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DNA damaging potential of *Ganoderma lucidum* extracts

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ABSTRACT

Ethnopharmacological relevance: *Ganoderma lucidum* (Lingzhi or Reishi) is a medicinal mushroom historically used in Asian countries to treat a wide variety of diseases and prolong life. In the last years, *G. lucidum* has been internationally recognized as an effective adjuvant in cancer treatment. Among active components, the most recent research indicates that polysaccharides modulate the immune response favoring the recovery from toxicity of chemo and radiotherapy while triterpenes are cytotoxic to tumoral cells mainly by altering gene expression. Beyond this body of evidence on the efficacy of *G. lucidum* in cancer treatment, it is not yet understood whether these extracts exert the same mechanisms of action than current antitumoral drugs.
Aim of the study: In this study, we tested the DNA damaging potential of *G. lucidum* extracts by the β-galactosidase biochemical prophage induction assay (BIA) using doxorubicin, a DNA intercalating agent, as a positive control. This assay was traditionally used to screen microbial metabolites towards antitumoral agents. Here, we used this bacterial assay for the first time to assess DNA damage of herbal drugs.
Results: After a bioguided assay, only a purified fraction of *G. lucidum* containing a mixture of C16 and C18:1 fatty acids exerted weak activity which could not be attributed to direct interaction with DNA. At the same concentrations, the induction observed for doxorubicin was clearly contrasting.
Conclusions: The micro BIA assay could be successfully used to demonstrate differences in cellular effects between *G. lucidum* extracts and doxorubicin. These results showed that *G. lucidum* extracts display weak DNA damaging potential. Since DNA injury promotes aging and cancer, our results substantiate the traditional use of this mushroom to prolong life.

1. Introduction

Historically known as the "mushroom of immortality", *Ganoderma lucidum* has received special attention among fungi. Many medicinal properties such as anti-inflammatory, anti-viral, anti-atherosclerotic, anti-diabetic and anti-cancer activity have been attributed to this mushroom which is included in both ancient and reference pharmacopoeias such as the USP (Bishop et al., 2015). *G. lucidum* has been used popularly as a complementary treatment for cancer therapy in traditional Chinese medicine and is internationally recommended for its efficacy as an adjuvant in cancer treatments (Jin et al., 2016). Different components of *G. lucidum* display different responses on both immune and tumoral cells. Polysaccharides are responsible of the immunomodulatory effects in animals and humans, exerting the anticancer activity indirectly by activation of the immune responses against tumors (Wasser, 2017). It has been demonstrated that the effect of the polysaccharides on immunomodulation is more relevant than the direct effect on tumoral cells *in vitro* (Sui et al., 2016), while triterpenes are the main cytotoxic components in *G. lucidum* (Yue et al., 2008). The mechanisms by which triterpenes exhibit anti-cancer activities include inhibition of cell proliferation through cancer-specific cell cycle arrest and apoptosis, and inhibition of metastasis by inhibition of pre-metastatic gene expression. According to the last update of the Cochrane database (Jin et al., 2016), it has been recognized that the incorporation of a *G. lucidum* preparation as an adjuvant in conventional chemo/radiotherapy regimens improves the response to the treatment. When *G. lucidum* is incorporated to the regimens, these are 1.25 times more likely to yield a better tumor response. Besides, the preparations of *G. lucidum* counter the immunosuppressive effect of chemo/radiotherapy, especially the T-lymphocyte depletion. The use of *Ganoderma* as an adjuvant for cancer therapy has raised the question whether its relationship with chemotherapy and radiotherapy is synergistic. It is

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currently accepted that traditional chemotherapy and radiotherapy are widely unspecific, causing cell damage to normal cells. Many antitumoral drugs of current clinical use display mechanisms of action based on initial binding to DNA and disruption of its structure, such as cisplatin and analogues, cyclophosphamide and doxorubicin. In contrast, herbal medicines traditionally used to prevent aging and cancer may not share such mechanisms since DNA damage promotes both aging and cancer according to well established theories (Hoeijmakers, 2009).

Although cellular effects of some *G. lucidum* components have been studied, the possible interaction with current cancer therapies is difficult to predict since the final effect resulting from the combined action of each component depends on the amount and biochemical features of many individual components. Although the apoptotic effects of triterpenes have been recognized, the biochemical pathways involved are still poorly understood. While the involvement of ROS generation and inhibition of antioxidative cell defenses was shown to be part of the mechanism of action of triterpenes (Liu et al., 2015), other studies showed that some components trigger more specific responses for example, by being recognized by specific transcription factors (J. Liu et al., 2007). This last hypothesis is supported by recent work (Zhao and He, 2018). However, it has not been demonstrated if *G. lucidum* also triggers unspecific mechanisms such as direct damage to DNA. Tests such as the comet assay and the DNA fragmentation assay evidenced DNA damage involved in apoptosis of eukaryotic cells after treatment with triterpenes from *G. lucidum* (Wu et al., 2012). The *β*-galactosidase induction assay, which is sensitive to agents that form DNA adducts and cause direct damage to DNA, would help to recognize if DNA damage occurs independently from apoptotic events. Aiming to assess the DNA damaging potential of *G. lucidum* extracts, we used an assay based on the induction of the SOS response in a genetically modified strain of *E. coli*.

Mutagens and radiation can induce the production of bacteriophages leading to cell lysis in some bacterial systems. Induction of prophage is one of the manifestations of the SOS response in bacteria, following their exposure to agents which damage or interact with DNA. Endo et al. (1963) described the relationship between tumoricidal agents with the induction of prophage in lysogenic bacteria. They found that the number of infective centers and the turbidity displayed a relationship with the chemical agent tested and its concentration. Since then, the lysogenic effect has been used to study antitumor antibiotics in tests such as the SOS inductest. Later, Elespuru and Yarmolinsky (1979) introduced the *lacZ* gene into an operon under the control of a lambda phage promoter developing a colorimetric method based on the expression of *β*-galactosidase. The quantitation of *β*-galactosidase is then a direct measure of SOS-induced gene expression. They constructed the BR513 strain with the *envA* mutation, which confers permeability to the chemical agents and substrates of *β*-galactosidase, and the *uvrB* mutation which leads to deficient DNA repair and increases sensitivity. Elespuru and White (1983) further described the induction activity of different substances known to interact with DNA and demonstrated that agents that act directly with the DNA require lower incubation times, while those causing indirect effects needed longer incubation periods. Since then, the BIA assay has been typically used to detect antitumoral antibiotic producers in actinomycete screening programs (Zazopoulos et al., 2003). One similar assay, the SOS inductest, has been used to assess the genotoxicity of herbal drugs (Sponchiado et al., 2016). Here, we use the BIA assay for the first time on fungal extracts to evaluate whether these interact directly with DNA, resembling the mechanism of doxorubicin, a typical DNA intercalating agent used in chemotherapy.

2. Materials and methods

2.1. Media and reagents

E. coli ATCC 33312 was grown in ATCC Medium 1065, supplemented with E Medium and glucose. LB medium contains (per L) 10 g Bacto-tryptone, 5 g yeast extract, 10 g sodium chloride, and 5 mL 1 M Tris. After autoclaving, the medium was supplemented with 4 mL of sterile 50 × medium E and 10 mL of 20% glucose. Buffer ZCM, medium A and o-nitrophenyl-β-D-galactopyranoside (ONPG, TECNOLAB SA) were prepared as described by Elespuru and White (1983).

2.2. Strains

Ganoderma lucidum strain E47 (CERZOS-UNS-CONICET, Bahía Blanca, Argentina) was cultivated on a sunflower seed hulls substrate (32.5% sunflower seed hulls, 5.0% barley, 2.0% CaSO4, 0.5% CaCO3, and 60% water, by weight) using a bag cultivation system (Bidegain et al., 2015). Bacterial suspensions of *Escherichia coli* BR513 ATCC 33312 (Elespuru and Yarmolinsky, 1979) were stored at −70 °C in LBE with glycerol 25%.

2.3. Extraction and chemical characterization

The basidiome of *G. lucidum* (100 g) was extracted with ethanol 96° to yield 4.7 L of alcoholic extract. The solvent was removed at low pressure on a rotatory evaporator. The resulting extract was sequentially extracted with hexane, ethyl-acetate, methanol and water. The hexanic sub-extract was subjected to open column silica gel chromatography, and eluted with mixtures of hexane and ethyl acetate to yield eight fractions. A purified fraction, F3 (7.1 mg), was dissolved in deuterated chloroform and analyzed by ^{1}H-RMN and ^{13}C-RMN using a Bruker AVANCE 300 spectrometer at 300 MHz. For the methylation of F3, an aliquot was poured into a clean glass tube and 1 mL of 10% methanolic HCl (v/v) was added. The reaction was held at 50 °C for 30 min. After cooling the samples to room temperature, fatty acid methyl esters were extracted with 1 mL of n-hexane. Fatty acid methyl esters were analyzed by GC–MS with a HP6890 chromatograph equipped with a mass spectrometer HP5972A. The ionization energy was 70 eV. Samples (1 μL) were injected into a HP-5 capillary column (30 m × 0.25 mm i.d. × 0.25-μm film thickness). The temperature was programmed from 85 °C to 250 °C at a rate of 4 °C/min and held at the final temperature for 15 min. The temperature of the injector and detector was 280 °C; and the carrier gas was helium at a flow rate of 1 mL/min and a split ratio of 20:1. Fatty acids were identified by comparing their retention times and mass fragmentation patterns with those of the chromatograph database.

2.4. Micro-BIA assay

The assay was performed in a 96-well microtiter plate according to Elespuru and Moore (1985). Before testing, stock solutions of doxorubicin (Sigma) were diluted 10 and 100-fold into water and further diluted to reach final concentrations of 10 μg/mL, 40 μg/mL, 100 μg/mL, 400 μg/mL and 1000 μg/mL. The solutions of the sub-extracts were prepared in DMSO (molecular biology grade, AppliChem) in the same manner. Bacteria was diluted 100-fold into LBE medium and an overnight culture was incubated. Then, it was diluted 10-fold into LBE medium and incubated for two hours to $A_{600} \approx 0.2$ (Griffith and Wolf, 2002). After addition of the test solutions to the microplate, the cell suspension was added, and the plate was incubated at 37 °C for 4 h without agitation. After incubation, the buffer ZCM and the substrate (ONPG) were added and the reaction was followed in a SUNRISE (TEKAN) microplate reader at 405 nm in kinetic mode for 30 min. The normalized absorbance was calculated by substracting the initial absorbance to avoid interference with color sample, and referred to the

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