ANTIMICROBIAL ACTIVITY PRESENT IN *Ganoderma curtisii* AQUEOUS EXTRACTS

Actividad antimicrobial de extractos acuosos de *Ganoderma curtissii*

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Abstract

Macroscopic mushrooms have proven to be excellent sources of protein. In addition to this, their medicinal properties have been noted, including their natural antitumor, antiviral, antimicrobial, and antioxidant action, as well as their role in immune system modulation. Although polysaccharides and triterpenoids have been identified as the main antimicrobial components of the *Ganoderma* species, some studies mention peptidic components that possess antimicrobial effects such as ganodermin, a 15-kDa antifungal peptide. In view of the growing demand for molecules with potential therapeutic applications that could help fight antibiotic-resistant microorganisms, the purpose of this work consists of studying the antimicrobial activity of aqueous extracts of *Ganoderma curtisii*, found in "La Primavera" Forest in Jalisco, Mexico. The aqueous extracts of *G. curtisii* showed specific dose-response antibacterial activity against *E. coli*. Furthermore, PAGE-SDS gels obtained from the pileus of the *G. curtisii* aqueous extracts show peptide bands measuring approximately 5 kDa, along with bands measuring between 10 and 15 kDa, which could represent the antimicrobial peptides that completely inhibited the growth of *E. coli* when the conventional CFU microdilution method was used. A preliminary conclusion is that the findings reported in this study can be seen as potentially useful and original for therapeutic application in the field of biomedicine.

Keywords: antimicrobial activity, antimicrobial peptides, fungal protein, SDS-PAGE, *Ganoderma curtisii*, Ganodermataceae

Resumen

Los hongos macroscópicos se han utilizado como excelentes fuentes de proteína. Además se han mencionado sus propiedades medicinales, tales como antitumorales, antivirales, antimicrobianas, antioxidantes naturales, así como agentes inmunomoduladores. Aunque se han mencionado polisacáridos

y triterpenoides como los principales componentes antimicrobianos de las especies de *Ganoderma*, algunos estudios mencionan componentes peptídicos que poseen efectos antimicrobianos; por ejemplo, "ganodermin" un péptido antifúngico de 15 kDa. Debido a la demanda creciente de moléculas con posibles aplicaciones terapéuticas que puedan ayudar en la lucha contra los microorganismos resistentes a los antibióticos convencionales, el objetivo de este trabajo fue estudiar la actividad antimicrobiana de extractos acuosos de *Ganoderma curtisii*, proveniente del bosque de "La Primavera" en Jalisco, México. Los resultados muestran que los extractos acuosos de *G. curtisii* exhibieron actividad antibacteriana específica potencial contra *E. coli* de una manera dosis-respuesta. Además, los geles de PAGE-SDS del píleo de extractos acuosos de *G. curtisii* muestran bandas de péptidos de aproximadamente 5 kDa, junto con otras bandas de entre 10 y 15 KDa, y podrían representar los péptidos antimicrobianos que inhibieron completamente el crecimiento de *E. coli* en el método de microdilución de UFC convencional. Se puede concluir de manera preliminar que los hallazgos reportados en este estudio, pueden considerase potencialmente útiles y originales para su aplicación terapéutica en el campo biomédico.

Palabras Clave: actividad antibacteriana, péptidos antimicrobianos, proteínas fúngicas, SDS-PAGE, *Ganoderma curtisii*, Ganodermataceae

Introduction

Macroscopic mushrooms have been a source of food for thousands of years. Due to their low fat content and the absence of cholesterol, many macroscopic mushrooms are excellent sources of protein. Furthermore, popular culture has made frequent reference to their medicinal properties. Now, from a scientific perspective, *Ganoderma* species complex have been reported as important sources of antimicrobial bioactive compounds. Besides the major secondary metabolites (terpenes, terpenoids and polyketides of farnesyl quonines types) with anti-tumor, anti-inflammatory, antioxidant, immune-enhancing, and antimicrobial activities; small peptides, polysaccharide, and chitosan also possess antimicrobial and anti-parasitic properties (Basnet et al., 2017). Although extensive researches on antimicrobial bioactive compounds have been carried out on *Ganoderma* sp; most of the studies are focused on few species, *Ganoderma lucidum* for instance, produces the 15-kDa anti-fungal peptide, ganodermin, which has inhibitory effects against common fungi *Botrytis cinerea* and *Fusarium oxysporum*, (Basnet et al., 2017; Wang et al., 2017; Wang y Ng, 2006). The European mushroom name, *Ganoderma lucidum*, has been misapplied to this species, reidentified as *G. lingzhi* (Dai et al., 2017). *Ganoderma curtisii* is a closely related species to *G. lingzhi* based on phylogenetic analysis (Costa-Rezende et al., 2017), and is frequently found in Mexico (Torres-Torres y Guzmán-Dávalos, 2005; López-Peña et al., 2016).

Fungi, in particular Basidiomycota, are a still underexplored, highly promising source of antimicrobial peptides. We are currently living in the "post-antibiotic" era, where both, the numbers and percentages of multi-resistant bacterial and fungal pathogens against the established antibiotics and synthetic antibacterial agents are drastically increasing, while the number of new therapeutic agents has decreased (Hyde et

al., 2019). It is known that macroscopic mushrooms contain multiple proteins with interesting biological activity for biomedical applications such antimicrobial qualities (Basnet et al., 2017). The bioactive proteins present in macroscopic mushrooms, however, will need to be isolated and analyzed to then prove their biomedical potential and their suitability for treating diseases.

The reports on antimicrobial peptides in *Ganoderma* species complex are scarce (Antimicrobial peptides data base 3–APD3) and in the *G. curtisii*, the bioactive compounds studies are focused on antioxidant activity of the phenolic and polysaccharides content (Huerta et al., 2016); lanostane triterpenoids and their anti-inflammatory activities (Jiao et al., 2016); chemical composition of main sterols (Islas-Santillán et al., 2017) and neuroprotective potential against epilepsy of soluble polysaccharides (León-Rivera et al., 2019). Experts around the world are now giving warnings about the serious consequences that the lack of antibiotics—in particular against the multi-resistant Gram negative human pathogenic bacteria—can have. After two decades of neglect, efforts of both the private and the academic sector on the discovery of new antibiotics have substantially increased (Hyde et al., 2019). The *Ganoderma* sp. medicinal mushroom is considered a key source of therapeutic agents to treat infectious bacteria, viruses and parasites (Basnet et al., 2017). This study shows the antimicrobial properties of *G. curtisii* collected in the protected natural area of "La Primavera, Jalisco".

Despite the limited studies on the activities of antimicrobial peptides in the *Ganoderma* species complex and in response to an increasing demand for molecules with potential therapeutic applications, the purpose of this work consists of studying the antimicrobial activity of aqueous extracts of *G. curtisii* found in "La Primavera" Forest in Jalisco, Mexico.

Materials and Methods

Fungal material

The basidiocarp of *Ganoderma curtisii* that was used for this study was collected in July 2016 at a location in "La Primavera" forest, located in the municipality of Zapopan in the Mexican state of Jalisco (L. Guzmán-Dávalos 11377, IBUG). The specimen was initially identified based on macro and micro-morphological characteristics of the basidiome using conventional taxonomical techniques, and its identification was confirmed based on molecular tools using the Internal Transcribed Spacer (ITS) region of the rDNA.

Preparation of the fungal extracts

Samples of the fresh fruiting body (pileus and stipe) were homogenized in a PBS pH 7,2 (1ml/mg) buffer solution. After centrifugation (10.000 X g. 30 minutes) the supernatant was used to analyze antimicrobial activity and electrophoretic band proteins.

Protein determination

Soluble protein content was determined by the Bradford method (Bradford, 1976) using a Bio-Rad protein assay reagent (Bio-Rad, USA). Bovine serum albumin (BSA) was used as a standard protein.

Determination of antimicrobial activity

The antimicrobial effect of the *G. curtisii* extract on *Staphylococcus aureus* (ATCC® 6538 ™), *Escherichia coli* (ATCC® 9637 ™) and *Candida albicans* (obtained from the Dermatological Institute of Jalisco) was assessed using the conventional micro-dilution method (Dalgaard et al., 1994) in nine bioassays. The minimum inhibiting concentration was analyzed using a Colony-Forming Unit (CFU) dilution assay (Ong et al., 2002).

Statistical analysis

The JMP4-type non-parametric median test was used to determine significant differences, considering a standard error with a confidence level of 0.05.

Electrophoresis testing of fungal extracts

The sample was prepared using a Laemmil 2X solution with 5% 2-mercaptoethanol/extract, 1:1. The dilution was heated at 100°C for 5 minutes [18] and placed at 20°C for 1 minute before loading. 15 µL of the 1:1 dilution were set aside, along with 4 µL of the Plus Protein TM Dual Xtra Standard® (BioRad) marker, using an SDS-PAGE 4-20% Mini-PROTEAN® TGXTM Precast Gel (Bio-Rad) with 1X Tris/Glycine SDS Buffer® (BioRad) pH 8.3. The electrophoretic testing was conducted using a HV Power Pac™ (BioRad) power source at 120 V, 18 mA for 113 min. The staining process was done using a Coomassie blue R-250 solution (Bollag y Edelstein, 1991).

Results and Discussion

Aqueous *G. curtisii* extracts obtained from the pileus showed specific dose-response antibacterial activity against *E. coli* (Figura 1).

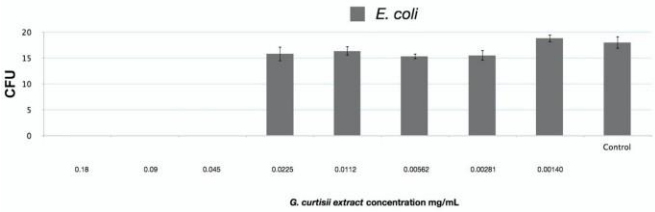

Figure 1. Bar graph of the average values obtained from nine inhibition-dose response bioassays, showing the Colony- Forming Units (CFU) of *E.coli* in contact with the aqueous extract of the *G. curtisii* pileus, compared to the control, using different *G. curtisii* aqueous extract concentrations. The first three concentrations (0.18, 0.09, 0.045 mg/mL) completely inhibited bacterial growth.

Of the eight concentrations that were tested, only the first three (0.18, 0.09, 0.045 mg/mL) showed strong antibacterial activity and inhibited bacterial growth completely. The following four concentrations had slight activity against bacterial growth, but it was statistically insignificant. *S. aureus* and C. *albican*s were not inhibited by the evaluated concentrations of *G. curtisii* extract. The antimicrobial properties of *G. curtisii* shown in this study are consistent with antibacterial peptide fractions from *G. lucidum* possessed substantial antibacterial activity against *E. coli* and *Salmonella typhi* (Mishra et al., 2018), *Bacillus subtilis*, *B. cereus*, *Staphylococus epidermidis*, *E. coli*, *Pseudomonas aeruginosa*, except *S. aureus* (Sa-ard et al., 2015) and our previous laboratory findings, which were based on a cultivated specimen of a *Ganoderma* sp. fruiting body of unknown origin. The aqueous extracts of that sample showed consistent antimicrobial properties against Gram-positive bacteria, such as *S. aureus*; Gram-negative bacteria, such as *E. coli*; and yeasts such

as *C. albicans* (data not published). Unlike the findings for *Ganoderma* sp., the *G. curtisii* aqueous extracts specifically inhibited *E. coli*, and did not inhibit *S. aureus* or *C. albicans*.

Figure 2 shows the *G. curtisii* aqueous extracts in a SDS-PAGE. A strongly stained band of approximately 5 kDa is evident in the P lanes, along with other bands of between 10 and 15 kDa.

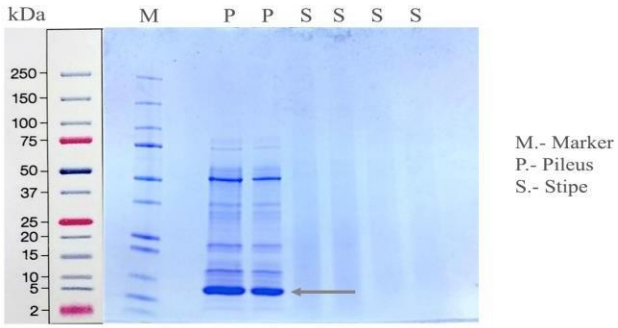

Figure 2. SDS-PAGE of *G. curtisii* aqueous extracts. M lane, BioRad protein marker; P lanes, aqueous *p*ileus extract; S lanes, aqueous *s*tipe extract.

These bands correspond to the aqueous pileus extracts, and could contain the antimicrobial peptides that completely inhibited the growth of *E. coli* in the conventional CFU microdilution method. The remaining S lanes correspond to the aqueous stipe extract, where no bands were detected. Interestingly, the strongly stained peptidic bands of low molecular weight of approximately 5 KDa and those between 10 and 15 KDa, which are shown in PAGE-SDS 4-20% (Figure 2), could correspond to antimicrobial peptides not previously described. Curiously, the electrophoretic behavior of the *G. curtisii* protein bands is similar to that of ganodermin, the isolated antifungal protein contained in fresh fruiting bodies of the "*Ganoderma lucidum*" medicinal mushroom, collected at the campus of the University of China in Hong Kong (Wang y Ng, 2006); the Lyophyllum (LAP) antifungal protein, with a molecular weight of 14 kDa, isolated from *Lyophyllum shimeji* (Ng y Lam, 2002), 16 kDa and 18 kDa proteins from *G. lucidum*, both proteins may be the reported lectins and hexameric with specific agglutination activity (Li et al., 2018) and 14 kDa protein in the mycelia and fruiting bodies protein extract of *G. lucidum* (Sa-ard et al., 2015). Of course, it will be necessary to sequence these bands and look for homologies with other closely related species, or determine whether they correspond to new molecules not yet described.

Finally, it is noteworthy that only the pileus extract of the *G. curtisii* shows electrophoretic bands that could be related to bioactive molecules, unlike the stipe extract, which does not show any such bands (Figure 2). In other cases, specific proteins and peptides, secondary metabolites and other potent bioactive molecules are confined to fruiting bodies and even to specific tissues and different stages of the mushroom's development (Kües y Badalyan, 2017). A preliminary conclusion is that the findings of this study can be seen as potentially useful and original for therapeutic application in the field of biomedicine.

Acknowledgements

Research supported by PRODEP, PFCE and P3e (2016-2018 UDG-CA-44).

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