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RESEARCH ARTICLE

Antiproliferative and antibacterial activity of extracts of *Ganoderma* strains grown in vitro

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Abstract In this bioprospecting study the biological activities of extracts of the in vitro culture of *Ganoderma* Mexican strains were evaluated. The extracts were tested by the Sulforhodamine B staining method for antiproliferative activity and the plate microdilution method for antibacterial activity. Extracts that proved bioactive in these two activities, the antioxidant activity (Galvinoxyl, ABTS, and DPPH) and total phenolic contents (Folin-Ciocalteu) were additionally determined, as well as acute toxicity (*Artemia franciscana*). In the antiproliferative activity *Ganoderma curtisii* strain (GH-16-015) obtained a remarkable value of $GI_{50} \leq 50$ µg/mL against tumor lines: A549, HBL-100, HeLa, and T-47D. *G. curtisii* strains (GH-16-012 and GH-16-015) showed MIC values = 500 µg/mL against *Staphylococcus aureus*. *G. curtisii* strain (GH-16-012) almost reduced by 50% the radical Galvinoxyl. Finally, *G. curtisii* strain (GH-16-023) presented the lowest level of toxicity with a LC_{50} of 490.881 µg/mL against *A. franciscana*. These results support the potential medicinal effects of Mexican strains of *G. curtisii*.

Keywords Antibacterial activity · Antioxidant activity · Antiproliferative activity · Brine shrimp · Molecular identification

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Introduction

Many of the medications that we know today have been derived from ancient folk remedies of natural origin, this due to their millenary use, which was based on the testimonies of their beneficial effects on human health (Harhaji et al., 2009). On the other hand, the bioprospecting of natural products is an alternative that allows us to understand the pharmacological, nutraceutical, and medicinal benefits which can be obtained from various organisms such as plants, bacteria, and fungi, the latter organisms having been historically proven their value in the field of pharmacology and in particular with the discovery of new therapeutic agents (Evidente et al., 2014; Nobili et al., 2009). Within the wide variety of fungi, we can find basidiomycete fungi of the genus *Ganoderma* (P. Karst.), which has been used as a natural

source in Traditional Asian Medicine for more than two centuries. Its use has been based mainly on observations and testimonies of its effects in the prevention and treatment of cancer, fighting bacterial infections, regulating the immune system, and blood pressure, among other medicinal effects (Bishop et al., 2015; Paterson, 2006). Today, *Ganoderma* is consumed as a nutraceutical, in soup/tea or other drinks (Bishop et al., 2015).

It is necessary to mention the scientific support of various studies worldwide on these fungi, that confirm that the medicinal effects of *Ganoderma* are largely due to the amount of bioactive compounds they synthesize, highlighting triterpenoids such as ganoderic acids with reports of the following activities: antioxidant, antimicrobial, and cytotoxicity (Li et al., 2012), and antiproliferative and antibacterial activity (Upadhyay et al., 2014). Other important compounds are polysaccharides such as β-glucans and the phenolic compounds with antioxidant and antiproliferative activity (Veljović et al., 2017). Finally, sterols such as ergosterol peroxide induce death effect in tumor cells (Li et al., 2016). *Ganoderma* studies are not limited to using only fruiting bodies. There are several reports that use mycelial isolates in vitro as an alternative for the evaluation of various biological activities. This type of isolation is an economical and fast alternative for the cultivation of these fungi (Ćilerdžić et al., 2016).

However, nowadays, despite the enormous progress in the field of medicine, the set of related diseases known as cancer is one of the leading causes of death around the world, with approximately 9.6 million deaths worldwide and 18.1 million of new cases per year, meanwhile, the types of cancer with the highest incidence are: lung, breast, colon, prostate, skin cancer (not melanoma), and stomach (Bray et al., 2018). On the other hand, bacterial diseases continue to be a major threat to global health, mainly due to the acquired drug resistance to various antibiotics. Resistance is a natural phenomenon; however, the irresponsible use of these drugs has accelerated the process. For this reason, the World Health Organization in 2017 published a list of the bacteria for which new antibiotics with critical, high and medium priority levels are urgently needed, where the bacteria can be found: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, Enterobacteriaceae, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, among others (WHO, 2017). In relation to the aforementioned problem, the present study aimed to evaluate the antiproliferative, antibacterial, antioxidant, and acute toxicity activity of crude extracts of the in vitro culture of *Ganoderma* strains from Veracruz, Mexico.

Materials and methods

Collection of biological material

The fruiting bodies of 30 specimens of the genus *Ganoderma* were collected during the months of July–September 2016. The collection sites included the cities of Xalapa (Lat. 19.526805, Long. − 96.923774), Jalacingo (Lat. 19.805374, Long. − 97.308817), and Coatepec (Lat. 9.454365, Long. − 96.959730) located in Veracruz, Mexico. The substrates from which fruiting bodies were collected corresponded to stumps and trees found in areas of *Quercus affinis* Scheidw., *Pinus patula* Schiede ex Schltdl. & Cham., *Alnus acuminata* Kunth, *Fagus grandifolia* subsp. *mexicana* (Martínez) A.E. Murray, *Liquidambar styraciflua* L., *Fraxinus uhdei* (Wenz.) Lingelsh., *Jacaranda mimosifolia* D. Don, and fruit trees of *Prunus persica* (L.) Batsch., *Prunus domestica* L., and *Persea americana* Mill. Table 1 shows the origin of all *Ganoderma* species collected in this investigation: *Ganoderma australe* (Fr.) Pat., *Ganoderma applanatum* (Pers.) Pat., *Ganoderma colossus* (Fr.) C.F. Baker, *Ganoderma curtisii* (Berk.) Murrill, *Ganoderma lobatum* (Schwein.) G.F. Atk., *Ganoderma oregonense* Murrill, and *Ganoderma resinaceum* Boud., Bull.

Morphological identification and mycelial isolation

The 30 specimens were identified by morphological description of the macroscopic and microscopic characteristics for the identification of *Ganoderma* species according to López-Peña et al. (2016), Torres-Torres et al. (2012), Torres-Torres et al. (2015), and the Index Fungorum (http://www.indexfungorum.org/Names/Names.asp). On the other hand, the mycelial isolation was performed by disinfecting small pieces of the fruiting bodies with sodium hypochlorite (NaClO 2%) and distilled water. The small pieces were inoculated in Petri dishes with potato dextrose agar (PDA) (MCD LAB, Edomex, Mexico) and incubated at 30 ± 2 °C for 8 days until inducing mycelial development (Fang and Zhong, 2002; Mendoza et al., 2015). The 30 isolates in vitro were deposited in the fungal strain collection of the Centro de Investigación en Micología Aplicada of the Universidad Veracruzana, Xalapa, Veracruz, Mexico.

Cultivation and extraction

The isolated strains were grown according to Fang and Zhong (2002), in a liquid medium, which contained the following components: sucrose (35 g/L), peptone (5 g/L), yeast extract (2.5 g/L), KH_2PO_4 H_2O (1 g/L), $MgSO_4$

Table 1 General list of *Ganoderma* specimens collected for this study

Species	Strain	Substrate	Geographic location
G. australe	GH-15-005	*J. mimosifolia*[a]	Xalapa
G. australe	GH-16-005	*F. grandifolia* subsp. *mexicana*[b]	Xalapa
G. australe	GH-16-033	*P. americana*[b]	Jalacingo
G. applanatum	GH-15-T3	Dead stump	Xalapa
G. applanatum	GH-16-003	*F. grandifolia* subsp. *mexicana*[b]	Xalapa
G. applanatum	GH-16-004	*F. grandifolia* subsp. *mexicana*[b]	Xalapa
G. applanatum	GH-16-006	Dead stump	Xalapa
G. applanatum	GH-16-025	Dead stump, zone of *P. patula*	Jalacingo
G. applanatum	GH-16-027	Dead stump	Jalacingo
G. applanatum	GH-16-028	Dead stump	Jalacingo
G. applanatum	GH-16-035	*L. styraciflua*[a]	Xalapa
G. colossus	GH-16-011	*L. styraciflua*[a]	Xalapa
G. colossus	GH-16-013	*F. uhdei*[a]	Xalapa
G. colossus	GH-16-030	Dead stump	Coatepec
G. curtisii	GH-16-007	*J. mimosifolia*[a]	Xalapa
G. curtisii	GH-16-012	Dead stump, zone of *P. domestica*	Xalapa
G. curtisii	GH-16-015	Dead stump, zone of *Q. affinis*	Jalacingo
G. curtisii	GH-16-018	Dead stump, zone of *A. acuminata*	Jalacingo
G. curtisii	GH-16-019	*P. persica*[a]	Jalacingo
G. curtisii	GH-16-021	Dead stump	Jalacingo
G. curtisii	GH-16-022	Dead stump, zone of *Q. affinis*	Jalacingo
G. curtisii	GH-16-023	Dead stump, zone of *Q. affinis*	Jalacingo
G. curtisii	GH-16-031	Dead stump	Jalacingo
G. lobatum	GH-15-004	Dead stump	Xalapa
G. lobatum	GH-16-010	Dead stump of *P. americana*	Jalacingo
G. oregonense	GH-15-001	Dead stump	Xalapa
G. oregonense	GH-16-008	Dead stump	Xalapa
G. oregonense	GH-16-037	Dead stump of *L. styraciflua*	Xalapa
G. resinaceum	GH-16-009	Dead stump	Xalapa
G. resinaceum	GH-16-020	Dead stump	Xalapa

[a]Live tree

[b]Dead tree

$7H_2O$ (0.5 g/L), and vitamin B_1 (0.05 g/L). The strains were cultivated in a total volume of 500 mL distributed in five Erlenmeyer flasks (500 mL) with 100 mL of medium each, inoculated with mycelium (approximately 1 cm^2). Then flasks were incubated at 30 ± 2 °C for 21 days, 8 days in agitation at 115 rpm and the rest under a static condition (Mendoza et al., 2015). After the incubation period, the biomass (pellets) was separated from the culture broth through Whatman No. 1 filter paper under vacuum. Both the biomass and the broth were frozen and lyophilized. Subsequently, the biomass and dehydrated broths were macerated with a mixture of chloroform–methanol (1:1 v/v) and concentrated in a rotary evaporator (40 °C) (Büchi R-124, Flawil, Switzerland) and the crude extracts obtained were centrifuged and filtered to finally be stored (− 4 °C) (Couttolenc et al., 2016).

Antiproliferative activity evaluation

The cell lines used were of human solid tumors: A549 and SW1573 (lung), HBL-100 and T-47D (breast), HeLa (cervix), and WiDr (colon), which were donated by Professor G.J. Peters (VU Medical Center, Amsterdam, The Netherlands). The culture of the cell lines was carried out as previously reported by Couttolenc et al. (2016). From the cultures obtained, cell suspensions were taken using the Orflo Moxi Z automatic counter (Orflow, Ketchum, ID, USA) and placed in a volume of 100 µL per well in 96-well plates at densities of 2500 (A549, HBL-100, HeLa, and SW1573), 5000 (T-47D and WiDr) cells per well, based on their doubling time. Antiproliferative activity evaluation was carried out following the protocol for antitumor assays (NCI-60) of the National Cancer Institute (NCI) with some

modifications, following the method of staining with Sulforhodamine B (SRB) (NCI, 2015; Skehan et al., 1990). Stock solutions of the 60 crude extracts were prepared (30 of which from the biomass and 30 from the broth) of the 30 isolated fungal strains, dissolving them in DMSO (Sigma-Aldrich, St. Louis, MO, USA), obtaining solutions 400 times the maximum concentration of the final test desired (i.e. 10 mg/mL) and diluted in each well of the microplates until a maximum concentration of 250 µg/mL was reached (Monks et al., 1991). Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). The treatment with the crude extracts began the next day after sowing in each well, incubation was for 48 h, after which period the cells were precipitated with 25 µl of cold trichloroacetic acid (50% w/v) and fixed at 4 °C for 1 h. A colorimetric test with the SRB was performed according to Skehan et al. (1990). The optical density (OD) of each well was measured at 530 nm, using a BioTek's PowerWave XS microplate absorption reader (BioTek, Winooski, VT, USA). The percentage growth (PG) was calculated with respect to the untreated control cells (C) for each of the concentration levels of the extract, as a function of the difference in OD at the start (Tz) and at the end of the exposure time to the extract (Ti), according to the 50% growth inhibition formula (GI_{50}) [(Ti-Tz)/(C-Tz)] × 100 = 50 established by the NCI (NCI, 2015). The results obtained were expressed as the extract concentration that caused 50% reduction in the proliferation of tumor cells (GI_{50}) (Skehan et al., 1990).

Antibacterial evaluation

The antibacterial evaluation was performed following the plate microdilution method established by the Clinical and Laboratory Standards Institute (CLSI, 2012) with modifications to evaluate fungal extracts as reported by Zengin et al. (2015). The antibacterial activity was determined for the strains that were bioactive in the antiproliferative activity assay. The crude extracts of the fungal strains were dissolved in DMSO, obtaining stock solutions at a concentration of 10 mg/mL. In 96-well microplates 75 µL of a solution of the fungal crude extract solubilized in Müller-Hinton broth (Dibico, D.F., Mexico) were placed and 75 µL of a bacterial suspension ($1.5X10^6$ CFU/mL) of each bacterial strain of medical importance: *Escherichia coli* (ATCC 35218), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 23853), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 25923). Serial double dilutions were made from row A to F of each microplate, starting at a concentration of 1000–31.25 µg/mL and wells in row G were used as growth controls (Müller-Hinton broth, bacterial suspension, and DMSO) and wells in row H as sterility controls (Müller-Hinton broth). Ampicillin, (Sigma-Aldrich, St. Louis, MO, USA) at initial concentration of 1000 µg/mL, was used as positive control. The microplates were incubated at 37 °C, for a period of 24 h. After the incubation period, 30 µL of Triphenyl Tetrazolium Chloride (1.0% TTC) (Sigma-Aldrich, St. Louis, MO, USA) were added to all wells. The results obtained were expressed as the Minimum Inhibitory Concentration (MIC), which was evidenced by the addition of TTC and a change in color to deep red indicated the presence of live bacteria and that least dilution that remained colorless corresponded to the MIC (Zengin et al., 2015). This evaluation was done in triplicate.

Molecular identification of bioactive strains

Molecular identification was made only for strains with antiproliferative and antibacterial activity, using the standard protocol for direct preparation of fungal DNA for PCR according to Izumitsu et al. (2012). The bioactive *Ganoderma* strains were molecularly identified by sequencing the ITS regions (ITS1-5.8S-ITS2) of ribosomal RNA (rRNA). The ITS region was amplified by PCR according to Shnyreva et al. (2018), using 2 primers: initial ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), the PCR products were purified and sequenced. Subsequently, an analysis of the sequences obtained was performed comparing them with the nucleotide database NCBI GenBank (http://www.ncbi.nlm.nih.gov/) using the local alignment tool (BLAST), from which 19 sequences of species of the genus *Ganoderma* and an external sequence to the genus as outgroup (*Trametes versicolor*, MH855444.1) were selected for the phylogenetic analysis. For this analysis, the sequences were edited using the BioEdit software. The dendogram was constructed by multiple sequence alignment CLUSTALW and the algorithm of Maximum likelihood (Juke-Cantor) of the MEGAX software, performing a Bootstrap analysis with 1000 repetitions (Couttolenc et al., 2016). The nucleotide sequences of *Ganoderma* strains obtained in this study were deposited in GenBank.

Antioxidant activity

As a complementary assay, the antioxidant activity was determined in the extracts that were bioactive in the antiproliferative and antibacterial activity evaluations. The fungal extracts were evaluated by measuring the free radicals Galvinoxyl, DPPH, and ABTS according to the methods described by Couttolenc et al. (2020). Trolox (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard antioxidant for calibration curves, and the linear

regression equation was used with which the necessary dose of the standard was obtained, that reduces the concentration of the radical by 50% known as the median effective concentration (EC_{50}) (Couttolenc et al., 2020). The results of the extracts were expressed as Trolox equivalent per 1 mg of extract (TE/mg). In addition, the total phenolic contents were also determined, that was expressed as equivalents of mg of gallic acid per mg of sample GAE/mg.

Galvinoxyl radical scavenging test

The scavenging test was carried out by mixing 1800 µL of galvinoxil (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol (10 mM) and 180 µL of a stock solution of fungal extract dissolved in methanol (1 mg/mL). The mixture was allowed to react for 20 min at 37 °C and the absorbance value was measured on a spectrophotometer at 428 nm (Perkin Elmer, Lambda 265, USA). The Trolox standard curve was prepared in concentrations within the range of 5–30 µM.

ABTS radical scavenging test

The test was carried out by mixing dissolved solutions in deionized water of ABTS 7.4 mM (Sigma-Aldrich, St. Louis, MO, USA) with 2.6 mM potassium persulfate (Sigma-Aldrich, St. Louis, MO, USA) in equal parts. This mixture was allowed to react for a period of 12 h until obtaining the ABTS radical and 1 mL of the radical obtained was added to 60 mL of methanol. After this 1900 µL of this last solution was mixed with 100 µL of stock solution of fungal extract. The mixture was allowed to react for 5 min and the absorbance value was measured in a spectrophotometer at 734 nm. The Trolox standard curve was prepared in concentrations within the range of 3.125–200 µM.

DPPH radical scavenging test

The scavenging test was developed by preparing a solution of the 0.1 mM DPPH radical (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 80% methanol (v/v). The solution was stirred for 40 min. Subsequently, 1500 µL of the DPPH solution was mixed with 500 µL of the stock fungal extract. The mixture was allowed to react for 30 min and the absorbance value was measured on a spectrophotometer at 517 nm. The Trolox standard curve was prepared in concentrations within the range of 6.25–50 µM.

Total phenolic contents determination

Total phenolic content was calculated by the method Folin-Ciocalteu with modifications. The assay was carried out by mixing 80 µL of the stock solution of fungal extract dissolved in methanol (1 mg/mL) or standard (gallic acid) and 80 µL of the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA), and 1040 µL of a 50% deionized water–methanol solution. After 6 min of reaction 800 µL of a 10.75% sodium carbonate solution dissolved in deionized water (w/v) was added. The mixture was allowed to react for 90 min, subsequently the absorbance value of each sample was measured in a spectrophotometer at 760 nm. The gallic acid standard curve was prepared in concentrations within the range of 25–200 mg/L.

Acute toxicity activity

The acute toxicity evaluation was determined following the method described by Kviecinski et al. (2008) and Meyer et al. (1982) with modifications. In this assay, *Artemia franciscana* Kellogg nauplii was used, which was obtained from the Faculty of Biology, Universidad Veracruzana (Xalapa, Veracruz, Mexico). 100 mg of brine shrimp cysts were added for its culture in 100 mL of artificial seawater (2.8% NaCl), the cysts were incubated at 27 ± 3 °C, with constant illumination and aeration for 48 h until hatching. Stock solutions of the fungal strains (extracts that were bioactive in the antiproliferative and antibacterial evaluations) were prepared at a concentration of 4 mg/mL using a mixture of 0.5% DMSO in artificial seawater. 200 µL of artificial seawater and 10 nauplii of brine shrimp per well were placed in 24 well microplates, and stock solutions of each extract were added until the concentrations of 1000, 100, and 10 µg/mL were obtained. Each concentration was evaluated in triplicate; artificial seawater with 0.5% DMSO was used as a negative control. Potassium dichromate was used as a positive control. The plates were incubated for 24 h at 27 ± 3 °C and lighting. At the end of the incubation period, the percentage of dead nauplii at the three concentrations of fungal extracts was determined. The results obtained were expressed as the mean lethal concentration (LC_{50}), which causes a lethal effect in 50% of the organisms exposed during a certain time (Meyer et al., 1982).

Statistical analysis

Statistical analysis to determine the LC_{50} was performed using the Probit method with a 95% confidence interval. The data were processed in the SPSS version 25 software. The statistical analysis for the antioxidant activity tests was the mean ± standard deviation (n = 3).

Results and discussion

Antiproliferative activity

As mentioned in the materials and methods, the antiproliferative activity assay included the evaluation of 60 crude extracts (30 biomass and 30 broths) obtained from 30 *Ganoderma* specimens. Five of the biomass extracts showed antiproliferative activity against the six tumor cell lines tested, with concentration values lower than 50 μg/mL (Table 2). On the other hand, the extracts from the broths were inactive with concentrations higher than the highest concentration tested (> 250 μg/mL) (data not shown). The *G. curtisii* strain (GH-16-015) showed remarkable inhibition values of: 47 μg/mL (A549), 45 μg/mL (HBL-100), 31 μg/mL (HeLa), and 43 μg/mL (T-47D), that are relevant for the preliminary selection of bioactive extracts with a value of $GI_{50} < 50$ μg/mL, suggests that this strain is a candidate for future analysis of its metabolites (Skehan et al., 1990; Monks et al., 1991). This strain presented a GI_{50} of 31 μg/mL against the HeLa cell line. This result is consistent with the antiproliferative activity reported by Taofiq et al. (2017), where extracts of fruiting bodies of *Ganoderma lucidum* with GI_{50} values equivalent to 73 μg/mL were evaluated against the HeLa cell line. According to Veljović et al. (2017), in vitro

Table 2 Antiproliferative activity of biomass of crude extracts of 30 strains of the genus *Ganoderma*

Species	Strain	GI_{50} by Cell lines (μg/mL)					
		A549 (Lung)	HBL-100 (Breast)	HeLa (Cervix)	SW1573 (Lung)	T-47D (Breast)	WiDr (Colon)
G. australe	GH-15-005	106	116	83	80	75	80
G. australe	GH-16-005	243	> 250	92	220	138	156
G. australe	GH-16-033	115	91	59	51	94	93
G. applanatum	GH-15-T3	174	184	81	157	79	85
G. applanatum	GH-16-003	59	58	55	90	66	71
G. applanatum	GH-16-004	68	59	58	79	60	59
G. applanatum	GH-16-006	> 250	> 250	> 250	> 250	> 250	> 250
G. applanatum	GH-16-025	112	100	59	54	82	91
G. applanatum	GH-16-027	232	221	86	168	134	106
G. applanatum	GH-16-028	125	133	75	115	145	121
G. applanatum	GH-16-035	192	185	94	112	120	109
G. colossus	GH-16-011	101	100	64	112	118	82
G. colossus	GH-16-013	65	60	54	76	52	55
G. colossus	GH-16-030	107	86	75	111	99	94
G. curtisii	GH-16-007	> 250	> 250	> 250	> 250	> 250	> 250
G. curtisii[a]	GH-16-012[a]	54	52	41	85	48	70
G. curtisii[a]	GH-16-015[a]	47	45	31	54	43	51
G. curtisii[a]	GH-16-018[a]	85	92	44	89	78	73
G. curtisii[a]	GH-16-019[a]	47	47	33	59	41	49
G. curtisii	GH-16-021	> 250	> 250	175	> 250	> 250	> 250
G. curtisii	GH-16-022	110	145	58	102	77	58
G. curtisii[a]	GH-16-023[a]	47	48	44	46	45	55
G. curtisii	GH-16-031	206	209	96	172	207	166
G. lobatum	GH-15-004	> 250	> 250	> 250	> 250	> 250	> 250
G. lobatum	GH-16-010	79	82	71	70	67	57
G. oregonense	GH-15-001	127	174	104	145	169	159
G. oregonense	GH-16-008	117	242	59	137	68	72
G. oregonense	GH-16-037	> 250	> 250	> 250	> 250	> 250	> 250
G. resinaceum	GH-16-009	222	174	75	190	101	151
G. resinaceum	GH-16-020	128	127	69	110	97	84

GI_{50} (μg/mL) concentration that causes a 50% reduction in tumor cell proliferation

[a]Bioactive extracts ($GI_{50} < 50$ μg/mL)

antiproliferative activity by *G. lucidum* extracts against HeLa cells may be due to the total phenolic content and the glucans. On the other hand, Upadhyay et al. (2014) attributed the inhibition of the HeLa cells proliferation to the ganoderic acids presented in extracts of *G. lucidum*. Likewise, Liu et al. (2009) reported antiproliferative activity against tumor lines in some species of the genus *Ganoderma*, attributing the activity to presence of triterpenoids, sterols, and nucleosides, which induce apoptosis through mitochondrial transmembrane depolarization and cell cycle arrest. In our study, the extracts of *G. curtisii* is likely to show such growth inhibitory mechanisms in tumor cells.

Antibacterial activity

Additionally, the biomass extracts of three *G. curtisii* strains (GH-16-012, GH-16-015, and GH-16-018) that demonstrated the antiproliferative activity, inhibited the growth of *S. aureus* (ATCC 25923) with values MIC $\leq$ 1000 µg/mL (Table 3). However, the broth extracts of these strains were inactive at the highest concentration tested (> 1000 µg/mL) (data not shown). The antibacterial activity of fungal extracts in this assay was evaluated according to O'Donnell et al. (2010), suggesting that the MIC > 1000 µg/mL denotes no bioactivity, the range of MIC 501–1000 µg/mL indicates mild bioactivity, the range of 126–500 µg/mL shows moderate bioactivity, the range of 26–125 µg/mL denotes good bioactivity, the range of 10–25 µg/mL shows strong bioactivity, and MIC < 10 µg/mL indicates very strong bioactivity. The extract of strain GH-16-018 showed a MIC in the range 501–1000 µg/mL, which indicates a mild bioactivity against the Gram-positive bacterium *S. aureus*. On the other hand, extracts of the fungal strains GH-16-012 and GH-16-015 showed a MIC in the range 126–500 µg/mL, that indicates moderate bioactivity against the same Gram-positive bacterium. These results were in coincidence with those reported by Ćilerdžić et al. (2016): mycelium extracts of various *Ganoderma* species manifested MICs of 1–4 mg/mL against *S. aureus* (ATCC 25923). According to Upadhyay et al. (2014), the growth inhibition of *S. aureus* may be attributed of ganoderic acids present in extracts of *G. lucidum*. Apart from ganoderic acids, phenolic compounds may also be responsible for antibacterial inhibition and antioxidant activity (Li et al., 2012). One of the possible mechanisms by which the *G. curtisii* extracts exerted antibacterial activity could be a direct action on bacterial membranes by changing their permeability and interrupting the electron transport chain and oxidative phosphorylation. Compounds such as fatty acids, terpenes, and phenols obtained from *Ganoderma* species have been reported with this antibacterial capacity (Cör et al., 2018; Desbois and Smith, 2010; Rempe et al., 2017).

Molecular identification of bioactive strains

Five strains of *Ganoderma* that were bioactive in the antiproliferative and antibacterial activity were genetically identified through the analysis of their ITS regions, and the sequences obtained were compared with the GenBank database, NCBI. The results obtained with the Maximum likelihood (Jukes-Cantor) algorithm confirmed in 99% identity with *G. curtisii* (Fig. 1). The species *G. curtisii* belongs to the *G. lucidum* lacquered complex and its distribution according to Torres-Torres et al. (2015), occurs in Japan, China, India, Africa, and North America. There are several records on *G. curtisii* in several states in Mexico, and it is common to find this species in pine-oak forests, with the main function to break down wood and woody debris (López-Peña et al. 2016; Torres-Torres et al., 2015). However, there is a problem that makes identification of *Ganoderma* specimens difficult, that is due to high morphological plasticity of the species, the overabundance of synonyms, and a misuse of species names (Smith and Sivasithamparam, 2003). So, in this study, it was necessary to complement the identification of bioactive species with molecular biology techniques.

Table 3 Antibacterial activity (MIC) of crude extracts from five strains of *G. curtisii*

Species	Strain	Extract	Human pathogenic bacteria MIC (µg/mL)				
			E. faecalis (ATCC 29212)	*S. aureus* (ATCC 25923)	*E. coli* (ATCC 25922)	*E. coli* (ATCC 35218)	*P. aeruginosa* (ATCC 23853)
G. curtisii	GH-16-012	Biomass	> 1000	500	> 1000	> 1000	> 1000
	GH-16-015	Biomass	> 1000	500	> 1000	> 1000	> 1000
	GH-16-018	Biomass	> 1000	1000	> 1000	> 1000	> 1000
	GH-16-019	Biomass	> 1000	> 1000	> 1000	> 1000	> 1000
	GH-16-023	Biomass	> 1000	> 1000	> 1000	> 1000	> 1000
Ampicillin			62.5	62.5	125	125	250

Minimum Inhibitory Concentration (MIC) µg/mL

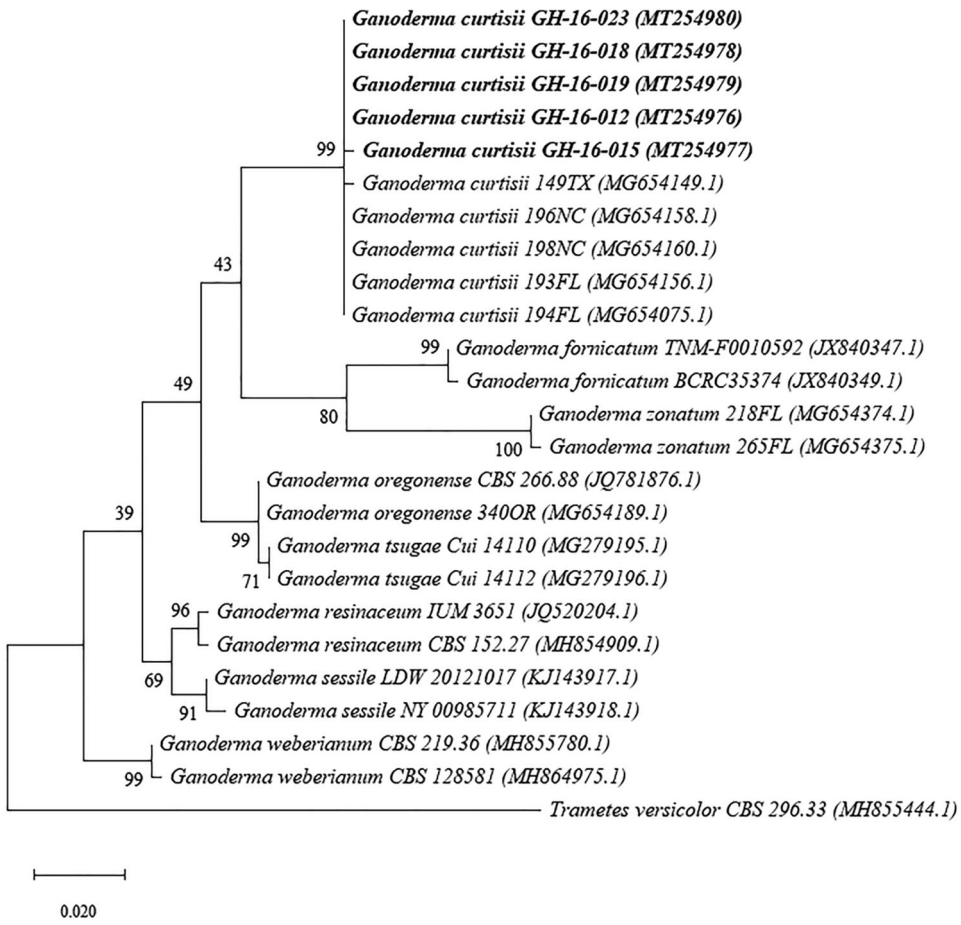

Fig. 1 Dendogram of the *Ganoderma* species. This was constructed using the Maximum likelihood (Jukes-Cantor) algorithm with bootstrap analysis of 1000 repetitions. The strains highlighted in bold are those that were sequenced in this study. *Trametes versicolor* species was added as an outgroup. The analysis was performed using the MEGAX software

Antioxidant activity

The results on the antioxidant activity of the biomass of five bioactive *G. curtisii* strains were obtained through evaluations of free radical measurement by the methods of Galvinoxyl, ABTS, and DPPH and were expressed as Trolox equivalents per 1 mg of extract (TE/mg) (Table 4).

For the Galvinoxyl method, a Trolox EC_{50} with a value of 23.30 ± 0.56 µM was obtained, a value obtained from the standard curve by interpolation of the linear regression analysis with a value of R^2 = 0.9848. Fungal extracts that presented a greater or equal cut-off value of 23.30 µM were considered as bioactive, the biomass extract of strain GH-16-012 obtained a remarkable value of 20.28 ± 2.47 µM TE/mg. On the other hand, with the ABTS method, a Trolox EC_{50} was obtained with a value of 144.28 ± 6.85 µM obtained from the standard curve by interpolation of the linear regression analysis with a value of R^2 = 0.9993. Fungal extracts that had a greater or equal cut-off value of 144.28 µM were considered as bioactive. The biomass extract of strain GH-16-012 obtained a remarkable value of 78.31 ± 4.94 µM TE/mg. Finally, using the DPPH method, a Trolox EC_{50} value of 29.51 ± 1.18 µM was obtained, a value obtained of the standard curve by interpolation of the linear regression analysis with a value of R^2 = 0.9782. Fungal extracts that had a greater or equal cut-off value of 29.51 µM were considered as bioactive, the biomass extract of strain GH-16-018 manifested a value of 20.62 ± 1.11 µM TE/mg. This result confirms the DPPH radical scavenging activity of *Ganoderma* extracts, previously reported in some investigations, such as that carried out by Veljović et al. (2017), where the reduction of the DPPH radical was evaluated by extracts obtained from *G. lucidum* fruiting bodies. In the case of *G. curtisii* extracts, the antioxidant activity is much likely to be attributed to phenolic compounds and polysaccharides, the compounds responsible for accepting or donating hydrogen to stabilize free radicals (Cör et al., 2018).

In addition, the results on total phenolic contents of the crude extracts of mycelial cultures were expressed as mg equivalent of gallic acid (GAE/mg) (Table 4); the biomass

Table 4 Antioxidant activity of crude extracts of five strains of *G. curtisii*, determined by the Galvinoxyl, ABTS, DPPH, and Total phenolic contents

Species	Strain	Extract	Galvinoxyl (μM TE/mg)	ABTS (μM TE/mg)	DPPH (μM TE/mg)	Total phenolic (GAE/mg)
G. curtisii	GH-16-012	Biomass	20.28 ± 2.47	78.31 ± 4.94	19.05 ± 1.35	6.31 ± 0.39
	GH-16-015	Biomass	8.78 ± 0.53	39.01 ± 3.19	10.97 ± 0.71	2.53 ± 0.50
	GH-16-018	Biomass	7.92 ± 0.23	71.76 ± 4.99	20.62 ± 1.11	3.41 ± 0.12
	GH-16-019	Biomass	5.82 ± 0.67	45.97 ± 2.50	6.55 ± 0.42	1.52 ± 0.51
	GH-16-023	Biomass	6.67 ± 0.40	54.04 ± 2.59	11.66 ± 1.21	1.90 ± 0.60
	(EC_{50}) Trolox		23.30 ± 0.56	144.28 ± 6.85	29.51 ± 1.18	NA

TE trolox equivalent/mg; *GAE* equivalents of gallic acid/mg. Each value is presented as mean ± standard deviation (n = 3); *EC_{50}* median effective concentration; *NA*: not applicable

extract of strain GH-16-012 manifested a value of 6.31 ± 0.39 GAE/mg. This result confirms the presence of phenolic compounds in the extracts of *Ganoderma* mycelial cultures, as well as in the studies reported by Ćilerdžić et al. (2016).

Acute toxicity activity

Finally, with the aim to relate the previous results, the acute toxicity activity was determined for the five bioactive biomass extracts of *G. curtisii* strains (Table 5). The five extracts showed effect with LC_{50} values below 1000 μg/mL considering them as toxic (Meyer et al., 1982). According to Mwangi et al. (2015), the lower the value of LC_{50}, the more toxic the extract is and, the opposite, the higher the LC_{50} value, the lower the toxicity. Extracts with LC_{50} values > 1000 μg/mL are considered non-toxic, the range of LC_{50} 500–1000 μg/mL denotes a weak toxicity, the range of 100–500 μg/mL indicates a moderate toxicity and, a range of LC_{50} < 100 μg/mL denotes high toxicity. The five fungal strains evaluated showed an LC_{50} 100–500 μg/mL, which indicated moderate toxicity. The fungal strain with the lowest toxicity corresponded to the one labeled with the code GH-16-023 which showed a LC_{50} of 490.881 μg/mL. To a certain extent, most antitumor compounds present toxicity, under this premise the bioassay with brine shrimp is considered a preliminary, fast and inexpensive analysis that relates toxicity, and it is suggested to be used by pharmacognosists and natural product chemists. Likewise, this assay is convenient to use especially at the crude plant or fungal extract stage when such extracts often contain a mixture of various compounds (Meyer et al., 1982).

In this bioprospecting study, five out of 30 *Ganoderma* strains cultivated in vitro showed remarkable antiproliferative activity against six tumor cell lines, as well as antibacterial, antioxidant activity, and some acute toxicity. However, although all five strains with the highest bioactivity were found to be of the same species (*G. curtisii*), these results indicate that both the substrate specificity and the different strains of the same species can be determining factors to show variation in the fungus bioactivity. On the other hand, our study showed that the five bioactive strains demonstrated moderate toxicity. This work prevents the possibility of indiscriminate use that can be given to a medicinal fungi in Mexico, but opens the possibility for further ongoing research with these strains to isolate

Table 5 Acute toxicity of crude extracts of five strains of *G. curtissi* against *A. franciscana*

Species	Strain	Extract	Total *A. franciscana*	% Mortality after 24 h			LC_{50} (μg/mL)
				10 μg/mL	100 μg/mL	1000 μg/mL	
G. curtisii	GH-16-012	Biomass	30	23.3	33.3	83.3	406.765
	GH-16-015	Biomass	30	23.3	30.0	83.3	425.656
	GH-16-018	Biomass	30	23.3	33.3	86.7	377.037
	GH-16-019	Biomass	30	20.0	30.0	80.0	475.343
	GH-16-023	Biomass	30	23.3	30.0	76.7	490.881
Potassium dichromate			30	46.7	77.7	100	19.277
Artificial seawater + DMSO			30	0	0	0	ND

LC_{50} (μg/mL) mean lethal concentration which causes a lethal effect in 50% of the organisms exposed during a certain time. *ND*: not detected

metabolites responsible for its potentially nutraceutical and medicinal properties.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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