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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.

Keywords. *Ganoderma lucidum*; biochemical induction assay; DNA damage; antitumoral; beta galactosidase; fatty acid

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 currently accepted that traditional chemotherapy and radiotherapy are widely unspecific, causing cell damage to normal cells. Many antitumoral drugs of current clinical 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 mechanism 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 (Liu J. et al., 2007). This last hypothesis is supported by recent work (Zhao & 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 x 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 & Yarmolinsky, 1979) were stored at -70 °C in LBE with glycerol 25%.

2.3. Extraction and chemical characterization

The basidiome of *G. lucidum* (100g) was extracted with ethanol 96º 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 & Wolf, 2002). After addition of the test solutions to the microplate, the cell suspension was added, and the plate was incubated at 37ºC for 4h 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 minutes. The normalized absorbance was calculated by substracting the initial absorbance to avoid interference with colour sample, and referred to the initial inoculum (A_{600}) for each run.

$$\boldsymbol{Normalized\ A} = \frac{(\boldsymbol{A\ 405_{t}} - \boldsymbol{A\ 405_{t=0}})\ \mathbf{x\ 100}}{\boldsymbol{A\ 600}}$$

2.5. Statistics

Normality was tested by the Shapiro Wilk's test (Shapiro & Wilk, 1965) and homoscedasticity by the Bartlett's test (Bartlett, 1937). The multiple comparison procedure with a control was performed by the test *t* with Bonferroni adjustment (Abdi, 2007). Linear regression was analyzed with the ULC 2.0 computer software (Boqué et al., 1994). Linear regression was evaluated from time = 10 min to time = 30 min.

3. Results

Due to the heterogeneity of components, the ethanolic extract of *G. lucidum* was difficult to dissolve in the amounts required for the test. To solve these limitations, the ethanolic extract of *G. lucidum* was sequentially extracted with organic solvents to yield four subextracts of different polarity. The highest percentages based on dry weight corresponded to the hexanic (39 % w/w) and methanolic subextracts (33 % w/w). All the subextracts were easily dissolved in DMSO to reach final concentrations ranging from 10 µg/mL to 1000 µg/mL, with a final maximal concentration of DMSO of 1 %. Doxorubicin was dissolved in sterile water and used as a positive control. The activity of both sterile water and DMSO 1 % were also tested in each run, as well as that of free LBE medium. Samples were incubated with fresh cultures of *E. coli* ATCC 33312 for 4 h, an incubation time suitable to detect agents that act directly on DNA. After incubation, buffers were added and the *β*-galactosidase reaction started with the addition of ONPG. The reaction was followed for 30 min in a microplate reader at 405 nm. The absorbances were normalized by substracting the absorbance at t=0, to avoid the effect of sample color, and values were adjusted to the initial inoculum

for each run. When the normalized values at 30 min were plotted against concentration, the response of the cells treated with the hexanic extract seemed to be above that of DMSO, while the most polar extracts were inactive at tested concentrations. Due to the variability of results, significant differences between the hexanic subextract and DMSO 1 % could not be found at any concentration. With the aim of purifying the samples and concentrate putative active components which could be at low concentration in this mixture, the hexanic extract was fractionated by column chromatography and fractions were tested at the same conditions. The bioguided assay led to the active fraction F3. This fraction could not be tested at the highest concentration due to poor water solubility. The values at 30 min were very close to that of DMSO, which was not expected to display activity at these concentrations (Elespuru and Moore, 1985). To better visualize the individual effects, we looked at the kinetic of the induction (Normalized A *vs* time) to compare treatments. In the last 20 min the curves showed a linear correlation with time, while values in the first 10 min were very variable and did not correlate linearly. As shown in Table 1, a significant and positive correlation between the normalized absorbance and time was found for all treatments at all the concentrations tested. The r^2 values were low at the lowest concentrations for F3 and doxorubicin, showing a decrease on linearity at low concentrations and associated lack of inducing effect, whereas the same behavior was observed for the hexanic extract at high concentrations, possibly due to instability of solutions in the microplate. Although a correlation could be probed, the r^2 values for DMSO were low.

Table 1. Linear regression of normalized absorbance versus time curves of doxorubicin, hexanic extract, F3 and DMSO [t=10 min to t=30 min].

		Doxorubicin		**Hexanic extract**		**F3**	
Conc. µg/mL	**Run**	**r^2**	**Correlation**	**r^2**	**Correlation**	**r^2**	**Correlation**
10	**1**	0.88	very good p < 0.005	0.98	very good p < 0.005	0.85	very good p < 0.005
	2	0.60	Good p < 0.05	0.98	Good p < 0.005	0.87	very good p < 0.005
40	**1**	0.76	Good p < 0.01	0.97	very good p < 0.005	0.97	very good p < 0.005
	2	0.61	Good p < 0.01	0.98	very good p < 0.005	0.83	very good p < 0.005
100	**1**	0.94	very good p < 0.005	0.98	very good p < 0.005	0.99	very good p < 0.005
	2	0.99	very good p < 0.005	0.98	very good p < 0.005	0.99	very good p < 0.005
400	**1**	0.99	very good p < 0.005	0.73	Good p < 0.01	0.99	very good p < 0.005
	2	0.94	very good p < 0.005	0.96	very good p < 0.005	0.99	very good p < 0.005
1000	**1**	0.91	very good p < 0.005	0.70	Good p < 0.01		
	2	0.97	very good p < 0.005	0.98	very good p < 0.005		
DMSO 1 %	**1**	0.6	Good p < 0.01				
	2	0.7	Good				

$p < 0.005$

As Figure 1 shows, the slopes of the curves of normalized absorbance *versus* time were dose-dependent for doxorubicin, while F3 displayed a weak effect and the slopes of the hexanic extract tended to decrease at higher concentrations, possibly due to lack of solubility.

Figure 1. Slopes of *β*-galactosidase reaction vs concentration (last 20 min) in different runs.

We further considered the values at 30 min *versus* concentration, following the original recommendations of Elespuru and Moore (1985) and compared the normalized absorbances of both F3 and hexanic extract with DMSO 1 % each, and doxorubicin with sterile water. The data followed a normal distribution and were homoscedastic for all the treatments. The multiple comparison with the control confirmed no significant effects between the hexanic extract and DMSO 1 % ($p > 0.1$), and significant effects of F3 against DMSO and doxorubicin against water (Fig. 2).

Figure 2. Inducing effect of the *G. lucidum* active fraction F3 and doxorubicin and statistical comparison with controls DMSO 1% and sterile water respectively [* $p<0.05$; ** $p<0.005$; *** $p<0.002$].

The interaction of simultaneous supply of doxorubicin and F3 was studied by exposing cells to different concentrations of doxorubicin in combination with a constant active concentration of F3 (400 μg/mL). Figure 3 shows the results of the combined exposure to doxorubicin and F3 compared with doxorubicin dissolved in water. The effects of DMSO on doxorubicin induction were also tested by adding DMSO 1% to doxorubicin treatments. At higher doxorubicin concentrations (100-1000 μg/mL), adding F3 400 μg/mL caused a reduction of the inducing effect of doxorubicin, while no significant differences were found at lower concentrations. The presence of DMSO 1 % positively affected doxorubicin induction at 400 μg/mL. This tendency could be observed at higher concentrations, although significant differences were not found. Overall, these results showed that the active fraction of *G. lucidum* diminished the induction of doxorubicin, being the interaction of DMSO irrelevant.

Figure 3. Interaction of *G. lucidum* active fraction F3 400 μg/mL and DMSO 1% on the inducing effect of doxorubicin [* $p<0.05$; ** $p<0.01$; *** $p<0.001$].

To identify the components of F3, the sample was characterized by ^{1}H NMR and ^{13}C NMR (Fig. 4). The proton spectrum of F3 yielded a multiple signal (m) at 5.33 ppm corresponding to olefinic protons and a broad prominent signal between 1.42 ppm and 1.19 ppm typical of the methylene groups of a linear carbonated chain. Other signals were 2.33 (t, 7.5 Hz), 2.01 (m), 1.62 (m) and 0.87 (m) ppm. The signal intensity ratio between ^{1}H NMR signals indicated that this fraction was a mixture of components.

Figure 4. Left: ^{1}H NMR spectrum of F3 and expansion of signals between 5.25 and 5.40 ppm. Right: ^{13}C NMR spectrum of F3 and chemical structures of major fatty acids detected by GC-MS.

The carbon spectrum showed two carbons at 179.73 and 179.70 ppm and two olefinic carbons at 129.9 and 129.7 ppm. ^{13}C NMR DEPT experiments confirmed the presence of methylene carbon signals typical of linear carbon chains and a methyl group at 14.1 ppm. The two signals with chemical shifts in the region of the free carboxylic acids were quaternary carbons while carbons at 129.9 and 129.7 were methynes, confirming the presence of two carboxylic groups and one double bond respectively. Accordingly, ^{1}H NMR signals at 2.33 and 1.62 ppm were assigned to H2 and H3 protons of a carbonated chain respectively. The integral of ^{1}H NMR signals at 2.01 ppm was proportional to the integral of signals at 5.33 ppm, indicating that the former protons were those adjacent to the double bond. Both ^{1}H NMR and ^{13}C NMR data indicated that F3 was a mixture of at least two fatty acids, where at least one of them was unsaturated. According to literature on the chemical metabolites of *Ganoderma*, fatty acids are common components of the lipids of these species (Zhao et al., 2012) and long chain fatty acids have been previously isolated from spores (Fukuzawa et al., 2008). The spectroscopic data of F3 was in accordance with that reported for C19 fatty acids from spores of *G. lucidum*, excepting differences in chemical shifts due to esterification of samples (Gao et al., 2012). Moreover, the spectral pattern of olefinic signals between 5.30 and 5.35 ppm of F3 (Fig. 4) was identical to that reported for C19:1 (Δ^{9}) from *G. lucidum*, thus supporting that a *cis*-9 fatty acid was a major component of F3.

As shown in Figure 5, the fatty acid profile of F3 revealed the presence of two major fatty acids, one of them unsaturated, in accordance with NMR data. Palmitic acid (hexadecanoic acid, C16) was the most abundant fatty acid followed by oleic acid (*cis*-9-octadecenoic acid, C18:1 *cis*-9). The mixture also contained minor quantities of other fatty acids but, contrary to that observed by Gao *et al* and in accordance with previous reports on fatty acid profiles of *Ganoderma* spp. (Liu X. et al., 2007), C19 fatty acids were not detected in F3.

Figure 5. Fatty acid profile of fraction F3 from the hexanic extract of *G. lucidum*.

3. Discussion

This assay is a rapid an easy method to understand the interaction of DNA with chemical agents. Its use could be adapted to support the toxicity tests of herbal drugs. However, reproducibility problems may arise from the manipulation of bacterial cultures. Considering that the β-galactosidase basal activity of uninduced cells increases with exponential growth, the use of synchronized cultures is critical for this assay. Elespuru and Moore (1985) observed that the main factors influencing both prophage induction and uninduced background are incubation time, enzymatic assay time, solvent concentration and cell density. From these, the induction is highly dependent on the inoculum. Small differences in cell density cause mayor changes in the induction effect. An increase in the sensitivity of the strain is also required to avoid the use of high percentages of DMSO or organic solvents that indeed interfere with the assay. The heterogeneity of the sample and the instability of the test solutions in aqueous media may increase the dispersion of results and limit testing of high concentrations. The values retrieved from the assay were statistically analyzed considering both values at specific time points as suggested in the original method and the kinetics of the β-galactosidase reaction. All this data showed a very weak effect of certain components of *G. lucidum* extracts on DNA. Being the incubation period equal for both F3 and doxorubicin treatments (4 h), it is likely that the weak effect displayed by F3 was caused by indirect effects on DNA such as those observed for agents that inhibit prokaryotic enzymes, RNA primer formation or bacterial cell wall synthesis (Elespuru & White, 1983). Our results showed that these effects exerted by F3 on *E. coli* cells were not additive to those exerted by doxorubicin, but negatively interfered with doxorubicin DNA damage induction instead. It is worth to consider here that any effect of F3 causing prokaryotic cell growth inhibition could prevent DNA replication, thus diminishing doxorubicin action.

The use of *G. lucidum* as an adjuvant in cancer therapy prompted research on the interaction between its components and chemo- and radiotherapy. Smina et al. (2015) reported that total triterpenes from *G. lucidum* protect DNA and membranes of human peripheral blood lymphocytes *ex vivo* induced by radiation. This mechanism could play a role in ameliorating the effects of radiotherapy. Yue et al. (2008) studied the interaction of *Ganoderma* triterpenes with doxorubicin in HeLa cells using a proteomic method. A change in the expression of certain proteins showed that triterpenes may sensitize cells to doxorubicin by enhancing apoptosis, ROS production and downregulating DNA repair proteins, thus evidencing a synergism between doxorubicin and triterpenes. Our results agree with these previous studies, showing that the cytotoxicity of *G. lucidum* extracts was not related to direct damage to DNA. In the present study, only a fraction containing fatty acids and not triterpenes exerted weak indirect damaging effects on prokaryotic cells, which interfered with DNA damage induced by doxorubicin. The cytotoxicity of *G. lucidum* components, which has been mainly attributed to triterpenes, may be a result of more specific mechanisms operating on eukaryotic cells. Under this hypothesis, the effects of ganoderic acid DM on DNA of human breast cancer cells evidenced by the comet assay and DNA fragmentation test could be a consequence of apoptosis more than a consequence of direct interaction with DNA (Wu et al., 2012).

Although the major components of the mushroom are polysaccharides and triterpenes, other components such as unsaturated long chain fatty-acids, appear to be responsible of the antitumoral activity of *Ganoderma* by inducing apoptosis (Gao et al., 2012). Our results agree with those reported by Fukuzawa et al. (2008), where the lipidic

components such as fatty acids from *G. lucidum* spores displayed activities that should not be underestimated. Moreover, it is worth to consider that the fatty acid content depends on the substrate used for cultivation of the mushroom. Consequently, different cultivating practices may lead to products with different biological activities.

4. Conclusion

The potential of the micro BIA assay to test both the toxicity of herbal drugs and the role of DNA interaction as a mechanism of action should be considered. We showed here that *G. lucidum* extracts do not act directly on DNA like doxorubicin does. Consequently, cytotoxicity observed on tumoral cells may be due to specific mechanisms triggering apoptosis. A mixture of fatty acids from *G. lucidum* extracts caused weak induction which was attributed to an indirect damage on DNA. According to evidence from previous reports, fatty acids should be considered as active components of *G. lucidum* formulations, together with triterpenes and polysaccharides. The presence of these fatty acids caused a reduction of doxorubicin effects in the BIA assay. Although this result contributes to the understanding of the *in vitro* activity of *G. lucidum* components, the clinical impact of this finding may be very low considering final effects on eukaryotic cells, the overlapping effects of major components of *G. lucidum* and the human metabolism of fatty acids. Finally, this study supports that traditional use of *G. lucidum* to prolonging life and its efficacy as an adjuvant for cancer treatment are based on the activation of specific cellular mechanisms that do not damage DNA, at least directly, thus differing from those mechanisms triggered by traditional chemotherapeutic agents.

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References

Abdi, H., 2007. Bonferroni and Šidák corrections for multiple comparisons, in: Salkind, N.J. (Ed.), Encyclopedia of measurement and statistics. SAGE Publications Inc., Thousand Oaks (CA), pp. 103-107.

Bartlett, M.S., 1937. Some examples of statistical methods of research in agriculture and applied biology. J. Royal Statist. Soc. S4, 137-183.

Bidegain, M.A., Cubitto, M.A., Curvetto, N.R., 2015. Optimization of the yield of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (Higher Basidiomycetes), cultivated on a sunflower seed hull substrate produced in Argentina: Effect of olive oil and copper. Int. J. Med. Mushrooms 17, 1095-105.

Bishop, K.S., Kao, C.H.J., Xu, Y., Glucina, M.P., Paterson, R.R.M., Ferguson, L.R., 2015. From 2000 years of *Ganoderma lucidum* to recent developments in nutraceuticals. Phytochemistry 114, 56-65.

Boqué, R., Rius, F.X., Massart, D.L., 1994. Straight line calibration: something more than slopes, intercepts, and correlation coefficients. J. Chem. Educ. 71, 230-232.

Elespuru, R.K., Moore, S.G., 1985. Micro-BIA, a colorimetric microtiter assay of λ prophage induction. Mutat. Res. 164, 31-40.

Elespuru, R.K., White, R.J., 1983. Biochemical prophage induction assay: A rapid test for antitumor agents that interact with DNA. Cancer Res 43, 2819-2830.

Elespuru, R.K., Yarmolinsky, M.B. 1979. A colorimetric assay of lysogenic induction designed for screening potential carcinogenic and carcinostatic agents. Environ. Mutagen. 1, 65-78.

Endo, H., Ishizawa, M., Kamiya, T., Sonoda, S., 1963. Relation between tumouricidal and prophage-inducing action. Nature 168, 258-260.

Fukuzawa, M., Yamaguchi, V., Hide, I., Chen, Z., Hirai, Y., Sugimoto, A., Yasuhara, T., Nakata, Y., 2008. Possible involvement of long chain fatty acids in the spores of *Ganoderma lucidum* (Reishi Houshi) to its anti-tumor activity. Biol. Pharm. Bull., 31, 1933-1937.

Gao, P., Hirano, T., Chen, Z., Yasuhar, T., Nakata, Y., Sugimoto, A., 2012. Isolation and identification of C-19 fatty acids with anti-tumor activity from the spores of *Ganoderma lucidum* (reishi mushroom). Fitoterapia 83, 490-499.

Griffith, K.L., Wolf Jr., R.E., 2002. Measuring β-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. Biochem. Biophys. Res. Commun. 290, 397-402.

Hoeijmakers, J.H.J., 2009. DNA Damage, Aging, and Cancer. N. Engl. J. Med. 361, 1475-1485.

Jin, X., Ruiz Beguerie, J., Sze, D.M.Y., Chan, G.C.F., 2016. *Ganoderma lucidum* (Reishi mushroom) for cancer treatment (Review). Cochrane Database of Systematic Reviews 4, 1-37.

Liu, J., Shimizu, K., Konishi, F., Kumamoto, S., Kondo, R., 2007. The anti-androgen effect of ganoderol B isolated from the fruiting body of *Ganoderma lucidum*. Bioorg. Med. Chem. 15, 4966-4972.

Liu, R.M., Li, Y.B., Liang, X.F., Liu, H.Z., Xiao, J.H., Zhong, J.J., 2015. Structurally related ganoderic acids induce apoptosis in human cervical cancer HeLa cells: Involvement of oxidative stress and antioxidant protective system. Chem. Biol. Interact. 240, 134-144.

Liu, X., Xu, S-P., Wang, V., Yuan, J-P., Guo, L-X., Li, X., Huang X-N., 2007. Characterization of ganoderma spore lipid by stable carbon isotope analysis: implications for authentication. Anal. Bioanal. Chem. 388, 723-731.

Lv, G.P, Zhao, J., Duan, J.A., Tang, Y.P., Li, S.P., 2012. Comparison of sterols and fatty acids in two species of *Ganoderma*. Chem. Cent. J. 6, 10.

Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples). Biometrika 52, 591-611.

Smina, T.P., Maurya, D.K., Devasagayam, T.P.A., Janardhanan, K.K., 2015. Protection of radiation induced DNA and membrane damages by total triterpenes isolated from *Ganoderma lucidum* (Fr.) P. Karst. Chem. Biol. Interact. 233, 1-7.

Sponchiado, G., Adam, M.L., Dadalt Silva, C., Silva Soley, B., de Mello-Sampayo, C., Almeida Cabrini, D., Correr, C.J., Otuki, M.F., 2016. Quantitative genotoxicity assays for analysis of medicinal plants: A systematic review. J. Ethnopharmacol. 178, 289-296.

Sui, X.C., Guo, Q.B., Xia, Y.M., Cui, S.W., Shen, J., Zhang, J., Ding, Z.Y., 2016. Structure features of the intracellular polysaccharide from *Ganoderma lucidum* and the irrelative immune-anticancer activities of GLPs. Bioact. Carbohydr. Diet. Fibre 8, 43-50.

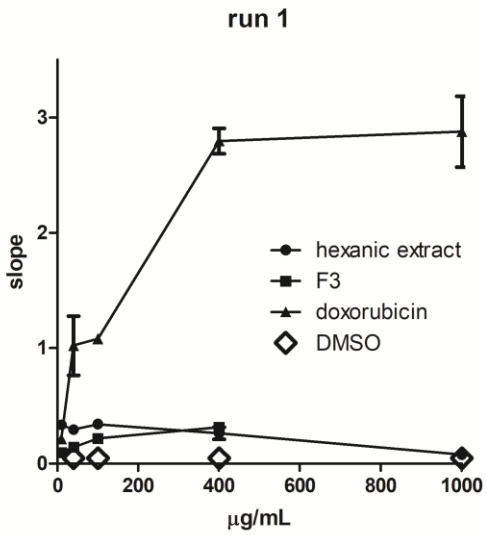
Wasser, S.P., 2017. Medicinal mushrooms in human clinical studies. Part I. Anticancer, oncoimmunological, and immunomodulatory activities: A review. Int. J. Med. Mushrooms 19, 279-317.

Wu, G.S., Lu, J.J., Guo, J.J., Li, Y.B., Tan, W., Dang, Y.Y., Zhong, Z.F., Xu, Z.T., Chen, X.P., Wang, Y.T., 2012. Ganoderic acid DM, a natural triterpenoid, induces DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells. Fitoterapia 83, 408-414.

Yue, Q.X., Xie, F.B., Guan, S.H., Ma, C., Yang, M., Jiang, B.H., Liu, X., Guo, D.A., 2008. Interaction of *Ganoderma* triterpenes with doxorubicin and proteomic characterization of the possible molecular targets of *Ganoderma* triterpenes. Cancer Sci. 99, 1461-470.

Zhao, R.L., He, Y.M., 2018. Network pharmacology analysis of the anti-cancer pharmacological mechanisms of *Ganoderma lucidum* extract with experimental support using Hepa1-6-bearing C57 BL/6 mice. J. Ethnopharmacol. 210, 287-295.

Zazopoulos, E., Huang, K., Staffa, A., Liu, W., Bachmann, B.O., Nonaka, K., Ahlert, J., Thorson, J.S., Shen, B., Farnet, C. M., 2003. A genomics-guided approach for discovering and expressing cryptic metabolic pathways. Nat. Biotechnol. 21, 187-190.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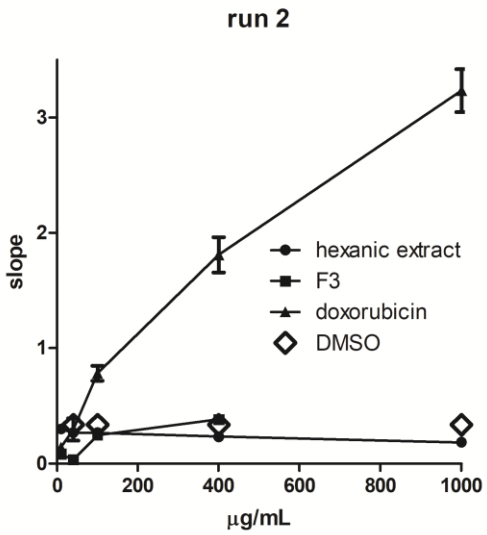
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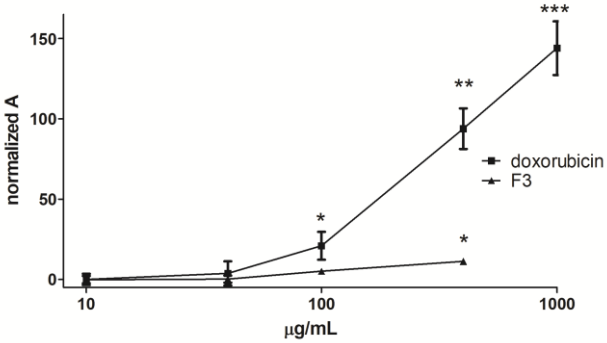
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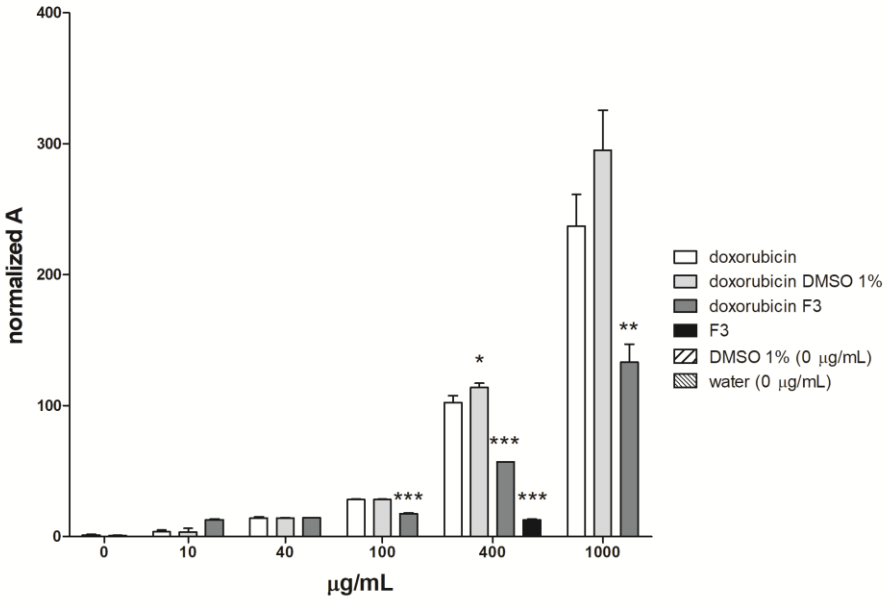

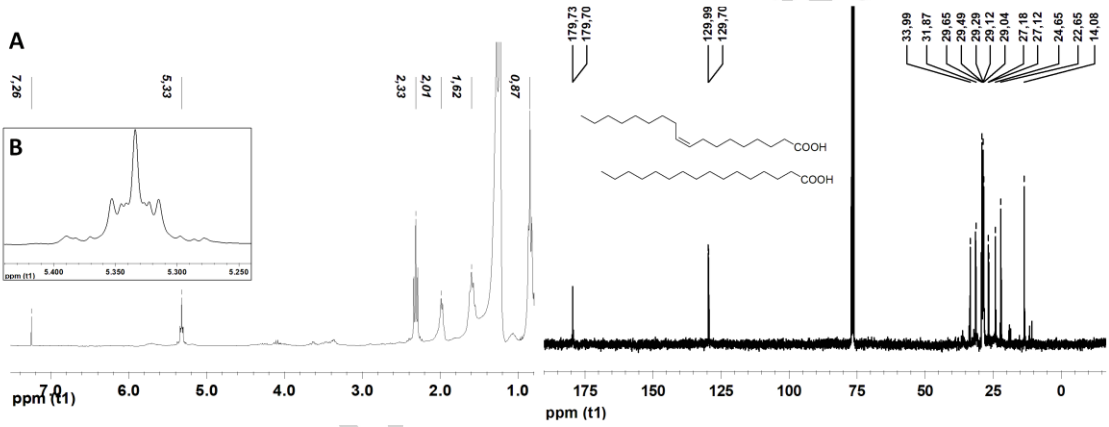

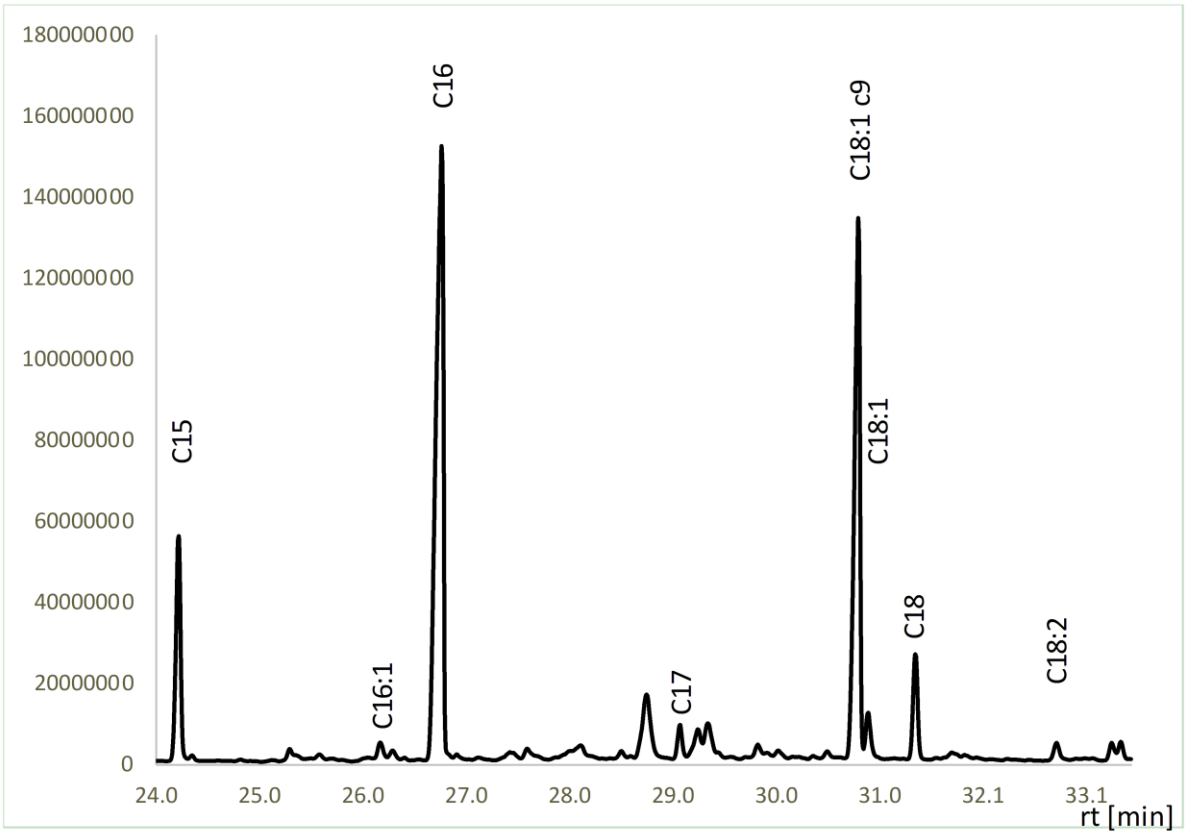
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