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Actividad anticancerígena y antioxidante *in vitro* de polisacáridos ácidos obtenidos desde hongos pertenecientes a la familia Hymenochaetaceae asociados a bosque nativo de Chile.

Por

Verónica Agustina Albornoz Muñoz

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Profesores integrantes Comisión Evaluadora:

Dr. Víctor Campos A.
Director de Tesis
Facultad de Ciencias Biológicas
Universidad de Concepción

Dr. José Becerra A.
Co-director de Tesis
Facultad de Ciencias Naturales
y Oceanográficas
Universidad de Concepción

Dr. Víctor Hernández S.
Facultad de Ciencias Naturales
y Oceanográficas
Universidad de Concepción

Dr. Carlos Smith G.
Facultad de Ciencias Biológicas
Universidad de Concepción

Dr. Roberto Abdala Díaz
Departamento de Ecología y Geología
Universidad de Málaga. España

Director
Facultad de Ciencias Biológicas
Universidad de Concepción

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“Existen derrotas, pero nadie está a salvo de ellas. Por eso es mejor perder algunos combates en la lucha por nuestros sueños que ser derrotados sin siquiera saber por qué se está luchando”.

Paulo Coelho – A orillas del río Piedra me senté y lloré.

INDICE

INDICE DE FIGURAS

INDICE DE TABLAS

RESUMEN

El cáncer es una de las principales causas de muerte a nivel mundial. Su padecimiento puede estar vinculado con factores hereditarios, o verse favorecido por malos hábitos como el consumo excesivo de alcohol, de tabaco, o la exposición prolongada a componentes químicos, entre otros factores. Estas condiciones aumentan la producción de especies reactivas de oxígeno (ROS), desencadenando stress oxidativo, el cual produce daños en el material genético que pueden inducir el desarrollo de cáncer. Los tratamientos convencionales aplicados para esta enfermedad, como la quimioterapia, presentan un alto costo monetario y un gran deterioro al estilo de vida de los pacientes; por lo que la búsqueda de nuevos tratamientos o compuestos, naturales o sintéticos, que puedan emplearse satisfactoriamente en el control del cáncer han despertado un gran interés en los científicos. Los polisacáridos de origen fúngico han demostrado presentar una amplia gama de actividades biológicas, tales como actividad antibacteriana, hipoglucémica, inmunomoduladora, antioxidante y anticancerígena. El nivel de actividad anticancerígena que presentan los polisacáridos fúngicos está relacionado con sus características físicoquímicas, incluyendo su composición monomérica y los grupos funcionales asociados a la cadena principal de glucanos. En este trabajo se evaluará la capacidad antioxidante y citotóxica contra líneas celulares tumorales, de los polisacáridos ácidos de cuatro cepas fúngicas, pertenecientes a la familia Hymenochaetaceae presente en bosques nativos del sur de Chile. Los polisacáridos ácidos serán obtenidos desde las cepas FQ1645 (*Nothophellinus andinopatagonicus*), FQ1626 y FQ1640 (*Phylloporia boldo*), y FQ1648 (posible *Fomitiporia sp*.), por medio de una precipitación selectiva con Cetavlon. Los polisacáridos obtenidos (NAAPs, PBAP26 y PBAP40, y APFQ48, respectivamente) fueron caracterizados por análisis infrarrojo con transformada de Fourier (FT-IR). La actividad anticancerígena *in vitro* fue determinada por análisis de citotoxicidad con MTT y citometría de flujo. Además, la capacidad antioxidante de los polisacáridos fue

evaluada usando los radicales libres sintéticos DPPH y ABTS. El análisis de FT-IR permitió identificar la presencia de enlaces α y β-glicosídicos tanto en PBAP40 como en APFQ48; mientras que los picos correspondientes a grupos sulfatos fueron evidenciados en NAAPs y APFQ48. Los ensayos de citotoxicidad contra líneas celulares tumorales mostraron que tres de los polisacáridos estudiados (NAAPs, PBAP40 y APFQ48) presentan un mayor efecto contra la línea celular HL-60, con valores de IC_{50} de 767.16 µg mL^{-1}, 127.85 µg mL^{-1} y 800 µg mL^{-1}, respectivamente. Por su parte, el polisacárido PBAP26 presenta una mayor actividad citotóxica contra la línea celular tumoral HCT-116, con un valor de IC_{50} de 535.83 µg mL^{-1}. La evaluación del efecto citotóxico de los polisacáridos contra una línea celular no tumoral permitió evidenciar que todos los polisacáridos ácidos analizados presentan un efecto proliferante en la línea celular HGF-1, obteniéndose valores de supervivencia mayores al 100% con las mayores concentraciones de polisacáridos usadas. En cuanto al efecto de los polisacáridos sobre el ciclo celular de la línea celular HL-60, se pudo observar que todos los polisacáridos producen un aumento de eventos en la fase Sub G1, suponiendo un efecto de arresto de células en esta fase del ciclo celular. Los ensayos de actividad antioxidante evidenciaron que los polisacáridos ácidos de las cuatro cepas en estudio presentan una mayor capacidad reductora del radical DPPH en comparación con el radical ABTS; y que los polisacáridos PBAP40 y APFQ48 presentan la mayor actividad antioxidante con valores de 24.53% y 27.31% de actividad antioxidante, respectivamente. Los polisacáridos analizados presentan una significativa actividad anticancerígena *in vitro* contra las líneas celulares empleadas, HCT-116, MCF-7 y HL-60, especialmente contra esta última. La bioactividad presentada por NAAPs, PBAP26, PBAP40 y APFQ48 podría estar dada por la presencia de grupos funcionales como grupos sulfatos, así como la existencia de β-glucanos.

ABSTRACT

Cancer is one of the leading causes of death worldwide. Cancer may be linked to hereditary factors or be favoured by bad habits such as excessive alcohol consumption, tobacco, or prolonged exposure to chemical components, among other factors. These conditions increase the production of reactive oxygen species (ROS), inducing oxidative stress, which produces damage to the genetic material that can induce the development of cancer. Conventional treatments applied for this disease, such as chemotherapy, have a high monetary cost and a great deterioration in the lifestyle of patients. Therefore, the search for new treatments or compounds, natural or synthetic, that can be successfully used in cancer control have generate great interest among scientists. Fungal polysaccharides have been shown to present a wide range of biological activities, such as antibacterial, hypoglycaemic, immunomodulatory, antioxidant and anticancer activity. The level of anticancer activity exhibited by fungal polysaccharides is related to their physicochemical characteristics, including their monomeric composition and the functional groups associated with the main chain of glucans. In this work, antioxidant, and cytotoxic capacity against tumour cell lines, of acid polysaccharides from four fungal strains belonging to the Hymenochaetaceae family present in the native forests of southern Chile, will be evaluated. Acid polysaccharides will be obtained from strains FQ1645 (*Nothophellinus andinopatagonicus*), FQ1626 and FQ1640 (*Phylloporia boldo*), and FQ1648 (possible *Fomitiporia sp.*), by selective precipitation with Cetavlon. The polysaccharides obtained (NAAPs, PBAP26, PBAP40, and APFQ48, respectively) were characterized by Fourier transform infrared analysis (FT-IR). *In vitro* anticancer activity was determined by MTT cytotoxicity assays and flow cytometry. Furthermore, the antioxidant capacity of polysaccharides was evaluated using the synthetic free radicals DPPH and ABTS. FT-IR analysis identified the presence of α and β-glycosidic linkages in both PBAP40 and APFQ48; while peaks corresponding to sulphate groups were evidenced in

NAAPs and APFQ48. Cytotoxicity assays against tumour cell lines showed that three of the polysaccharides studied (NAAPs, PBAP40 and APFQ48) have a greater effect against the HL-60 cell line, with IC_{50} values of 767.16 µg mL^{-1}, 127.85 µg mL^{-1} and 800 µg mL^{-1}, respectively. On the other hand, the PBAP26 polysaccharide has a greater cytotoxic activity against the tumour cell line HCT-116, with an IC_{50} value of 535.83 µg mL^{-1}. The evaluation of the cytotoxic effect of polysaccharides against a non-tumour cell line showed that all acid polysaccharides analysed have a proliferative effect on the HGF-1 cell line, obtaining survival values greater than 100% with the highest concentrations of polysaccharides used. Regarding the effect of polysaccharides on the cell cycle of the HL-60 cell line, it was observed that all polysaccharides produce an increase in events in the Sub G1 phase, assuming a cell arrest effect in this phase of the cell cycle. The antioxidant activity assays showed that the acid polysaccharides of the four strains under study have a greater reducing capacity of the DPPH radical compared to the ABTS radical; and that the polysaccharides PBAP40 and APFQ48 present the highest antioxidant activity with values of 24.53% and 27.31% of antioxidant activity, respectively. The polysaccharides analysed show significant in vitro anticancer activity against the cell lines used, HCT-116, MCF-7 and HL-60, especially against the latter. The bioactivity presented by NAAPs, PBAP26, PBAP40 and APFQ48 could be due to the presence of functional groups such as sulphate groups, as well as the existence of β-glucans.

CAPÍTULO I: INTRODUCCIÓN

El cáncer es una patología que cobra relevancia por ser una de las principales causas de mortalidad a nivel mundial, estimándose mas de 22 millones de muertes para 2032 atribuibles a esta causa [1,2]. En el caso de Chile, las muertes por cáncer se posicionan en segundo lugar, sólo por debajo de las muertes por enfermedades cardiovasculares [1]. El cáncer es una enfermedad que se produce a partir de secuencias de ADN que presentan una mutación, causando la modificación de vías que regulan la homeostasis tisular, la supervivencia y la muerte celular [3]. Estas mutaciones pueden adquirirse por el traspaso vertical del material genético dañado, o pueden producirse por exposición a factores externos como la radiación solar, el consumo excesivo de alcohol o tabaco, la exposición prolongada a compuestos químicos e incluso una dieta desbalanceada [2]. En algunas oportunidades, la exposición a estos factores externos genera un desequilibrio en la cantidad de especies reactivas de oxígeno (ROS) en el ambiente celular, lo que deriva en un estrés oxidativo [4,5]. A pesar de que las ROS son mensajeros subcelulares en las vías de regulación de genes y transducción señales [6], en condiciones de estrés oxidativo los radicales libres pueden dañar los ácidos grasos, las proteínas e incluso el ADN, lo que puede causar enfermedades y daños neurodegenerativos, además de cáncer [5,6]. Hoy en día, existen variados tratamientos para los diferentes tipos de cáncer; sin embargo, muchos presentan un elevado costo económico y producen efectos secundarios que deterioran la calidad de vida de los pacientes [1,7,8]. Por esto, la búsqueda e investigación de nuevos compuestos naturales o sintéticos que puedan postularse como un posible tratamiento para alguno de los cánceres existentes, cobra una gran relevancia.

Los hongos son un grupo diverso de organismos que incluye mohos, levaduras y setas [9,10]. Pueden producir una gran cantidad de metabolitos primarios y

secundarios, muchos de ellos con una destacable actividad biológica [11]. Motivo por el cual han sido usado por cientos de años en la medicina tradicional China [12]. Dentro de los metabolitos que se han podido aislar de algunos hongos se encuentran los triterpenos, ergoesteroles, ácidos grasos, cumarinas, y en mayor proporción, los polisacáridos [13]. Los polisacáridos de origen fúngico son una importante macromolécula, que presenta una efectiva actividad antioxidante, anticancerígena, antiinflamatoria y inmunomoduladora [5,14]. De acuerdo con Chen *et al.* [13], los polisacáridos obtenidos desde *Phellinus linteus* presentan una destacable actividad inmunomoduladora, induciendo la proliferación de linfocitos T y regulando la producción de anticuerpos primarios. Por su parte, los polisacáridos de *Phellinus baumii* aumentan significativamente la producción de óxido nítrico en los macrófagos Raw 264.7 cuando son usados en una concentración de 400 µg mL^{-1} [15]. En relación con la actividad anticancerígena, los polisacáridos de *P. baumii* en concentración de 400 µg mL^{-1} han demostrado inhibir la proliferación de las células HepG2 en un 61.2%, causando el arresto de las células en la fase S del ciclo celular e induciendo la apoptosis [15]. Además, los polisacáridos obtenidos desde *Inonotus obliquus* y *P. linteus* son capaces de inhibir el crecimiento de las células SGC-7901 de cáncer gástrico humano y las células de Sarcoma-180 de murino, respectivamente [16,17]. Por otra parte, los polisacáridos obtenidos desde diferentes especies pertenecientes al género *Phellinus* han presentado una alta capacidad antioxidante frente a radicales superóxido, hidroxilo, DPPH (2,2-diphenyl-1-picrylhydrazyl) y ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) [18-20].

Los polisacáridos son macromoléculas que presentan un gran poder bioactivo, y su efectividad depende de sus características físicas y químicas, tales como estructura, composición y peso molecular [21,22]. Pueden clasificarse en base a criterios estructurales como homoglicanos o heteroglicanos, lineales o ramificados; también pueden clasificarse como neutros, catiónicos o aniónicos

[23]. De acuerdo con Sun *et al.* [24], la extracción ácida de los polisacáridos permite la obtención de un mayor rendimiento de polisacáridos bioactivos. Los polisacáridos ácidos conforman un importante grupo bioactivo dentro de los polisacáridos fúngicos [25]. De acuerdo con Ukai *et al.* [26], los polisacáridos ácidos obtenidos desde *Tremella fuciformis* evidenciaron una importante actividad anticancerígena, inhibiendo hasta en un 91.7% el crecimiento de sarcoma S180 en ratones. Por otra parte, los polisacáridos ácidos obtenidos desde *Cordyceps sinensis* mostraron una significativa acción inmunomoduladora, al estimular la fagocitosis, la producción de NO y la producción de citoquinas en la línea celular de macrófago Raw 264.7 [25].

En el presente trabajo se evaluará la actividad anticancerígena y antioxidante *in vitro*, que presentan los polisacáridos ácidos de cuatro cepas fúngicas (FQ1645, FQ1626, FQ1640 y FQ1648) pertenecientes a la familia Hymenochaetaceae, asociadas a bosque nativo de Chile; y se determinará el posible mecanismo de acción como anticancerígeno directo. De acuerdo con los reportes de Rajchenberg [27], y de Fisher & Binder [28], muchas especies de la familia Hymenochaetaceae, se encontraban clasificadas dentro del género *Phellinus s.l.*. Sin embargo, este género está conformado por numerosos géneros dentro de un mismo clado [27]; provocando que algunas especies fueran distribuidas en grupos monofiléticos como un género separado [28]. En base a análisis realizados con anterioridad por Rajchenberg *et al.* [29,30], las cepas en estudio fueron identificadas como *Nothophellinus andinopatagonicus* (FQ1645), *Phylloporia boldo* (FQ1626; FQ1640) y *Fomitiporia sp.* (FQ1648). *Nothophellinus andinopatagonicus* se caracteriza por producir pudrición blanca en diferentes especies de *Nothofagus,* arboles distribuidos en el sur de Chile y Argentina. De acuerdo con Aqueveque *et al.* [31], el extracto total de *N. andinopatagonicus* presenta una importante actividad antimicrobiana. Por su parte, el hongo *P. boldo* restringe su distribución geográfica a la distribución de su hospedero *Peumus boldus,* especie endémica de Chile. Se ha evidenciado

que esta especie biosintetiza compuestos derivados de hidroquinolonas cloradas [32]. En el caso de las especies del género *Fomitiporia,* los estudios de Guo *et al.* [33] y Zan *et al.* [34], han indicado que corresponden a hongos de gran importancia medicinal. En Chile, la especie *Fomitiporia chilensis* (Rajchenb. And Pildain) fue descrita por Rajchenberg *et al.* [30] como un hongo causante de pudrición blanca, asociado a las especies *P. boldus* y *Cryptocarya alba.* Aunque las especies han sido estudiadas anteriormente, los ensayos realizados en este trabajo son el primer acercamiento que evidencia la capacidad bioactiva que presentan los polisacáridos ácidos de las especies *N. andinopatagonicus, P. boldo* y *Fomitiporia sp..*

CAPÍTULO II: ANTECEDENTES GENERALES

II.1. EL CÁNCER

El cáncer es una enfermedad que agrupa distintas manifestaciones clínicas [2]; pero en general se puede considerar como el crecimiento anormal y desordenado de las células del cuerpo [2,35]. Está causada por alteraciones celulares, a partir de secuencias de ADN que presentan una mutación, causando la modificación de vías que regulan la homeostasis tisular, la supervivencia y la muerte celular [3]; de esta forma, la célula cancerosa pierde el control de su propio desarrollo, dividiéndose a una velocidad mayor que el resto de las células que conforman el tejido [35]. Este crecimiento anormal de células forma masas de tejidos denominados tumores.

Es una patología que cobra relevancia por ser una de las principales causas de mortalidad a nivel mundial, estimándose más de 22 millones de muertes para 2032 atribuibles a esta causa [1,2]. En el caso de Chile, las muertes por cáncer se posicionan en segundo lugar, sólo por debajo de las muertes por enfermedades cardiovasculares [1].

II.1.a. Factores de riesgo del cáncer

El proceso por el cual las células normales se transforman en células cancerígenas se denomina carcinogénesis [2]; y, como se mencionó anteriormente, se origina por alteraciones en secuencias de ADN responsables de controlar el crecimiento y multiplicación de las células [2,35]. Se han identificado que las mutaciones que dan origen a la carcinogénesis pueden adquirirse por herencia o por exposición a factores externos [2,36].

Herencia: Sólo un pequeño porcentaje de casos (5%-10%) ocurren por esta causa. Se produce debido al traspaso vertical (herencia) de genes anormales, lo que provocará el desarrollo de cáncer en varios miembros de una misma familia, por lo general a una edad temprana [35]. Esta herencia se producirá

sólo si las alteraciones genéticas se presentan en las células reproductoras de los padres [37].

Factores externos: La exposición a diversos factores externos puede desencadenar en un estrés oxidativo (por un desequilibrio en la cantidad de ROS) que induce un daño celular. los ROS atacan a numerosas sustancias biológicas, entre ellas el ADN, produciendo variadas patologías como el cáncer [4]. Algunos de estos factores externos son el tabaquismo, el consumo de alcohol, la exposición solar, la exposición a sustancias químicas y la dieta [36].

El consumo de tabaco es el principal factor de riesgo para el desarrollo de cáncer de pulmón, faringe, laringe y riñón siendo el cáncer de pulmón el que causa más muertes en el mundo [35,36]. Por su parte, el consumo de bebidas alcohólicas favorece el desarrollo de cáncer de laringe, esófago y de la cavidad oral. La exposición solar, más específicamente la exposición a la radiación ultravioleta, induce el desarrollo de cáncer de piel en las zonas de mayor exposición. Mientras que el consumo de una dieta no balanceada puede provocar el desarrollo de cáncer de estómago (por exceso de almidón), cáncer de recto y colon (por exceso de azúcares), entre otras [35,37].

Normalmente, el sistema inmunológico se encarga de eliminar las células que presentan algún tipo de daño, previniendo el desarrollo de cáncer y otras enfermedades. Sin embargo, bajo algunas condiciones (consumo de inmunodepresores o enfermedades como SIDA), el sistema inmune no logra actuar de manera correcta, presentándose un estado de inmunodeficiencia, que es incapaz de controlar el desarrollo de cáncer [37].

Inmunodeficiencia: A pesar de que el sistema inmune es complejo, su función se resume principalmente en la búsqueda y eliminación de microorganismos invasores. Cuando no funciona adecuadamente, se genera una respuesta inmune inapropiada, provocando enfermedades inmunitarias [38]. La

inmunodeficiencia puede ser causada por deficiencias genéticas, inanición, inmunodepresión inducida por fármacos o enfermedades como el SIDA; y puede estar afectando el funcionamiento de células T, células B, fagocitos o el complemento [10,39,40].

La inmunodeficiencia puede considerarse un factor que contribuye al desarrollo de cáncer debido a que los antígenos tumorales marcan las células cancerosas para que sean destruidas; y células inmunitarias como los linfocitos T, las células NK y los macrófagos son los responsables de llevar a cabo dicha tarea [36]. A pesar de que las células cancerosas se producen con frecuencia, el sistema inmune se encarga de eliminarlas; sin embargo, presentar inmunodeficiencia o tener las defensas inmunológicas debilitadas a causa de la avanzada edad aumenta la probabilidad de desarrollar cáncer [10,39].

II.1.b.- Tipos de cáncer

Existe una gran cantidad de tipos de cáncer. La clasificación de estos puede basarse en el tipo de célula que lo conforma; agrupándolos en carcinoma, sarcoma, leucemia, linfoma, mieloma o melanoma [37]. Por otra parte, pueden nombrarse de acuerdo con el tejido donde se están desarrollando; como cáncer de pulmón, cáncer de próstata, cáncer de mama, entre otros [36]. En esta investigación se evaluará la actividad anticancerígena haciendo uso de tres líneas celulares tumorales, correspondientes a cáncer de colon, cáncer de mama y leucemia.

Cáncer de colon: Corresponde al tercer tipo de cáncer más común en el mundo [41]. Se produce por el desarrollo de células cancerosas en los tejidos del colon. Su ocurrencia está relacionada principalmente por exposición a factores externos, siendo sólo un 5% de los casos causados por herencia. La dieta es el factor externo más relevante en el desarrollo de cáncer de colon; siendo el

consumo excesivo de carne roja, el abuso en el consumo de alcohol y la grasa abdominal los hábitos más perjudiciales [42].

Cáncer de mama: Es la principal causa de muerte por cáncer en mujeres a nivel mundial [43,44], y la segunda a nivel país (Chile); además, corresponde al 1% de los casos de cáncer en hombres [45]. Tanto en mujeres como en hombres, los factores de riesgo están asociados a desórdenes hormonales (por un aumento en los niveles de estrógeno circulante), antecedentes familiares y mutaciones en genes determinados [44,45]. A su vez, los desórdenes hormonales puedes verse favorecidos por el consumo excesivo de alcohol (que disminuye la síntesis de testosterona en hombres) o drogas como la marihuana [45].

Leucemia: Es un cáncer de las células primitivas productoras de sangre, principalmente de los glóbulos blancos (linfocitos); sin embargo, puede afectar a otras células sanguíneas [46]. La leucemia mielógena aguda (LMA) abarca a muchos trastornos que se diferencian por la morfología de la célula leucémica, las alteraciones cromosómicas específicas y patrones de reordenamiento genético [47]. Un sub-tipo de la LMA es la leucemia promielocítica aguda (LPA), que representa entre un 10-20% del total de LMA [48,49]. Al igual que otras leucemias, la LPA presenta síntomas de anemia, sangrado asociado a trombocitopenia y una disminución en el recuento de neutrófilos; no obstante, el síntoma que la diferencia es el desarrollo de una coagulopatía grave, lo que provoca que este sub-tipo de LMA sea altamente mortal [48]. En relación con los factores de riesgo, Pulsoni *et al.* [49] sugirió que la exposición a campos electromagnéticos puede favorecer el desarrollo de leucemia promielítica aguda; además de los factores de riesgo en común con otras LMA, tales como el tabaquismo, el envejecimiento, exposición a radiación, exposición a ciertos compuestos químicos (por ejemplo, el benceno) y el tratamiento con algunos medicamentos de quimioterapia [46].

II.1.c.- Tratamientos contra el cáncer

El tipo de tratamiento oncológico que se decida aplicar depende de diferentes características del tumor, tales como el estadio del tumor, su tamaño o si se presenta metástasis [36]. Entre los tratamientos más aplicados se encuentran:

Cirugía: Puede ser de carácter preventiva, diagnóstica, curativa o paliativa. La cirugía ofrece la mayor posibilidad de curarse del cáncer, y es aplicable a una gran cantidad de tipos de cáncer. Consiste en la extirpación quirúrgica del tumor, de forma total o parcial [37].

Radioterapia: Consiste en la aplicación de partículas de rayos X o rayos Gama sobre la zona que presenta el cáncer, con el fin de eliminar o dañar las células cancerígenas [37]. Puede usarse en diferentes estadios de la enfermedad, y en algunos casos se emplea de forma complementaria con otro tratamiento, como la cirugía o la quimioterapia. Esta alternativa puede presentar efectos secundarios desgastantes para el paciente; tales como fatiga, estomatitis, alopecia, náuseas y vómitos, diarrea, y pérdida del apetito, entre otras [35,37].

Quimioterapia: Consiste en el uso de fármacos para tratar el cáncer. Los fármacos antineoplásicos y citotóxicos actúan eliminando las células que se reproducen con rapidez; sin embargo, este tratamiento no es selectivo, por lo que se ven afectadas de igual forma algunas células no cancerígenas que se dividen rápidamente, como los folículos pilosos o las células del aparato reproductor [37]. Este tratamiento trae consigo un amplio número de efectos secundarios tales como la alopecia, pérdida de peso y apetito, náuseas, vómito, fatiga, daños cardiacos, daños pulmonares, daños al tejido reproductor [35].

Terapia hormonal: Consiste en el empleo de fármacos que interfieren con la producción de hormonas, o en la extirpación quirúrgica de glándulas secretoras de hormonas que puedan estar vinculadas con el desarrollo del tumor; con el fin de eliminar o reducir el crecimiento de las células cancerígenas [46]. Presentan

menos efectos secundarios que la quimioterapia; sin embargo, en algunos casos se puede presentar sofocos y sudoración, aumento de peso, cambios de humor, calambres musculares y cefaleas [36,46].

Inmunoterapia: Se considera a cualquier terapia que incluya componentes del sistema inmunitario [35]. Consiste en promover o reforzar la respuesta del sistema de defensa natural del cuerpo, haciendo uso de sus componentes para eliminar selectivamente las células cancerígenas (marcadas como dianas) [50].

Los tratamientos más convencionales aplicados para el control del cáncer pueden convertirse en una enorme carga financiera y un deterioro a la calidad de vida de los pacientes oncológicos [1,7,8]. Debido a esto, nuevas alternativas de drogas anticancerígenas o de tratamientos anticáncer son investigadas continuamente.

II.2. LOS HONGOS

Se han descrito alrededor de 80.000 a 120.000 especies de hongos; sin embargo, se estima que podrían existir cerca de 1,5 millones de especies [10,51,52], por lo que son considerados como el recurso de biodiversidad menos explorado [53]. Los hongos son un grupo diverso de organismos tanto macro como microscópicos, pudiendo incluir mohos, levaduras y setas [9,10]. La mayoría de los hongos son multicelulares y forman un entramado de filamentos que se conoce como hifas; el crecimiento conjunto de las hifas da lugar a la formación del micelio [9]. Las setas corresponden a macrohongos en donde el desarrollo del micelio forma una estructura reproductiva denominada cuerpo fructífero en el cual se desarrollan las esporas [10]. Se clasifican de manera separada de las plantas y animales, y una de sus características diferenciales es la presencia de una pared celular con alto contenido de quitina [9]; la quitina es un polisacárido resistente pero flexible [54].

La gran mayoría corresponden a hongos terrestres; no obstante, existen algunos de agua dulce o marinos [54]. A pesar de que muchos pueden ser patógenos, afectando tanto a plantas como a animales; existen también aquellos que establecen relaciones beneficiosas con otros microorganismos; por ejemplo, la asociación con plantas (micorrizas) [10,54]. La mayoría de los hongos son saprófitos, por lo que obtienen los nutrientes a partir de la descomposición de materia orgánica; haciendo favorable su desarrollo en lugares oscuros y húmedos, aunque también se les puede encontrar creciendo sobre plantas [54].

Desde el punto de vista humano, los hongos presentan gran relevancia tanto en términos de beneficio como de perjuicio, en el ámbito ecológico como económico [9,54]. Algunos hongos producen compuestos alucinógenos o tóxicos, como alcaloides, que pueden presentar un riesgo para el ser humano; de igual forma, su acción patógena sobre algunos cultivos provoca el deterioro de los alimentos, lo que genera grandes pérdidas económicas [9]. Sumado a esto, algunas especies de hongos son patógenos animales, incluido el humano, lo que causa variadas enfermedades fúngicas [9,10]. Sin embargo, los beneficios obtenidos desde los hongos son mayores a los perjuicios. El hábito saprófito que presenta gran parte de los hogos permite que estos descompongan la materia orgánica que se encuentra presente en los suelos; aprovechando algunos nutrientes, y a su vez liberando el resto de los nutrientes al suelo donde son aprovechados por otras especies (principalmente vegetales) que crecen en el lugar. Por otra parte, los seres humanos han aprovechado los cuerpos fructíferos de muchos hongos como fuente de alimento [55], tales como el champiñón, las trufas y las colmenillas. También se ha hecho uso de los hongos (levaduras) en la elaboración de alimentos tales como el pan, el vino o la cerveza [54]. Además, los hongos son productores de una gran cantidad de metabolitos, muchos de los cuales presentan una relevante actividad biológica [11,55]. Esto ha permitido emplear los hongos en medicina, industria y ciencia, donde han sido usados como fuente de antibióticos (siendo la penicilina el antibiótico más conocido),

inmunosupresores (ciclosporina) y enzimas (celulasas, pectinasas y proteasas) [9,54,56].

Los metabolitos primarios y secundarios obtenidos desde diferentes especies de hongos han sido ampliamente estudiados, y una vasta gama de actividades biológicas han sido descritas.

II.2.a Actividad biológica de metabolitos fúngicos

A través de numerosas investigaciones, una gran variedad de metabolitos provenientes de hongos ha sido identificados. Un ejemplo de esto es el hongo medicinal *Phellinus linteus,* que de acuerdo con Chen *et al.* [13], a partir de él se ha podido aislar diferentes constituyentes; entre ellos, polisacáridos (que se presentan en mayor proporción), flavonas, cumarinas, ácidos grasos, ergoesteroles, triterpenos, ácidos aromáticos, aminoácidos, y enzimas como la catalasa, ureasa, esterasa, lactasa, etc.. Los hongos se han usado en la medicina tradicional China por cientos de años, debido a que las setas han demostrado poseer beneficios a la salud, tales como actividad antiinflamatoria, inmunomoduladora, antioxidante, hypoglicémica, anticancerígena, antimicrobiana y anticoagulante [12,21,55,57-60].

Actividad antiinflamatoria: La inflamación es la respuesta biológica ante una lesión, que puede haber sido causada por agentes como patógenos, irritantes e incluso células dañadas [61,62]. De acuerdo con Yuan *et al.* [62], la inflamación presenta cuatro signos clásicos, el enrojecimiento, calor, dolor e hinchazón; con el fin de tratar estos signos se requiere el uso de compuestos antiinflamatorios. Sumado a esto, la prolongación del estado de inflamación puede desencadenar mutaciones genéticas por la acumulación de especies reactivas de oxígeno, lo que derivaría en enfermedades como artritis, esclerosis múltiple o cáncer [63]. De acuerdo con Chen *et al.* [13], una fracción en n-butanol del extracto etanólico obtenido del cuerpo fructífero de *Phellinus linteus* mostró un efecto

antiinflamatorio induciendo la hemo oxigenasa 1 en macrófagos RAW 264.7, y reduciendo la producción de óxido nítrico (NO). De igual forma, según lo informado por Du *et al.* [64], el extracto acuoso del cuerpo fructífero de *Cordyceps militaris*, conformado principalmente por β-glucanos, redujo significativamente la producción de NO, TNF-α e IL-6 en macrófagos Raw 264.7 estimulados por lipopolisacárido (LPS); además, los β-glucanos del hongo *Pleurotus ostreatus* suprimen la secreción de TNF-α e IL-6 en ratones expuestos a LPS *in vivo*.

Actividad inmunomoduladora: El sistema inmunológico es la primera barrera con la que cuentan los organismos para prevenir o combatir las enfermedades. Los inmunomoduladores pueden definirse como agentes modificadores de esta respuesta biológica [10]. Los inmunomoduladores pueden ser inmunoestimulantes, capaces de activar el sistema inmune; o, inmunosupresores, capaces de inhibir el sistema inmune (presentan uso en cirugías de trasplante). El complejo polisacárido-proteína (PPC) obtenido desde *P. linteus* presenta una significativa actividad inmunoestimulante, induciendo la proliferación de esplenocitos, específicamente la proliferación de células B, aumentando hasta ocho veces su proliferación al compararla con células B no expuestas al PPC [65]. A su vez, Kim *et al.* [65] evidenció que el PCC aumentaba la producción de citoquinas IL-6 y TNF-α en macrófagos peritoneales. Por su parte, Jiang *et al.* [66] demostró que un exopolisacárido obtenido desde *Phellinus pini* es capaz de estimular la producción de NO, TNF-α y ROS en macrófagos peritoneales, cuando es empleado en concentraciones superiores a 100 µg mL^{-1}. De acuerdo con Chen *et al.* [13], los polisacáridos de *P. linteus* son capaces de inducir la proliferación de linfocitos T, regular la respuesta de los anticuerpos primarios dependientes e independientes de células T, y actúan como un activador policlonal de células B. Por otra parte, los macrófagos Raw 264.7 expuestos a 400 µg mL^{-1} de polisacáridos parcialmente purificados obtenidos desde micelio de *Phellinus baumii* dieron como resultado un aumento significativo en la producción de NO, alcanzando una producción cinco veces más grande que

el de los controles no tratados con polisacáridos [15]. De manera similar, Li *et al.* [67] evidenció que diferentes fracciones de polisacáridos obtenidos desde *P. baumii* cultivado por diferentes métodos, generan un aumento en la producción de NO en macrófagos Raw 264.7 dependiente de la dosis de polisacárido usado.

Actividad antioxidante: Las especies reactivas de oxígeno (ROS) son una especie química caracterizadas por poseer electrones desapareados, y que se generan espontáneamente en las células vivas producto del metabolismo; y que participan en rutas de señalización celular [5,59]. Sin embargo, al presentarse en grandes cantidades y generar un desequilibrio celular, son causantes de estrés oxidativo, lo que puede dañar macromoléculas del cuerpo humano como las proteínas, ácidos grasos y el ADN, desencadenando enfermedades cardiovasculares, neurodegenerativas, cáncer y trastornos asociados a la edad [5]. Se ha descrito variados metabolitos fúngicos con capacidad antioxidante; entre ellos, la hispidina, aislada desde el caldo de cultivo micelial de *P. linteus*, la cual en concentración de 1.0 mM presentó efecto antioxidante sobre el radical anión superóxido, el radical hidroxilo y el radical DPPH en porcentajes de 56.8%, 95.3% y 85.5% respectivamente [13]. De acuerdo con Chen *et al.* [68], una fracción de polisacárido extracelular obtenido desde la especie *Fusarium oxysporum* en concentración de 10 mg mL^{-1} presenta una destacable capacidad reductora de radical hidroxilo, superóxido y DPPH, alcanzando una actividad de 90.2%, 89.2% y 88.2% respectivamente. Por otra parte, los polisacáridos purificados obtenidos desde *Grifola frondosa* evidenciaron una actividad antioxidante frente al radical DPPH de un 33.33% cuando una concentración de 1 mg mL^{-1} fue usada [69].

Actividad anticancerígena: El cáncer está causado por alteraciones celulares (mutación del material genético) [2], y corresponde a la principal causa de muerte a nivel mundial [1,2]. De acuerdo con Chen *et al.* [13], los polisacáridos obtenidos desde *P. linteus* evaluados en concentraciones de 0 a 1 mg mL^{-1} suprimieron la proliferación de las células HepG2 en hasta un 50% de forma dosis dependiente,

además se determinó que los polisacáridos de *P. linteus* inducen la formación de morfología apoptótica en las células HepG2 y producen un arresto en la fase S del ciclo celular. Por otra parte, según Nakamura *et al.* [17], las fracciones obtenidas desde un extracto etanol-acetona del micelio de *P. linteus* presentaron actividad antitumoral *in vivo* frente a tumores implantados en ratones; la fracción FIII-1 obtenida por precipitación con NaOH al 24% v/v y compuesta por 49.4% de proteína y un 39.3% de azúcares presentó la mayor actividad, reduciendo en un 81.2% el tamaño del tumor. De acuerdo con Xue *et al.* [15], los polisacáridos solubles en agua obtenidos desde *P. baumii* son capaces de reducir la proliferación de las células HepG2 en un 9.5%, 31.9% y 61.2%, cuando las células son tratadas con 100, 200 y 400 µg mL^{-1} de polisacárido, respectivamente; además, se determinó por citometría de flujo que la inhibición de la proliferación celular de HepG2 estaba vinculada con un arresto de las células en la fase S del ciclo celular y con la inducción de la muerte celular por apoptosis.

Otras actividades biológicas: De acuerdo con Hwang *et al.* [70], compuestos polifenólicos aislados por fraccionamiento con acetato de etilo de un extracto etanólico del cuerpo fructífero de *P. baumii*, presentaron la capacidad de inhibir la infección del virus de la influenza (H1N1) *in vitro*, inhibiendo la neuraminidasa del virus de forma dosis dependiente. Por su parte, los extractos crudos obtenidos desde el cultivo micelial de *Fomitiporia sp.* y de *Inonotus pachyphloeus* presentaron actividad antimicrobiana frente a la especie clínica *Bacillus subtilis*, con valores de MIC de 300 µg mL^{-1} cada extracto [71].

II.2.b. Especies en estudio

Familia Hymenochaetaceae.

Los poliporos de la familia *Hymenochaetaceae* (*Hymenochaetales*) es un grupo que contiene muchas especies fitopatógenas de importancia mundial, así como

también otras especies tienen un alto valor medicinal [29,57,72]. Debido a esto, muchos de sus taxa han sido estudiados y caracterizados; entre ellos *Phellinus* Quél. [29]. *Phellinus sensu lato*, es uno de los géneros más grandes de la familia *Hymenochaetaceae*, con distribución cosmopolita [27,72,73], que contiene hongos tanto saprófitos como parásitos viviendo en una gran variedad de bosques [28]; con numerosas especies comprendiendo aproximadamente 220 especies conocidas, de las cuales al menos 26 especies han sido usadas como hongos medicinales y/o comestibles en los países asiáticos [74].

De acuerdo con Rajchenberg [27], el género *Phellinus,* basado en la especie tipo *Polyporus igniarius* L.: Fr. (*Phellinus igniarius*), podía describirse morfológicamente como un hongo con basidiocarpo poroide, perenne, resupinado, pileado y sésil, leñoso, solitario o varios imbricados, de color castaño ferrugíneo a marrón oscuro, xantocroico. La superficie himenial de color castaño leonina, umbrina a amarillenta. Sistema hifal dimítico (que lo distingue de los géneros poroides "*Hymenochaetoides"*), más raramente monomítico o trimítico, constituido por hifas generativas con septos simples y paredes hialinas, amarillentas o ligeramente castañas, delgadas a ligeramente engrosadas. Basidiosporas cilíndricas, elipsoidales a globosas, con paredes delgadas a engrosadas, hialinas, amarillentas o castañas, cianófilas o acianófilas. Por otra parte, el género *Fomitiporia* Murrill (Hymenochaetaceae, Hymenochaetales, Basidiomycota), separado desde *Phellinus s.l.* como un clado monofilético en base a datos moleculares [75], se caracteriza por un hábito perenne, dextrinoide y basidiosporas cianófilas, con ausencia de setas en la mayoría de las especies [76].

Estudios filogenéticos demostraron que el género *Phellinus s.l*. es una entidad sumamente heterogénea, conformada por numerosos géneros dentro de un mismo clado [27]. Por esto, basándose en las diferencias morfológicas, pigmentos y citología, las especies del género *Phellinus* s.l. fueron colocados en

grupos monofiléticos más pequeños, cada una designada como un género separado [28].

Muchas especies de este género han sido consideradas hongos medicinales con diversas funciones biológicas, y han sido empleados en medicina tradicional oriental a lo largo de los años [18,57,66]. Hwang *et al*. [70] menciona en su investigación que el extracto de *Phellinus baumii*, especie empleada como medicina y alimento, presenta diversa actividad biológica de tipo antioxidante, hipoglicémica y antiinflamatoria; mientras que *Phellinus linteus* presenta propiedades biológicas entre las que destaca su actividad antitumoral, antiinflamatoria e inmunomoduladora [58].

Género *Phylloporia*

El género *Phylloporia* Murrill (Hymenochaetaceae), fue descrita a partir de una especie de poliporo inusual, *P. parasitica* Murrill (1904) [77,78]. *Phylloporia* comenzó como un género monofilético; sin embargo, actualmente corresponde a un género paralifilético, con integrantes ampliamente diversos [77]. Morfológicamente, incluye especies con basidioma anuales o perennes; con hábitos resupinados, sésil o estipitado, con sistema hifal dimítico o monomítico, y basidiosporas elipsoides a subglobosas, raramente subcilíndricas, amarillentas, con paredes ligeramente engrosadas [77-80].

Se han descrito 56 especies pertenecientes a este género [77,79], las cuales presentan una distribución cosmopolita, abarcando principalmente las zonas tropicales [78,80]. De acuerdo con Ferreira [78], abarca especies con hábitos saprofíticos y parasíticos.

De acuerdo con Cheng *et al.* [81], las especies del género *Phylloporia* han sido empleadas como alimento antiinflamatorio en la medicina tradicional china; demás, sus metabolitos han demostrado poseer actividad antitumoral,

antioxidante, neuroprotectora e inmunomoduladora. El cuerpo fructífero de la especie *Phylloporia ribis* ha sido usado como ingrediente funcional en el tratamiento de faringitis, laringitis e hiperglicemia; y se ha demostrado que el micelio fermentado no presenta toxicidad al ser administrado en ratas como parte de su dieta en una dosis del 10% del alimento [82]. Y de acuerdo con Zhao *et al.* [11], el extracto de *P. ribis* contiene compuestos con capacidad antioxidante, tales como el aminoácido ergotioneína y el ácido cafeico, capaces de reducir el radical DPPH en un 69.78% y un 31.77%, respectivamente, cuando son aplicados en una concentración de 5 mg L^{-1}.

Phylloporia boldo Rajchenb. and Pildain es una especie descrita por Rajchenberg *et al.* [30], la palabra “boldo” hace referencia al nombre común del hospedero sobre el cual crece el hongo, la especie vegetal endémica de Chile, *Peumus boldus*. Hasta ahora, presenta una distribución geográfica restringida a la distribución de su hospedero [30]. Crece en los tallos vivos de *P. boldus*, y es causante de pudrición blanca [30,79]. Morfológicamente, el hongo *P. boldo* presenta basidioma perenne, sésil, pileado, piramidal, a veces ungulado, adherido al hospedero sólo por una pequeña porción del basidioma, basidiomas solitarios o poco imbricados, con sistema hifa dimítico, con hifas generativas septadas simples; las basidiosporas son elipsoides a subglobosas, de pared gruesa [30,79]. Riquelme *et al.* [32], evidenció la capacidad de *P. boldo* de biosintetizar compuestos derivados de hidroquinonas cloradas e hidroquinonas bromadas, planteándolo como una especie relevante en la industria farmacéutica; sin embargo, la bioactividad de sus metabolitos no ha sido evaluada aún.

Género *Nothophellinus*

Nothophellinus Rajchenb. y Pildain, gen. nov., es un nuevo género monotípico del sur de América del Sur [29]. La especie tipo *Nothophellinus*

andinopatagonicus (sinónimo *Phellinus andinopatagonicus*), se caracteriza por causar pudrición blanca en varias especies del género *Nothofagus*, presentes en el sur de Chile y Argentina; siendo un patógeno importante de la especie *Nothofagus pumilio* [29,83].

Morfológicamente se describen como hongos con basidiomas pileados, perennes, con una cutícula gruesa en el píleo, un contexto grueso que puede presentar cordones hifales blancos, con un sistema hifal de di a trimítico, con hifas generativas simples septadas e hifas setales ausentes [29]. Las basidiosporas son de cilíndricas a obclavadas, de pared delgada y acianófilas [29].

De acuerdo con Aqueveque *et al.* [31], el extracto total obtenido desde el cultivo micelial de *N. andinopatagonicus* presenta una importante actividad antimicrobiana contra bacterias de interés clínicos; sin embargo, otra actividad biológica para esta especie no ha sido descrita.

Género *Fomitiporia*

El género *Fomitiporia* (Hymenochaetaceae) fue descrito por Murrill en 1907, e incluyó 17 especies; las cuales, basados en la especie tipo *F. langloisii* Murrill (sinónimo *Fomitiporia punctata*), se describen como hongos con basidiomas perennes, resupinados, efuso-reflejados, pileados; con sistema hifal dimítico o seudodimítico; setas himeniales raras a ausentes, con contexto de color bronce a marrón; además, con esporas subglobosas a globosas, con paredes delgadas a gruesas, hialinas, dextrinoides y cianófilas [84,85].

Las especies de este género crecen sobre la madera de árboles gimnospermas o angiospermas, vivos o muertos, y son responsables de causar pudrición blanca [84]. Se distribuyen de manera cosmopolita en regiones tropicales y templadas [30,84].

Las especies del género *Fomitiporia* son hongos de gran importancia medicinal, presentando metabolitos bioactivos como triterpenoides, flavonoides, ergoesteroles, polisacáridos y otros componentes funcionales [33,34]. De acuerdo con Liu et al. [86], los extractos de la especie *F. punctata* han sido empleados para tratar enfermedades cardiacas y la angina de pecho. En cuanto a los polisacáridos obtenidos desde *F. punctata,* Liu *et al.* [86] evidenció que presentan una significativa actividad antioxidante contra el radical DPPH, mostrando una actividad del 51% con una concentración de 250 µg mL^{-1} de polisacárido. De acuerdo con Zan *et al.* [34], a partir de la especie *Fomitiporia ellipsoidea* B.K. Cui & Y.C. Dai se obtuvo un extracto anaranjado denominado Phelligridin K (8,9-dihydroxy-[4'-(7',8'-dihydroxyphenyl)-furanone]- 1H,6H-pyrano[4,3-c][2]-benzopyran-1,6-dione), el cual presenta una potente actividad antioxidante contra el radical catiónico ABTS, al nivel de 3.56 mM de Trolox, y fue propuesto como un posible antioxidante alimentario natural.

En Chile, Rajchenberg *et al.* [30] describieron una nueva especie de *Fomitiporia. Fomitiporia chilensis* Rajchenb. and Pildain fue descrito como un hongo que crece sobre tocones o ramas caidas de *Peumus boldus* y *Cryptocarya alba*, especies vegetales endémica y nativa del Chile, respectivamente; condicionando su distribución geográfica a la que presentan sus hospederos. Su desarrollo sobre el tejido de estos árboles provoca pudrición de madera de tipo blanca y fibrosa [30]. Actualmente no se ha descrito algún tipo de actividad biológica asociada a esta especie.

II.3. LOS POLISACÁRIDOS

Los polisacáridos son los polímeros naturales más abundantes en la tierra, ya que están presentes en animales, plantas, hongos y microorganismos [23,87]. Junto con los ácidos nucleicos, los lípidos y las proteínas, los polisacáridos son las macromoléculas que determinan la vida, y presentan funciones fisiológicas en

cada una de las especies [88,89]. La unidad básica que conforma a los polisacáridos corresponde a una azúcar simple o monosacárido; este monosacárido está dado por la fórmula química $C_n H_{2n} O_n$; en donde "*n*" corresponde a un número entre 3 y 10 [87,89]; siendo los más abundantes aquellos de 5 (furanosa) y 6 (piranosa) carbonos [88]. La unión de dos de estos monómeros por medio de uno de los cuatro tipos de enlaces glucosídicos (α-α'/ α-β'/ β-α'/ β-β') da origen a un disacárido [89]. Si los enlaces glicosídicos se producen entre 3-20 monosacáridos, se forma un oligosacárido [87]. Por su parte, los polisacáridos están formados por más de 20 hasta 60,000 monómeros unidos por enlaces glicosídicos; y podrían ser lineales o presentar ramificaciones, las cuales pueden ser cortas o ramificaciones largas con estructura arbustiva [87].

Según Di Donato *et al.* [23], los polisacáridos pueden clasificarse de acuerdo con sus características químicas y estructurales, o en base a su rol biológico. A partir de sus características estructurales, podemos clasificar los polisacáridos en heteroglicanos, cuya estructura está formadas con dos o más tipos de monosacáridos; o, en homoglicanos, con una estructura formada en base a un tipo de unidad monomérica [23]. También pueden clasificarse en base a su carga, como polisacáridos neutros o cargados, pudiendo ser catiónicos o aniónicos. En cuanto a su rol biológico, los polisacáridos pueden clasificarse en estructurales o polisacáridos de almacenamiento de energía [23].

A pesar de que las unidades monosacáridas pueden repetirse en diferentes polisacáridos, los diferentes tipos de enlaces y el grado de ramificación que presentan las diferentes cadenas que se forman, les otorgan las propiedades características a los polisacáridos [89]. De acuerdo con Maiti & Jana [87], los polisacáridos han mostrado un amplio rango de aplicaciones farmacéuticas, como propiedades antioxidantes, antiinflamatorio, anticancerígeno y anticoagulante. Esta actividad biológica depende principalmente de las características estructurales de los polisacáridos, como su composición química

(secuencia de monosacáridos, largo de la cadena y tipo de enlace) y los componentes asociados (proteínas o grupos sulfatos), y sus propiedades físicas (como el peso molecular y la solubilidad) [22,58,90-93]. Los polisacáridos no digeribles, especialmente los β-glucanos, son componentes bioactivos muy beneficiosos para la salud humana [22,93]. De acuerdo con Zhang *et al.* [94], los β-glucanos insolubles en agua obtenidos por extracción con álcali caliente desde los esclerocios de *Pleurotus tuber-regium* presentan una potente actividad antitumoral *in vitro* e *in vivo*, asociada a la activación del sistema inmune en complemento con una actividad citotóxica directa sobre las líneas celulares. De igual manera, se ha evidenciado mediante estudios clínicos que los β-glucanos presentan actividad inmunomoduladora y antitumoral, aplicable en tratamientos contra el cáncer, como fármacos adjuvantes [95].

Según Wang *et al.* [25], los polisacáridos ácidos son el grupo más grande de polisacáridos, los cuales contienen uno o más grupos carboxílicos, éster sulfúrico o grupos fosfatos; siendo los más comunes aquellos que presentan grupos carboxílicos de ácido urónico. Los grupos ácidos presentes en estos polisacáridos forman asociaciones con biomoléculas presentes en las células del hospedero; lo que hace presumir que tiene una mayor bioactividad que los polisacáridos neutros [25]. Chen *et al.* [96] demostró que la fracción ácidos de los polisacáridos del hongo *Cordyceps sinensis* estimulan la fagocitosis en células de macrófagos Raw 264.7, además de incrementar la proliferación de NO. Además, Wang *et al.* [25] determinó que los polisacáridos ácidos de *C. sinensis* aumentan también la producción de citoquinas proinflamatorias (TNF-α, IL-1β y IL-6) y antiinflamatoria (IL-10) cuando el polisacárido es aplicado en bajas concentraciones; sin embargo, al evaluar concentraciones mayores (250 μg mL^{-1}) sólo la producción de IL-10 fue estimulada.

II.3.a. Los polisacáridos fúngicos

La pared celular de los hongos es un específico y complejo organelo celular [97], está compuesto sobre todo por polisacáridos y proteínas; y las proteínas se encuentran mayormente asociadas a polisacáridos dando lugar a glicoproteínas [98]. Entre los polisacáridos que forman parte de la pared fúngica se encuentran principalmente la quitina, los glucanos y los galactomananos [98], pudiendo encontrarse también xilosa y fucosa [13,67].

Quitina: Es un carbohidrato natural sintetizado por la enzima quitin sintasa a partir de N-acetil glucosamina [87,98]. Corresponde al segundo polisacárido más abundante en la naturaleza [99]. La quitina se posiciona en el espacio próximo al citoplasma; y su porcentaje dentro de la pared del hongo depende del estado morfológico que este presenta, variando entre un 1% y un 30% del peso seco de la pared celular; presentándose en menor porcentaje en levaduras, mientras que en los hongos filamentosos alcanza los porcentajes mayores [97-99].

Glucanos: Corresponde al polisacárido estructural más importante en la pared del hongo; representando hasta el 60% del peso seco de la estructura [97,98]. Se compone de unidades de glucosa con enlaces β-1,3; β-1,6; β-1,4; α-1,3 y α-1,4 [13,98]; y puede encontrarse formando parte de la pared celular de levaduras, hongos y cereales [4]. El β-1,3-D-glucano es el componente estructural más importante en la pared de los hongos, es sintetizado en la membrana plasmática por un complejo de enzimas denominadas glucano sintasas [97]. De acuerdo con Brown & Gordon [100], los β-glucanos son un grupo heterogéneo, consistente en una cadena lineal de polímeros de glucosa unidos por enlaces β(1→3), enlazadas con cadenas laterales por enlaces β(1→6); capaces de formar estructuras terciarias complejas por medio de la unión de cadenas de glucanos a través de enlaces de hidrógeno. Diversos estudios han evidenciado las propiedades fisicoquímicas de los glucanos, demostrando que poseen beneficios

a la salud como reductores de colesterol en la sangre, un efecto protector contra infecciones, reductor de radicales libres y propiedad antitumoral [4].

Galactomananos: Son polisacáridos ubicados en la capa más externa de la pared celular. Se componen de residuos de α-(1→2) ó α-(1→6) manosilo y β-(1→5) ó β-(1→6) galactofuranosilo [101,102]. En hongos se han identificado dos tipos de galactomananos, el galactomanano de tipo fúngico (FTGM) y el galactomanano de tipo O-manosa (OMGM) [101]. El FTGM se encuentra en tres formas: soluble (el cual se libera al medio), asociado con otros polisacáridos de la pared celular por enlaces no covalentes, o anclado en la membrana celular por un glicosil fosfatidil inositol [101,102]. Por su parte, la estructura del OMGM presenta un β-(1→5)-galacto furano tetraosa unido al extremo terminal no reductor de la O-manosa con un residuo de serina o treonina como base del esqueleto [101].

CAPÍTULO III: HIPÓTESIS, OBJETIVO GENERAL Y OBJETIVOS ESPECÍFICOS

Hipótesis

1. Los polisacáridos ácidos obtenidos a partir del micelio de cuatro cepas fúngicas de la familia Hymenochaetaceae, del centro-sur de Chile, poseen actividad anticancerígena por medio de la captación de radicales libres, disminuyendo el estrés oxidativo.

2. Los polisacáridos ácidos obtenido desde cuatro cepas de hongos de la familia Hymenochaetaceae, presentan actividad citotóxica contra líneas celulares tumorales.

Objetivo general

Determinar el potencial y elucidar el posible mecanismo de acción de los polisacáridos ácidos de cuatro cepas de hongos chilenos de la familia Hymenochaetaceae, como agentes anticancerígenos directos.

Objetivos específicos

1. Obtener y caracterizar los polisacáridos ácidos de las cepas fúngicas en estudio.
2. Evaluar la actividad antioxidante de los extractos de polisacáridos de los cuatro hongos de la familia Hymenochaetaceae.
3. Determinar el potencial citotóxico de los polisacáridos ácidos de las cepas fúngicas, sobre líneas celulares tumorales.

CAPÍTULO IV: EVALUATION OF CYTOTOXIC EFFECT AGAINST TUMOUR CELLS OF THE ACIDIC POLYSACCHARIDES OF THE FUNGUS *Nothophellinus andinopatagonicus*

Verónica Albornoz, Virginia Casas-Arrojo, Fabián Figueroa, Víctor Hernández, Claudia Pérez, Mario Rajchenberg, Carlos T. Smith, José Becerra, Jaime R. Cabrera-Pardo, Víctor L. Campos, and Roberto T. Abdala-Díaz

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ABSTRACT

Fungal polysaccharides possess an important bioactive potential, including antioxidant and anticarcinogenic activity. The aim of this work was to determine the antioxidant activity and cytotoxicity against tumour and non-tumour cell lines acidic polysaccharides (NAAPs) of the fungus *Nothophellinus andinopatagonicus*. The effect of NAAPs on tumour cells lines was evaluated by MTT assay and flow cytometry. The analyses determined that glucose was the most abundant monomer and IR spectrum showed the typical peaks of β-glucans in the NAAPs. The cell viability assays revealed significant activity of NAAPs against HL-60, HCT-116 and MCF-7 tumour cell lines (IC_{50} = 767,16 µg mL^{-1}, 1256 µg mL^{-1} and 4241,7 µg mL^{-1}, respectively); but a much lower cytotoxicity against the non-tumour cell line HGF-1 (outside the range of the highest concentration tested (>10 mg mL^{-1})). NAAPs affected the cell cycle of HL-60 tumour cells, increasing the percentage of cells in the sub G1 phase and reducing it in the S/G2/M phases. Moreover, low concentrations of NAAPs also showed an effective cytotoxic activity against tumour cell lines while the non-tumour cell line was unaffected, maintaining a viability close to 100%. The

antioxidant activity of the highest NAAPs concentration tested was 6.24% and 4.63%, for DPPH and ABTS method, respectively.

1. INTRODUCTION

Cancer is a disease exhibiting the second-largest mortality rate at a worldwide level, being surpassed only by cardiovascular diseases [1,58,103]. The development of cancer is mainly associated with exposure to different carcinogens, which generate genetic alterations and the loss of normal regulatory processes, impeding the antitumoral immune response [104-105].

Free radicals produced by the cell's metabolism play an essential role in the regulation and expression of genes. An imbalance in their production induces irreversible oxidative damage resulting in various diseases, such as cardiovascular diseases, Alzheimer's disease, reduced immune function, muscle degeneration, and cancer [59,106]. The treatments used to control the development and progress of cancer cause secondary effects that deteriorate the patients 'quality of life and have elevated cost [1,7]. The search for new compounds capable of protecting tissues from the oxidative stress and/or having the capacity to eliminate or control cancer cells require all possible attention [59].

Polysaccharides are macromolecules showing an elevated bioactive potential and their activity is determined by their structure, composition, and size [18,21,22,74]. They can be obtained from plants, animals, fungi, and microorganisms [5,58,74]. Polysaccharides produced by fungi, mainly *Basidiomycetes*, have been extensively studied because they are one of the most abundant fungal cell components, and they have potential biomedical use as antioxidant, immunomodulator, anticoagulant, anticarcinogenic, hypoglycaemic, anti-inflammatory, antimicrobial, and cytotoxic agents [14,17,21,22,58,59,60,74,90,91].

Acidic polysaccharides constitute an important group of fungal polysaccharides and usually their bioactivity exceeds that of neutral polysaccharides [25]. Acid polysaccharides produced by *Tremella fuciformis*, have shown an important anticarcinogenic activity, inhibiting up to 91.7% the growth of sarcoma S180 cells in affected mice [26,107]. Many fungi have been used during centuries by different cultures as a source of foodstuff and medication [18,60,108], including many species belonging to the genus *Phellinus sensu lato* (*Hymenochaetaceae*; Div. *Basidiomycota*) [55,58,74,91].

The polysaccharides of certain species belonging to the genus *Phellinus s.l.* have potential pharmacological use as immunomodulators, activating cells of the immune system (such as T lymphocytes, B lymphocytes, natural killer (NK) cells, and macrophages); as anti-inflammatory compounds, inhibiting the production of pro-inflammatory cytokines; as anticarcinogens, slowing the growth of tumours and metastasis, and as antioxidants [5,14,20,25,60,74,108,109]. *Nothophellinus andinopatagonicus* (J.E. Wright & J.R. Deschamps) Rajchenb. & Pildain (Hymenochaetaceae) Syn. *Phellinus andinopatagonicus* (J.E. Wright & J.R. Deschamps) Ryvarden is a fungus endemic from southern South America, belonging to a monotypic genus. It has been found associated with several *Nothofagus* species, such as *N. antarctica, N. dombeyi* and *N. pumilio* and it is described as an important pathogen causing wood decay [27,29]. Aqueveque et al. [31] reported that total extracts obtained from mycelial cultures of this species showed an important antimicrobial activity against bacteria of clinical interest. However, no other biological activity has been reported for this species.

Therefore, considering, as mentioned above, the potential pharmacological uses of polysaccharides obtained from members of the *Phellinus* genus, including anticarcinogenic activity, and that some fungal acidic polysaccharides have shown better bioactivity than neutral of fungi, the aim of the present study was

to characterize the acidic fraction of the polysaccharides obtained from an *in vitro* culture of *N. andinopatagonicus* and to evaluate their possible antioxidant and antitumor potential against human colorectal carcinoma, mammary adenocarcinoma and leukaemia cell lines.

2. MATERIALS AND METHODS

2.1. Biological material

The strain of *Nothophellinus andinopatagonicus* used in this study was collected at Coyhaique (Aysén Region, Chile) (46°12'13.3"S 72°48'46.5"W) in 2016. It was identified by Dr. Mario Rajchenberg (Centro de Investigación y Extensión Forestal Andino Patagónico, Chubut, Argentina) and assigned the code FQ1645 of the Laboratory of Chemistry of Natural Products (University of Concepcion, Concepcion, Chile). It was maintained by culturing it, at 20±2°C, *in vitro* using YMG agar medium (5 g yeast extract (BD Biosciences, San José, USA), 10 g malt extract (BD Biosciences, San José, USA), 15 g glucose and 20 g agar per litter of medium). To improve the yield of mycelial mass, fragments of mycelium were transferred to liquid YMG medium (1% w/v glucose, 1% w/v malt extract, 0.4% w/v yeast extract) at pH 5.8 and incubated at 20 °C for 1 to 2 months under constant agitation (120 rpm). Finally, the mycelia were lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain).

2.2. Extraction of polysaccharides

The acidic polysaccharides were obtained from lyophilized mycelia of the FQ1645 strain according to Abdala-Díaz *et al*. [110] and Figueroa *et al*. [111], with minor modifications. Briefly, 100 g of lyophilized mycelia was cut and boiled in sterile distilled water for 1 h with constant agitation. After cooling, the mixture was filtered using sterile muslin cloth. An acidic polysaccharide selective precipitation was prepared adding a cold solution of 2% (w/v) n-cetylpyridinium

bromide (Cetavlon) (Merck, Darmstadt, Germany). Acidic polysaccharides were precipitated by centrifugation at 10000 g for 20 min. The pellet was purified using a 4 M NaCl solution and centrifuged at 4000 g for 15 min and the supernatant recovered. Polysaccharides were flocculated with 96% (v/v) ethanol (1:1 etanol:extract) and centrifuged at 4000 g for 20 min. Polysaccharides of the pellet were dialyzed using 2M NaCl solution and finally lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain). The *N. andinopatagonicus* acidic polysaccharides extracted will be henceforth referred as NAAPs.

2.3. Fourier-transform infrared spectroscopy

Infrared spectra were obtained using a Fourier transform infrared spectrophotometer (FT-IR spectra). To analyse the NAAPs, disks (16 mm diameter) were prepared with a polysaccharide and potassium bromide (KBr) (1% w/w) mixture, pressed at 15.0 T hydrostatic pressure for 5 min. Subsequently, the discs were measured using a Thermo Nicolet Avatar 360 IR spectrophotometer (Thermo Electron Inc., Waltham, USA) with a resolution of 4 cm^{-1}, a DTGS detector and the OMNIC 7.2 software (bandwidth 50 cm^{-1}, enhancement factor 2.6). The 4000–450 cm^{-1} region was analysed. Baseline adjustment was generated using the Nicolet OMNIC Software (Version 7.2, Thermo Nicolet, Waltham, USA) (bandwidth 50 cm^{-1}, enrichment factor 2.6), to flatten the baseline of each spectrum. The OMNIC correlation algorithm was used to compare the spectra of the sample with those of the spectra library (Thermo Fischer Scientific, Waltham, USA).

2.4. Characterization of NAAPs by HPLC-IR

NAAPs (100 mg) were subjected to acidic hydrolysis using H2SO4 (72% v/v), following the protocol described by Peredo *et al.* [112]. An aliquot of the hydrolysate was filtered (0,45µm sterile syringe filter) and the sample analysed by HPLC-IR (Young Lin YL Clarity 9000, Korea) with a 0.6 mL min^{-1} flow of the

mobile phase (5 mM H_2SO_4) and 20 µL injection in an Aminex HPX-87H column (300 mm x 7.8 mm), at a temperature of 35 °C. According to the retention times, the spectra obtained were compared to standards of glucose, xylose, arabinose, rhamnose and mannose (Sigma-Aldrich, San Luis, USA).

2.5. Characterization of NAAPs by Gas Chromatography-Mass Spectrometry (GC-MS)

Polysaccharides previously hydrolysed with H_2SO_4 (72% v/v) and then dried were acetylated using acetic anhydride, as described by Meng *et al*. [113], with modifications. The hydrolysed sample (100 mg) was dissolved in 1 mL pyridine anhydrous, and an excess of acetic anhydride was added to obtain the acetylated derivatives and the mixture was agitated during 6 h at room temperature. Once the derivatization was completed, the mixture was diluted with a NaCl solution, and extracted with dichloromethane. The organic phases obtained were combined and dried adding anhydrous sodium sulphate. The solvent was eliminated by distillation using a Heidolph rotavapor (Heidolph Instruments GmbH & CO. KG Schwabach, Germany). GC-MS analysis was conducted with an Agilent Technologies 7890A/5975C instrument, using a HP-5MS capillary column (30m × 0.250 mm × 0.25 µm). The initial column temperature was kept at 70 °C for 4 min, increased to 200 °C at 3 °C min^{-1}, then increased to 300 °C at 10 °C min^{-1}, and finally, the temperature was maintained for 5 min. The ionization potential was 70 eV and the temperature of the ion source was 280 °C. Peak assignments were made based on retention times and mass spectra with the aid of a NIST17 mass spectral library.

2.6. Cell lines

HCT-116 human colorectal carcinoma, MCF-7 human mammary adenocarcinoma, HL-60 human leukaemia cells and non-tumoral HGF-1 human gingival biopsy cells (all from ATCC, Manassas, Virginia, USA), were used in

this study. The HCT-116, MCF-7 and HGF-1 cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Biowest, Nuaillé, France) supplemented with 10% fetal calf serum (FCS) (Biowest, South America Origin, S1810), 1% penicillin-streptomycin solution 100X (Biowest, Nuaillé, France) and 0.5% amphotericin B (Biowest, Nuaillé, France). HL-60 cells were cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 20% FCS, 1% 100X penicillin-streptomycin solution and 0.5% amphotericin B. Cells were kept sub-confluent at 37 °C in the presence of 5% CO_2. Adherent cells were collected when the confluence reached 75%. Suspended cells were collected by centrifugation at 600g during 5 min [110].

2.7. Anticarcinogenic activity of NAAPs

To assay the antitumoral effect of NAAPs, HL-60, HCT-116 and MCF-7 tumour cells were independently incubated with serial dilutions of NAAPs including 10, 5, 2.5, 1.25, 0.625 or 0.3125 mg mL^{-1}. As control of cytotoxicity of NAAPs on a non-tumour cell line, HGF-1 cells were subjected to the same concentrations of NAAPs mentioned above. A total of $1x10^4$ MCF-7 or HL-60 cells, $4x10^3$ HCT-116 cells or $5x10^3$ HGF-1 cells in 100 µL of the same culture media described in the previous section (including FCS and antibiotics) were placed in wells of a 96 wells microplate and subjected to the different concentrations of NAAPs during 72 h at 37 °C and 5% CO_2. Proliferation of the cell lines was evaluated by adding 10 µL of MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, San Luis, USA) solution (5 mg mL^{-1} MTT in phosphate buffered saline (PBS) pH 7.5) per well [114]. Microplates were incubated at 37°C for 4 h and the insoluble purple formazan crystals formed were dissolved by addition of sulphated isopropanol (150 µL 0.04 N HCl in 2-propanol) and measured spectrophotometrically at 550 nm (Micro Plate Reader 2001, Whittaker Bioproducts, Dauphin, USA). Finally, the relative cell viability was expressed as the mean percentage of viable cells compared to untreated cells. The same cell

lines not exposed to NAAPs were used as positive controls. This experiment was made in triplicate, inoculating three wells of the microplate per variable analysed.

2.8. Cell cycle analysis by flow cytometry

The tumour cell line showing the greatest susceptibility to the cytotoxic effect of NAAPs was selected for this assay. Changes in the progression of the cell cycle of the selected cell line were analysed by flow cytometry, following the procedure of Afrin and co-workers [115] with modifications. A volume of 1.5 mL containing $5x10^5$ cells were placed in each well of 6 wells microplates and incubated at 37 °C in the presence of 5% CO_2 until reaching sub-confluence. Then, different NAAPs concentrations were added in fresh culture media. Three concentrations of NAAPs, the first one corresponding to the half maximal inhibitory concentration (IC_{50}), the second one equal to 4X IC_{50} and the third one equal to ¼ IC_{50}, were used. After incubating overnight in the presence of NAAPS, cells were harvested, centrifuged and the resulting pellets washed using PBS (pH 7.5) and fixed with 70% ethanol for 1 h, at -20 °C. Finally, cells were centrifuged at 600g during 5 min and washed twice using PBS before been suspended in the staining solution containing 40 µg mL^{-1} propidium iodide (Sigma-Aldrich, San Luis, USA) and 0.1 mg mL^{-1} RNase-A (Sigma-Aldrich, R6513) in PBS. This mixture was incubated for 30 min at 37°C in darkness. Samples were analysed in a FACS VERSETM (BD Biosciences, San José, USA) flow cytometer and the results analysed using the Kaluza analysis software version 2.1 (Beckman Coulter, Brea, USA). A 2-methoxyestradiol (20µM) (Sigma-Aldrich, San Luis, USA) solution was used as positive control for changes in the cell cycle.

2.9. Antioxidant activity of NAAPs (DPPH·)

The antioxidant activity of NAAPs was evaluated following the procedure of Brand-Williams *et al*. [116], with modifications. The previously prepared DPPH

(2,2-diphenyl -1-picryl-hydrazyl-hydrate) (Sigma-Aldrich, San Luis, USA) radical was adjusted to an absorbance of 0.3 at 517nm. Serial dilutions of the NAAPs sample were prepared from a 40 mg mL^{-1} stock solution in sterile distilled water. Mixtures of 180 µL DPPH radical plus 20 µL of each respective concentration of NAAPs were prepared in a 96 wells microplate. Samples were incubated for 30 min in darkness and the absorbance read at 517nm using a microplate spectrophotometer (Epoch BioTek (BioTek Instruments Inc., Winooski, VT, USA)). A control including 180 µL DPPH radical plus 20 µL of sterile distilled water was used.

The antioxidant activity was calculated using the following equation and it was expressed in Trolox (µg mL^{-1}) (Thermo Fisher Scientific, Loughborough, United Kingdom) equivalents in accordance with a standard curve (concentrations from 0 to 100 µg mL^{-1} Trolox),

AA% = ((Abs (control) – Abs (sample)) / Abs (control)) x 100

Where, AA% is the percentage of antioxidant activity, Abs (control) is the absorbance of the DPPH radical plus water and Abs (sample) is the absorbance of the DPPH radical plus the different concentrations of NAAPs used.

The antioxidant activity was also determined by the ABTS method, as described by Re *et al*. [117] with some modifications. The ABTS (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, San Luis, USA) solution was adjusted to an absorbance of 0.7 at 725 nm. The antioxidant activity of the different concentrations of NAAPs was determined by adding 190 µL ABTS solution plus 10µL of NAAPs in wells of a 96 wells microplate at the respective concentration. A control containing 190 µL ABTS plus 10 µL sterile distilled water was used. The mixture was incubated for 8 min at room temperature and its absorbance measured at 725 nm using a microplate spectrophotometer (Epoch BioTek (BioTek Instruments Inc., Winooski, VT, USA)).

The percentage of antioxidant activity of the different NAAPs concentrations was calculated using the following equation and expressed in Trolox (µg mL^{-1}) equivalents in accordance with a standard curve (concentrations from 0 to 100 µg mL^{-1} Trolox)

AA% = ((Abs (control) – Abs (sample)) / Abs (control)) x 100

Where, AA% is the percentage of antioxidant activity, Abs (control) is the absorbance of the ABTS plus water and Abs (sample) is the absorbance of the DPPH radical plus the different concentrations of NAAPs.

2.10. Statistical analysis

All results were expressed as mean ± standard deviation of three experiments (n=3). One-way analysis of variance (ANOVA) with post-hoc Tukey HSD test was used to compare the differences of cell viability and antioxidant activity. Previous to these analyses, normality and homogeneity of variance assumptions were evaluated. Differences were considered as significant when $p<0.05$. All analyses were done using the Statistica 12.0 software (TIBCO Data Science, Palo Alto, USA). Graphs were obtained using the SigmaPlot software version 12.0, 2015 (Systat Software Inc., Chicago, USA).

3. RESULTS

3.1. NAAPs yield

The extraction of NAAPs from *N. andinopatagonicus* using a selective precipitation method with Cetavlon from the mycelial mass in aqueous solution yielded 20.22 mg of NAAPs per g of wet mycelia, corresponding to 20% of dry mycelia.

3.2. Infrared analysis

The IR spectrum of the polysaccharides showed absorption peaks characteristic for polysaccharides` compounds (Figure 4.1). In particular, a wide and strong peak was present at 3445.67 cm^{-1} associated to the stretching produced by O-H bonds and another small peak at approximately 2922 cm^{-1} attributed to the stretching vibration produced by C-H bonds. On the other hand, the contraction vibration detected at 1637.96 cm^{-1} can be ascribed to the C=O bond of carboxyl groups. The small peak at approximately 1431 cm^{-1} was attributed to the bending contraction of the C-H bond. The presence of a vibration at 1250 cm^{-1} allowed the assumption sulphate groups, while the absence of a peak at nearly 1730 cm^{-1} indicated the absence of uronic acid in the NAAPs. Furthermore, a peak between 1200 and 1000 cm^{-1} is characteristic of the presence of β-glucans, whose vibration can be attributed to the presence of β bonds in their glycosidic chain, as well as the presence of pyranose rings [18,21,74].

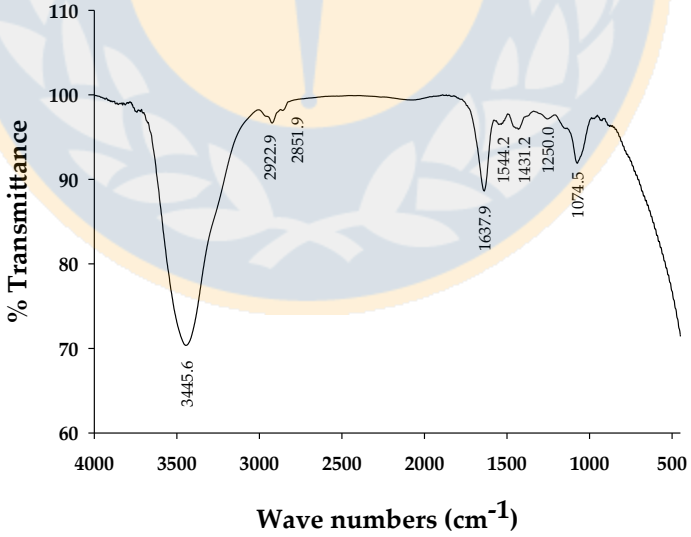

Figure 4.1 Fourier-transform infrared spectroscopy (FT-IR) of the acidic polysaccharides obtained from *N. andinopatagonicus* FQ1645 strain.

3.3. Characterization of monosaccharides of NAAPs

The retention times obtained by HPLC-IR analysis of a sample of NAAPs allowed the identification of at least three monosaccharides: arabinose, xylose, and a derivative of glucose (Table 4.1), the last being the one present in the highest percentage. On the other hand, the GC-MS analysis allowed the elucidation of the presence of acetylated monomers in the NAAPs, which, according to their respective retention times, included arabinose, mannose, D-glucose, mannitol, myo-inositol and trehalose (Table 4.2).

Table 4.1 Monosaccharides identified in the acidic polysaccharides obtained from *Nothophellinus andinopatagonicus* FQ1645 strain by HPLC-IR analysis.

Compound	**Retention Time (min)**	**Amount (%)**
Glucose derivate	7.675	49.3
Xylose	9.342	30.7
Arabinose	10.225	20.0

Table 4.2 Acetylated derivatives of saccharides present in acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain determined by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Compound	**Retention Time (min)**	**Amount (%)**
Methyl-2,3,5-tri-O-acetyl-L-arabinofuranoside	12.40	0.27
α-D-Mannopyranoside-methyl tetraacetate	15.04	0.77
D-Glucose, 2,3,4,5,6-pentaacetate	15.89	7.42
Mannitol hexaacetate	16.78	0.51
Trehalose octaacetate	25.78	70.03

3.4. Effect of NAAPs on the cell viability of tumour cell lines

This assay evaluated the effect of the tested NAAPs concentrations on the cell viability of the three human tumour cells lines (HL-60 human leukaemia, HCT-116 human colorectal carcinoma and MCF-7 human mammary adenocarcinoma) and on the non-tumour cell line (HGF-1 human gingival biopsy), which was used as control. The results demonstrated that the cytotoxic activity of NAAPs on the tumour cell lines increased, observed as reduced survival percentages, when the cells were exposed to higher NAAPs concentrations (Figure 4.2 A, B and C). The highest NAAPs concentration evaluated (10 mg mL^{-1}) resulted in a survival of 4.94±0.8% for HL-60 cells (Figure 4.2 A), 5.30±1.45% for HCT-116 cells (Figure 4.2 B) and 15.55±1.26% for MCF-7 cells (Figure 4.2 C). When testing the 0.312 mg mL^{-1} concentration of NAAPs, the lower concentration evaluated, the survival of tumour cells achieved 74,3±3.5% for HL-60 cells, 74.5±0.4% for HCT-116 cells and 69.7±1% for MCF-7 cells (Figure 4.2).

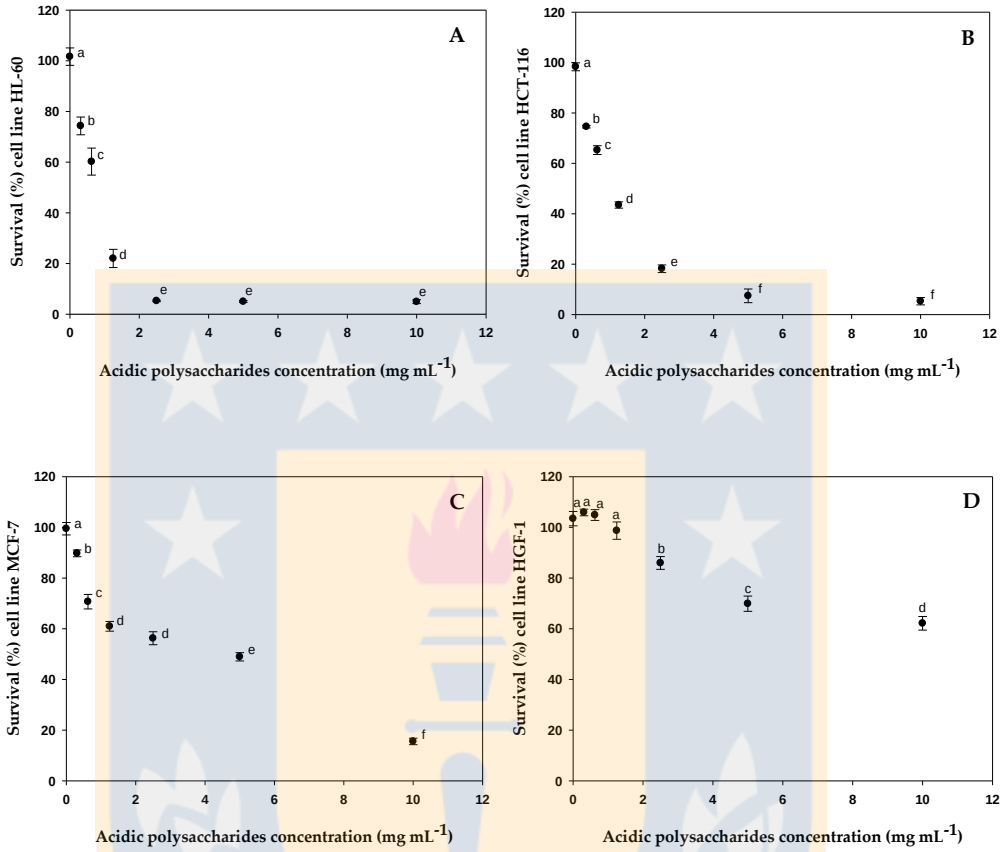

Figure 4.2 Cell survival (%) of cell lines exposed to 10, 5, 2.5, 1.25, 0.625, 0.3125 mg mL^{-1} of acidic polysaccharides of *Nothophellinus andinopatagonicus* (NAAPs) FQ1645 strain. (**A**) Survival of tumour cell line HL-60; (**B**) Survival of tumour cell line HCT-116; (**C**) Survival of tumour cell line MCF-7; (**D**) Survival of non-tumour cell line HGF-1. A same letter indicates that there are no significant differences between the different concentrations (Tukey, $p<0.05$).

With all the concentrations of NAAPs tested, including the lowest one (0.312 mg mL^{-1}), none of the three human tumour cell lines reached survivals over 75%. When comparing the above-mentioned figures obtained for the tumour cell lines with those of the non-tumour HGF-1 cell line, it was possible to observe that the cytotoxic effect of NAAPs affected to a lesser degree the non-tumour cell line (Figure 4.2 D), which can be considered an encouraging result. The viability of

the HGF-1 cell line in the presence of the highest NAAPs concentration tested (10 mg mL^{-1}) was 62.15±2.6% (equivalent to a mortality of 37.85±2.6%), a cell survival four times better than that of the tumour cell line MCF-7 (viability of 15.55±1.26%) when exposed to the same NAAPs concentration, the one showing the best survival of the three tumour lines tested. Regarding the viability of the non-tumour cell line HGF-1 when exposed to the lower NAAPs concentrations assayed, the viability of this cell line was approximately 100% in the presence of the three lower NAAPs concentrations (0.312, 0.625 and 1.25 mg mL^{-1}), a viability not significantly different to that in the absence of NAAPs. On the contrary, at the same low concentrations of NAAPs not affecting the viability of the non-tumour cell line, the viability of tumour cell lines was significantly reduced, ranging approximately from 20% to 60%. The actual viability figures for the tumour cell lines were 22.00±3.5% for HL-60 cells, 43.47±1.2% for HCT-116 cells and 60.95±1.9% for MCF-7 cells. The IC_{50} figures for the NAAPs isolated from *N. andinopatagonicus* FQ1645 strain were 767.16 µg mL^{-1} for HL-60 cells, 1256 µg mL^{-1} for HCT-116 cells and 4241.7 µg mL^{-1} for MCF-7 cells, while the IC_{50} for the non-tumour cell line HGF-1 could not be calculated because it is outside the range of highest concentration used (>10 mg/mL^{-1}). Therefore, NAAPs were capable to reduce the survival of tumour cell lines to a much greater extent than the non-tumour cell line.

3.5. Effect of NAAPs on the cell cycle of tumour cells

HL-60 cells were treated with different concentrations of de NAAPs. For positive control, 2-methoxyestradiol (20 µM) was used. The different NAAPs concentrations showed dose dependent responses. The highest NAAPs concentrations tested (3068.6 µg mL^{-1}) showed an increase of cells in apoptotic phase (sub. G1) of 46.30±1,99%, exceeding the negative control (sub G1 13,49±0,87%) by 32,81%. Regarding cells in the G0/G1 phase, there was a significant decrease from 54.45±1.01% for untreated cells to 28.87±1.82% for

cells subjected to the highest NAAPs concentration. Similarly, the percentage of cells in the S/G2/M phases was reduced to a 24.83±0,17% in NAAPs treated cells as compared to 32.39±1.88% in the negative control (untreated cells) (Figure 4.3 A and C). Lower NAAPs concentrations caused non-significant differences in the distribution of cells in the different phases of the cell cycle when compared to the untreated cells (data not shown). On the other hand, 20 µM 2-methoxyestradiol caused, as expected, an increased number of cells in phases S/G2/M, reaching 45.65±3.35%; while figures for phases G0/G1 and sub G1 were 30.51±0.17% and 23.84±3.19% respectively (Figure 4.3 B).

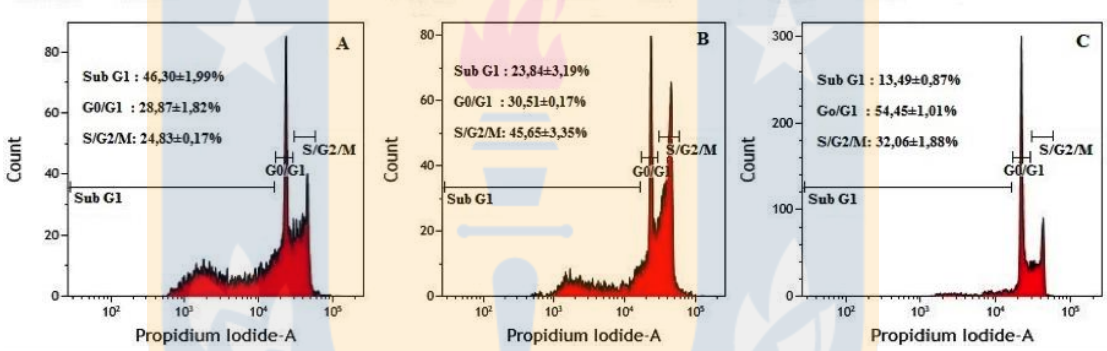

Figure 4.3 Subpopulations of tumour HL-60 cells at different cell cycle phases after 16 h treatment with (**A**) four-fold the IC_{50} concentration of acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain. (**B**) For the positive control 2-methoxyestradiol was used. (**C**) For the negative control the cells with RPMI-1640 was used. The percentages are the mean ± SD of three independent experiments.

3.6. Antioxidant activity of NAAPs

Results shown in Figure 4.4 demonstrated that the bioactivity of NAAPs against DPPH and ABTS radicals was directly related to the concentration of the NAAPs tested. The assays performed indicated that only the higher concentrations of NAAPs evaluated (20 and 40 mg mL^{-1}) exhibited a significant antioxidant activity. The results obtained for the highest NAAPs concentration tested (40 mg mL^{-1}) showed that, for the DPPH radical method, the figure was 6.24% while the ABTS

radical method only showed an antioxidant activity at the same NAAPs concentration of 4.63%.

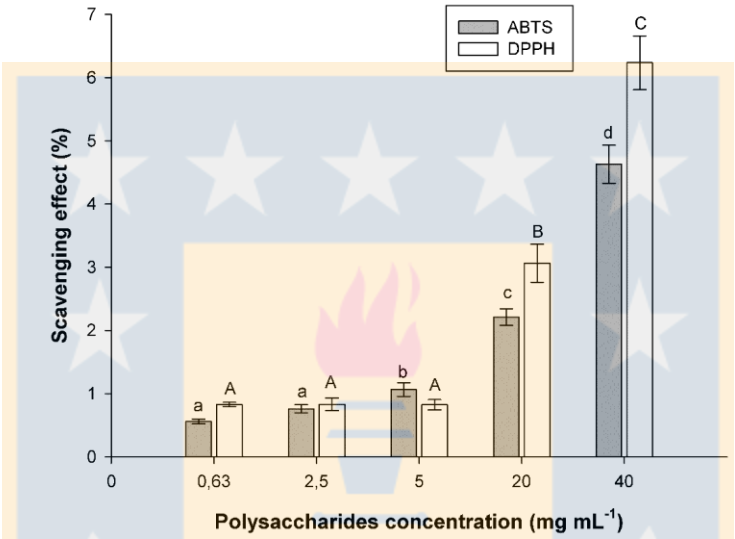

Figure 4.4 Scavenging effects (%) of acidic polysaccharides of *Nothophellinus andinopatagonicus* FQ1645 strain on ABTS and DPPH radical. The data are the mean of 3 replicates measurements ± standard error. Similar letters indicate that there are no significant differences between the different concentrations.

4. DISCUSSION

Polysaccharides are among the most important metabolites produced by representatives of genus *Phellinus s.l..* So far, a number of polysaccharides having anticarcinogenic activity have been isolated from the fruiting body and from mycelial cultures [58,67,92]. Nevertheless, besides the antimicrobial activity reported by Aqueveque *et al.* [31] for the total extract of by *Nothophellinus andinopatagonicus*, there are no reports regarding their

biological activity, either as total polysaccharides or some of their fractions, against any tumoral cell line or of any other cell type.

The biological activity of polysaccharides depends mostly on their structural characteristics, such as chemical composition and associated components (i.e. proteins or sulphate groups), and their physical properties (i.e. solubility and molecular weight) [22,90-93]. The IR spectra of the NAAPs isolated from *N. andinopatagonicus* FQ1645 strain included sulphate groups and β-glucans. Fungal β-glucans are polymers constituted by D-glucopyranosyl residues linked by β-1,3 and β-1,6 glycosidic bonds [94]. Several studies have demonstrated that β-glucans possess bioactivity, including antitumoral, immunomodulatory and anti-inflammatory activities, which are directly related to the length of the glucan chain and its interaction with functional groups [93,95]. Polysaccharides having sulphate groups in their composition considerably increase the anticarcinogenic activity of these polymers because they facilitate the interaction of the polysaccharide with the surface of tumour cells, reducing the IC_{50} when compared to non-sulphated glucan chains or achieving a synergistic effect with anticarcinogenic compounds against breast cancer (MCF-7), lung cancer (NCI-H460), colon cancer (HT-29) and leukaemia (CEM) cell lines [94,118].

According to Ferreira *et al*. [119], the polysaccharides constituting the cell wall of fungi can possess a variety of at least eleven monosaccharides, with D-glucose, N-acetylglucosamine and D-mannose the most frequently found [120]. The remaining monosaccharides, such as xylose, galactose, fucose, arabinose, ribose and rhamnose, can be found in lesser amounts. The HPLC-IR and GC-MS results showed that the NAAPs are heteroglycans constituted by at least glucose, arabinose, mannose and xylose, being D-glucose the most abundant monomers present in the sample. In fact, D-glucose is the main component of the polymers present in the cell wall of fungi [121,122]. On the other hand, the presence of myo-inositol and mannitol in the GC-MS spectrum may be explained

because these poly-alcohols are present in the cell membrane of fungi or serve as storage of carbohydrates, respectively [121,122]. The GC-MS analysis allowed the identification of trehalose as the main component of the NAAPs. This glucose dimer (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is synthetized by numerous organisms and serves as a cell membrane protecting agent against freezing. In fungi, it is involved in carbon translocation from mycelia to fruiting bodies [122-124].

The cell viability assays showed that the NAAPs significantly reduced the viability of the three tumour cell lines we assayed, reducing the survival of these cells up to more than 90% when subjected to the highest NAAPs concentration tested (10 mg mL^{-1}). On the other hand, the viability of the non-tumour cell line was reduced by only 37.85% when exposed to the same NAAPs concentration (10 mg mL^{-1}) and at lower NAAPs concentrations the viability of the non-tumour cell line reached survival figures close to 100%. A mortality close to 0% of the non-tumour cell line in the presence of NAAPs concentrations up to 1.25 mg mL^{-1} while the tumour cell lines showed significant mortalities at the same NAAPs concentrations probably deserves further analysis in the future. Results of cell viability assays testing polysaccharides obtained from other species of *Phellinus s.l*. reported mortality rates of tumour cell lines in the order of 90-96% using concentrations under 200 µg mL^{-1} [74,91,109]. Nevertheless, most studies evaluated the bioactivity of crude polysaccharides; thus, not allowing a fair comparison with NAAPs. Analysis of the IC_{50} values evidenced that the cytotoxic bioactivity of NAAPs is dependent on the tumour cell line tested. The NAAPs IC_{50} values for the tumour cell lines HCT-116 and MCF-7 were two and six times, respectively, that of cell line HL-60. The molecular weight of the polysaccharides [103] plus particular characteristics of the cell lines evaluated, such as their adherence, cell morphology and the expression and/or mutation of specific oncogenes [125-127]; could be responsible for the difference in IC_{50} observed in cell lines HCT-116 y MCF-7, when compared to cell line HL-60.

In accordance with Yan *et al.* [58], the anticarcinogenic activity associated to fungal polysaccharides, particularly those obtained from *Phellinus s.l*. species, can be classified on the basis of their mechanism of action. It can be preventive (avoiding the development of oncogenes), inducing soluble mediators and activating the cells of the immune system; or by the direct activity on tumour cells, causing their apoptosis or inhibiting their metastasis. The results obtained by flow cytometry showed that leukaemia HL-60 cells treated with NAAPs increased the events in Sub. G1 phase and decreased the events in cell growth and division phases. It is possible that the mechanism of action of NAAPs causing a high "mortality" rate of these tumour cells could be associated to a cytotoxic event, resulting in a decrease of cells capable to divide by mitosis. The alteration of the cell cycle is one of the causes of carcinogenesis, which induces cell proliferation; thus, mechanisms effecting cell viability may play a key role in the control of cancer progression [115]. Kudo *et al.* [123] and Allavena *et al*. [124] reported that trehalose possesses antiproliferative activity against several human tumour cell lines, which is attributed to a blocking effect on phases G2/M of the cell cycle and activation of apoptosis. Since one of the main components obtained from our *N. andinopatagonicus* culture was trehalose, the same antiproliferative activities reported by those authors may be present in our NAAPs. Moreover, previous studies reported that polysaccharides of *Phellinus s.l*. species can induce the cell cycle arrest of tumour cell lines at different stages of the cycle and/or induce their apoptosis [22,58]. One example is polysaccharides obtained from *Phellinus baumii* and *Phellinus linteus*, which are capable of suppressing proliferation and stimulating apoptosis of murine melanoma cells and induce apoptosis of sarcoma (S-180) cells [74,109]. The anticarcinogenic activity of these fungi is mostly due to a combination of mechanisms acting directly or indirectly on tumour cells. Polysaccharides containing glucose or mannose could show immunomodulator activity besides having direct antitumoral activity because human innate immunity cells have

receptors highly specific for these two monomers [58,95,116,128]. Therefore, it is possible to suggest that *N. andinopatagonicus* polysaccharides may induce a cell mediated anticarcinogenic activity in addition to reducing the viability of tumour cell lines observed in our *in vitro* assays. Future analyses may contribute to bring light into thus issue.

Oxidative stress has been identified as a crucial factor of oncogenesis proliferation. This idea is based on the finding that carcinogenic cells have greater amounts of reactive oxygen species (ROS) when compared to healthy cells and that ROS are responsible for maintaining the carcinogenic phenotype overexpressing certain genes [21,106,129]. According to both methods used in the present study (DPPH and ABTS) the NAAPs obtained from *N. andinopatagonicus* showed significant antioxidant activity only at the highest concentrations tested (40 and 20 mg mL^{-1}) inhibiting less than 10% of the radical, equivalent to <7.3 µg mL^{-1} Trolox for the DPPH assay and <16 µg mL^{-1} Trolox for the ABTS assay. These concentrations are very high when compared with polysaccharides previously studied from other species of *Phellinus s.l*. genus. Those studies demonstrated a strong capacity to capture free radicals (i.e. being potent antioxidant agents), even at low concentrations (IC_{50} in the order of 2-5 mg mL^{-1}), when assayed using DPPH and ABTS assays, the same methods used in this study [5,59,130]. According to Wang *et al*. [131], several studies postulate that proteins or other compounds (such as phenolic compounds) present in crude extracts of fungal polysaccharides could be responsible for trapping free radicals, making crude extracts better antioxidants than purified fractions. Thus, the low free radicals trapping activity exerted by NAAPs of the strain studied could be the consequence of its purification process.

5. CONCLUSIONS

The present study constitutes the first report regarding the biological activity of the acidic fraction of the polysaccharides produced by *N. andinopatagonicus*. On the basis of the results provided by different assays, it is possible to conclude that NAAPs possess anticarcinogenic activity, reducing the cell viability of leukaemia (HL-60), colon (HCT-116) and breast (MCF-7) tumour cell lines. Moreover, NAAPs were shown to be less cytotoxic to the non-tumour cell line (HGF-1) than for the three human tumour cell lines (HL-60, HCT-116 and MCF-7), a result encouraging further studies on the bioactivity of the acidic polysaccharides produced by *N. andinopatagonicus*.

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CAPÍTULO V: *In vitro* CYTOTOXIC CAPACITY AGAINST TUMOUR CELL LINES AND ANTIOXIDANT ACTIVITY OF ACIDIC POLYSACCHARIDES ISOLATED FROM THE ANDEAN PATAGONIAN FUNGUS *Phylloporia boldo*.

Verónica Albornoz, Virginia Casas-Arrojo, Fabián Figueroa, Víctor Hernández, Claudia Pérez, Mario Rajchenberg, Carlos T. Smith, José Becerra, Víctor L. Campos, Roberto T. Abdala-Díaz and Jaime R. Cabrera-Pardo

Natural Product Research (enviado) (2022)

ABSTRACT

Phylloporia boldo, a basidiomycete fungus, grows associated to the Chilean endemic species *Peumus boldus*. Fungal polysaccharides possess a broad biological activity, including cytotoxic, immunomodulating and antioxidant activities. This work aimed to evaluate the cytotoxic and antioxidant activity of the acidic polysaccharides of two *P. boldo* strains (named PBAP26 and PBAP40). The cytotoxic activity of PBAP26 and PBAP40 were evaluated determining the viability of tumour cell lines (HCT-116, MCF-7 and HL-60) by the MTT assay and by flow cytometry and their antioxidant activity was evaluated using the DPPH and ABTS assays. The most active acidic polysaccharide was characterized by FT-IR. PBAP40 was more cytotoxic than PBAP16 for the tumour cell lines evaluated. Results also suggest that *P. boldo* acidic polysaccharides arrested tumour cells in the cell cycle Sub G1 phase. The acidic polysaccharides of both strains were not cytotoxic for the non-tumour HGF-1 cell line. PBAP40 also showed a better antioxidant activity than PBAP26, reducing a 24.54% of the free radical DPPH. The FT-IR analysis of PBAP40 detected the presence of glucans having α- and β- type glycosidic bonds, perhaps being the latter, the main mediators of the bioactivity shown by the assays performed.

1. INTRODUCTION

Fungi are organisms which produce a large variety of metabolites, many of them having relevant biological activity [11]. Fungal-derived natural products are pharmaceutically prolific, having been developed into several important applications in medicine ranging from highly potent toxins to approved drugs [56]. Polysaccharides are chain polymeric carbohydrates found in the three domains of life and play a central role on cell-cell interactions [132] and cell architecture. In both prokaryotic and eukaryotic microbial pathogens, polysaccharides are a major cell wall component critical for host–pathogen interactions. Moreover, these carbohydrate polymers are also involved in both pathogenic mechanisms as well as immune responses during fungal infections [133]. As opposed to nucleotides and amino acids that only have one binding hotspot, monosaccharides can bind to each other at different points, leading to a range of combinations for branched and linear polysaccharides [134]. Thus, due to their chemical plasticity, polysaccharides show high levels of structural versatility that enable them to display a range of pharmaceutically relevant attributes. Recently, fungal polysaccharides have captured the attention of the scientific community due to their remarkable biological activities including immunomodulating, antioxidant, anticarcinogenic, antimicrobial and cytotoxic abilities [21,22,58,67].

Polysaccharides can be classified depending on their chemical structure or biological role. Based on structural criteria, polysaccharides can be categorized as: homoglycans, heteroglycans, linear and branched. They can also be neutral or charged (cationic or anionic) [23]. Several studies have found that acidic extraction allowed for more efficient cleavage of glycoside bonds, yielding bioactive low-molecular-weight polysaccharides in a high rate [24]. According to Wang *et al*. [25], the acidic fraction of fungal polysaccharides constitutes a relevant group of bioactive molecules. Their biological activity may be stronger

than the one shown by neutral polysaccharides, due to the acidic groups that can form associations with target biomolecules. As an example, the fucose containing acidic polysaccharides from the submerged fermentation of *Agaricus blazei* Murill could induce secretion of TNF-α from RAW264.7 macrophages [135]. Moreover, the acid polysaccharide fraction extracted from *Cordyceps sinensis* displayed remarkable modulating effects on murine macrophage cell line RAW264.7 (stimulation of phagocytosis, NO production and secretion of cytokines) [25,96]. Additionally, citric acid extracted *Laminaria japonica* polysaccharide (LJP-CA) showed high antioxidant activity with remarkable superoxide and hydroxyl radical scavenging abilities [24].

Polypore fungi of the Hymenochaetaceae Donk family (Hymenochaetales, Basidiomycota) comprise a group known to include several forest pathogens [29,30] as well as species with a medicinal potential which have been used in oriental traditional medicine for a long time [18,57,66,136]. Polysaccharides obtained from species belonging the genus *Phellinus* have demonstrated a high antioxidant capacity, capturing superoxide, hydroxyl, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) type radicals [14,18-20]. In addition, polysaccharides obtained from *Inonotus obliquus* and *Phellinus linteus* were shown to inhibit the growth of SGC-7901 human gastric cancer cells and murine Sarcoma-180 cells, respectively [16,17]. Furthermore, polysaccharides extracted from *Phellinus igniarius* showed cytotoxic activity against cancer cell lines HepG2 (human hepatocellular carcinoma) and SW480 (human colon adenocarcinoma), reducing their cell viability up to 39% and >50%, respectively [67]. The genus *Phylloporia* Murril (1904) presently includes a large variety of polypore fungi with a worldwide distribution, whose species are mainly parasitic or saprophytic [77,137]. The presence of bioactivity in metabolites produced by this genus, represented by the species *Phylloporia ribi*, has been previously reported. According to Lu *et al*. [82], a chemical analysis of extracts obtained from this species revealed the

presence of bioactive compounds, such as β-glucans. *Phylloporia ribi* glucans can improve the immune response of mice due to a reduction of the oxidative stress and showed to be non-toxic when orally administered [82].

Andean Patagonian ecosystems in the south of Chile represent one of the few undisturbed ecosystems in the world with little human intervention. These environments have unique ecosystems, promoting high levels of endemism [138]. Mycological and chemical studies in Andean Patagonian forests have been limited mainly due to challenges in accessibility and harsh weather conditions [139]. One of our research programs is focused on exploring new fungal strains and molecules with relevant biological activities in Andean-Patagonian ecosystems. *Phylloporia boldo* Rajchenb & Pildain (Hymenochaetaceae) is a basidiomycete fungus that has been recently described in the south of Chile [30]. This fungus is responsible for causing white rot in infected trees and was found associated to the stem of the endemic Chilean species *Peumus boldus* Molina (1782) (common name “boldo”) (Monimiaceae, Laurales). Studies about natural products in *P. boldo* are very limited [32]. To the best of our knowledge, there are no studies reporting the biological activity of polysaccharides isolated from *P. boldo.* In this study, we purified acidic polysaccharides from two strains of *P. boldo* collected at the Bio Bío region, Chile. These polysaccharides were evaluated to determine their cytotoxic activities against three human tumour cell lines and the effect on the cell cycle. We also evaluated their antioxidant capacity by means of DPPH and ABTS.

2. MATERIALS AND METHODS

2.1. Fungus collection and culture

Two strains of *P. boldo* were collected during autumn/winter 2016 at the “Nature Sanctuary” sector of the Hualpén peninsula (Bio Bio region, Chile). Nature Sanctuaries are defined by Chilean law as terrestrial or marine sites that offer special or unique conditions and possibilities for geological, paleontological, zoological, botanical, or ecological studies and research, whose conservation is of interest to science or to the country. Basidiomata of *P. boldo* were found and codified as FQ1626 and FQ1640 and kept at the Chemistry of Natural Products Lab. (Department of Botany, Faculty of Natural and Oceanographic Sciences, University of Concepcion, Concepcion, Chile). The *in vitro* maintenance of mycelia was done at 20±3°C in YMG agar medium (5 g yeast extract (BD Biosciences, San José, CA, USA), 10 g malt extract (BD Biosciences, San José, CA, USA), 15 g glucose (Merck, Darmstadt, Germany) and 20 g agar (BD Biosciences, San José, CA, USA) per litre of medium. Mycelia subcultures were maintained in YMG broth (0,4% w/v yeast extract, 1% w/v malt extract, 1% w/v glucose) at room temperature and constant agitation (120 rpm) during 1 to 2 months.

2.2. Cell lines

Three tumour cell lines, HCT-116 (human colorectal carcinoma), MCF-7 (human mammary adenocarcinoma) and HL-60 (human promyelocytic leukaemia), and a non-tumour cell line, HGF-1 (human primary gingival cells), were used for the cytotoxicity and anticarcinogenic activity assays. All four cell lines were obtained from ATCC (Manasas, VA, USA). Cell lines, HCT-116, MCF-7 and HGF-1 were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Biowest, Nuaillé, France) supplemented with 10% foetal bovine serum (FBS) (Biowest, Nuaillé, France), 1% penicillin-streptomycin 100X solution (Biowest, Nuaillé, France) and

0.5% amphotericin B (Biowest, Nuaillé, France). The cell line HL-60 was cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 20% FBS, 1% penicillin-streptomycin 100X solution and 0.5% amphotericin B. Cultures were incubated at 37° C in the presence of 5% CO_2 until reaching a 75-85% confluence. Cells were harvested and collected by centrifugation (600 g for 5 min) [110].

2.3. Acidic polysaccharides extraction

The acidic polysaccharides were obtained as described by Abdala-Díaz *et al*. [110] and Parages *et al*. [151], with modifications. One hundred g of mycelia of each strain were grinded and suspended in sterile distilled water, boiled during 1 h under constant agitation and then filtered to eliminate the debris. The acidic fraction of polysaccharides was obtained by selective precipitation adding 2% w/v n-cetylpyridinium bromide (Cetavlon) (Merk, Darmstadt, Germany) in an 8:1 ratio polysaccharides:cetavlon and centrifugation at 10,000 g for 20 min. The pellet was dissolved in 4 M NaCl heated to 100°C, then centrifuged at 4,000 g during 15 min and the supernatant recovered. Acidic polysaccharides were flocculated adding cold 96% ethanol (v/v) (1:1 ethanol:extract) and centrifuged at 4,000 g for 20 min. The pellet was dialyzed using a membrane and 2 M NaCl and flocculated again using cold 96% ethanol. Finally, after centrifuging at 4,000 g for 15 min, the acidic polysaccharides were frozen for 24 hrs and lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain). The acidic polysaccharides obtained from *P. boldo* strains FQ1626 and FQ1640 were hereafter named PBAP26 and PBAP40, respectively.

2.4. PBAP26 and PBAP40 cytotoxicity on cell lines

The effect of PBAP26 and PBAP40 on the viability of tumour (HCT-116, MCF-7 and HL-60) and non-tumour (HGF-1) human cell lines was evaluated by means of the MTT assay as described by Zhao *et al*. [152] and Meng *et al*. [113]. Cell

suspensions ($4x10^3$ HCT-116 cells per well or $1x10^4$ MCF-7 or HL-60 cells per well or $5x10^3$ HGF-1 cells per well) in a final volume of 100 µL per well were placed in 96 wells microplates and incubated for 72 h at 37°C in the presence of 5% CO_2 with different concentrations of the acidic polysaccharides PBAP26 or PBAP40. Serial dilutions of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 or 0.04 mg mL^{-1} PBAP26 or PBAP40 were evaluated. Cells not exposed to the acidic polysaccharides were used as control. At the end of the incubation period, cell viability was determined adding MTT solution (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Luis, MO, USA). Ten µl of MTT solution (5 mg mL^{-1} MTT in phosphate buffered saline (PBS) pH 7.5) were added per well. Microplates were incubated for further 4 h at 37°C and then 150µL isopropanol (0.04 N HCl in 2-propanol) were added to dissolve the formazan crystals produced. The absorbance of each well was measured at 550 nm in a MicroPlate Reader 2001 (Whittaker Bioproducts, Dauphin, PA, USA). The percentage of viability of cell lines and the IC_{50} were determined by comparison with the control.

2.5. Effect of PBAP26 or PBAP40 on the cell cycle of a selected tumour cell line

The effect of PBAP26 or PBAP40 on the cell cycle of a selected tumour cell line was evaluated using flow cytometry, as described by Afrin *et al*. [115]. The tumour cell line showing the greatest susceptibility to the cytotoxic activity of PBAP26 or PBAP40 (i.e., the smallest IC_{50}) was selected for this assay. Results (see below) demonstrated that HL-60 cells satisfied this criterion (PBAP40 IC_{50} = 127.85 µg mL^{-1}). Therefore, HL-60 cell suspensions ($5x10^5$ cells per well) were cultured in 6 wells plates in a total volume of 1.5 mL RPMI-1640 medium, supplemented with 20% FBS, 1% penicillin-streptomycin 100X solution and 0.5% amphotericin B; during 24 h at 37°C in the presence of 5% CO_2 and the culture medium was eliminated by centrifugation (600 g for 5 min). Cells were

resuspended in RPMI-1640 medium supplemented with 20% FBS, 1% penicillin-streptomycin 100X solution and 0.5% amphotericin B plus one of three different concentrations of PBAP26 or PBAP40. The concentrations of acidic polysaccharides were calculated on the basis of the IC_{50} for HL-60 cells. One concentration was equal to the IC_{50} (PBAP26 = 1140.04 µg mL^{-1}; PBAP40 = 127.85 µg mL^{-1}), and the other two were equivalent to ¼ IC_{50} (PBAP26 = 285.01 µg mL^{-1}; PBAP40 = 31.96 µg mL^{-1}) or 4x IC_{50} (PBAP26 = 4560.16 µg mL^{-1}; PBAP40= 551.4 µg mL^{-1}). Cells not treated with PBAP26 or PBAP40 were used as negative control while 20µM 2-methoxyestradiol (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control. After overnight incubation cells were centrifuged, the pellet washed using cytometry PBS (1X PBS, 1% FBS 1% v/v, 10mM Hepes buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), cells fixed using cold 70% ethanol during 1 h at 4°C, centrifuged at 600 g for 5 min and washed twice using cytometry PBS. Finally, cells were resuspended in 40 µg mL^{-1} propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg mL^{-1} RNase (Sigma-Aldrich, R6513) in PBS and the mixture incubated in the darkness during 30 min in a water bath adjusted at 37°C. Samples were analysed using a FACS VERSETM flow cytometer (BD Biosciences, San Jose, CA, USA), counting 10,000 events, and the results processed using the Kaluza version 2.1 software (Beckman Coulter, Brea, USA).

2.6. Antioxidant activity of PBAP26 and PBAP40

The antioxidant activity of PBAP26 or PBAP40 extracts was determined using the DPPH and ABTS capture of free radicals assays following the procedures described by Brand-Williams *et al*. [116] and Re *et al*. [117], respectively, with modifications. PBAP26 or PBAP40 were prepared at a concentration of 30 mg mL^{-1} in 0.1 M phosphate buffer pH 6.5. A DPPH (2,2-diphenyl -1-picryl-hydrazyl-hydrate) (Sigma-Aldrich, St. Louis, MO, USA) solution in 90% v/v methanol was adjusted (either adding DPPH or methanol) until obtaining an absorbance of 0.3

at 517nm using a spectrophotometer (UV/Vis Pharo 300 Spectroquant (Merk, Darmstadt, Germany)). A mixture of 150 µL DPPH and 50 µl of acidic polysaccharide, either PBAP26 or PBAP40 (30 mg mL^{-1}), were incubated for 30 min in the darkness at room temperature and then their respective absorbances were measured at 517 nm (UV/Vis Pharo 300 Spectroquant spectrophotometer). For the ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma-Aldrich, St. Louis, MO, USA) assay, the radical was prepared in 0.1M phosphate buffer at pH 6.5 and the solution adjusted (using phosphate buffer) until obtaining an absorbance of 0.7 at 727 nm (UV/Vis Pharo 300 Spectroquant spectrophotometer). Nine hundred and fifty µL of the ABTS solution were mixed with 50µL of 30 mg mL^{-1} PBAP26 or PBAP40 prepared as described above. The mixture was incubated during 8 min at room temperature and the absorbance measured at 727 nm using the same spectrophotometer.

In both assays, a mixture of the radical and the phosphate buffer were used as control. The antioxidant activity was calculated using the equation shown below and the result expressed as Trolox equivalents (µg mL^{-1}) (Thermo Fisher Scientific, Loughborough, United Kingdom) according to a previously obtained standard curve (Trolox concentrations from 0 to 100 µg mL^{-1}).

AA% = ((Abs (control) – Abs (sample))/ Abs (control)) x 100;

where AA% corresponds to the percentage of antioxidant activity, Abs (control) corresponds to absorbance of the respective radical in phosphate buffer and Abs (sample) corresponds to the absorbance of the respective radical plus the concentration of PBAP26 or PBAP40 being tested.

2.7. Fourier transform infrared spectroscopy (FT-IR)

The acidic polysaccharides obtained from the strain showing the best cytotoxic effect on the tumour cell lines were selected to be characterized by FT-IR.

Therefore, according to the results of cytotoxic capacity (see below), the acidic polysaccharides of the strain PBAP40 were analysed. The acidic polysaccharides were analysed preparing discs (16 mm diameter) with a mixture of PBAP40 and 1% KBr using a hydrostatic press at 15.0 T during 5 min. Discs were measured in a Thermo Nicolet Avatar 360 IR spectrophotometer (Thermo Electron Inc., Waltham, USA) with a 4 cm-1 resolution, a DTGS detector and the OMNIC 7.2 version software (Thermo Nicolet, Waltham, USA) (50 cm-1, bandwidth and 2.6 enrichment factor). The region analysed included the range from 4000 to 450 cm^{-1}. The baseline of each spectrum was flattened using the same version of the Nicolet OMNIC software. The correlation algorithm OMNIC was used to compare the spectrum of the sample with a spectra library (Thermo Fischer Scientific, Waltham, USA).

2.8. Statistical analysis

Results were expressed as the mean ± standard deviation of three experiments. The normality and homogeneity of variance of data was analysed previously to the one-way variance analysis (ANOVA). The Tukey HSD test was performed to compare the media of the results and to determine the statistical significance ($p<0.05$) among each concentration of PBAP26 or PBAP40 evaluated in the cytotoxicity and anticarcinogenic activity assays. The statistical analyses were done using the Statistica 12.0 software (TIBCO Data Science, Palo Alto, CA, USA) and the graphics were made using the SigmaPlot version 12.0 software (Systat Software Inc., Chicago, IL, USA).

3. RESULTS

3.1. Cytotoxic capacity of PBAP26 and PBAP40 against tumour cell lines and a primary cell line

Assays to evaluate the cytotoxic activity of PBAP26 and PBAP40 demonstrated that the viability of the three tumour cell lines (HCT-116, MCF-7 and HL-60) decreased as the acidic polysaccharide's concentration increased (Figure 5.1). The survival percentages when using high PBAP26 concentration (5 mg mL^{-1}) were 5.08±0.15% for HCT-116 cells, 32.34±0.15% for MCF-7 and 5.85±0.87% for HL-60 cells (Figure 5.1 A, B and C, respectively). Survivals in the presence of the same concentration of PBAP40 (5 mg mL^{-1}) were 4.02±1.37% for HCT-116 cells, 22.62±1,09% for MCF-7 cells and 5.85±0.58% for HL-60 cells (Figure 5.1 A, B and C, respectively).

On the other hand, when the low acidic polysaccharides concentration (0.04 mg mL^{-1}) was tested, the survival percentages of tumour cells were 91.31±1.39% for HCT-116 cells, 92.25±0.48% for MCF-7 cells and 100.00±0.30% for HL-60 cells, when PBAP26 was evaluated (Figure 5.1 A, B and C, respectively). When the same concentration of PBAP40 was evaluated, survival percentages were 79.72±1.04% for HCT-116 cells, 91.72±2.38% for MCF-7 cells and 92.83±0.92% for HL-60 cells (Figure 5.1 A, B and C, respectively).

Interestingly, the cytotoxicity of the acidic polysaccharides PBAP26 and PBAP40 on non-tumour primary HGF-1 cell line is rather mild (Figure 5.1 D). In the presence of 0.04 mg mL^{-1} of acidic polysaccharides, the survival percentage of HGF-1 cells was 90.6±0.99% in the case of PBAP26 and 82.86±2.64% in the case of PBAP40. Moreover, as the concentrations of PAPB26 or PAPB40 increased, the viability of the non-tumour primary HGF-1 cells showed survival percentages above 100% (159.3±0.35% and 109.86±2.1% in the presence of 5

mg mL^{-1} of PBAP26 or PBAP40, respectively), as compared with the negative control cells not treated with the acidic polysaccharides (Figure 5.1 D).

Regarding the IC_{50} figures for the tumour cell lines subjected to the PBAP26 acidic polysaccharides, they were 535.83 µg mL^{-1} in the case of HCT-116 cells, 3375.32 µg mL^{-1} for MCF-7 cells and 1140.04 µg mL^{-1} in the case of HL-60 cells. When PBAP40 was assayed under the same conditions; IC_{50} figures were reduced to only 236.41 µg mL^{-1}, 2566.72 µg mL^{-1} and 127.85 µg mL^{-1} for the same cell lines, respectively. These results indicate that PBAP40 shows more cytotoxic activity against human tumour cell lines than PBAP26.

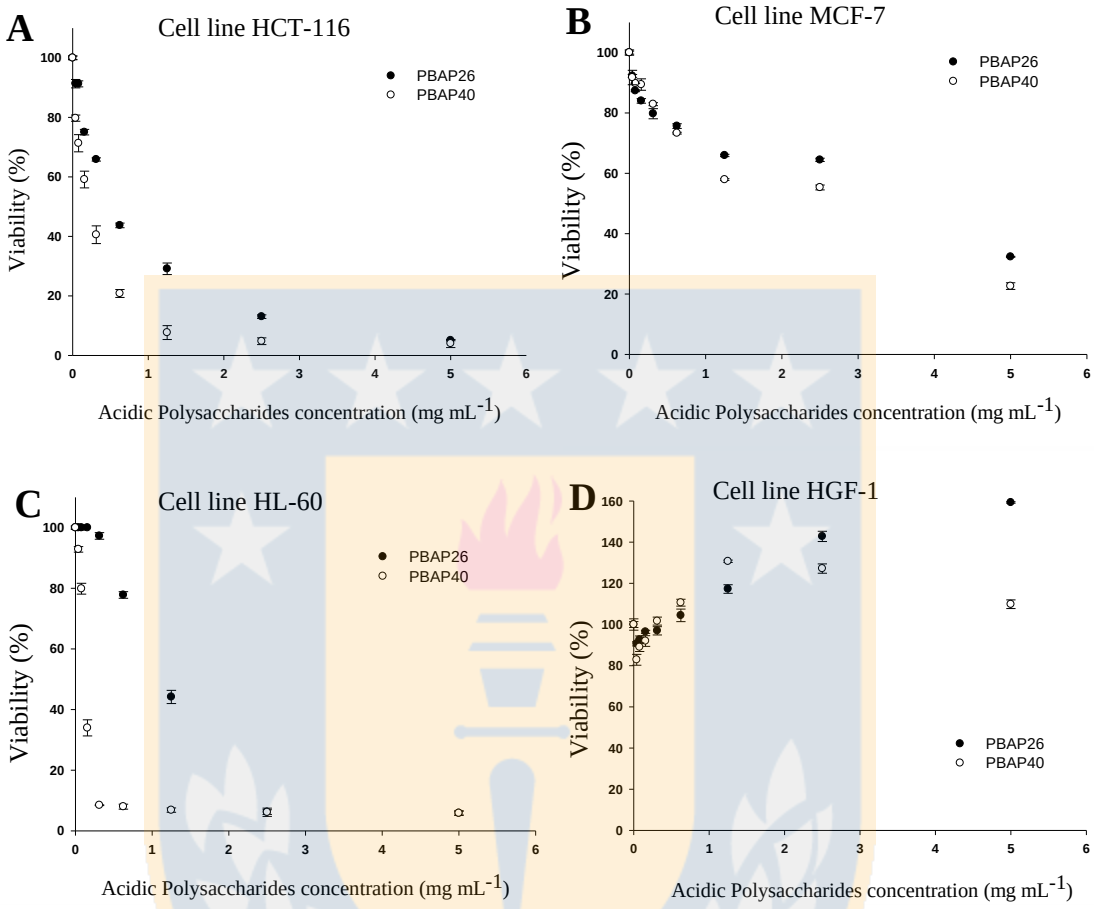

Figure 5.1 Viability (%) of the tumour cell lines and the primary cell line when subjected to different concentrations (5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 or 0.04 mg mL^{-1}) of acidic polysaccharides PBAP26 or PBAP40 obtained from de fungus *P. boldo*; (**A**) tumour cell line HCT-116 (human colorectal carcinoma); (**B**) tumour cell line MCF-7 (human mammary adenocarcinoma); (**C**) tumour cell line HL-60 (human promyelocytic leukaemia); (**D**) non-tumour primary cell line HGF-1 (human gingival cells). Bars indicate standard deviation.

3.2. Effect of PBAP26 or PBAP40 on the cell cycle of HL-60 tumour cell line

Flow cytometry studies of the HL-60 cell line subjected to different PBAP26 or PBAP40 concentrations allowed to determine the dose dependent effect of acidic polysaccharides on the cell cycle. The lower concentrations of acidic

polysaccharides used, corresponding to the IC_{50} figures (PBAP26= 1140.04 µg mL^{-1}; PBAP40= 127.85 µg mL^{-1}) and to ¼ IC_{50} figures (PBAP26= 285.01 µg mL^{-1}; PBAP40= 31.96 µg mL^{-1}) for the HL-60 human promyelocytic leukaemia cell line, showed no significant differences when compared to those of the negative control (data not shown). The highest concentrations of the acidic polysaccharides assayed (4X IC_{50}) showed differences in the percentages of events in the cell cycle phases when compared to the control. A 4560.16 µg mL^{-1} concentration of PBAP26 caused an increase in the percentage of events in the apoptosis phase (Sub G1) reaching 56.25±0.75%, approximately 43% over than that shown by the negative control (13.49±0.87%). Concomitantly, there was a 30% decrease of events in phases G0/G1 (PBAP26= 24.20±1.17%; Control= 54.45±1.01%) and 13% in the phases S/G2/M (PBAP26= 19.56±0.43%; Control= 32.06±1.88%), respectively (Figure 5.2 A and C). On the other hand, PBAP40, at a 511.4 µg mL^{-1} concentration, caused a moderate increase of Sub G1 phase events percentage, reaching 24.90±3.57% (11% above the control) (Figure 5.2 B and C) and also a smaller reduction of G0/G1 phase events (40.10±3.1%) and S/G2/M phase events (29.00±0.56%) with respect to the control when compared with the effect of PBAP26. As expected, the exposure of the cell line HL-60 to 20 µM 2-methoxyestradiol caused an increase of events in phases S/G2/M, reaching a percentage of 45.65±3.35% (13% over the control), an increase of events in the phase Sub G1 (23.84±3.19%) and a decrease of events in the phase G0/G1 (30.51±0.17%) (Figure 5.2 D).

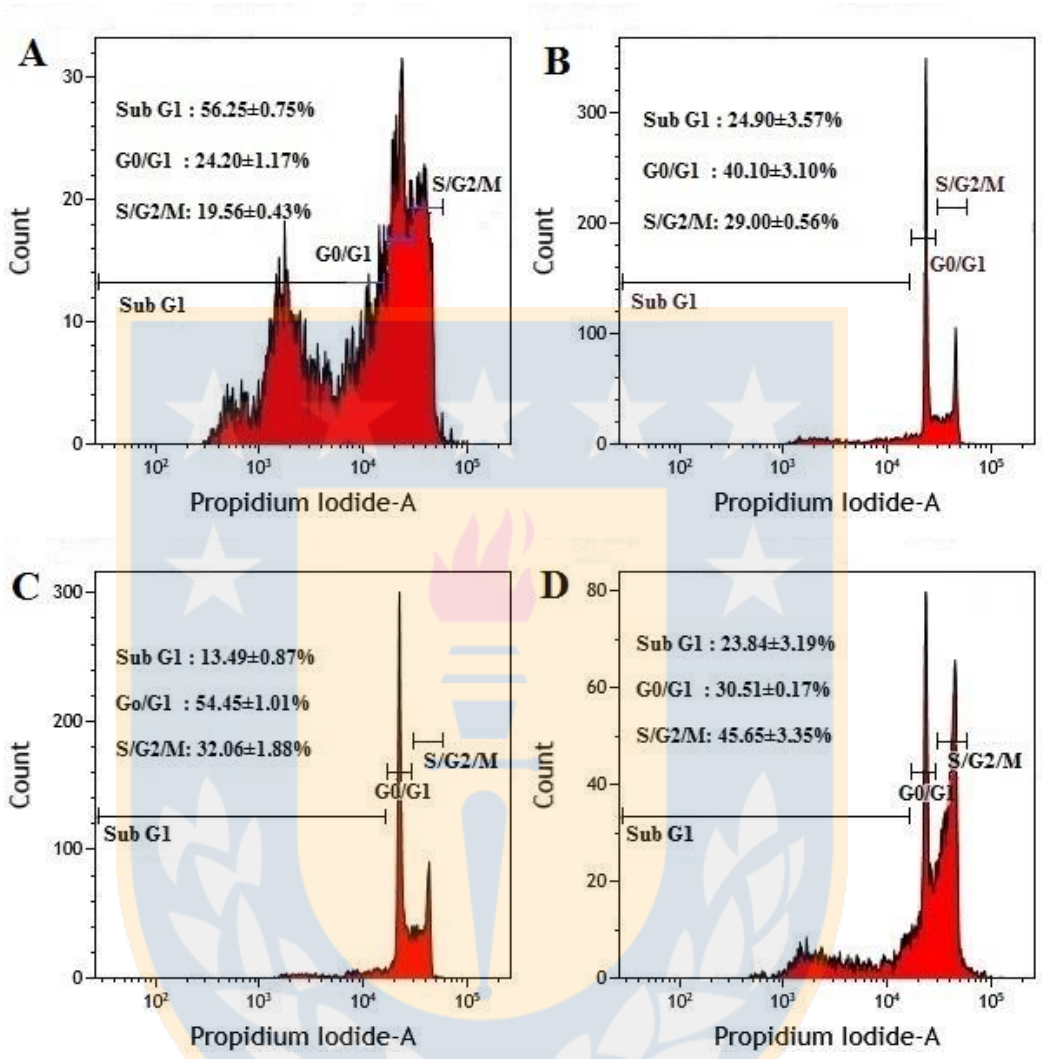

Figure 5.2 Subpopulations of tumour HL-60 cells at different cell cycle phases after 16 h treatment with (**A**) 4X IC_{50} concentration of acidic polysaccharides of *P. boldo* FQ1626 strain; (**B**) 4X IC_{50} concentration of acidic polysaccharides of *P. boldo* FQ1640 strain; (**C**) negative control (cells not exposed to acidic polysaccharides); (**D**) positive control (2-methoxyestradiol). Percentages correspond to the mean ± SD of three independent experiments.

3.3. Antioxidant capacity of PBAP26 and PBAP40

Results of the antioxidant effect of PBAP26 and PBAP40 on the free radicals DPPH and ABTS are shown in Table 5.1. PBAP26, evaluated at a concentration

of 30 mg mL^{-1}, showed an antioxidant activity of 17.04±0.40% on DPPH and 8.31±0.18% on ABTS, with no significant differences when compared to the control. On the contrary, PBAP40, evaluated at the same concentration of PBAP26, showed a better antioxidant activity; reaching percentages of 24.53±1.36% on DPPH and 10.20±1.04% on ABTS, showing significant differences on antioxidant activity when compared to the control. When the antioxidant activity of PBAP26 and PBAP40 was analysed in comparison with a Trolox calibration curve it was determined that none of the acidic polysaccharides exceeded he activity of 1 µg mL^{-1} Trolox (Table 5.1).

Table 5.1 Antioxidant activity (%) of 30 mg mL^{-1} acidic polysaccharides PBAP26 and PBAP40 on radicals DPPH and ABTS.

Acidic polysaccharides	**Antioxidant activity (%)**		**Trolox equivalents (µg mL^{-1})**	
	DPPH	**ABTS**	**DPPH**	**ABTS**
Control (-)	0*	0▪	0*	0▪
PBAP26	17.04 ± 0.40*▪	8.31 ± 0.18▪*	0.54 ± 0.02*▪	0.48 ± 0.01▪*
PBAP40	24.53 ± 1.36▪	10.20 ± 1.04*	0.83 ± 0.05▪	0.59 ± 0.06*

Table shows media ± standard deviation of antioxidant activity and Trolox equivalents (n=3).

DPPH (2,2-diphenyl -1-picryl-hydrazyl-hydrate), ABTS: (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)). Symbols (▪ or *) indicate statistical significance. Different signs indicate significant difference.

3.4. FT-IR spectrometric analysis

The FT-IR spectrometric analysis in the range from 4000 to 450 cm^{-1} was used to identify functional groups. The FT-IR spectra of PBAP40 polysaccharides is shown in Figure 5.3. The typical absorption bands of water-soluble polysaccharides were obtained in the spectrum of PBAP40 [142]. Specifically, an intense peak at 3432.13 cm^{-1} was observed from the stretching vibrations of

OH due to inter- and intra-molecular hydrogen bonds [141]. The absorption bands observed at approximately 2937.77 cm^{-1} correspond to CH absorptions (CH, CH2 and CH3 stretching and bending vibrations) [142]. The peaks at 1652 cm^{-1} and 1547.20 cm^{-1} were attributed to the absorbance corresponding to stretching vibration of the carbonyl bond of the amide group and the bending vibration of the N-H bond. The band at 1422 cm^{-1} was attributed to C–H bending vibration [141]. Characterization of PBAP40 by FT-IR analysis showed the typical absorption of pyranose ring at 1155.77 cm^{-1}, 1076.22 cm^{-1} and 1035.08 cm^{-1} [142]. The characteristic peaks around 888.86 cm^{-1} and 932.87 cm^{-1} indicated the existence α- and β-type glycosidic bonds in the structure in PBAP40 [143].

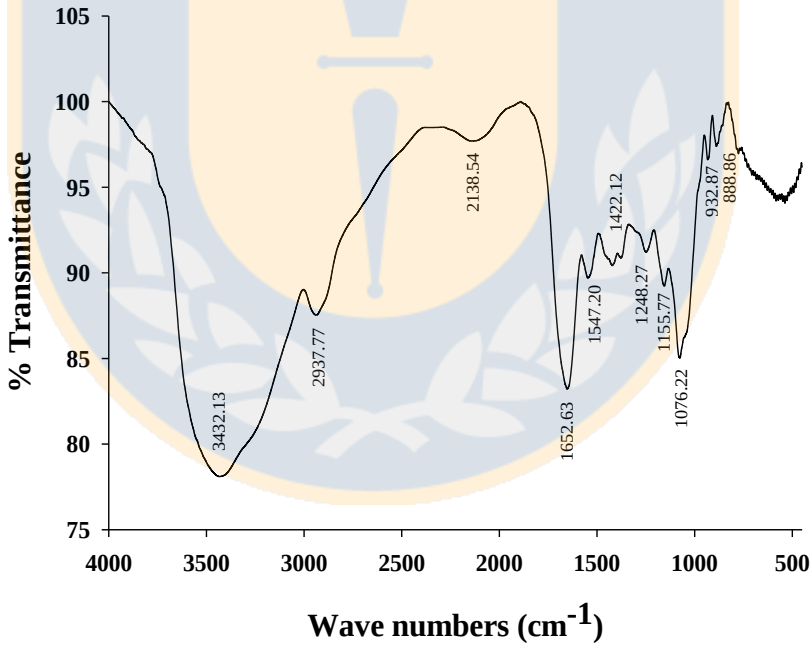

Figure 5.3 Spectrum obtained by means of Fourier transform infrared spectroscopy (FT-IR) after analysing the PBAP40 sample

4. DISCUSSION

Fungal polysaccharides display remarkable biological activities and represent a prolific source of chemicals that can potentially contribute to the development of new medicines [90,144]. According to Lu *et al.* [82], glucans isolated from the fruiting body of *P. ribis* can improve the immunological function and reduce the oxidative stress in mice. Indeed, this fruiting body is used as a functional ingredient to treat various diseases [82]. Considering the favourable interaction of acidic groups with target biomolecules present in cells, fungal acidic polysaccharides may have a better bioactivity than their neutral counterparts [25]. Kiho *et al*. [145] reported that the acidic polysaccharides fraction obtained from the fungus *Tremella aurantia* showed a strong hypoglycaemic effect in mice. Furthermore, it has also been reported that acidic polysaccharides of the ascomycete fungus *C. sinensis* have a significant immunostimulant activity, increasing the levels of cytokines on macrophage-like Raw 264.7 cells [25,96]. These results make acidic polysaccharides promising candidates as bioactive therapeutic agents.

According to Qin *et al*. [77], certain species of *Phylloporia* are restricted to a specific host, limiting their geographic distribution to that of the respective host. One of these examples is *Phylloporia crataegi*, which only grows on *Crataegus*, and *Phylloporia gabonensis*, found only on *Dichostemma. Phylloporia boldo* was described by Rajchenberg *et al.* [30] growing on *P. boldus*, an endemic species of the Andean Patagonian ecosystem of southern Chile [32]. To the best of our knowledge, *P. boldo* is restricted to that host. The chemical characterization of polysaccharides and other metabolites from *P. boldo* as well as the study of their biological activity are currently very limited [32]. The present work aimed to evaluate the cytotoxic activity of acidic polysaccharides isolated from *P. boldo* against three human tumour cell lines and determine their effect on the cell cycle. We also evaluated their antioxidant capacity by means of DPPH and ABTS.

Our results show that acidic polysaccharides obtained from both strains of *P. boldo* were able to significantly reduce the viability of the human tumour cell lines in a dose dependent manner. Interestingly, the viability of primary cell lines proved to be unaffected by the acidic polysaccharides. At elevated concentrations (5 mg mL^{-1}), the viability of the HCT-116 cell line was reduced to 95% in the case of PBAP26 and a 96% in the case of PBAP40, while the viability of MCF-7 cells was reduced by 68% (PBAP26) and 78% (PBAP40). In the case of the HL-60 cell line, both acidic polysaccharides reduced the cell viability in a 94%. The highest mortality was suffered by HCT-116 cells (95.98%) in the presence of PBAP40. According to Liu *et al.* [109], the cytotoxic capacity of 0.6 mg mL^{-1} crude polysaccharides obtained from *Phellinus baumii*, another polypore species in the Hymenochaetaceae family, can inhibit the proliferation of HeLa cells up to 90.63%. Similarly, Mei *et al.* [74], reported that 200 µg mL^{-1} of *P. linteus* polysaccharides reduced the survival of murine Sarcoma-180 cells by 93.6%. Although the concentrations of polysaccharides reported in previous works were lower than those used to assay PBAP26 and PBAP40, most studies evaluated the bioactivity of total or crude fungal polysaccharides, making it difficult to compare their activity with that of *P. boldo* acidic polysaccharides. After calculating the IC_{50} for each extract for each tumour cell line assayed, PBAP40 showed a smaller IC_{50} than PBAP26 against all tumour cell lines assayed, indicating its better cytotoxic activity against these cells. It must be considered that concentrations required to reduce the viability of the assayed tumour cell lines to a 50% may be the consequence of the composition of the acidic polysaccharides of *P. boldo* as well as the inherent features of the tumour cell lines assayed such as adherence, expression of genes and mutation of specific oncogenes [125-127]. As opposed to the cytotoxic activity of the acidic polysaccharides on the tumour cell lines, except for the lowest concentration tested (0.04 mg mL^{-1}), PBAP26 and PBAP40 induced a significant proliferation of the non-tumour primary HGF-1 cell line which increased as the concentration

of acidic polysaccharides increases. Similar results were also observed with the acid fraction of polysaccharides from *Cordyceps militaris* cultured on silkworm pupa (CM-jd(Y)-CPS2), where CM-jd(Y)-CPS2 produced statistically significant promotion of proliferation of mouse splenocytes at a concentration of 200 μg mL^{-1} [146]. These results suggest that the effect of the acidic polysaccharides of *P. boldo*, PBAP26 and PBAP40, might be selective on at least certain tumour cell lines. This biological effect has also been observed by other research groups [147].

The inhibition of tumour cell lines by fungal polysaccharides may be the consequence of different mechanisms [58]. To determine if the cytotoxic effect of PBAP26 and PBAP40 was associated to an arrest of the cell cycle, we evaluated the effect of *P. boldo* acidic polysaccharides on the phases of the cell cycle of HL-60 cells by flow cytometry experiments. The percentage of HL-60 cells in phases G0/G1 and S/G2/M decreased after been subjected to the acidic polysaccharides when compared with the negative control. These results allow to suggest that PBAP26 and PBAP40 can arrest the cell cycle of the tumour cell tested in the Sub G1 phase, inducing the death of HL-60 cells [109,148]. Other studies have shown a similar effect of the fungal polysaccharides on cell cycle of tumour cells lines. As an example, according with Mei *et al*. [74], the percentages of apoptotic cells of the *P. linteus* polysaccharides in concentration of 200 μg mL^{-1} were 118.2% higher than the positive control, this could indicate that antitumor activity of *P. linteus* polysaccharides is related to apoptosis *in vitro*. Similarly, the percentage of HeLa cells in G0/G1 phase significantly increased from 27.11% to 48.17%when were treatment with polysaccharide from *P. baumii* (PPB), this could imply that PPB was able to induce cell cycle of HeLa arrest at G0/G1phase [109].

The assays to evaluate the antioxidant activity of PBAP26 and PBAP40 against free radicals showed that both have a better antioxidant activity against the

DPPH radical (reaching percentages of 17% and 24%, respectively) than that against ABTS. According to Martysiak-Żurowska & Wenta [149], the interactions between antioxidant and DPPH radical might be determined by the structural composition of the antioxidant (in this case the acidic polysaccharides of *P. boldo*), being the reduction of radicals proportional to the hydroxyl groups of the antioxidant [21,149]. Studies performed with other fungi belonging to the Hymenochaetaceae family, such as *P. linteus* and *P baumii*, have shown that their total polysaccharides are effective reducers of free radicals, reaching an 86.9% antioxidant activity at a 10 mg mL^{-1} concentration in the case of *P. linteus* and 80.29% at a 1.2 mg mL^{-1} concentration in the case of *P. baumii* against the free radical DPPH [19,21]. Crude polysaccharides can be conjugated with other components, such as proteins, lipids, pigments, polyphenols, and other residues [144]. These residues of proteins or polyphenols can be responsible of the radical reducing effect, causing crude polysaccharides to show a greater antioxidant activity than that shown after fractionation and purification of the samples [111,144].

The biological activity shown by fungal polysaccharides is conditioned by their physic-chemical characteristics. The structure, molecular weight, solubility, length, and composition of the saccharides chain are some of the factors determining their medicinal properties [21,150]. Results of the FT-IR analysis of PBAP40 indicated the presence of functional groups characteristic for polysaccharides, such as pyranose ring and hydroxyl groups which may be the ones associated to their bioactivity observed in the cytotoxic and antioxidant activities. The FT-IR analysis may allow to infer the presence of α- and β-glucans in PBAP40 by the presence of bands characteristic of α- and β-types glycosidic bonds [143]. Previous studies have reported that β-glucans are one of the main bioactive components present in fungal polysaccharides and strong anti-tumoral, immunomodulating and antioxidant activities have been attributed to them [21,93,95,150].

Thus, we report for the first time the isolation and biological evaluation of acidic polysaccharides from *P. boldo*. We observed a selective cytotoxicity of PBAP26 and PBAP40 against tumoral cell lines (HCT-116, MCF-7 and HL-60) as compared to their cytotoxicity activity against non-tumoral cell lines (HGF-1). Also, we evaluated the effect of PBAP26 and PBAP40 on the cell cycle of HL-60 cell line, the result shown that both polysaccharides could exhibit the capacity of arrest the cells in a specific phase of cell cycle (Sub G1). On the other hand, we tested the antioxidant ability of PBAP26 and PBAP40 by means of DPPH and ABTS, rendering excellent results. Finally, we believe our work contributes to expand polysaccharide research isolated from Andean-Patagonian fungi and the development of new chemical probes to control cancer.

5. CONCLUSIONS

This study characterized and demonstrated the cytotoxic and antioxidant activities of the acidic polysaccharides of two strains of the fungus *P. boldo* against human tumour cell lines. *Phylloporia boldo* acidic polysaccharides PBAP40 (obtained from the strain FQ1640) showed a better cytotoxic and antioxidant activities than PBAP2026 (obtained from strain FQ1626). Both PBAP26 and PBAP40 were highly effective and selective to reduce the viability of human colorectal carcinoma HCT-116, mammary adenocarcinoma MCF-7 and promyelocytic leukaemia HL-60 cell lines. Interestingly, PBAP26 and PBAP40 did not show cytotoxicity against non-tumour cell line HGF-1 primary cell line, at the concentrations tested. Analysis of the HL-60 tumour cell line by flow cytometry revealed the effect of PBAP26 and PBAP40 on its cell cycle, which determined the arrest of tumour cells in the Sub G1 phase. Antioxidant studies were also performed. PBAP40 showed a better antioxidant capacity than PBAP26 at the concentration tested (30 mg mL^{-1}). To determine the mechanisms by which these acidic polysaccharides exert their cytotoxic and antioxidant activities will require further research.

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CAPÍTULO VI: *In vitro* ANTITUMOUR POTENTIAL AND ANTIOXIDANT ACTIVITY OF THE MYCELIAL ACIDIC POLYSACCHARIDES OF *Fomitiporia sp.* STRAIN FQ1648, AN ANDEAN PATAGONIAN FUNGUS OF SOUTHERN CHILE.

Verónica Albornoz, Virginia Casas-Arrojo, Cristian Riquelme, Fabián Figueroa, Víctor Hernández, Claudia Pérez, Carlos T. Smith, José Becerra, Jaime R. Cabrera-Pardo, Víctor L. Campos and Roberto Abdala-Díaz

ABSTRACT

Fungi are a source of various bioactive metabolites; fungal polysaccharides have been shown to possess antioxidant and antitumor abilities, among many other biological properties. The fungal strain FQ1648 was described by macroscopic and microscopic observation. The *in vitro* cytotoxic and antioxidant capacities of acid polysaccharides from strain FQ1648 were evaluated. Acid polysaccharides (APFQ48) were analysed by FT-IR. The effect of APFQ48 on tumour cell lines was evaluated by MTT assay and flow cytometry. The antioxidant activity was determined by the DPPH and ABTS methods. Assays determined that APFQ48 has a higher cytotoxic activity against the HL-60 tumour cell line (IC_{50}= 800.00 µg mL^{-1}), compared to the other cell lines evaluated (IC_{50}= 1833.65 µg mL^{-1} for HCT-116 and 6091.75 µg mL^{-1} for MCF-7). On the other hand, against the non-tumour HGF-1 cell line, no cytotoxic effect is observed (IC_{50} outside the evaluated range). The highest antioxidant activity presented by APFQ48 in a concentration of 30 mg mL^{-1}, was 27.31±3.26% against the free radical DPPH; equivalent to the antioxidant activity of 0.94±0.13 µg mL^{-1} of Trolox.

1. INTRODUCTION

Cancer is the second cause of death worldwide; it being surpassed only by cardiovascular diseases [58,103]. In accordance with Yan *et al*. [58], carcinogens, such as tobacco, alcohol, or chemicals, can increase the risk of contracting cancer. Similarly, to other chronic diseases, such as diabetes, Alzheimer and cardiovascular pathologies, cancer can be induced by an increase of reactive oxygen species (ROS) in the organism which accelerate cell aging causing damage to tissues and cells [4,59]. On the contrary, appropriate levels of ROS play an important role in the maintenance of vital activities, acting as subcellular messengers in gene regulation and signal transduction pathways [6,86]. Therefore, the search for potential antioxidant and/or antitumoral compounds which can control or treat the effects of free radicals is a relevant one.

Polysaccharides are the main bioactive macromolecules present in plants, microorganisms, and animals [58]. They are involved in several biological processes, such as cell communication, cellular and humoral immunity, and infection of hosts by bacteria, viruses, and fungi [25,86]. With respect to their structure, they are polymers having at least ten monosaccharides linked by glycosidic bonds [133]. According to Liu *et al*. [86], polysaccharides have shown to possess antioxidant, antitumoral, immunomodulatory, anti-inflammatory and antidiabetic activities. The type and degree of bioactivity of polysaccharides depend on the physico-chemical characteristics of the molecules, such as chemical composition, glycosidic bonds, molecular weight, and the degree of branching [90,146].

Fungi are a plentiful source of bioactive polysaccharides [12]. According to Wang *et al.* [25], Wu *et al*. [146] and Cheung *et al*. [12], fungal polysaccharides have nutraceutical and pharmaceutical uses, and they are extensively used in traditional medicine due to their antitumoral and immunomodulating properties.

Certain fungal polysaccharides, such as lentinan and schizophyllan, have been used as adjuvants or immunomodulators in anticancer therapies [135,146]. The acidic fraction of fungal polysaccharides is a large and important group [25]. Acidic polysaccharides contain one or more carboxylic groups, phosphate groups or sulphuric ester, and they are usually more bioactive than neutral polysaccharides due to the electronic interactions of the acidic groups with other biomolecules [25]. Some acidic polysaccharides obtained from the fermentation of *Agaricus blazei* might be able to induce TNF-α secretion by the macrophage cell line RAW264.7 [135], while the acidic fraction of polysaccharides obtained from the mycelium of *Cordyceps sinensis* stimulates phagocytosis in this same cell line [153].

Among the fungal bioactive polysaccharides, the Hymenochaetaceae Donk poroid group includes several species with a potential medicinal application [29]. The species *Phellinus linteus* is a medicinal mushroom whose extracts have shown to include biomacromolecules having antitumoral, antioxidant, anti-inflammatory and immunomodulating activities [14]. Also, the polysaccharides obtained from *Fomitiporia punctata* (fraction G1, a homogenous polysaccharides purified by using a Sephadex G-75 column which was eluted with distilled water) showed a significant antioxidant activity (51% uptake of the DPPH radical and a 68.75 uptake of superoxide radical at 250 and 200 μg mL^{-1} polysaccharides concentrations, respectively) [86]. The aim of the present work was to determine the cytotoxic and antioxidant capacities of the acidic polysaccharides obtained from a mycelial culture of the FQ1648 strain, collected from the stump of *Laurelia serpenvirens* (endemic of Chile and Argentina).

2. MATERIALS AND METHOD

2.1. Biological material and culture

The basidiocarp, identified as FQ1648 according to the records of the Laboratory of Chemistry of Natural Products (Department of Botany, Faculty of Natural and Oceanographic Sciences, Universidad de Concepción, Chile), was collected in December 2017 at Temuco (Araucanía region, Chile). The fungus was found growing on the stump of *Laurelia serpenvirens* (Ruiz & Pav) Tul (Atherospermataceae) (common name “laurel chileno”). The mycelium was maintained *in vitro* in YMG agar medium at a temperature of 20±2°C [154]. Mycelium subcultures were maintained in YMG broth (0,4% w/v yeast extract, 1% w/v malt extract, 1% w/v glucose) at room temperature and under constant agitation (120 rpm). Subcultures were kept for periods of 1 to 2 months to obtain the biggest possible mycelium mass. To characterize the *in vitro* culture of FQ1648 strain, the mycelium was cultured according to Rajchenberg *et al.* [30], placing a small piece of mycelium at the border of a 90 mm Petri dish containing 2% w/v malt extract agar (BD Biosciences, San José, CA, USA) and kept in the darkness at 20±2 °C for 6 weeks.

2.2. Macro and microscopic characterization of the fungus

The description of the morphology of the basidiome was carried out following the works de Rajchenberg *et al.* [29,30] and Drechsler–Santos *et al.* [155]. Microscopic observations were made using distilled water, KOH 3% w/v and Melzer’s reagent. All measurements were made in KOH 3% w/v preparations. Colour nomenclature followed the ISCC–NBS colour system [156].

2.3. Cell lines cultures

The cytotoxic activity of the acidic polysaccharides extract obtained from FQ1648 strain was assayed using three tumour cell lines (HCT-116 (colorectal carcinoma),

MCF-7 (mammary adenocarcinoma) and HL-60 (promyelocytic leukaemia)) and the HGF-1 non-tumour cell line (human gingival fibroblasts) cell line. All cell lines were obtained from ATCC (Manassas, Virginia, VA, USA). Cell lines HCT-116, MCF-7 and HGF-1 were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Biowest, Nuaillé, France) supplemented with 10% foetal bovine serum (FBS) (Biowest, Nauillé, France), 1% penicillin-streptomycin 100X solution (Biowest, Nuaillé, France) and 0.5% amphotericin B (Biowest, Nuaillé, France). Cell line HL-60 was cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 20% FBS and penicillin-streptomycin and 0.5% amphotericin B as described above. All cell lines were incubated at 37°C in the presence of 5% CO_2. Cells were harvested and centrifuged (600 g for 5 min) when they reached a confluence of 75-85% [157].

2.4. Extraction of acidic polysaccharides

The acidic fraction of the polysaccharides of the FQ1648 strain were obtained as described by Abdala-Díaz *et al.* [157] and Parages *et al.* [151], with modifications. The culture of the fungus was filtered, and 100 g of mycelium were grinded and washed using 0.01M phosphate buffered saline (PBS) at pH 7.4. The mycelium was resuspended in distilled water, kept at 100 °C during 1 h under constant agitation and then filtered to eliminate the mycelium debris. The acidic fraction of the polysaccharides was obtained by selective precipitation, adding a 2% w/v cold aqueous solution of n-cetylpyridinium bromide (Cetavlon) (Merk, Darmstadt, Germany) in a extract:cetavlon 8:1 proportion followed by centrifugation at 10,000 g for 20 min. The pellet containing the acidic polysaccharides was resuspended in a 4 M NaCl solution and heated to 100 °C. After cooling to room temperature, the suspension was centrifuged at 4,000 g for 15 min and the supernatant recovered. The polysaccharides were flocculated adding 96% ethanol v/v cold in a ethanol:extract 1:1 proportion and centrifuged at 4,000 g for 20 min. The acidic polysaccharides contained in the pellet were dialysed in a membrane subjected

to 2 M NaCl, precipitated adding 96% v/v cold ethanol centrifuged at 4,000 g for 15 min. The acidic polysaccharides obtained were stored overnight at -20 °C and then lyophilized (Lyophilizer Cryodos, Telstar, Terrasa, Spain) and maintained at 4 °C for their posterior use. The acidic polysaccharides of the FQ1648 strain will be hereon referred as APFQ48.

2.5. Analysis of Fourier transform infrared spectroscopy (FT-IR)

The structural analysis of the APFQ48 acidic polysaccharides was achieved preparing discs (16 mm in diameter) with a mixture of APFQ48 and 1% KBr using a hydrostatic press (15.0 T for 5 min). The discs were analysed in a Thermo Nicolet Avatar 360 IR spectrophotometer (Thermo Electron Inc., Waltham, USA) with a 4 cm^{-1} resolution, a DTGS detector and the OMNIC 7.2 version software (Thermo Nicolet, Waltham, USA) (50 cm^{-1}, bandwidth and 2.6 enrichment factor). The analysis was done in the 4,000 - 450 cm^{-1} region. The baseline of each spectrum was flattened using the same version of the Nicolet OMNIC software. The spectrum obtained for APFQ48 was compared with those of a spectra library using the correlation algorithm OMNIC (Thermo Fischer Scientific, Waltham, USA).

2.6. Cytotoxic activity of APFQ48 against cell lines

The cytotoxic capacity of APFQ48 against tumour cell lines (HCT-116, MCF-7 and HL-60) and the non-tumour cell line (HGF-1) was measured using the (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, San Luis, USA) (MTT) assay to evaluate cell viability [113]. Cell suspensions were prepared in 96 wells plates ($4x10^3$ HCT-116 cells per well, $1x10^4$ MCF-7 or HL-60 cells per well or $5x10^3$ HGF-1cells per well, respectively, in 100 µL of their culture medium) containing the following APFQ48 concentrations: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 or 0.078 mg mL^{-1}. Cells not treated with APFQ48 were used as positive control of cell viability. Cultures were incubated at 37 °C in the presence of 5%

CO_2 for 72 h, the culture medium containing the different concentrations of acidic polysaccharides replaced by culture medium alone, 10µl MTT solution (5 mg mL^{-1} MTT in PBS pH 7.5) added per well and incubated at 37 °C for 4 h. The culture medium of each well was discarded and replaced by 150µL 0.04 N HCl in 2-propanol to dissolve the purple formazan crystals resulting from tetrazolium reduction and the absorbances measured at 550 nm using a Micro Plate Reader 2001 (Whittaker Bioproducts, Dauphin, USA). Viability percentages and IC_{50} were calculated based on the results of the positive control.

2.7. Effect of APFQ48 on the cell cycle stages of a tumoral cell line evaluated by flow cytometry

The effect of the acidic polysaccharides of the strain FQ1648 was evaluated using the most sensitive tumour cell line used in the present work. According to Afrin *et al*. [115], the effect of APFQ48 on the cell cycle of the selected tumoral cell line was evaluated by flow cytometry. A cell suspension of $5x10^5$ cells in RPMI-1640 of the selected tumour line in 1.5 mL per well of 6 wells microplates were incubated at 37 °C in the presence of 5% CO_2 during 24 h. Then, the culture medium was removed by centrifugation at 400 g for 5 min and cells resuspended in RPMI-1640 (supplemented with 20% FBS, 1% penicillin-streptomycin 100X solution and 0.5% amphotericin B) plus one of three different concentrations of APFQ48 (¼X IC_{50}, 1X IC_{50} or 4X IC_{50}) determined based on the IC_{50} calculated from the MTT assay to evaluate the cell viability of the selected cell line described in Section 2.6. Cells not treated with APFQ48 were used as negative control while cells subjected to 20µM 2-methoxyestradiol (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control. Cultures were incubated overnight and then cells centrifuged and washed using cytometry PBS (1X PBS, 1% FBS 1% v/v, 10 mM Hepes buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)). Cells were fixed using 70% v/v cold during 1 h at 4 °C, then they were centrifuged at 600 g for 5 min and washed twice using cytometry PBS. Next, cells were resuspended

in 40 µg mL^{-1} propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg mL^{-1} RNase (Sigma-Aldrich, R6513) in PBS, incubated in a bath at 37 °C for 30 min in the darkness and finally analysed using a flow cytometer FACS VERSETM (BD Biosciencies, San José, CA, USA) and the results processed using the Kaluza software, version 2.1 (Beckman Coulter, Brea, USA).

2.8. Antioxidant activity of APFQ48

The antioxidant activity of APFQ48 was evaluated using the DPPH free radical assay as described by Brand-Williams *et al.* [116] and the ABTS cationic radical assay reported by Re *et al.* [117]. APFQ48 acidic polysaccharides were prepared at a 30 mg mL^{-1} concentration in 0.1M buffer phosphate pH 6.5. A DPPH radical (2,2-diphenyl -1-picryl-hydrazyl-hydrate) (Sigma-Aldrich, San Luis MO, USA) in 90% v/v methanol was prepared and adjusted to an absorbance of 0.3 at 517nm using a spectrophotometer (UV/Vis Pharo 300 Spectroquant (Merk, Darmstadt, Germany). A mixture of 150 µL DPPH plus 50 µl 30 mg mL^{-1} APFQ48 was prepared, incubated in the darkness at room temperature for 30 min and the absorbance of the mixture measured at 517nm. On the other hand, the ABTS radical (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma-Aldrich, San Luis, USA) was prepared in 0.1M buffer phosphate pH 6.5 and its absorbance adjusted to 0.7 at 727 nm. A mixture of 950 µL of ABTS radical and 50 µl of 30 mg mL^{-1} APFQ48 solution was prepared, incubated in the darkness for 8 min at room temperature and the absorbance measured at 727 nm. Mixtures of the radical and phosphate buffer in accordance with the respective protocols were used as negative controls.

The percentage of antioxidant activity (AA%) was calculated using the following equation,

AA% = ((Abs (control) – Abs (sample))/ Abs (control)) x 100;

where AA% corresponds to *antioxidant activity* percentage, Abs (control) to the absorbance of the control (radical in phosphate buffer) and Abs (sample) to the absorbance of sample being evaluated (radical + APFQ48).

Also, the absorbance measured in each sample analysed was expressed as Trolox equivalents (µg mL^{-1}) (Thermo Fisher Scientific, Loughborough, United Kingdom) based on a standard curve previously done.

2.9. Statistical analysis

Data was subjected to a one-way analysis of variance (ANOVA) after their normality and homogeneity of variance were determined. The significant differences ($p<0.01$) between the results of the cytotoxic and antioxidant activities of APFQ48 and their respective controls were determined using the Tukey's HSD test. All assays were done in triplicate and their results expressed as their means. Statistical analyses were done using the Statistica 12.0 software (TIBCO Data Science, Palo Alto, USA) and the graphics using SigmaPlot version 12.0 software (Systat Software Inc., Chicago, USA).

3. RESULTS

3.1. Macro and microscopic description of the fungus

Observed samples of strain FQ1648 presented a basidiome perennial, pulvinated, forming an indurated margin, up to 10 cm long, 7 cm wide and 3 cm thick (Figure 6.1 A). Pilear surface light yellowish brown to strong yellowish-brown darkening with KOH, pores round to angular, 0.11–0.15 mm. Context up 7 mm thick. Tubes up to 8 mm long. Consistency woody. Hyphal system dimitic. Skeletal hyphae 2.48 to 3.82 µm in diameter, strong orange yellow in water, becoming strong yellowish brown to dark yellowish brown in KOH. Setoid elements absent. Basidia clavate up to 8 µm, with four sterigmata. Basidiospores globose to subglobose, 3.92–6.46 × 3.85–5.54 (5.40 ± 0.25 × 4.98 ± 0.11, aveQ = 1.08, n = 40), with a small apiculus, thick walled, hyaline in water and KOH, dextrinoid (Figure 6.1 C and D).

Associated wood-rot: white.

Ecology and hosts: on *Laurelia sempervirens* (Atherospermataceae, Laurales).

Distribution: Temuco, Cautín Province, Chile.

Culture description: approximate growth of 75 mm per week. Regular fan-shaped margin, pale yellow. Homogeneously fluffy growth, presenting in some plate's areas of woolly growth with a darker coloration, reaching a light brown hue (Figure 6.1 E). The reverse of the plate does not show changes in texture. Culture without characteristic odour.

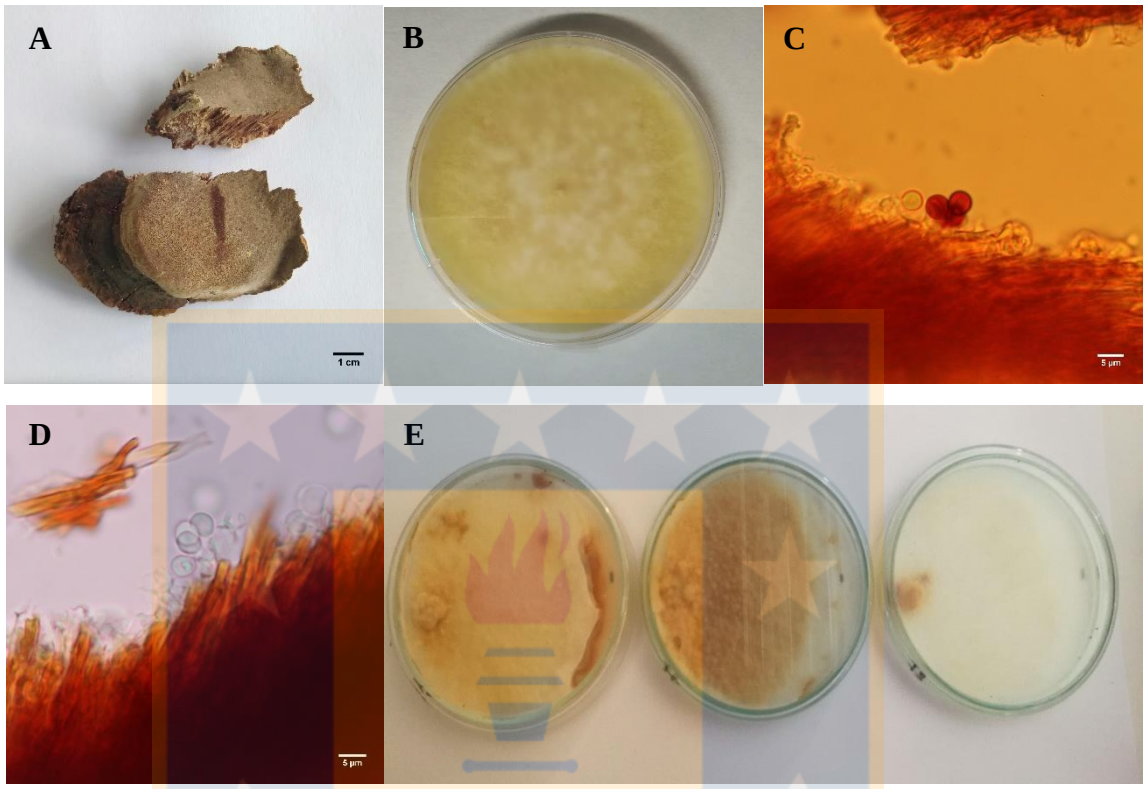

Figure 6.1 Macroscopic and microscopic features of basidiome of FQ1648 strain. (**A**) Holotype showing pulvinate habit. (**B**) *In vitro* culture of a 2-month-old mycelium, in YMG medium. (**C**) Basidiospores showing dextrinoid reaction of their walls. (**D**) Hyaline basidiospores in water (**E**) Macroscopic features of cultures, FQ1648 strain, 6 weeks culture assay in Petri dishes (9 cm diameter). Bars: A= 1 cm; C and D= 5 µm

3.2 Yield of APFQ48

After culturing the mycelial mass of FQ12648 strain in broth, the acidic polysaccharides (APFQ48) were obtained by selective precipitation with Cetavlon. The yield of APFQ48 was 0.48 mg per g of wet mycelium, equivalent to 0.5% of dry mycelium.

3.3 Infrared analysis

As shown in Figure 6.2, the IR spectrum obtained from the APFQ48 extract allowed to detect peaks characteristic for polysaccharides. A wide and marked peak, corresponding to the stretching of O-H bonds, was detected at 3411.06 cm^{-1}; while a peak, which can be associated to the stretching vibrations produced by the C-H bonds, was detected at 2926.59 cm^{-1}. On the other hand, a small peak observed at 1745.11 cm^{-1} can be ascribed to the stretching vibration of C≡O in the protonated carboxylic acid of uronic acid [25]. In addition, the contraction vibration detected at 1649.21 cm^{-1} can be attributed to the C=O bonds present in carboxyl groups. At 1419.13 cm^{-1} it is possible to observe a small peak corresponding to the bending contraction of the C-H bond, and at 1248.63 cm^{-1} a vibration attributed to the presence of sulphates. The presence of marked peaks between 1200 and 1000 cm^{-1} (1156.39 cm^{-1} and 1026.50 cm^{-1}) indicates the presence of pyranose rings [142,143]. Absorption bands detected at 930.78 cm^{-1} and at 854.58 cm^{-1} indicate the presence of both α- and β-type glycosidic bonds in the structure of acidic polysaccharides present in APFQ48 [143].

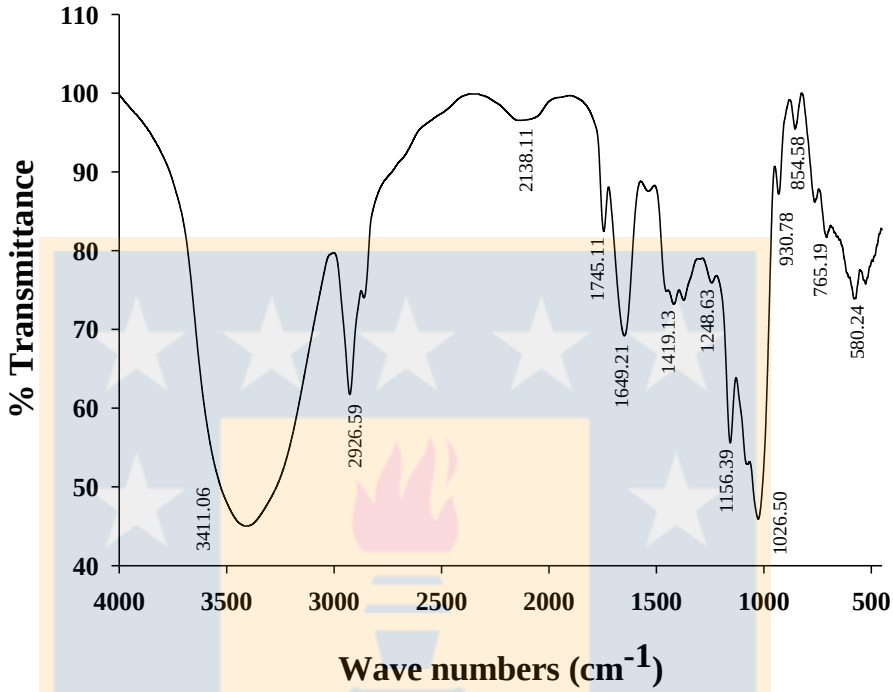

Figure 6.2 Fourier-transform infrared spectroscopy (FT-IR) of the acidic polysaccharides obtained from the polypore fungus FQ1645 strain.

3.4 Cytotoxic capacity of APFQ48 against cell lines

The effect of APFQ48 on the tumour cell lines (HCT-116, MCF-7 and HL-60) and the non-tumour cell line (HGF-1) was determined evaluating the viability of each cell line by means of the MTT assay. Results showed that APFQ48 possess a dose-dependent cytotoxic effect against all three tumour cell lines tested, increasing the mortality of cells as the concentration of acidic polysaccharides increased (Figure 6.3 A, B and C). The viability of the tumour cell lines subjected to the highest APFQ48 concentration (10 mg mL^{-1}) was reduced to nearly 10% in the case of cell lines HCT-116 and MCF-7, reaching figures of 13.49±3.49% or 10.59±0.45%, respectively (Figure 6.3 A and B). With respect to cells of the HL-60 tumour line subjected to the same 10 mg mL^{-1} APFQ48 concentration, their viability was reduced, even more, to 3.75±0.48% (Figure 6.3 C). When treated

with the lowest APFQ48 concentration assayed (0.078 mg mL^{-1}) cell viability of cells was reduced to 99.87±5.5% in HCT-116 cells, to 80.18±4.48% in MCF-7 cells and 84.81±3.24% in HL-60 cells. Thus, the best cytotoxic effectivity of APFQ48 at both, high and low concentrations, was exerted on MCF-7 and HL-60 cells. When the effect of APFQ48 was evaluated on the non-tumour cell line HGF-1 (Figure 6.3 D), results showed that it had a proliferative effect at high and low concentrations on this primary culture cell line, reaching percentage viabilities of 300.35±1.86% and of 114.27±2.57% when subjected to 10 or 0.078 mg mL^{-1} APFQ48, respectively, when compared to the control.

Regarding the IC_{50} figures for the cytotoxic activity of APFQ48, calculations indicated that its best cytotoxic activity occurred against the HL-60 cell line (IC_{50} = 0.8 mg mL^{-1}) if compared to those for the HCT-116 cell line (IC_{50} = 1.83 mg mL^{-1}) or the MCF-7 cell line (IC_{50} = 6.09 mg mL^{-1}). As indicated in Materials and Method and considering the IC_{50} values above reported for the three tumour cell lines, the HL-60 cell line (IC_{50} = 0.8 mg mL^{-1}) was selected to evaluate the effect of APFQ48 on the cell cycle of a tumour cell line. Due to the proliferative effect of APFQ48 on the non-tumour cell line HGF-1, the figure calculated for the IC_{50} was outside the range of acidic polysaccharides evaluated in the present work and therefore, it was not possible to calculate the IC_{50} for this cell line.

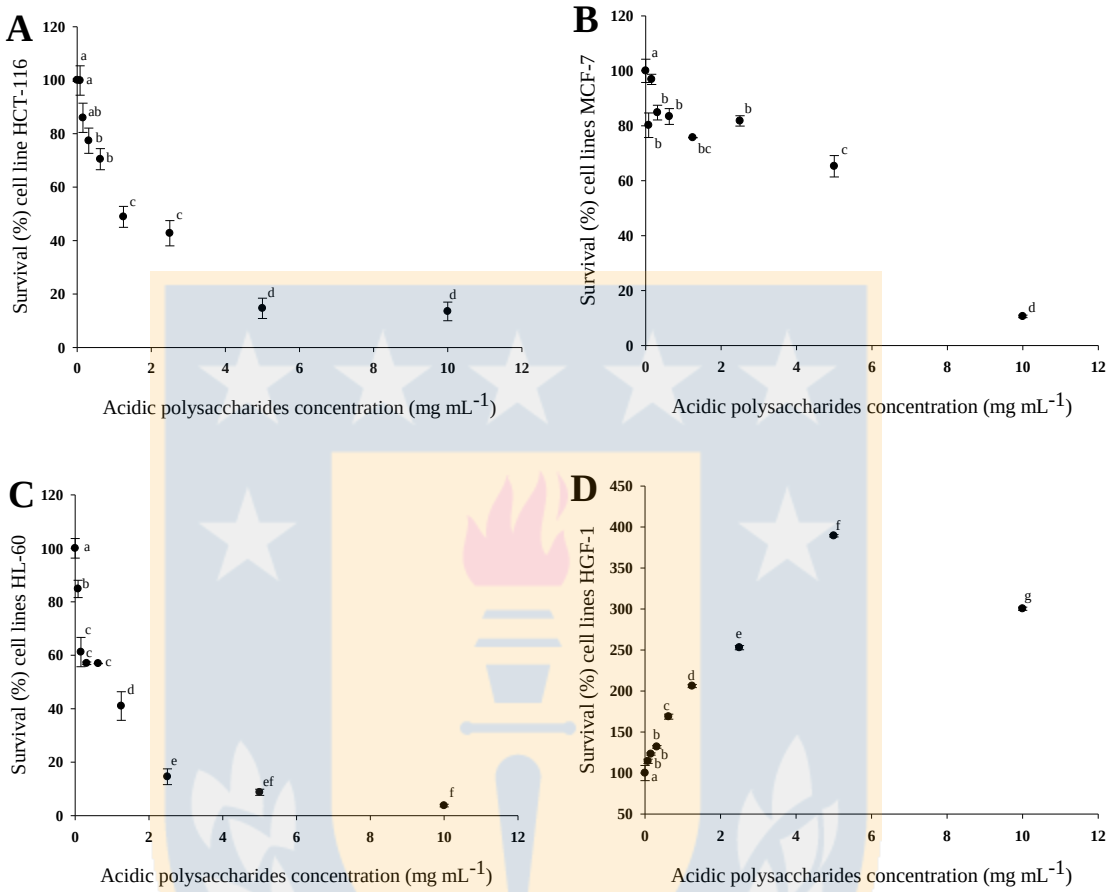

Figure 6.3 Cell survival (%) of cell lines exposed, for 72 hours, to 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 mg mL^{-1} of acidic polysaccharides of FQ1648 strain (APFQ48). (**A**) Survival of tumour cell line HCT-116; (**B**) Survival of tumour cell line MCF-7; (**C**) Survival of tumour cell line HL-60; (**D**) Survival of non-tumour cell line HGF-1. A same letter indicates that there are no significant differences between the different concentrations (Tukey, $p<0.001$).

3.5 Effect of APFQ48 on the cell cycle of the HL-60 cell line

To determine the effect of APFQ48 on the cell cycle of a tumour cell line, the most susceptible one those tested in the present work was selected, using the IC_{50} figures calculated based on the cytotoxic effect of the acidic polysaccharides. Therefore, the cell line HL-60 was selected. HL-60 cells were treated with three

different concentrations of APFQ48 (¼X, 1X or 4X the IC_{50} for this cell line), with 20 µM 2-methoxyestradiol (positive control) or not treated with APFQ48 (negative control). Results indicated that the effect of both lower APFQ48 concentrations tested (¼X IC_{50}= 0.20 mg mL^{-1} and 1X IC_{50}= 0.80 mg mL^{-1}) was concentration dependent and not significantly different to that observed in the negative controls (data not show). On the contrary, the highest APFQ48 concentration tested (4X IC_{50}= 3.20 mg mL^{-1}) significantly increased the percentage of events detected in Sub G1 phase (reaching 43.85±4.62% in comparison to 13.49±0.87% in the negative control, i.e., untreated cells) (Figure 6.4 A and B). With respect to the other phases of the cell cycle, APFQ48 reduced the percentage of events when compared to the negative control. These percentages were 34.28±6.18% for the G0/G1 phase and 21.87±1.56% for the S/G2/M phase while in the negative control they were 54.45±1.01% and 32.06±1.88%, respectively (Figure 6.4 A and B). Regarding the positive control, 20 µM 2-methoxyestradiol significantly increased the percentage of events in Sub G1 and S/G2/M phases of the cell cycle, (23.84±3.19% and 45.65±3.35%, respectively), but it significantly reduced the percentage of events in G0/G1 phase (30.51±0.17%) when compared to the negative control (Figure 6.4 C).

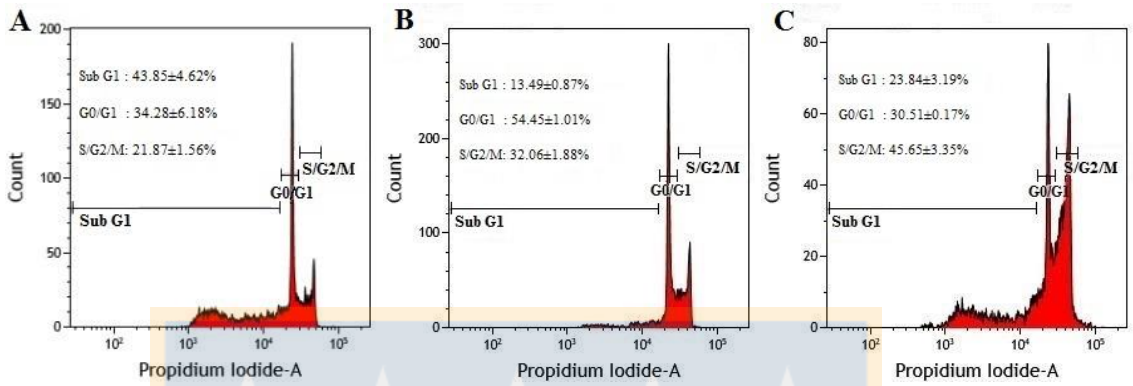

Figure 6.4 Subpopulations of tumour HL-60 cells at different cell cycle phases after 16 h treatment with (**A**) Cells treated with 4X IC_{50} concentration of acidic polysaccharides of FQ1648 strain. (**B**) Negative control, cells with culture medium alone. (**C**) Positive control (20 µM 2-methoxyestradiol). Percentages are the mean ± SD of three independent experiments.

3.6 Antioxidant capacity of APFQ48 on free radicals

The results of the antioxidant activity exerted by APFQ48 demonstrated that the acidic polysaccharides extracted from FQ1648 strain possess a significant reducing potential ($p<0.01$) against DPPH and ABTS free radicals when compared to the negative control (radical + phosphate buffer). A 30 mg mL^{-1} concentration of APFQ48 exerted antioxidant activities of 27.31±3.26% against DPPH and of 11.34±2.19% against ABTS. When the antioxidant activity of APFQ48 was expressed as Trolox equivalents, making use of a previously prepared calibration curve, it was observed that the activity of APFQ48 against DPPH or ABTS did not exceeded that of 1 µg mL^{-1} Trolox with figures of 0.94±0.13 µg mL^{-1} Trolox equivalent for DPPH radical and 0.66±0.14% µg mL^{-1} Trolox equivalent for the ABTS radical.

4. DISCUSSION

The results obtained from the FT-IR analysis allowed to identify the presence of characteristic functional groups of polysaccharides, such as the existence of α and β-type glycosidic bonds, and pyranose rings; at the same time, it was possible to evidence the presence of sulphate groups and uric acid residues, characteristic of acid polysaccharides. According to Kozarski *et al*. [21] and Mei *et al.* [74], the biological activity of macromolecules, such as polysaccharides, is related to the structure, the composition of monomers, the molecular weight and even the types of bonds present in the biomolecule; β-glucans being the most bioactive fungal polysaccharides [21]. Research by several authors has shown the bioactivity of fungal β-glucans; standing out the antitumor and immunomodulatory activity, with anti-proliferative effects of tumour cells in vitro and activation of immune cells (increase of cytokines and ROS production) [21,74,95]. Fungal β-glucans also stand out the antioxidant activity against the DPPH free radical [21,74,95]. A widely studied example is lentinan obtained from the fungus *Lentinus edodes* (Shiitake), a polysaccharide with the conformation of (1→3/1→6)-β-D-glucan, which has antitumor and immunomodulatory activity and, has been used in therapies against human cancer in combination with conventional treatments [25,158].

In the case of the acid polysaccharides obtained from the FQ1648 strain, the results of the cytotoxic activity assay indicated that they have significant activity against the tumour cell lines HCT-116 (colorectal carcinoma), MCF-7 (mammary adenocarcinoma) and HL-60 (promyelocytic leukaemia); reaching percentages of inhibition of cell proliferation between 87-97% with the highest concentration of APFQ48 evaluated (10 mg mL^{-1}). Most previous research evaluating the antitumor capacity of fungal polysaccharides has shown that these macromolecules strongly inhibit in vitro proliferation of tumour cell lines, even at low concentrations. According to Mei *et al*. [74] the polysaccharide PLPS-1 obtained from *Phellinus*

linteus was able to inhibit the proliferation of S-180 cells by 93.6% when applied at a concentration of 200 µg mL^{-1}. Similarly, the research by Liu *et al*. [109] showed that *Phellinus baumii* polysaccharides (PPB) inhibit the proliferation of the HeLa cell line by 90.63% at a concentration of 0.6 mg mL^{-1}. Research by Cao *et al.* [92], indicated that a fraction of polysaccharides obtained from Lentinus edodes (LMP2) managed to inhibit the proliferation of the Hep2 cell line by 37.2% with the highest concentration evaluated (200 µg mL^{-1}). Similarly, the acid polysaccharides obtained from *Nothophellinus andinopatagonicus* (NAAPs) were able to inhibit the proliferation of the HL-60 cell line by ~95%, in a concentration of 10 mg mL^{-1} [154]. However, sometimes polysaccharides (β-glucans) can have negative effects on the host [100]; for which the evaluation of the cytotoxic activity against non-tumour cell lines acquires great relevance. The results obtained with APFQ48 against the non-tumour cell line indicated that this acid polysaccharide did not present a cytotoxic effect against HGF-1; conversely, all the concentrations evaluated (0.078 – 10 mg mL^{-1}) induced cell proliferation, obtaining survival percentages greater than 100%. On the other hand, when comparing the IC_{50} values obtained for each of the cell lines, it was possible to determine that APFQ48 is more effective against the HL-60 cell line at lower concentrations, achieving an IC_{50} of 0.8 mg mL^{-1} compared to the values obtained with the cell lines HCT-116 (1.83 mg mL^{-1}) and MCF-7 (6.09 mg mL^{-1}). This notable difference between IC_{50} values may be due to physical characteristics of the polysaccharide (molecular weight, functional groups); as well as specific characteristics of the cell lines used, such as their adherence, morphology, and gene expression and mutation [125-127].

Mechanisms by which fungal polysaccharides exert their anticancer activity can be indirect (by activating cells of the immune system) or of direct action (by inducing apoptosis or inhibiting metastasis of tumour cells) [58]. According to Li *et al*. [135], extracellular polysaccharides from the fungi *Aspergillus aculeatus*, *Aspergillus terreus* and *Trichoderma sp*. can inhibit the proliferation of HeLa cell

line, arrest the cell cycle on certain phases, and induce *in vitro* cell apoptosis. Liu *et al*. [109] shown that *P. baumii* polysaccharides (PPB) are capable of inducing HeLa cell cycle arrest in the G0/G1 phase, increasing the percentage of events in this phase from 27.11% (control) to 48.17 % after treatment. Analysis by flow cytometry showed the effect of APFQ48 on the cell cycle of the HL-60 cell line. After treatment with the polysaccharide APFQ48, percentage of events in the Sub G1 phase increased from 13.48% to 43.85%; furthermore, the percentages in the G0/G1 and S/G2/M phases decreased. This suggests that one of the mechanisms of APFQ48 against the HL-60 tumour cell line could be the arrest of cell cycle in the Sub G1 phase.

Oxidative stress is one of the crucial factors in the proliferation of oncogenesis, this is due to the damaging effect of reactive oxygen species on cells [106]. For this reason, the antioxidant action of fungal polysaccharides becomes relevant. According to Liu *et al*. [86], the G1 fraction from *Fomitiporia punctata* polysaccharides has significant antioxidant activity against the DPPH radical, reaching 51% activity at a concentration of 250 µg mL^{-1}. Also, Wu *et al*. [146] showed that the CM-dj-CPS2 and CM-dj(Y)-CPS2 fractions obtained from *Cordyceps militaris* acid polysaccharides can reach 94% of DPPH-scavenging activity at a concentration of 8 mg mL^{-1}. The results obtained in the antioxidant activity assays of APFQ48 allowed us to observe that the acid polysaccharides from FQ1648 strain a significant ($p<0.001$) antioxidant activity against DPPH and ABTS free radicals with the only concentration evaluated (30 mg mL^{-1}); when compared with the control (phosphate buffer). The antioxidant effect of APFQ48 on the DPPH radical was 27.31%, equivalent to the activity of 0.94 µg mL^{-1} of Trolox. On the other hand, the effect against the ABTS cationic radical was 11.34%, equivalent to the activity of 0.66 µg mL^{-1} of Trolox. The antioxidant activity obtained in the present investigation is lower than the antioxidant activity reported by other authors for fungal polysaccharides. However, the composition of the polysaccharides, as well as the presence of other components associated with

the sugar chains (generating a synergistic effect) could be responsible for the difference in the antioxidant capacity observed. To determine the mechanisms of action and the differences that the acid polysaccharide APFQ48 presents, new assays are required.

5. CONCLUSION

This research allowed us to determine the antitumour and antioxidant capacity of the fungal strain FQ1648, collected from a specimen of *Laurelia sempervirens*, an endemic tree from Chile and Argentina. The results indicated that the acid polysaccharides (APFQ48) extracted from the mycelial culture have significant antitumour activity against the tumour lines evaluated (HCT-116, MCF-7 and HL-60), being much more effective against the HL-60 cell line of human leukaemia (IC_{50}= 0.80 mg mL^{-1}). Flow cytometry analysis showed a possible effect of cell arrest in the Sub G1 phase of the cell cycle of the HL-60 line treated with APFQ48, observing an increase in the percentage of events that occurred in this phase, compared to the percentage obtained in the negative control (HL-60 cells not treated with acid polysaccharide). In addition, the acidic polysaccharides APFQ48 had no cytotoxic effect against the non-tumour cell line HGF-1 at the concentrations tested; on the contrary, an increase in the cell proliferation of the HGF-1 line was evidenced. Acid polysaccharides APFQ48 have a significant capacity to reduce DPPH and ABTS free radicals, proving to be more effective against the DPPH free radical with 27.31% activity; on the other hand, APFQ48 only reached 11.34% activity against the ABTS cationic radical. The results obtained in this work allow elucidating a fragment of the bioactivity of APFQ48. However, in order to know in detail, the mechanisms of action and the bioactive potential that the acid polysaccharides of the FQ1648 strain may present, it is necessary to carry out new future research.

6. REFERENCIAS

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CAPITULO VII: DISCUSIÓN GENERAL

Los polisacáridos son polímeros naturales que presentan una gran relevancia debido a que corresponden al recurso renovable más abundante en la tierra [89], además de presentar una destacable capacidad bioactiva, contribuyendo al desarrollo de nuevos medicamentos [90]. A la fecha se han aislado una gran cantidad de polisacáridos con actividad anticancerígena y antioxidante a partir del cuerpo fructífero y el cultivo micelial de diversas especies pertenecientes a la familia Hymenochaetaceae [58,67,82,92]. Además, basados en la interacción de los grupos ácidos existentes en las cadenas de polisacáridos con diferentes biomoléculas dianas presentes en las células [25], se considera que la fracción ácida de los polisacáridos presentaría una actividad biológica mucho más relevante que la fracción neutra. En base a esto, y a estudios de actividad biológica de polisacáridos ácidos de hongos, como el de Kiho *et al.* [145] donde se evidencia que los polisacáridos ácidos obtenidos desde el hongo *Tremella aurantia* actúan como un poderoso hipoglicemiante en ratones; y los trabajos de Wang *et al.* [25] y Chen *et al.* [96] que demuestran que la fracción ácida de los polisacáridos de *Cordyceps sinensis* presentan potencial inmunoestimulante, aumentando la producción de citoquinas en macrófagos Raw 264.7; se decidió evaluar la capacidad antitumoral y antioxidante de los polisacáridos ácidos de cuatro cepas de hongos asociados al bosque nativo de Chile. Las especies estudiadas en este trabajo, *Nothophellinus andinopatagonicus, Phylloporia boldo* y *Fomitiporia sp.*, no presentaban una determinación previa de la bioactividad de sus polisacáridos, ni ácidos, ni totales; convirtiendo esta investigación en la primera descripción de la capacidad antitumoral y antioxidante de estos hongos.

Los resultados de los ensayos de citotoxicidad contra líneas celulares tumorales demostraron que los polisacáridos ácidos obtenidos desde las cuatro cepas en estudio poseen una alta capacidad anticancerígena directa; reduciendo

significativamente la proliferación celular de las líneas HCT-116, MCF-7 y HL-60, de manera dosis dependiente. A su vez, se pudo determinar que esta acción citotóxica presume ser selectiva contra células tumorales, no presentando efecto anti-proliferativo relevante en la línea celular no tumoral HGF-1 aún con la mayor concentración de polisacárido evaluada. En base a los valores de IC_{50}, se determinó que tres de los polisacáridos ácidos presentan un efecto anticancerígeno más elevado contra la línea celular HL-60; requiriendo una concentración de sólo 127.85 µg mL^{-1} de PBAP40, 767.16 µg mL^{-1} de NAAPs y 800 µg mL^{-1} de APFQ48 para reducir la supervivencia celular a un 50%. Por su parte, PBAP26 presenta un efecto citotóxico mayor contra la línea celular HCT-116, con un valor de IC_{50} de 535.83 µg mL^{-1}. La composición química de los polisacáridos, así como su peso molecular o largo de la cadena, sumados a características particulares de las líneas celulares, como su adherencia, y la expresión de determinados genes, además de la presencia de mutaciones en oncogenes específicos, podrían facilitar la acción citotóxica de los polisacáridos ácidos de las cepas FQ1640, FQ1645 y FQ1648 sobre la línea celular HL-60 [103,125-127]. De acuerdo con Mei *et al.* [74] y Liu *et al.* [109], los polisacáridos de origen fúngico presentan una significativa acción anticancerígena; siendo capaces de reducir significativamente la proliferación de las líneas celulares tumorales, cuando son tratadas con pequeñas concentraciones del polisacárido evaluado. Ejemplo de esto es el extracto crudo de polisacáridos de la especie *Phellinus baumii,* que en una concentración de 600 µg mL^{-1} tiene la capacidad de reducir la viabilidad de la línea celular HeLa, alcanzando un porcentaje de inhibición de un 90% [109]. Por su parte, los polisacáridos obtenidos desde *Phellinus linteus* en concentración de 200 µg mL^{-1} son capaces de inhibir la proliferación de las células de sarcoma-180 de murino en un 93% [74]. Sin embargo, cuando analizamos el efecto anticancerígeno de una fracción de los polisacáridos del hongo *Lentinus edodes*, podemos observar que alcanza un porcentaje de inhibición de la supervivencia de las células Hep2 cercana al 37%

con una concentración de polisacárido de 200 µg mL^{-1} [92]. Este resultado parece ser más cercano a lo observado con los polisacáridos ácidos de las especies evaluadas en esta investigación, haciendo suponer que la presencia de otros componentes en los extractos crudos, tales como complejos proteína-polisacárido, péptidos, pigmentos u otros metabolitos, podrían ser los responsables de la elevada actividad biológica que presentan dichos polisacáridos; diferenciándolos de los polisacáridos ácidos analizados.

Con el fin de clarificar el mecanismo de acción por el cual los polisacáridos ejercen su efecto anticancerígeno, se realizó un análisis por citometría de flujo con la línea celular tumoral de mayor susceptibilidad a estos polisacáridos ácidos. Al determinar el efecto de los polisacáridos sobre el ciclo celular de la línea HL-60, se pudo evidenciar que los cuatro polisacáridos ácidos afectan las fases del ciclo celular, concentrando el mayor porcentaje de eventos en la fase Sub G1 cuando los polisacáridos PBAP26, NAAPs y APFQ48 fueron aplicados en concentración igual a 4 veces la concentración IC_{50}. Por su parte, el polisacárido PBAP40 aumentó considerablemente el porcentaje de eventos en la fase G0/G1. Estos resultados sugieren que los polisacáridos analizados inducen un arresto de las células en una determinada fase del ciclo celular, pudiendo de esa forma reducir la proliferación celular o inducir la muerte de las células [109,148]. Resultados similares han sido descritos con anterioridad para polisacáridos de origen fúngico. De acuerdo con Mei *et al.* [74] y Liu *et al.* [109], los polisacáridos de *P. baumii* y *P. linteus* son capaces de generar un arresto del ciclo celular, inhibiendo la proliferación celular y provocando una posterior apoptosis en células de melanoma de murino y en células de sarcoma S-180. De igual manera, se ha demostrado que los polisacáridos extracelulares de las especies *Aspergillus aculeatus, Aspergillus terreus* y *Trichoderma sp.* son capaces de inhibir la proliferación de celular HeLa, y provocar el arresto del ciclo celular en una determinada fase, induciendo la apoptosis *in vitro* [135]. Según Yan *et al.* [58], los mecanismos de acción por los cuales los polisacáridos

ejercen la actividad anticancerígena pueden clasificarse como preventivos, directos e indirectos. De acuerdo con el efecto de los polisacáridos sobre el ciclo celular de la línea HL-60, podemos determinar que uno de los mecanismos anticancerígenos que presentan es de acción directa, provocando el arresto del ciclo celular, e induciendo una posterior apoptosis de las células tumorales.

Como un mecanismo de acción anticancerígeno preventivo, se encuentra la eliminación de radicales libres por parte de agentes antioxidantes [58]. Los resultados de los ensayos antioxidantes hacen presumir que este tipo de mecanismo no sería aplicable para los polisacáridos de las cuatro cepas analizadas. Los porcentajes de actividad antioxidante obtenidos contra los radicales DPPH y ABTS son bajos en comparación con la actividad presentada por otros polisacáridos fúngicos. El polisacárido NAAPs en concentración 40 mg mL^{-1} presentó la menor actividad antioxidante, con porcentajes de 6.24% frente al radical DPPH y un 4.63% frente al radical ABTS. Mientras que el polisacárido que evidenció la mayor actividad antioxidante fue APFQ48 en concentración de 30 mg mL^{-1}, con un 27.31% y un 11.34% de actividad, frente al radical DPPH y ABTS respectivamente. De acuerdo con los resultados reportado por otros autores, la actividad antioxidante demostrada por los polisacáridos ácidos de las cepas FQ1645, FQ1526, FQ1640 y FQ1648, no resultaría significativa. De acuerdo con Liu *et al.* [86], una fracción de los polisacáridos de *Fomitiporia punctata* alcanza una actividad antioxidante del 51% frente al radical DPPH cuando es evaluada en una concentración de 250 µg mL^{-1}; de igual manera, fracciones obtenidas desde polisacáridos ácidos de *C. militaris* en concentración de 8 mg mL^{-1}, presentaron una actividad antioxidante de un 94% contra el radical DPPH [146].

Como se ha mencionado anteriormente, la actividad biológica de los polisacáridos depende de sus características estructurales y propiedades físicas [21,22,90-93,150]. El análisis de las muestras de polisacáridos por medio

de FT-IR permitió identificar la presencia de grupos funcionales y enlaces glucosídicos que pueden favorecer la capacidad bioactiva del extracto de polisacáridos ácidos evaluado. El análisis de la muestra obtenida de *N. andinopatagonicus* cepa FQ1645 (NAAPs) evidenció la presencia de grupos sulfatos y un anillo de piranosa, elementos característicos de una muestra de polisacáridos. Por su parte, la muestra de los polisacáridos ácidos de *P. boldo* cepa FQ1640 (PBAP40) presentó un pico atribuido a un anillo de piranosa, y además se observó la presencia de enlaces glicosídicos de tipo α y β. En el caso de la muestra de la cepa FQ1648 (APFQ48), fue posible identificar la presencia de un anillo de piranosa, residuos de ácido urónico (pico del carboxilo a los 1745 cm^{-1}) y residuos de grupos sulfatos; sumado a esto, fue posible identificar la presencia de enlaces α y β glicosídicos. De acuerdo por Kozarski *et al.* [21], los β-glucanos corresponden a los polisacáridos de origen fúngico más bioactivo. Se ha demostrado en diversos estudios que las muestras de polisacáridos con un alto contenido de β-glucanos cuentan con una relevante actividad biológica; entre las que destacan la capacidad antitumoral (inhibiendo la proliferación de las células tumorales), inmunomoduladora (induciendo la secreción de citoquinas y estimulando la fagocitosis en macrófagos) y antioxidante (reduciendo a los radicales libre superóxido, hidroxilo y DPPH), siendo el largo de la cadena de glucanos y la presencia de grupos funcionales factores determinantes en el tipo de bioactividad [21,25,74,93,95,150,158]. Además, de acuerdo con Zhang *et al.* [94] y Cheng *et al.* [118], la presencia de grupos sulfatos en las cadenas de polisacáridos contribuye favorablemente a la interacción de la macromolécula con la superficie de las células tumorales, provocando un aumento en la capacidad anticancerígena presentada por los polisacáridos asociados a grupos sulfatos. Las características estructurales evidenciadas por medio de los análisis de FT-IR para cada una de las muestras; principalmente la presencia de β-glucanos, podrían ser las principales

responsables de la favorable actividad anticancerígena presentada por NAAPs, PBAP26, PBAP40 y APFQ48 contra las 3 líneas celulares tumorales evaluadas.

Debido a su seguridad y sencillez, la extracción con agua caliente es una de las metodologías más empleadas para la obtención de polisacáridos desde el cuerpo fructífero o cultivo micelial de hongos [58, 159]. Sin embargo, el uso de altas temperaturas por periodos prolongados puede causar la degradación de los polisacáridos, reduciendo el rendimiento y dificultando la determinación de componentes de la muestra de polisacáridos [58]. Los rendimientos de polisacáridos obtenidos por extracción con agua caliente y posterior precipitación ácida realizado en esta investigación, evidenciaron una clara diferencia entre las cuatro cepas estudiadas; con equivalencias desde un 0.5% del peso seco del micelio en el caso de la cepa FQ1648 (*Fomitiporia sp.*) y un 38% del peso seco del micelio para la cepa FQ1626 (*Phylloporia boldo*) (dato no mostrado anteriormente). Mientras que las otras dos cepas presentaron un rendimiento de polisacáridos de un 3,49% del peso seco micelial (cepa FQ1640; *P. boldo*) y un 20% del peso seco micelial (cepa 1645; *Nothophellinus andinopatagonicus*). Según lo reportado por Kiho *et al.* [160], la obtención de polisacáridos crudos desde *Cordyceps sinensis* por medio de extracción con agua caliente generó un rendimiento de 3.83%; mientras que el rendimiento para *Hohenbuehelia serotina* a través del mismo método de extracción permitió la obtención de 10.57% de polisacáridos totales [159]. En base a las cifras señaladas, los porcentajes de polisacáridos ácidos obtenidos desde los hongos de esta investigación podrían considerarse dentro del rendimiento promedio. Sin embargo, de acuerdo con Li & Wang [159], el método de extracción empleado para la obtención de los polisacáridos tiene un impacto importante no solo en el rendimiento del polisacárido obtenido; sino también, en el nivel de bioactividad que presentan dichos polisacáridos. Esto podría ser un punto a considerar para futuras investigaciones sobre actividad biológica de polisacáridos de estos u otros hongos.

CAPITULO VIII: CONCLUSIONES

Los polisacáridos de origen fúngico han despertado un gran interés en investigaciones científicas, debido a su potencial bioactivo. Sin embargo, el estudio de la fracción ácida de estos polisacáridos no ha sido ampliamente explorado aún. La presencia de residuos proteicos y pigmentos en los extractos de polisacáridos crudos obtenidos desde micelio y/o carpóforos, han sugerido una acción sinérgica al momento de evaluar la actividad anticancerígena, y sobre todo antioxidante en trabajos previos de diversos autores; esto podría considerarse una desventaja al momento de evaluar la bioactividad de una fracción de polisacáridos que ha sido sometida a protocolos para eliminar dichos residuos, como es el caso de los polisacáridos ácidos analizados en esta investigación.

A pesar de esto, los polisacáridos de las cuatro cepas estudiadas (NAAPs, PBAP26, PBAP40 y APFQ48) demostraron poseer una significativa actividad anticancerígena contra las tres líneas celulares tumorales empleadas en los ensayos (HCT-116, MCF-7 y HL-60), destacándose la actividad citotóxica presentada contra la línea celular HL-60 (leucemia humana). Aún más, resulta de gran relevancia el bajo o nulo efecto citotóxico que los polisacáridos en estudio mostraron frente a la línea celular no tumoral (HGF-1), en la cual se observó un aumento de la proliferación celular por sobre el 100% de viabilidad con al menos 3 de los polisacáridos evaluados, lo que sugiere una posible acción selectiva de las células blanco. De esta forma, no se rechaza la hipótesis que postula que los polisacáridos ácidos obtenidos desde las cepas en estudio presentan un efecto citotóxico frente a líneas celulares tumorales.

Además, en base a los resultados obtenidos desde los ensayos de citometría de flujo, se puede sugerir que el mecanismo de acción de los polisacáridos ácidos de las cepas FQ1626, FQ1640, FQ1645 y FQ1648, podría ser el arresto de las células de la línea celular HL-60 en la fase Sub G1 del ciclo celular,

reduciendo de esta forma la proliferación y generando de manera posterior la muerte celular. No obstante, para poder asegurar que el método de acción de los polisacáridos NAAPs, PBAP26, PBAP40 y APFQ48 corresponde al mencionado anteriormente, nuevos ensayos complementarios serían necesarios.

Por su parte, los ensayos de actividad antioxidante mostraron que los polisacáridos ácidos evaluados presentan una significativa actividad antioxidante cuando se compara los resultados con el control negativo; sin embargo, corresponde a un efecto bajo en relación con las actividades reportadas por otros polisacáridos fúngicos. Este rendimiento puede ser consecuencia de la escasa o nula presencia de residuos de otras moléculas fúngicas, como péptidos y fenoles, en la fracción ácida de los polisacáridos, como se sugiere anteriormente. No obstante, los resultados recabados en este trabajo permiten disponer de un primer registro de la capacidad reductora de radicales libres que estos cuatro polisacáridos ácidos presentan, como un punto de partida para futuras investigaciones relacionadas. A partir de estos resultados, podemos concluir que tampoco se rechaza la hipótesis que plantea a los polisacáridos ácidos de las cepas en estudio como anticancerígenos indirectos, por medio de la reducción del estrés oxidativo.

En relación con la caracterización de los polisacáridos NAAPs, PBAP26, PBAP40 y APFQ48 por medio de análisis con FT-IR, podemos concluir que la presencia en las muestras de enlaces β-glicosídicos, y por consiguiente de β-glucanos, podría ser la responsable de la actividad anticancerígena y antioxidante evidenciada en los diferentes ensayos, en base a la variada bioactividad atribuida a este tipo de polisacárido en estudios previos.

Al comparar los ensayos de actividad anticancerígena de los cuatro polisacáridos evaluados, y basados en los valores de IC_{50} obtenidos para cada

una de las líneas celulares tumorales, se puede evidenciar que el polisacárido PBAP40 presenta una mayor actividad citotóxica que los otros tres polisacáridos, presentando los menores valores de IC_{50} para las líneas celulares HCT-116, MCF-7 y HL60. Por el contrario, al evaluar los resultados de la actividad antioxidante, es el polisacárido APFQ48 el que presenta una capacidad antioxidante superior, tanto con DPPH como con ABTS.

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