–500. https://doi.org/10.1016/j.csbj.2020.02.009

Turchetti, G., and Paz, C. (2019). *Aristotelia chilensis* (Mol.) Stuntz: A natural source of bioactive compounds. *Current Traditional Medicine*, *5*(1), 66–74. https://doi.org/10.2174/2215083804666181002095249

Zúñiga, G. E., Tapia, A., Arenas, A., Contreras, R. A., and Zúñiga-Libano, G. (2017). Phytochemistry and biological properties of *Aristotelia chilensis* a chilean blackberry: a review. *Phytochemistry Reviews*, *16*(5), 1081–1094. https://doi.org/10.1007/s11101-017-9533-1

DISCUSION GENERAL

En relación con la respuesta fotosintética de las hojas de *Aristotelia chilensis*, se obtuvieron valores de F_v/F_m dentro de los rangos normales tanto en hojas apicales y basales en las tres estaciones del año evaluadas (invierno, primavera y verano). Estos valores se explican debido a que *A. chilensis* es tolerante a la alta intensidad lumínica, presentan gran plasticidad fenotípica asociada a economía hídrica y que estas plantas no se encontraban sometidas a estrés ambiental (Moya et al., 2019; Molina-Montenegro et al., 2012). Respecto a los valores de ETR y qP presentaron una correlación positiva a la intensidad de la luz, obteniendo valores más altos en primavera y verano.

Con respecto a los parámetros fotosintéticos Acosta-Motos et al., (2015) estudiaron el efecto de la alta intensidad lumínica en hojas apicales y basales de plantas de *Myrtus communis* (mirto). Estos autores encontraron que con bajo alta intensidad de luz, las hojas basales de plantas de mirto mostraron valores más altos para qP, Fv/Fm y NPQ que las hojas apicales. Estos resultados coinciden parcialmente con los del presente estudio, ya que los valores de Fv/Fm de las hojas de *A. chilensis* estuvieron influenciados por el tipo de hoja, sin presentar condiciones de estrés, con valores más altos en las hojas basales que en las apicales en condiciones de mayor intensidad lumínica ambiental (primavera y verano). Por otro lado, el NPQ se comportó de forma diferente según el tipo de hoja, pero no se registraron cambios significativos en los valores de este parámetro durante las diferentes estaciones del año evaluadas. La estabilidad de NPQ sugiere la capacidad de las hojas de *A. chilensis* para utilizar el exceso de energía lumínica en el proceso de fotosíntesis. Por el contrario, en las hojas de mirto basal se observaron altos valores de NPQ durante los periodos de alta intensidad lumínica (Acosta-Motos et al., 2015). Todas estas diferencias de los parámetros fotosintéticos en las estaciones de estudio se ven

reflejados en el metabolismo secundario, específicamente con la cuantificación de compuestos fenólicos y su actividad antioxidante.

Al evaluar el contenido de fenoles totales (siglas en inglés, TPC) y la actividad antioxidante en las hojas de individuos de *A. chilensis*, crecientes en campo en las diferentes estaciones del año; los resultados indicaron que el mayor contenido de fenoles totales se encuentra en las hojas basales de primavera y la mayor actividad antioxidante en las hojas basales de invierno. Rawat et al., (2011) ha reportado la proporcionalidad directa en la relación entre el TPC y la actividad antioxidante. Sin embargo, Kabra et al., (2019) indicaron que en los extractos de hojas de *Myrica esculenta*, la relación entre los compuestos fenólicos y la actividad antioxidante podría estar influenciada por la composición fitoquímica de cada extracto y el uso de dos mecanismos diferentes de barrido de radicales (ensayos DPPH y ABTS). Además, el TPC puede estar influenciado por la cantidad y la calidad de esta sustancia activa, el tipo de hábitat de la planta, la estación del año y las condiciones en las que se cultivan las plantas (Stanković et al., 2017). En este estudio, la intensidad de luz en la estación de primavera podría ser favorable para la síntesis de estos compuestos activos proveniente del metabolismo secundario de las hojas de *A. chilensis*. En este contexto, se analiza el estado de desarrollo de las hojas y su estructura foliar frente a la síntesis de metabolitos activos (de tipo fenólico), dado por las diferentes condiciones de crecimiento.

Los resultados anatómicos de las hojas adultas y hojas originadas bajo condiciones de cultivo *In vitro* de *A. chilensis* no mostraron mayor diferencia en cuanto a estructuras. En las hojas adultas de *A. chilensis*, las células epidérmicas de has y envés están cubiertas por una fina y ligera cutícula. Mientras que, en las hojas de *A. chilensis* generadas *In vitro*, sólo se distingue la epidermis sin protección cuticular en ambas superficies. De acuerdo con los resultados obtenidos en las pruebas histoquímicas, las hojas adultas y las hojas generadas *In vitro* de *A. chilensis* presentan compuestos identificados en este estudio y congruentes con la literatura existente de la especie (Turchetti & Paz, 2019).

Según la identificación realizada es este estudio mediante HPLC-MS, las hojas adultas (*ex vitro*) e *In vitro* de *A. chilensis* mostraron la presencia de quercetina, catequina, kaempferol y ácido 3-cafeilquínico. Estos resultados coincidieron con varios autores que reportaron la presencia de quercetina, catequina y kaempferol en hojas adultas de maqui (Céspedes, et al., 2017; González-Villagra et al., 2019; Muñoz et al., 2011; Vidal et al., 2013).

En los análisis cuantitativos realizados en HPLC-DAD-ESI (Ion Trap)-MS^n, los extractos de hojas de *A. chilensis* mostraron un mayor contenido de polifenoles totales en las muestras de extractos de hojas obtenidas en cultivo *In vitro* con respecto a los extractos de hojas *ex vitro*.

Se identificaron y cuantificaron un total de 16 compuestos fenólicos en las hojas originadas *In vitro* y 20 en los extractos de las hojas *ex vitro* de *A. chilensis mediante* HPLC-DAD-ESI (Ion Trap)-MS^n. Dentro de los compuestos los grupos de familias identificados se encuentran en orden descendente los derivados del ácido gálico, el ácido cafeoilquínico, los derivados del ácido elágico y los elagitaninos. En el caso de las hojas basales de primavera mostraron un nivel ligeramente superior a los derivados de ácido gálico, ácidos cafeolquínicos, elagitaninos y derivados de flavonoides, en comparación con las hojas apicales de primavera (Crisóstomo-Ayala et al.,2021). Mientras que en los extractos de las hojas basales de invierno las concentraciones de los compuestos mencionados son menores. Diversos estudios realizados en hojas de *A. chilensis* coinciden con la presencia de polifenoles en su composición (Céspedes et al., 2017; González-Villagra et al., 2019). Sin embargo, anteriormente no existía un estudio acabado de las diferencias en el contenido de polifenoles en hojas de *A. chilensis,* en relación con la estación del año o condiciones de crecimiento de las hojas y su ubicación dentro de su estructura. Los resultados mencionados de este estudio coinciden con las pruebas histoquímicas realizadas de fenoles totales en hojas *ex vitro* e *In vitro*, detectadas mediante

sulfato ferroso y formalina. La histoquímica mostró que los polifenoles se almacenan en las células del mesófilo de las hojas de *A. chilensis*. En general, la planta responde los cambios medioambientales con el incremento de la actividad de enzimas clave de la ruta de los fenilpropanoides, mediante la cual se sintetizan metabolitos secundarios como los polifenoles, que tienen un papel estructural y de defensa en las plantas. En el caso de las plantas con propiedades antioxidantes, se ve incrementado el uso de azúcares para la síntesis de compuestos fenólicos, esto es posible ya que el transporte de los azúcares se realiza mediante vía floemática (Lagunes-Fortiz & Zavaleta-Mejía, 2015). De esta forma es posible dilucidar un mecanismo de protección de la planta frente a un posible estrés dado por la estación del año o condiciones de cultivo, utilizando la vía de transporte del floema para la disponibilidad inmediata de carbohidratos y posterior síntesis de polifenoles necesarios.

La identificación de los compuestos fenólicos presentes en hojas coincide con lo reportado por otros autores anteriormente (Céspedes et al., 2017; Turchetti & Paz, 2019). Dentro de los resultados obtenidos por HPLC-DAD-ESI (Ion Trap)-MS^n, es necesario destacar que la granatina B descrita previamente en frutos de *A. chilensis* con función protectora contra la radiación UVB (Rodríguez et al., 2021, Chen et al., 2020), nunca antes había sido descrita en las hojas de *A. chilensis*, hasta este estudio.

Se ha descrito que las hojas de *A. chilensis* son una fuente de alcaloides indólicos policíclicos como aristotelina, aristotelinina, aristotelona, aristona, makonina, aristotelinina, 8-oxo-9-dehidrohorbatina, 8-oxo-9-dehidromakomakina, horbatina, aristoquinolina, serratolina, makomakina y sorrelina (Turchetti & Paz, 2019). En este estudió, mediante métodos histoquímicos con el reactivo de Dragendorff, se observó la presencia de alcaloides totales en la vaina del haz, el parénquima en empalizada y el parénquima esponjoso en las hojas adultas y las originadas bajo cultivo *In vitro* de *A. chilensis*.

Adicionalmente, los compuestos terpenoides han sido reportados en estudios con hojas de *A. chilensis* por GC-MS y maceración de hojas en la fracción n-hexano (Céspedes et al., 2017; Muñoz & Ramos, 2016). El estudio realizado por Muñoz & Ramos, (2016) destaca la presencia de β-sitosterol seguido de campesterol, sitostanol y campestanol en concentraciones insignificantes. Céspedes et al., (2017) indicaron en la fracción n-hexano de las partes aéreas de *A. chilensis* la presencia de β-sitosterol y friedelano. En este estudio, la identificación de terpenoides se realizó mediante GC-MS, donde se detectó la presencia de α-tocoferol, β-sitosterol, ácido linoleico y ácido linolénico tanto en hojas *In vitro* como *ex vitro* de *A. chilensis*. Además, presencia de terpenoides fue identificada mediante pruebas histoquímicas, observando estos compuestos en las células del mesófilo de las hojas adultas y las hojas originadas bajo cultivo *In vitro* de *A. chilensis*.

En este contexto, este estudio les otorga una mayor importancia fitoquímica a las hojas de *A. chilensis*, bajo un contexto relacionado con la síntesis de metabolitos secundarios presentes en diferentes condiciones de estacionalidad y/o condiciones de cultivo *In vitro*. Además, en el futuro poder desarrollar protocolos para la síntesis de estos compuestos de interés farmacológico de las hojas adultas desarrolladas en condiciones de alta intensidad lumínica y las hojas desarrolladas bajo cultivo *In vitro*. Se ha desarrollado en los últimos años considerablemente la investigación sobre los efectos de los polifenoles de la dieta en la salud humana y lo que se respalda firmemente en el papel de los polifenoles en la prevención de las enfermedades degenerativas, especialmente las cardiovasculares y el cáncer (Cory et al., 2018; Swallah et al., 2020). Por lo tanto, la búsqueda de metodologías nuevas o mejoradas tanto para la extracción y el aislamiento de metabolitos secundarios (polifenoles) como para la separación, la identificación y la cuantificación de polifenoles con potenciales propiedades antioxidantes, es esencial para dar a conocer esta excelente fuente de estos fitoquímicos y contribuir a la mejora de la calidad de vida en la salud humana (Swallah et al., 2020).

CONCLUSIONES GENERALES

Las hojas basales de provenientes de individuos creciendo en condiciones de campo mostraron un Fv/Fm óptimo por lo que indican que no se encuentran bajo estrés ambiental, aunque estaban en presencia de alta intensidad lumínica, lo que indujo a un mayor contenido de fenoles totales.

Con una alta intensidad luminosa en las estaciones de primavera y verano, en las hojas basales y apicales de individuos adultos, los valores de NPQ incrementaron lo que indica que esta especie presentaría un mecanismo seguro para disipar el exceso de energía lumínica. Además, se observó un incremento de los valores de qP y ETR en las estaciones de primavera y verano, lo que evidencia mayor eficiencia para operar del PSII en las hojas de *A. chilensis*.

La identificación y cuantificación de los polifenoles de los 23 compuestos entre el material vegetal de hojas *In vitro* y hojas *ex vitro* realza la importancia de la especie nativa *A. chilensis* y su utilidad en la extracción de compuestos con posibles propiedades antioxidantes. Se realizo la cuantificación de 16 compuestos fenólicos en las hojas originadas *In vitro* y 20 en los extractos de las hojas adultas de *A. chilensis.* Los compuestos se clasificaron de acuerdo de los grupos de derivados de ácido gálico, ácido cafeoilquínico, elagitaninos, derivados de ácido elágico y derivados de flavonoides.

El mayor contenido de fenoles totales se obtuvo en los extractos de hojas crecientes obtenidas bajo condiciones de cultivo *In vitro* a diferencia de las hojas adultas estudiadas (hojas primavera e invierno), donde se obtuvieron los valores más bajos.

La identificación de los compuestos histoquímicos de las hojas de *A. chilensis* contribuye a ampliar la información sobre las especies chilenas que se encuentran en una expansión comercial creciente. La descripción anatómica refleja que las estructuras principales de las hojas adultas de *A. chilensis* se conservan en las hojas obtenidas bajo cultivo *In vitro*. El uso de la prueba histoquímica demostró la acumulación de algunos metabolitos generales como polifenoles, terpenoides y alcaloides en las hojas adultas y hojas obtenidas *In vitro* de *A. chilensis*.

De esta manera, queda en evidencia que la hipótesis planteada "Hojas de *Aristotelia chilensis* (Mol.) Stuntz con diferente edad fisiológica, aumentan su producción de compuestos fenólicos con capacidad antioxidante", se rechaza. Esto se debe a que el comportamiento de los polifenoles de las hojas adultas de *A. chilensis* en las diferentes estaciones del año estudiadas y las hojas generadas *In vitro*, dependen de la disponibilidad de la intensidad lumínica y de la cantidad y calidad de la síntesis de metabolitos secundarios con propiedades antioxidantes.

REFERENCIAS

Acosta-Motos, J.R., P. Diaz-Vivancos, S. Álvarez, N. Fernández-García, M.J. Sánchez-Blanco, Hernández, J.A. (2015). NaCl-induced physiological and biochemical adaptative mechanisms in the ornamental *Myrtus communis* L. plants. *Journal of Plant Physiology* 183: 41–51. doi: 10.1016/j.jplph.2015.05.005.

Albornoz, V. (2012). Callogénesis y cuantificación de *trans*-resveratrol en *Aristotelia chilensis* (Molina) Stuntz y *Fragaria x ananassa* duc cv. camarosa. Memoria para optar al título de Ingeniera en Biotecnología Vegetal. Concepción, Chile. Universidad de Concepción, Facultad de Ciencias Forestales.

Apóstolo, N. M., Brutti, C. B., & Llorente, B. E. (2005). Leaf anatomy of *Cynara scolymus* L. in successive micropropagation stages. *In vitro Cellular and Developmental Biology - Plant*, 41(3), 307–313. https://doi.org/10.1079/IVP2004606

Arias, H. R., Ortells, M. O., Feuerbach, D., Burgos, V., & Paz, C. (2019). Alkaloids Purified from *Aristotelia chilensis* Inhibit the human α3β4 nicotinic acetylcholine receptor with higher potencies compared with the human α4β2 and α7 subtypes. *Journal of Natural Products*, 82(7), 1953–1960. https://doi.org/10.1021/acs.jnatprod.9b00314

Azofeifa, A. (2009). Problemas de Oxidación y oscurecimiento de explantes cultivados *In vitro*. *Agronomía Mesoamericana*, 20(1), 153-175.

Barallobre-Barreiro, J., Chung, Y. L., & Mayr, M. (2013). La proteómica y la metabolómica: los mecanismos de la enfermedad cardiovascular y el descubrimiento de biomarcadores. *Revista Española de Cardiología*, 66(8), 657–661. https://doi.org/10.1016/j.recesp.2013.04.010

Barros, J., & Dixon, R. A. (2020). Plant Phenylalanine/Tyrosine Ammonia-lyases. *Trends in Plant Science*, 25(1), 66–79. https://doi.org/10.1016/j.tplants.2019.09.011

Barupal, M., Kataria, V., & Shekhawat, N. S. (2018). *In vitro* growth profile and comparative leaf anatomy of the C3–C4 intermediate plant *Mollugo nudicaulis* Lam. *In vitro Cellular and Developmental Biology - Plant*, 54(6), 689–700. https://doi.org/10.1007/s11627-018-9945-7

Bastías-Montes, J. M., Choque-Chávez, M. C., Alarcón-Enos, J., Quevedo-León, R., Muñoz-Fariña, O., & Vidal-San-Martín, C. (2019). Effect of spray drying at 150, 160, and 170 °C on the physical and chemical properties of maqui extract (*Aristotelia chilensis* (Molina) Stuntz). *Chilean Journal of Agricultural Research*, 79(1), 144–152. https://doi.org/10.4067/S0718-58392019000100144

Belchí-Navarro, S., Almagro, L., Sabater-Jara, A. B., Fernández-Pérez, F., Bru, R., & Pedreño, M. A. (2013). Induction of trans-resveratrol and extracellular pathogenesis-related proteins in elicited suspension cultured cells of *Vitis vinifera* cv Monastrell. *Journal of Plant Physiology*, 170(3), 258–264. https://doi.org/10.1016/j.jplph.2012.10.003

Bonga, J. M., Klimaszewska, K. K., & Von Aderkas, P. (2010). Recalcitrance in clonal propagation, in particular of conifers. *Plant Cell, Tissue and Organ Culture*, 100(3), 241–254. https://doi.org/10.1007/s11240-009-9647-2

Borsani, O., Gonzalez-Neves, G., Ferrer, M., & Monza, J. (2010). Anthocyanins accumulation and genes-related expression in berries of cv. Tannat (*Vitis vinifera* L.). *Journal of Applied Horticulture*, 12(1), 3–9. https://doi.org/10.37855/jah.2010.v12i01.01

Brauch, J. E., Reuter, L., Conrad, J., Vogel, H., Schweiggert, R. M., & Carle, R. (2017). Characterization of anthocyanins in novel Chilean maqui berry clones by HPLC–DAD–ESI/MSn and NMR-spectroscopy. *Journal of Food Composition and Analysis*, 58, 16–22. https://doi.org/10.1016/j.jfca.2017.01.003

Bucchetti, B., Matthews, M. A., Falginella, L., Peterlunger, E., & Castellarin, S. D. (2011). Effect of water deficit on Merlot grape tannins and anthocyanins across four seasons. *Scientia Horticulturae*, 128(3), 297–305. https://doi.org/10.1016/j.scienta.2011.02.003

Buchanan, B., Gruissem, W., & Jones, R. (2015). Biochemestry & Molecular Biology of Plants (John Wiley & Sons (ed.); Second Edition).

Bustamante, R., Simonetti, J., Grez A.A, & San Martín, J. (2005). Fragmentación y dinámica de regeneración del bosque Maulino: diagnóstico actual y perspectivas futuras. *Smith-Ramírez C, JJ Armesto & C Valdovinos* (Eds) Historia, Biodiversidad y Ecología de Los Bosques Costeros de Chile. Editorial Universitaria, Santiago, Chile., 529–539.

Céspedes, C., Mancinelli, P., Orellana, B. & Silva, M. (1995). Cultivo *In vitro* de *Aristotelia chilensis* (Mol.) Stuntz Elaeocarpaceae. *Gayana-Botánica*, 52(2), 77–82. https://doi.org/10.1007/978-94-011-0737-2_6

Céspedes, C. L., Alarcón, J., Ávila, J. G., & El-Hafidi, M. (2010). Anti-inflammatory Activity of *Aristotelia chilensis* Mol. (Stuntz) (Elaeocarpaceae). *Boletín Latinoamericano y Del Caribe de Plantas Medicinales y Aromáticas*, 9(6), 432–439.

Céspedes, C. L., Balbontín, C., Ávila, J. G., Domínguez, M., Alarcón, J., Paz, C., Burgos, V., Ortiz, L., Peñaloza-Castro, I., Seigler, D. S., & Kubo, I. (2017). Inhibition on cholinesterase and tyrosinase by alkaloids and phenolics from *Aristotelia chilensis* leaves. *Food and Chemical Toxicology*, 109, 984–995. https://doi.org/10.1016/j.fct.2017.05.009

Céspedes, C. L., El-Hafidi, M., Pavón, N., & Alarcón, J. (2008). Antioxidant and cardioprotective activities of phenolic extracts from fruits of chilean blackberry *Aristotelia chilensis* (Elaeocarpaceae), Maqui. *Food Chemistry*, 107(2), 820–829. https://doi.org/10.1016/j.foodchem.2007.08.092

Céspedes, C. L., Pavón, N., Domínguez, M., Alarcón, J., Balbontín, C., Kubo, I., El-Hafidi, M., & Ávila, J. G. (2017). The Chilean superfruit black-berry *Aristotelia chilensis* (Elaeocarpaceae), Maqui as mediator in inflammation-associated disorders. *Food and Chemical Toxicology*, 108, 438–450. https://doi.org/10.1016/j.fct.2016.12.036

Chen, L., Zhou, G., Meng, X. S., Fu, H. Y., Mo, Q. G., & Wang, Y. W. (2020). Photoprotection of maqui berry against ultraviolet B-induced photodamage *In vitro* and *in vivo*. *Food & Function*, 11(3), 2749–2762. https://doi.org/10.1039/c9fo01902b

Chen, X., Qi, X., & Duan, L. X. (2015). Overview. In plant metabolomics: methods and applications. https://doi.org/10.1007/978-94-017-9291-2_1

Cory, H., Passarelli, S., Szeto, J., Tamez, M., & Mattei, J. (2018). the role of polyphenols in human health and food systems: a mini-review. *Frontiers in Nutrition*, 5(87). https://doi.org/10.3389/fnut.2018.00087

Crisóstomo-Ayala, K., Hernández de la Torre, M., Pedreño, M.A., Hernández, J.A., Pérez, C., Bustos, E., Sánchez-Olate, M., & Ríos, D. (2021). Seasonal changes in photosynthesis, phenolic content, antioxidant activity and anatomy of apical and basal leaves of *Aristotelia chilensis* (Mol.) Stuntz. *Biologia Plantarum.* art.no. 6728: Manuscript accepted

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15(10), 7313–7352. https://doi.org/10.3390/molecules15107313

Damascos, M. A., & Prado, C. H. B. A. (2001). Leaf phenology and its associated traits in the wintergreen species *Aristotelia chilensis* (Mol.) Stuntz (Elaeocarpaceae). *Revista Chilena de Historia Natural*, 74(4), 805–815. https://doi.org/10.4067/s0716-078x2001000400008

Davinelli, S., Bertoglio, J. C., Zarrelli, A., Pina, R., & Scapagnini, G. (2015). A randomized clinical trial evaluating the efficacy of an anthocyanin–maqui berry extract (Delphinol®) on oxidative stress biomarkers. *Journal of the American College of Nutrition*, 34(October), 28–33. https://doi.org/10.1080/07315724.2015.1080108

Di Lorenzo, A., Sobolev, A. P., Nabavi, S. F., Sureda, A., Moghaddam, A. H., Khanjani, S., Di Giovanni, C., Xiao, J., Shirooie, S., Tsetegho Sokeng, A. J., Baldi, A., Mannina, L., Nabavi, S. M., & Daglia, M. (2019). Antidepressive effects of a chemically characterized maqui berry extract (*Aristotelia chilensis* (molina) stuntz) in a mouse model of Post-stroke depression. *Food and Chemical Toxicology*, 129(April), 434–443. https://doi.org/10.1016/j.fct.2019.04.023

Fiehn, O. (2002). Metabolomics- the link between genotypes and phenotypes. *Plant Molecular Biology*, 48, 155–171.

Finkelstein R (2013) Abscisic acid synthesis and response. *Arabidopsis Book* 11, e0166.

Fredes, C., Yousef, G. G., Robert, P., Grace, M. H., Lila, M. A., Gómez, M., Gebauer, M., & Montenegro, G. (2014). Anthocyanin profiling of wild maqui berries (*Aristotelia chilensis* [Mol.] Stuntz) from different geographical regions in Chile. *Journal of the Science of Food and Agriculture*, 94(13), 2639–2648. https://doi.org/10.1002/jsfa.6602

Fuentealba, J., Dibarrart, A., Sáez-Orellana, F., Fuentes-Fuentes, M. C., Oyanedel, C. N., Guzmán, J., Pérez, C., Becerra, J., & Aguayo, L. G. (2012). Synaptic silencing and plasma membrane dyshomeostasis induced by amyloid-β peptide are prevented by *Aristotelia chilensis* enriched extract. *Journal of Alzheimer's Disease*, 31(4), 879–889. https://doi.org/10.3233/JAD-2012-120229

García-Díaz, D. F., Jiménez, P., Reyes-Farías, M., Soto-Covasich, J., & Costa, A. G. V. (2019). A Review of the Potential of Chilean Native Berries in the Treatment of Obesity and its Related Features. *Plant Foods for Human Nutrition*, 74(3), 277–286. https://doi.org/10.1007/s11130-019-00746-6

Garrido Makinistian, F., Gallo, L., Sette, P., Salvatori, D., & Bucalá, V. (2019). Nutraceutical tablets from maqui berry (*Aristotelia chilensis*) spray-dried powders with high antioxidant levels. *Drying Technology*, 0(0), 1–12. https://doi.org/10.1080/07373937.2019.1629589

Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50(1): 151-158.

Gironés-Vilaplana, A., Huertas, J. P., Moreno, D. A., Periago, P. M., & García-Viguera, C. (2016). Quality and microbial safety evaluation of new isotonic beverages upon thermal treatments. *Food Chemistry*, 194, 455–462. https://doi.org/10.1016/j.foodchem.2015.08.011

Gironés-Vilaplana, Amadeo, Calín-Sánchez, Á., Moreno, D. A., Carbonell-Barrachina, Á. A., & García-Viguera, C. (2015). Novel maqui liquor using traditional pacharán processing. *Food Chemistry*, 173, 1228–1235. https://doi.org/10.1016/j.foodchem.2014.10.062

Gironés-Vilaplana, A., Baenasa, N., Villaño, D., Speisky, H., García-Viguera, C., & Moreno, D. (2014a). Evaluation of Latin-American fruits rich in phytochemicals with biological effects. *Journal of Functional Foods*, 7(1), 599–608. https://doi.org/10.1016/j.jff.2013.12.025

Gironés-Vilaplana, Mena, P., Moreno, D. A., & García-Viguera, C. (2014b). Evaluation of sensorial, phytochemical and biological properties of new isotonic beverages enriched with lemon and berries during

shelf life. *Journal of the Science of Food and Agriculture*, 94(6), 1090–1100. https://doi.org/10.1002/jsfa.6370

González-Villagra, J., Cohen, J. D., & Reyes-Díaz, M. M. (2019). Abscisic acid is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress. *Physiologia Plantarum*, 165(4), 855–866. https://doi.org/10.1111/ppl.12789

Heldt, H.W., & Piechulla, B. (2011). Plant biochemistry. In F. Heldt (Ed.), Elsevier (Fourth edi, Vol. 144, Issue 2). https://doi.org/10.1046/j.1469-8137.1999.00571-1.x

Hussain, A., Ahmed, I., Nazir, H., & Ullah, I. (2012). Plant tissue culture: current status and opportunities. *Recent advances in plant In vitro culture*, 1–28. https://doi.org/10.5772/50568

Kabra, A., Sharma, R., Hano, C., Kabra, R., Martins, N., Baghel, U.S. (2019). Phytochemical composition, antioxidant, and antimicrobial attributes of different solvent extracts from *Myrica esculenta* Buch.-Ham. ex. D. Don Leaves Atul. *Biomolecules.* 9, 357. https://doi.org/10.3390/biom9080357

Lagunes-Fortíz, E., & Zavaleta-Mejía, E. (2015). Función de la lignina en la interacción planta-nemátodos endoparásitos sedentarios. *Revista Mexicana de Fitopatología.* https://doi.org/10.18781/r.Mex.Fit.1506-7

López de Dicastillo, C., López-Carballo, G., Gavara, R., Muriel Galet, V., Guarda, A., & Galotto, M. J. (2019). Improving polyphenolic thermal stability of *Aristotelia chilensis* fruit extract by encapsulation within electrospun cyclodextrin capsules. *Journal of Food Processing and Preservation*, 43(8), 1–11. https://doi.org/10.1111/jfpp.14044

López De Dicastillo, C., Rodríguez, F., Guarda, A., & Galotto, M. J. (2016). Antioxidant films based on cross-linked methyl cellulose and native Chilean berry for food packaging applications. *Carbohydrate Polymers*, 136, 1052–1060. https://doi.org/10.1016/j.carbpol.2015.10.013

Lloyd, G., & McCown, B. (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot tip culture. *Proceedings of the International Plant Propagators Society* 30:421-427.

Mendoza, C. A., Turrión, M. B., Aceñolaza, P. G., Gallardo, J. F., & Pando, V. (2014). Retranslocación de nutrientes en especies dominantes de bosques del Espinal Mesopotámico (Argentina). *Bosque*, 35(2), 185–193. https://doi.org/10.4067/S0717-92002014000200006

Misle, E., Garrido, E., Contardo, H., & González, W. (2011). Maqui [*Aristotelia chilensis* (Mol.) Stuntz]-the Amazing Chilean Tree: A Review. *Journal of Agricultural Science & Technology B*, 1(4b), 473–482. http://search.ebscohost.com/login.aspx?direct=true&db=aph&AN=67153785&site=ehost-live

Molina-Montenegro, M.A., C. Torres-Díaz, F. Carrasco-Urra, L.A. González-Silvestre, & E. Gianoli. (2012). Plasticidad fenotípica en dos poblaciones antárticas de *Colobanthus quitensis* (caryophyllaceae) bajo un escenario simulado de cambio global. *Gayana - Bot.* 69(1): 152–160. doi: 10.4067/S0717-66432012000100015.

Moreno. (2016). Atributos Morfofisiológicos de *Aristotelia chilensis* (Mol.) Stuntz en un gradiente altitudinal de la Región del Biobío. Memoria para optar al grado de Ingeniero en Biotecnología Vegetal, Facultad de Ciencias Forestales, Universidad de Concepción, Chile.

Mouradov, A., & Spangenberg, G. (2014). Flavonoids: A metabolic network mediating plants adaptation to their real estate. *Frontiers in Plant Science*, 5(November), 1–16. https://doi.org/10.3389/fpls.2014.00620

Moya, M., B. González, U. Doll, J.A. Yuri, & H. Vogel. (2019). Different covers affect growth and development of three maqui clones (*Aristotelia chilensis* [Molina] Stuntz). *Journal of Berry Research* 9(3): 449–458. doi: 10.3233/JBR-180377.

Muñoz, O., Christen, P., Cretton, S., Backhouse, N., Torres, V., Correa, O., Costa, E., Miranda, H., & Delporte, C. (2011). Chemical study and anti-inflammatory, analgesic and antioxidant activities of the leaves of *Aristotelia chilensis* (Mol.) Stuntz, Elaeocarpaceae. *Journal of Pharmacy and Pharmacology*, 63(6), 849–859. https://doi.org/10.1111/j.2042-7158.2011.01280.x

Muñoz, O., & F. Ramos. (2016). Quantitative analysis of phytosterols in *Aristotelia chilensis* (Maqui) leaves using GC/MS. *International Food Research Journal* 23(2): 822–826.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *Journal of Experimental Botany*, 64(13), 3983–3998. https://doi.org/10.1093/jxb/ert208

Narayani, M., & Srivastava, S. (2017). Elicitation: a stimulation of stress in *In vitro* plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochemistry Reviews*, 16(6), 1227–1252. https://doi.org/10.1007/s11101-017-9534-0

Navarrete. (2019). Multiplicación *In vitro* de *Aristotelia chilensis* en sistema de inmersión temporal SETIS. Memoria para optar al título de Ingeniero Agrónomo. Facultad de Ciencias Agrarias, Universidad de Talca, Chile. https://doi.org/.1037//0033-2909.I26.1.78

Ojeda, J., Jara, E., Molina, L., Parada, F., Burgos, R. A., Hidalgo, M. A., & Hancke, J. L. (2011). Effects of *Aristotelia chilensis* berry juice on cyclooxygenase 2 expression, NF-κB, NFAT, ERK1/2 and PI3K/Akt activation in colon cancer cells. *Boletín Latinoamericano y Del Caribe de Plantas Medicinales y Aromáticas*, 10(6), 543–552.

Orta-Salazar, E., Cuellar-Lemus, C. A., Díaz-Cintra, S., & Feria-Velasco, A. I. (2014). Marcaje colinérgico en la corteza cerebral y el hipocampo en algunas especies animales y su relación con la enfermedad de Alzheimer. *Neurologia*, 29(8), 497–503. https://doi.org/10.1016/j.nrl.2012.10.007

Phillips, G. C., & Garda, M. (2019). Plant tissue culture media and practices: an overview. *In vitro Cellular and Developmental Biology* - Plant, 55(3), 242–257. https://doi.org/10.1007/s11627-019-09983-5

Quispe-Fuentes, I., Vega-Gálvez, A., Aranda, M., Poblete, J., Pasten, A., Bilbao-Sainz, C., Wood, D., McHugh, T., & Delporte, C. (2020). Effects of drying processes on composition, microstructure and health aspects from maqui berries. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-020-04260-5

Quispe-Fuentes, I., Vega-Gálvez, A., & Campos-Requena, V. H. (2017). Antioxidant compound extraction from maqui (*Aristotelia chilensis* [Mol] Stuntz) berries: Optimization by response surface methodology. *Antioxidants*, 6(1). https://doi.org/10.3390/antiox6010010

Rawat, S., Jugran, A., Giri, L., Bhatt, I.D., & Rawal. R.S. (2011) Assessment of antioxidant properties in fruits of Myrica esculenta: A popular wild edible species in Indian himalayan region. - Evidence-based. *Complementary and Alternative Medicine* 2011, 512787, 2011. https://doi.org/10.1093/ecam/neq055.

Repetto-Giavelli, F., Cavieres, L. A., & Simonetti, J. A. (2007). Respuestas foliares de *Aristotelia chilensis* (Molina) stuntz (Elaeocarpaceae) a la fragmentación del bosque maulino. *Revista Chilena de Historia Natural*, 80(4), 469–477. https://doi.org/10.4067/s0716-078x2007000400008

Reyes-Farías, M., Vásquez, K., Fuentes, F., Ovalle-Marín, A., Parra-Ruiz, C., Zamora, O., Pino, M. T., Quitral, V., Jiménez, P., García, L., & García-Diaz, D. F. (2016). Extracts of Chilean native fruits inhibit oxidative stress, inflammation and insulin-resistance linked to the pathogenic interaction between

adipocytes and macrophages. *Journal of Functional Foods*, 27, 69–83. https://doi.org/10.1016/j.jff.2016.08.052

Ringuelet, J., & Viña, S. (2013). Productos naturales vegetales. Universidad Nacional de La Plata (Vol. 1). http://sedici.unlp.edu.ar/bitstream/handle/10915/27885/Documento_completo__.pdf?sequence=1

Rodríguez Beraud, M., Tampe Pérez, J., Hormazábal Vásquez, N., Araneda Durán, X., Tighe Neira, R., & Cárcamo-Fincheira, P. (2017). Efecto de la escarificación y estratificación sobre la germinación *In vitro* de *Aristotelia chilensis* (Molina) Stuntz. *Gayana - Botânica*, 74(2), 282–287. https://doi.org/10.4067/S0717-66432017000200282

Rodriguez, L., Trostchansky, A., Wood, I., Mastrogiovanni, M., Vogel, H., Gonzalez, B., Marostica Junior, M., Fuentes, E., & Palomo, I. (2021). Antiplatelet activity and chemical analysis of leaf and fruit extracts from *Aristotelia chilensis*. *PLoS One*, 16(4), e0250852. https://doi.org/10.1371/journal.pone.0250852

Rojo, L. E., Ribnicky, D., Logendra, S., Poulev, A., Rojas-Silva, P., Kuhn, P., Dorn, R., Grace, M. H., Lila, M. A., & Raskin, I. (2012). *In vitro* and *in vivo* anti-diabetic effects of anthocyanins from Maqui Berry (*Aristotelia chilensis*). *Food Chemistry*, 131(2), 387–396. https://doi.org/10.1016/j.foodchem.2011.08.066

Romero, F., Palacios, J., Jofré, I., Paz, C., Nwokocha, C. R., Paredes, A., & Cifuentes, F. (2019). Aristoteline, an indole-alkaloid, induces relaxation by activating potassium channels and blocking calcium channels in isolated rat aorta. *Molecules* (Basel, Switzerland), 24(15). https://doi.org/10.3390/molecules24152748

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., & Shene, C. (2011). Extracts of maqui (*Aristotelia chilensis*) and murta (*Ugni molinae* Turcz.): Sources of antioxidant compounds and α-glucosidase/α-amylase inhibitors. *Journal of Agricultural and Food Chemistry*, 59(5), 1630–1637. https://doi.org/10.1021/jf103461k

Sadino Riquelme, M. C. (2015). Estrategias de cultivo e inducción *In vitro* de células de maqui para la obtención de antocianinas. Memoria para optar al título de Ingeniero Civil en Biotecnología, Facultad de Ciencias Físicas y Matemáticas, Departamento de Ingeniería Química y Biotecnología, Universidad de Chile.

Salinas, J., & Caballé, G. (2020). Maqui, el fruto silvestre de mayor importancia en Chile. Instituto Forestal, Ed.

Sánchez, A.B., & Suárez E. (2011). Morfo-anatomía de vitroplantas de zábila (Aloe vera L. Burm.f.). *Revista de La Facultad de Agronomía*, 28(1), 139–149.

Sandoval, V., Femenias, A., Martínez-Garza, Ú., Sanz-Lamora, H., Castagnini, J. M., Quifer-Rada, P., Lamuela-Raventós, R. M., Marrero, P. F., Haro, D., & Relat, J. (2019). Lyophilized maqui (*Aristotelia chilensis*) berry induces browning in the subcutaneous white adipose tissue and ameliorates the insulin resistance in high fat diet-induced obese mice. *Antioxidants*, 8(9). https://doi.org/10.3390/antiox8090360

Scossa, F., & Fernie, A. R. (2020). The evolution of metabolism: How to test evolutionary hypotheses at the genomic level. *Computational and Structural Biotechnology Journal*, 18, 482–500. https://doi.org/10.1016/j.csbj.2020.02.009

Steiner, F. (2020). Plant abiotic stress tolerance. *In Plant Abiotic Stress Tolerance*. https://doi.org/10.46420/9786588319024

Stanković, M., S. Ćurčić, N. Zlatić, & B. Bojović. (2017). Ecological variability of the phenolic compounds of Olea europaea L. leaves from natural habitats and cultivated conditions. *Biotechnology & Biotechnological Equipment* 31(3): 499–504. doi: 10.1080/13102818.2016.1275804.

Stohs, S. J., & Hartman, M. J. (2015). Review of the safety and efficacy of *Moringa oleifera*. *Phytotherapy Research*, 29(6), 796–804. https://doi.org/10.1002/ptr.5325

Swallah, M. S., Sun, H., Affoh, R., Fu, H., & Yu, H. (2020). Antioxidant potential overviews of secondary metabolites (polyphenols) in fruits. *International Journal of Food Science*, 2020, 9081686. https://doi.org/10.1155/2020/9081686

Taiz, L., Zeiger, E., Møller, I., & Murphy, A. (2018). Plant physiology and development. Sixth Edition. Oxford University Press.

Tenci, M., Rossi, S., Giannino, V., Vigani, B., Sandri, G., Bonferoni, M. C., Daglia, M., Longo, L. M., Macelloni, C., & Ferrari, F. (2019). An *in situ* gelling system for the local treatment of inflammatory bowel disease (IBD). the loading of maqui (*Aristotelia chilensis*) berry extract as an antioxidant and anti-inflammatory agent. *Pharmaceutics*, 11(11), 1–18. https://doi.org/10.3390/pharmaceutics11110611

Tortosa. (2018). Multi-omics response to *Xanthomonas campestris* pv. *campestris* in *Brassica oleracea*. Memoria para optar al grado de Doctor en Biotecnología, Universidad de Vigo, España. http://www.investigo.biblioteca.uvigo.es/xmlui/bitstream/handle/11093/1040/multi_omics_response_xanthomonas_campestris.pdf?sequence=1

Turchetti, G., & Paz, C. (2019). *Aristotelia chilensis* (Mol.) Stuntz: A Natural Source of Bioactive Compounds. *Current Traditional Medicine*, 5(1), 66–74. https://doi.org/10.2174/2215083804666181002095249

Vidal, J. L., Avello, L. M., Loyola, C. C., Campos, P. J., Aqueveque, M. P., Dungan, R. S., Galotto, L. M., & Guarda, M. A. (2013). Microencapsulation of maqui (*Aristotelia chilensis* [Molina] Stuntz) leaf extracts to preserve and control antioxidant properties. *Chilean Journal of Agricultural Research*, 73(1), 17–23.

Zhou, G., Chen, L., Sun, Q., Mo, Q. G., Sun, W. C., & Wang, Y. W. (2019). Maqui berry exhibited therapeutic effects against DSS-induced ulcerative colitis in C57BL/6 mice. *Food and Function*, 10(10), 6655–6665. https://doi.org/10.1039/c9fo00663j