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Anti-tumor and anti-oxidant effects of *Ganoderma lucidum* extracts on oral squamous cell carcinoma and skin squamous cell carcinoma *in vitro*

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ABSTRACT

Ganoderma lucidum has had great importance in healthcare in the Chinese for many decades. Polysaccharides and triterpenoids are one of the most important components of *G. lucidum*. Biological evidence indicates that this mushroom can be a promising treatment in many cancer cases and reduce the toxic effect of chemotherapy and radiotherapy if taken synchronously. This study highlights preparing an extract with therapeutic effects capable of removing free radicals and protecting cell membranes from hyperoxidation effects. The antitumor effect was evaluated on two types of cancer cell lines *in vitro*, Human Oral Squamous Cell Carcinoma (HOSCC) and Human Skin Squamous Cell Carcinoma (HSSCC), at different concentrations 0.0-1600 µg/mL. Antioxidant by DPPH assay was assessed at concentrations from 0.0-500 µg/mL, in addition to their effects on cytogenetic indicators such as mitotic index (MI), blastogenic index (BI) and total chromosomal aberration (TCAs) for three types of extracts (ethanol extract, water extract and acidified water extract). The results recorded an increase in the antioxidant activity of the three extracts, and that the acidified water extract had the highest effect, reaching a killing rate of 78.53% for the acidified water extract on the HOSCC cell line. Chromosomal stability indicates that the three extracts enhanced the cytogenetics parameters and supported chromosomal stability by increasing both mitotic index (MI) and blastogenic index (BI), while reducing chromosomal aberrations.

INTRODUCTION

Chinese people have traditionally used ganoderma mushrooms for several health-related purposes. The primary chemical components of Ganoderma – polysaccharides and triterpenoids - have drawn a lot of interest in recent years [1]. Additionally, the *Ganoderma lucidum* (*G. lucidum Leyss. ex Fr.*) Karst mushroom (GL), also known as the "plant of immortality" – "Reish" in Japan, and "Ling-Zhi" in China, is traditionally used in Asiatic folk medicine to enhance human vitality, promote longevity, and to treat a number of human diseases, including cancer [2,3].

Given that some solid carriers, such as silica, have been linked to autoimmune diseases, lung cancer, silicosis, renal failure and systemic sclerosis [4], natural materials have drawn a lot of interest as a support to solidify L-SNEDDS. A number of diseases, including cancer, atherosclerosis, diabetes, obesity and gut dysbiosis, have been claimed to be treated by the polysaccharides found in mushrooms, such as *Ganoderma lucidium* extract paste (GLEP) [5]. likewise probiotics (PBs) aid in lowering insulin resistance by reducing lipopolysaccharide secretion [6]. Many of the anti-tumor properties of GL have been linked to particular substances found in this mushroom, such as its polysaccharides, primarily -glucans, water-soluble heteropolysaccharides or glycoproteins, the pharmacological mechanisms of which prevent the growth of tumor cells and induce their death [7]. Acute myeloid leukemia, colon cancer, hepatocellular carcinoma and breast cancer are just a few of the cancer cell lines that research has revealed that GL polysaccharides (GLPS) can mitigate tumor growth and metastasis [8].

Meroterpenoids, a newly found structural family in Ganoderma, had not met with research interest until recently. In Ganoderma, a phenol moiety and a terpenoid residue make up the meroterpenoids. Meroterpenoids are diverse, complicated and fascinating due to the variety of structural forms that result from the terpenoid group. From 2009 to

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2019, 396 meroterpenoids from Ganoderma species had been described; of them, 40 novel structures had been isolated from *G. lucidum*, according to a recent study of fungal metabolites [9]. With increasing numbers of meroterpenoids characterized by Ganoderma, such a compound's beneficial or evil role could not be ignored anymore. Although modern spectroscopic approaches provide powerful tools for clarifying the congregation structures of natural products, a significant challenge still exists [10].

Therefore, the study aims to prepare extracts with therapeutic effects capable of removing free radicals and protecting cell membranes from hyperoxidation.

MATERIALS AND METHODS

Ganoderma lucidum

The powder of the fruiting body of the fungus *Ganoderma lucidum* was obtained from DXN (Malaysia), in the form of capsules weighing 500 mg/capsule (Figure 1).



Figure 1. *Ganoderma lucidum* capsules DXN, Malaysia

Extracts Preparation

Five grams of mushroom powder were soaked in 250 milliliters of different solvents (cold distilled water) and (acidified water containing 0.1 M HCl and (70% ethanol) for 24 hours at 4°C with continuous stirring. The extract was filtered with medical gauze and filter papers (Whatman No. 1), then the infusion was centrifuged at 30000 rpm for 30 minutes at 4°C. The residue was discarded, and the filtrate was removed. The solvent was evaporated in the incubator at 40°C for 24 hours, and the remaining powder was kept in sterile and opaque vials at a temperature of 4°C to study the antioxidant and toxic effects of cancer cells [11].

Antioxidant assay

The method mentioned in [12] was followed in conducting an oxidative stress test using the 2, 2-diphenyl-1-picryl-hydrazylhydrate DPPH method. Herein, 0.024 grams of DPPH were added to 50 milliliters of absolute ethyl alcohol and dissolved well on a magnetic mixer without heat. The volume was completed to 100 milliliters with absolute ethyl alcohol to give a final concentration equal to 0.024 mg/milliliter. Half a milliliter of serial concentrations of the three extracts was then taken and added to a mixture of DPPH (0.5) mM and mL (3.3) of absolute ethanol. After 100 minutes of the reaction taking place at room temperature, the degree of color change was determined using a spectrophotometer at a wavelength of 515 nm. The blank tube included 3.3 mL of absolute ethanol and 0.5 mL of the sample, as opposed to 3.3 mL of absolute ethanol and 0.5 mL of DPPH in the control tube, the antioxidant activity (%) was calculated according to equation number (1).

$$\text{AntioxidantActivity} = 100 - \frac{\text{sampleabsorbency}}{\text{controlabsorbency}} \times 100\% \quad (1).$$

Studied cancer cell lines

The toxicological effect of Ganoderma aqueous, ethanolic, and acidified aqueous extracts at concentrations (0.0, 10, 15, 20, 25, 50, 100, 200, 400, 800, and 1600 µg/ml) was studied on two types of cancer cell lines: human skin squamous carcinoma cell line (HSSCC) and human oral squamous carcinoma cell line (HOSCC), and in passes 27 and 22, respectively. Cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Calf Serum (FCS). To conduct this test, the toxic effect was studied by culturing cells in tissue culture dishes with multiple holes (96-Microtiter plates) and flat bottoms. The experiment included three stages:

1. Cells Seeding

After the cancer cells were activated and multiplied for 24 hours, the growth monolayer was treated with a Trypsin-Versen solution. Twenty-five milliliters of RPMI-1640 medium prepared with serum were added to each container, and the number of cells was adjusted to (104 × 1) cell/mL using a Neubauer chamber slide count. A volume of 100 µL of cell suspension was taken and distributed to the holes of the tissue culture dish. The dishes were incubated after covering them with sterile adhesive paper at a temperature of 37°C for a period of time of 24 hours to allow the cells to adhere to the glass.

2. Serial concentrations of the test substance

   Serial concentrations of the test material were prepared using a tissue culture medium devoid of fetal calf serum. These were added to the wells containing adhering cancer cells, taking into account the preparation of material solutions immediately upon use, using six replications for each treatment. The culture medium was poured onto the tissue culture dishes. Column No. 1 was considered a negative control, and 200 µL of serum-free culture medium was added to it, while columns 2-12 were appended with graduated concentrations of 200 µL/hole. The dishes were covered and incubated at 37°C for different exposure times (24, 48, 72 hours).

3. Cytotoxicity assay

   After the end of the prescribed incubation period, the contents of the dishes (the culture medium and the suspended cells) were poured out and then washed with phosphate-buffered saline three times to ensure the removal of any trace of the test material and non-adherent cells. A 10 µL of MTT dye solution (with a final concentration of 0.5 mg/ml) was added to each well and left for 4 hours at 37°C in a carbon dioxide incubator. The cells were subsequently washed several times with a phosphate buffer until the excess dye was removed. After the dishes were dry completely, 100 µL of DMSO was included within each dish to ensure the complete dissolution of the violet granules (Formazan). The results were read with an ELISA microplate spectrophotometer (Elisa Reader – Dana 3200, UK) at a wavelength of 500 nm.

The inhibition rate was calculated according to equation number (2).

$$IR = 100 - \frac{Abtest}{Abcontrol} \times 100\% \quad (2),$$

whereas:
IR = Inhibitory Rate
A = Absorbency for Negative Control
B = Absorbency for Test.

Chromosomal analysis

Whole blood from 25 years old healthy man was collected by heparin-coated sterile syringe, and pre-stimulated peripheral blood lymphocytes with 10 µg/mL phytoheamoglutnine (PHA) were incubated for 72 hours at 37°C and 5% CO_2 atmospheric concentration. Subsequently, 0.5 mL of blood was added to 4.5 mL of completed culture medium RPMI-1640, supplemented with 10% fetal bovine serum, and a mixture of antibiotic (penicillin and streptomycin). The incubated cells were then exposed to 10 µg/mL Colchicine for 20 minutes, after which hypotonic KCl 0.075 M was used for 20 minutes. Following centrifugation for 5 minutes at 3000 rpm, the supernatant was discarded, and the pellet was washed three times with fixative (3:1 methanol to glacial acetic acid). The transparent cell suspension was then drooped on clean cold slides and air dried overnight, then stained with Giemsa stain. Mitotic index, blastogenic index, and chromosomal aberrations were scored for both non-exposed and exposed cells to different concentrations of the three botanical extracts [13].

Statistical analysis

ANOVA I by SPSS software was used to analyze all data. P values were set at ($p \leq 0.05$) to measure significance.

RESULTS AND DISCUSSION

Natural products such as plants, animals, and fungi are an important source of new treatments, and research is continuing on new alternatives to highly toxic or expensive drugs. About 300 mushrooms have been found to show significant medical effects [14]. Studies indicate that mushroom extracts benefit the human body as antifungals, antibacterials and anti-inflammatory and antiviral agents, besides their activity in reducing hyperglycemia [15]. The current study tested three types of *G. lucidum* extracts (Ethanolic, Watery, and Acidified water extract) to evaluate the antioxidant activity using the dpph method. A concentration-dependent increase in the antioxidant activity (Figure 2) was noted, which was at its highest levels in the acidified water extract, as the percentage of free radical removal reached its highest level of 78.56%. Various extracts of *G. lucidum* have antioxidant action by scavenging ions O^{2-}, HO, SO, H_2O_2, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) *in vitro*. *G. lucidum* extracts can stimulate superoxide dismutase (SOD), phase II detoxification enzyme, catalase and glutathione S-transferase P1 (GSTP1) by the Nrf2-mediated signals pathways in different types of tumors *in vitro* [16-19].

In our work, we studied the anti-tumor effects on two types of cancer cell lines (HSSCC and HOSCC) in different concentrations 0.0, 10, 15, 20, 25, 50, 100, 200, 400, 800, and 1600 µg/mL and three exposure times 24, 48, and 72 hours. The study results revealed that the three extracts induced an increase in the activity of cancerous cells at low concentrations of 0.0-50 µg/mL. At the same time, this effect became inhibited at concentrations higher than 50 µg/mL. The three extracts demonstrated the same inhibited pattern with a slight difference in the inhibition rate. The acidified water extract, however, had a more significant effect than the other extracts, and the killing rate on HOSCC cells was more effective than that on HSSCC (Tables 1 and 2, Figures 3 and 4). The killing rate was also correlated with concentration and exposure time.

Metastasis is one of the characteristics of malignant tumor cells and their migration through different tissues, and it is the leading cause of death in cancer patients. The proliferation and migration of malignant cells have received tremendous interest in chemotherapies. Studies showed that the *G. lucidum* extracts significantly reduced the migration invasive of cancer cells *in vitro* and metastasis *in vivo*. In several studies, *G. lucidum* extracts was found to inhibit β1-integrin manifestation in MT-1 breast cancer and prostate PC-3 cancer cells, depending on the concentration [20-22]. For example, cyclophosphamide doses cause metastasis of the lung C57BL/6 cancer cells in mice, and this is effectively repressed by inserting *G. lucidum* into the diet [23,24]. In our work, the results of the chromosomal study showed that there was a significant increase in the value of the mitotic index (MI) of peripheral blood lymphocytes (PBL) and an increase in the blastogenic index (BI), which was the highest in the acidified water extract (Table 3, Figure 5 and 6). From the results, it is noted that there was no genotoxicity wxhibited (chromosomal breakages, translocations, and deletions), or any toxic effects on human peripheral blood lymphocytes in the used concentrations, and this indicates that the aqueous and alcoholic extracts of *G. lucidum* are largely safe [19].

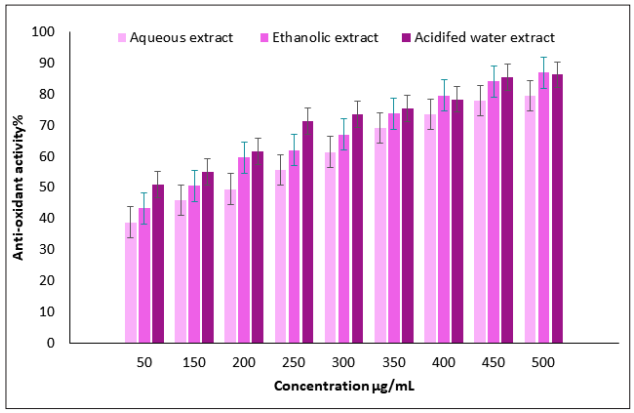
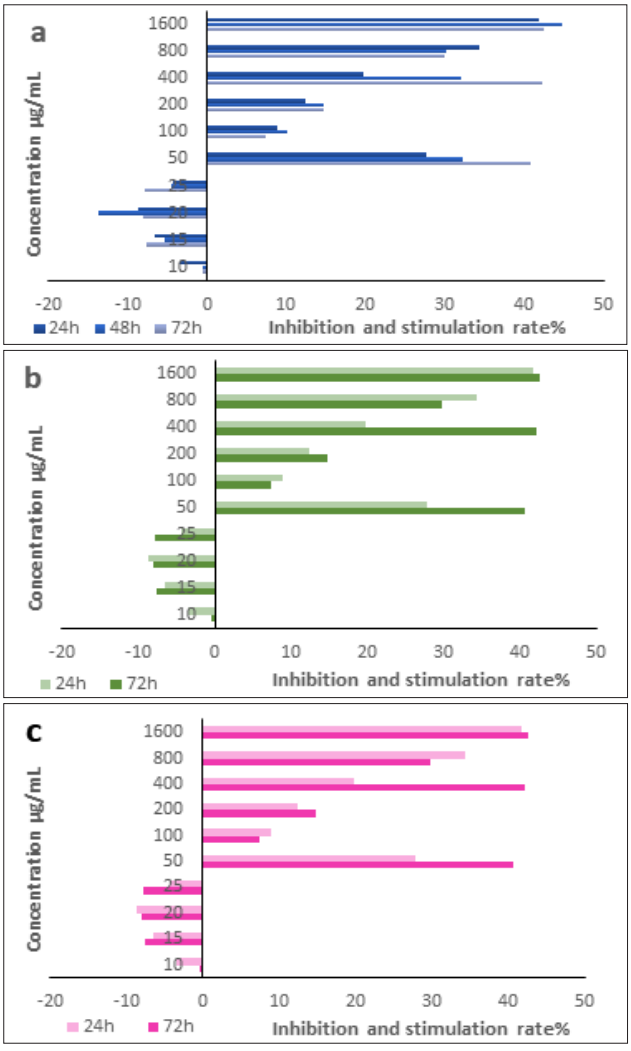


Figure 2. Antioxidant effects of *Ganoderma lucidium* extracts by DPPH assay at different concentrations

Table 1. Optical density (OD500nm) of G. Lucidum, ethanol extract, water extract and acidified water extract at different concentrations and different exposure times (24, 48, and 72 h) on Human Skin Squamous Cell Carcinoma cell line (HSSCC)

Concentration (µg/mL)	Human Skin Squamous Cell Carcinoma (HSSCC)								
	Ethanol extract			Water extract			Acidified water extract		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.0	1.74 ±0.02	1.86 ±0.01	1.96 ±0.01	1.76 ±0.02	1.87 ±0.02	1.95 ±0.02	1.76 ±0.01	1.87 ±0.01	1.97 ±0.02
10	1.77 ±0.01	1.92 ±0.01	2.16 ±0.02	1.82 ±0.02	1.91 ±0.02	2.15 ±0.01	1.82 ±0.02	1.88 ±0.01	1.98 ±0.02
15	1.82 ±0.01	1.97 ±0.01	2.21 ±0.01	1.87 ±0.02	1.94 ±0.02	2.24 ±0.02	1.94 ±0.02	1.98 ±0.02	2.13 ±0.01
20	1.95 ±0.02	2.14 ±0.02	2.33 ±0.01	1.90 ±0.01	2.10 ±0.01	2.23 ±0.02	2.11 ±0.01	2.25 ±0.02	2.3 ±0.01
25	2.15 ±0.02	2.28 ±0.02	2.60 ±0.01	2.13 ±0.01	2.21 ±0.01	2.35 ±0.01	2.2 ±0.02	2.35 ±0.01	2.48 ±0.02
50	1.71 ±0.01	1.64 ±0.01	1.49 ±0.02	1.77 ±0.02	1.72 ±0.01	1.67 ±0.01	1.59 ±0.02	1.59 ±0.02	1.47 ±0.02
100	1.63 ±0.01	1.56 ±0.01	1.36 ±0.02	1.68 ±0.02	1.62 ±0.01	1.48 ±0.02	1.45 ±0.01	1.43 ±0.01	1.36 ±0.02
200	1.46 ±0.01	1.33 ±0.01	1.17 ±0.02	1.57 ±0.1	1.44 ±0.01	1.35 ±0.01	1.27 ±0.01	1.22 ±0.01	1.16 ±0.01
400	1.15 ±0.02	0.90 ±0.01	0.78 ±0.01	1.44 ±0.01	1.35 ±0.02	1.25 ±0.01	1.02 ±0.01	0.83 ±0.00	0.67 ±0.02
800	0.86 ±0.02	0.79 ±0.01	0.75 ±0.01	1.34 ±0.01	1.24 ±0.02	1.12 ±0.01	0.67 ±0.02	0.58 ±0.01	0.47 ±0.01
1600	0.66 ±0.01	0.63 ±0.01	0.55 ±0.01	0.93 ±0.01	0.83 ±0.01	0.75 ±0.01	0.39 ±0.01	0.32 ±0.01	0.27 ±0.01
P value	0.9948			0.9934			0. 9937		

Each number represents Mean ±SD for three replicates
water extract at different concentrations and different exposure times (24, 48, and 72 h) on Human Skin Squamous Cell Carcinoma cell line (HSSCC)



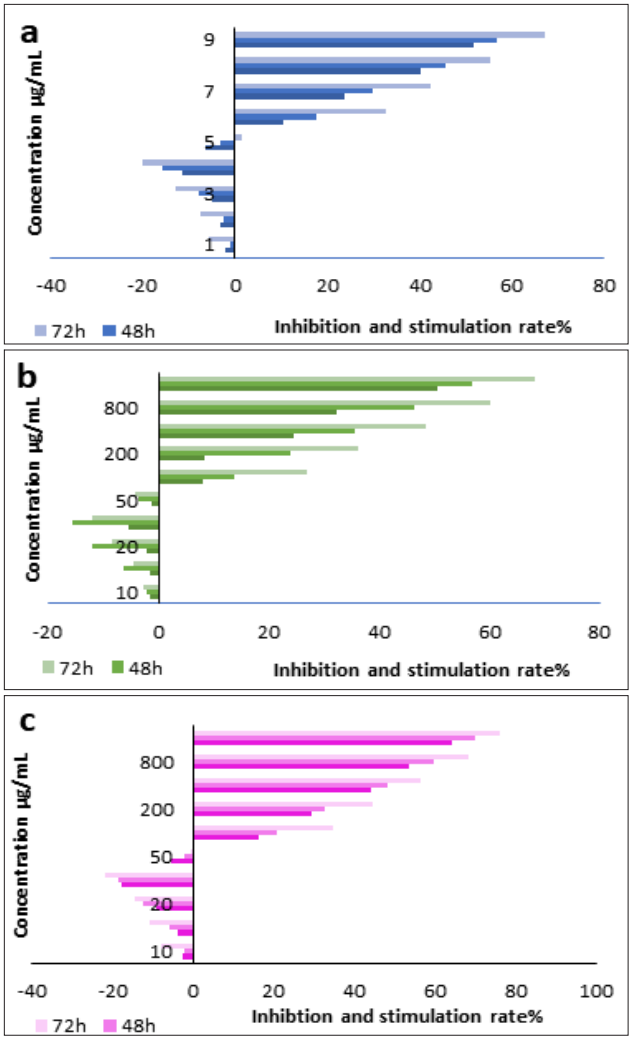
a) ethanol extract b) water extract c) acidified water extract at different concentrations and different exposure time (24, 48 and 72 h) on Human Skin Squamous Cell Carcinoma cell line (HSSCC)

Figure 3. Inhibition and stimulation rate% of *G. lucidum*

Table 2. Optical density (OD $^{500\ nm}$) of *G. lucidum*, ethanol extract, water extract and acidified water extract at different concentrations and different exposure times (24, 48 and 72 h) on Human Oral Squamous Cell Carcinoma cell line (HOSCC)

Concentration (µg/mL)	Human Oral Squamous Cell Carcinoma (HSSCC)								
	Ethanol extract			Water extract			Acidified water extract		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.0	1.84 ±0.01	1.91 ±0.02	2.11 ±0.02	1.86 ±0.02	1.93 ±0.01	2.14 ±0.02	1.86 ±0.02	1.93 ±0.02	2.11 ±0.01
10	1.88 ±0.02	1.93 ±0.02	2.23 ±0.01	1.88 ±0.01	1.96 ±0.02	2.19 ±0.02	1.91 ±0.01	1.97 ±0.01	2.29 ±0.02
15	1.91 ±0.02	1.96 ±0.01	2.27 ±0.02	1.89 ±0.01	2.05 ±0.01	2.23 ±0.02	1.94 ±0.01	2.05 ±0.02	2.3 ±0.02
20	1.94 ±0.02	2.06 ±0.02	2.39 ±0.01	1.92 ±0.02	2.15 ±0.02	2.31 ±0.01	2.04 ±0.01	2.18 ±0.02	2.42 ±0.01
25	2.0 ±0.02	2.22 ±0.02	2.53 ±0.01	1.95 ±0.02	2.22 ±0.02	2.39 ±0.01	2.19 ±0.01	2.29 ±0.02	2.57 ±0.02
50	1.96 ±0.01	1.97 ±0.02	2.08 ±0.02	1.88 ±0.02	1.99 ±0.02	2.042 ±0.02	1.97 ±0.01	1.98 ±0.02	2.11 ±0.02
100	1.64 ±0.02	1.58 ±0.02	1.42 ±0.02	1.71 ±0.01	1.66 ±0.02	1.56 ±0.01	1.56 ±0.01	1.54 ±0.02	1.38 ±0.01
200	1.39 ±0.01	1.342 ±0.02	1.22 ±0.02	1.55 ±0.02	1.46 ±0.01	1.37 ±0.02	1.32 ±0.01	1.31 ±0.02	1.18 ±0.01
400	1.1 ±0.02	1.04 ±0.02	0.94 ±0.01	1.42 ±0.02	1.25 ±0.02	1.15 ±0.01	1.04 ±0.02	0.99 ±0.02	0.92 ±0.01
800	0.89 ±0.02	0.83 ±0.01	0.69 ±0.02	1.26 ±0.01	1.04 ±0.01	0.86 ±0.02	0.87 ±0.02	0.78 ±0.01	0.67 ±0.01
1600	0.69 ±0.01	0.63 ±0.01	0.55 ±0.01	0.915 ±0.01	0.83 ±0.01	0.69 ±0.01	0.66 ±0.01	0.58 ±0.01	0.5 ±0.01
P value	0.938			0.983			0.956		

Each number represents Mean ±SD for three replicates



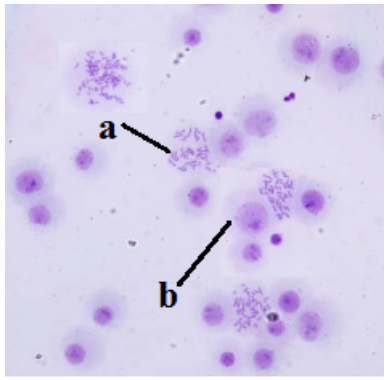
a) ethanol extract b) water extract c) acidified water extract at different concentrations and different exposure time (24, 48 and 72 h) on Human Oral Squamous Cell Carcinoma cell line (HOSCC)

Figure 4. Inhibition and stimulation rate% of *G. lucidum*

Table 3. Chromosomal analysis of *G. Lucidum* ethanol extract, water extract and acidified water extract at different concentrations of peripheral blood lymphocytes

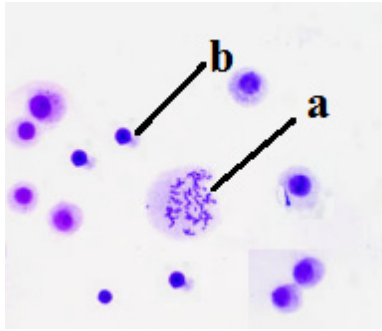
Concentration (µg/mL)	Ethanolic extract			Watery extract			Acidified water extract		
	BI	MI	TCA	BI	MI	TCA	BI	MI	TCA
0.0	63.41 ±0.56	1.88 ±0.02	0.25 ±0.01	63.41 ±0.44	1.88 ±0.03	0.25 ±0.01	63.41 ±0.55	1.88 ±0.02	0.25 ±0.01
10	68.81 ±0.77	1.95 ±0.03	0.21 ±0.01	65.15 ±0.68	1.91 ±0.04	0.23 ±0.01	71.13 ±0.68	2.01 ±0.02	0.15 ±0.01
15	71.25 ±0.39	2.12 ±0.03	0.13 ±0.01	70.22 ±0.75	1.98 ±0.02	0.18 ±0.01	76.48 ±0.91	2.44 ±0.01	0.10 ±0.01
20	77.12 ±0.45	2.55 ±0.05	0.0	72.28 ±0.84	2.21 ±0.01	0.0	79.55 ±0.85	2.91 ±0.01	0.0
25	82.55 ±0.89	2.61 ±0.09	0.0	75.13 ±0.36	2.44 ±0.02	0.0	85.19 ±0.67	2.94 ±0.02	0.0
50	87.18 ±0.66	2.87 ±0.07	0.0	80.47 ±0.456	2.63 ±0.02	0.0	87.63 ±0.72	2.97 ±0.01	0.0

Each number represents Mean ±SD for three replicates



a) mitotic cell b) blast cell

Figure 5. Peripheral blood lymphocytes stimulated by 50 µg/mL of acidified water extract of *G. lucidum*



a) mitotic cell, b) unstimulated lymphocytes

Figure 6. Peripheral blood lymphocytes: untreated

CONCLUSIONS

The studies discussed in this article provide compelling evidence for the anti-tumor and anti-oxidant effects of *Ganoderma lucidum* extracts on HOSCC and HSSCC *in vitro* models. The ability of *G. lucidum* to inhibit cancer cell proliferation, induce apoptosis, suppress angiogenesis and mitigate oxidative stress highlights its potential as a valuable therapeutic agent in managing these cancers.

The results indicate that three extracts of *G. lucidum* had a toxic effect on two cell lines (HSSCC and HOSCC) *in vitro* when the concentration was 50 µg/mL and above. Moreover, the three extracts had antioxidant activity and enhanced the cytogenetics parameters. The effect was mainly dependent on concentration. Further research, including *in vivo* studies and clinical trials, is warranted to validate these findings and explore the full therapeutic potential of *Ganoderma lucidum* in the treatment of HOSCC and HSSCC.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

ETHICAL APPROVAL

The experimental procedure was approved by bio-ethical committee of university of technology Baghdad, Iraq.

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