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ORIGINAL ARTICLE

Anti-proliferative Effect of *Ganoderma Lucidum* Polysaccharide and Triterpenoid Fractions against Cancer Cells

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ABSTRACT

Objectives: To evaluate the anti-cancer activity of *Ganoderma lucidum* polysaccharide (GLPS) and tritrepenoid fraction (GLTT), against five different cell lines.

Methods: The collected *Ganoderma lucidum* was authenticated and extracted with water and methanol to obtain GLPS and GLTT fractions. Cell lines (Vero, MCF-7, HEp-2, HeLa and A-549) were cultured and maintained as per standard procedure. Anti-proliferative activity of GLPS and GLTT were studied by standard MTT and SRB assays. Long term survival studies, nuclear staining and microtubule dynamic studies of GLPS and GLTT were investigated using HeLa cells.

Results: Both GLPS and GLTT showed better results and specificity towards HeLa cells followed by MCF-7, HEp-2, A-549 and Vero respectively. Concentration of GLPS and GLTT at 100 µg/ml (below the CTC_{50} value) was selected for nuclear staining and molecular dynamic studies. Nuclear staining experiments, Cancer cells showed severe damage with characteristic apoptotic morphological alterations such as condensation of nuclear and cytoplasm, reduction of cell volume and fragmentation. Further, GLPS, GLTT were tested for microtubule dynamic assay against HeLa cells specifically inhibited microtubule network.

Conclusion: The current studies demonstrate the strong inhibitory effect of GLPS and GLTT on cancerous cell lines at low concentrations.

Keywords: *Ganoderma lucidum*, mushroom, polysaccharide, triterpenoid, anti-cancer activity

INTRODUCTION

In Chinese tradition *Ganoderma lucidum* is recognized as one of the important kinds of mushroom to cure every kind of disease. Many clinical studies in Asia have used *G. lucidum* along with other herbal drugs in combination or as a single agent[1, 2]. For the development of pharmaceutical product it is important to isolate the active ingredients from *G. lucidum* and to prove its mechanism of action. A total of 140 different triterpenes have been identified from *G. lucidum*[3]. Major triterpenes isolated from *G. lucidum* include ganoderols, lucidones, applanoxidic and ganoderenic acids and ganoderols[4]. Many studies have proved that triterpenes are one of the important fractions for the therapeutic efficiency of *G. lucidum*[5]. Further, *G. lucidum* and its extracts have been reported to be safe in animal studies[6, 7]. On the other hand, because of its significant cytotoxicity towards cancer cells, there is a possibility of adverse effect from the derivatives of triterpenes on long term use[8, 9]. The latest research works on Ganoderma were reported in the area of antioxidant activity, cardio protective and liver based studies. Xia et al., 2013 have investigated tritrepenoids isolated from *G. resinaceum* on H_2O_2 induced toxicity in HepG2 cells[10]. Effect of *G. lucidum* on isoproterenol induced myocardial infraction in rat models were investigated by Cherian et al., 2009 [11]. Researchers have also reported anti-cancer activity of triterpene from *G. lucidum* against cancer cells and their signaling pathway[4, 12, 13].

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Polysaccharides from G. *lucidum* are another active fraction with promising anticancer properties. Anti-oxidant activity of the polysaccharide fraction from G. *lucidum* was reported by Deepalakshmi et al[14]. FAS /caspase signaling pathway and effect of polysaccharide fraction of G. *lucidum* on cancer cells was reported by Liang et al. Mushroom polysaccharides are also acknowledged for its immunomodulating and antitumor properties[15]. However, mushrooms are known to be sensitive to the climatic conditions. Studies also indicated that chemical composition of *G. lucidum* fruit bodies differ from one region to another. Environmental and cultivation conditions of mushroom will definitely have an impact on the biological activity of the active constituents[16]. It has been reported that cultivation condition and strains will have direct impact on the physiological and distinct properties of *G. lucidum*[16]. Little attention has been paid towards the Malaysian origin mushrooms and the molecular mechanisms underlying the anti-cancer property. Therefore, we evaluated the anti-cancer activity of G. *lucidum*, cultivated in northern region of Malaysia. We studied the effect of *G. lucidum* polysaccharide (GLPS) and tritrepenoid fraction (GLTT) against five different cell lines.

MATERIAL AND METHODS

Materials

High purity inorganic solvents, F12 Coons medium and β- tubulin antibody FITC conjugated, Antibiotic solution (Streptomycin 100 µg/ml, Pencillin100 U/ml and Amphotericin B 250 ng/ml), Dulbecco's Modified Eagles Medium (DMEM), Ethidium bromide and Crystal violet were procured from Sigma Aldrich Co., St. Louis, USA 3-(4, 5 dimethyl thiazole-2 yl) - 2, 5-diphenyl tetrazolium bromide (MTT), SRB Sulphorhodamine B, was obtained from Acros Organics., New Jersey, USA. New Born Calf Serum (NBCS) and Fetal Bovine Serum (FBS) was obtained from PAA Labs, Austria. GLPS and GLTT were dissolved in DMSO (Dimethyl Sulfoxide) not exceeding the concentration of 0.1% (v/v), and the volume was made up to 10 ml with appropriate medium. Prepared stock solution of 1 mg/ml concentration was membrane filtered and stored at -20°C for further use. Pure sample of *Ganoderma lucidum* was gifted by DXN Pharma Sdn Bhd., Malaysia.

Panel of cell lines and medium

MCF-7 Human breast cancer cell line, Vero African green monkey kidney cells, HEp-2 Caucasian male larynx epithelial carcinoma cells, A-549 Human small cell lung carcinoma cells, HeLa Human cervix carcinoma cells were obtained from Life Technologies, Bio-diagnostics Sdn Bhd, Malaysia. A-549 cells were maintained in F12 Coon's cell culture medium containing L-Glutamine and 10% of fetal calf serum. All other cells were maintained in DMEM supplemented with 10% New born calf serum, Streptomycin (100 µg/ml), Penicillin (100 IU/ml) and Amphotericin B (5 µg/ml) at 37°c temperature and 5% humidified atmosphere with Co_2.

Preparation of polysaccharide and triterpene extracts

The preparation of polysaccharide extract was performed according to the modified protocol of Ke Pan *et al*., 2013[17]. The *G. lucidum* was collected, authenticated and supplied by DXN Pharma Sdn Bhd. Malaysia. The obtained sample was dried and coarse powdered in mortar. 200 g of the powder was soaked in half liter distilled water, followed by refluxing for 3 h and cooling. The sample was further filtered using Whatman 41 filter followed by 0.45µm membrane filter. The filtrate was freeze dried to get a final yield of 20 g of *G. lucidum* polysaccharide extract (GLPS). Triterpene extract was prepared from 350 g of coarse powder subjected to soxhlet extraction using methanol (1 L) for 20 h. The extract obtained was concentrated, dried under reduced pressure and controlled temperature to yield a dark semisolid mass (28 g, 7%) of G. *lucidum* triterpene extract (GLTT) which was preserved in chilled conditions for further studies.

Anti-proliferative activity assay

Cells in exponential growing phase were trypsinized and seeded into maintenance medium. The cells were distributed in 96 well titre plate (SPL Life sciences, Korea) at 10000 cells/well and incubated for 24 h during for the formation of partial monolayer. They were treated with various concentrations of GLPS and GLTT and the control wells received only maintenance medium. Titre plates were incubated at 37°C with 5% CO_2 in a humidified incubator for a time of 72 h. Inverted microscope was used to study the morphological changes at different time intervals. Cellular viability was determined by using MTT[18]and SRB[19] assays at the end of 72 h of incubation.

Long term survival studies

Cultures were prepared in 25 cm^2 tissue culture flask (SPL Life sciences, Korea) by seeding 5x 10^4 HeLa cells/ml and incubate for 48 h. Later the medium was decanted and the cultures were exposed to different concentrations of GLPS and GLTT, below CTC_{50} value and incubated at 37°C for a time of 72 h. Later the cultures were trypsinized and seeded into 24 well micro titre plates at a concentration of 5000 cells/ml. Throughout the study, the control groups were maintained in growth medium. The cultures were incubated and the numbers of colonies greater than 32 cells were considered to have retained their regenerative capacity.

Table 1: Cytotoxicity profile of GLPS and GLTT against five different cell lines

	CTC_{50} in Cell Line (µg/ml)									
	Vero		MCF-7		Hep-2		HeLa		A-549	
	MTT	SRB	MTT	SRB	MTT	SRB	MTT	SRB	MTT	SRB
GLPS	375.38±4.14	357.22±3.05	168.54±1.88	175.32±2.49	176.47±2.49	184.13±2.45	121.65±2.90	134.56±1.21	195.24±3.13	200.48±2.15
GLTT	354.06±2.35	360.34±1.35	176.43±2.71	187.19±3.43	196.00±2.21	198.07±4.06	136.70±3.66	145.37±2.91	200.44±2.46	207.46±2.71

The cells were exposed to various concentrations of GLPS and GLTT. At the end of 72 h cellular viability was determined by using MTTand SRB. Number of independent experiments =3, 5 replicates, mean±SD

Crysal violet (1%) in absolute ethanol was used for staining the colonies[20].

Acridine orange staining

Different morphological changes occurred during apoptosis and visualized using acridine orange[21]. The cells were seeded at low density (5 x 10^4 HeLa cells/wells) on coverslip in 6 well microtitre plate (SPL Life sciences, Korea) and incubated for 24 h. Test compounds GLPS and GLTT were added to the cells. Control wells maintained cells without any treatment. After 24 h of incubation the microtitre plates were washed with phosphate buffered saline (PBS) twice and then fixed with ice cold methanol for 10 min followed by acetone for 10 sec. The cells were rinsed with PBS and stained with 0.1 % acridine orange in PBS for 20 min at room temperature. Lastly, the cells were washed with PBS thrice and analyzed under fluorescence microscope.

Microtubule dynamic study

Cells were seeded at low density (5 x 10^4 HeLa cells/wells) in 6 well microtitre plates with coverslip and incubated for 24 h. Test compounds GLPS and GLTT were added to the cells. Control wells maintained cells without any treatment. After 24 h, the microtitre plates were washed with phosphate buffered saline (PBS) twice followed by ice cold methanol treatment for 10 min and acetone for 10 S for fixing the cells. The cells were rehydrated with PBS containing triton X-100 (1%) at 37°C for 30 min, followed by treatment with 100 µl of FITC conjugated mouse monoclonal anti β-tubulin antibody (Sigma) for 45 min at 37°C. The cells were washed with PBS thrice and analyzed under fluorescence microscope[22].

Statistical analysis

All Statistics data are represented as mean±SEM of the mentioned number of testing. One way ANOVA (Graph Pad Prism 5.00, Instat Software, San Diego, CA, USA) followed by Tukey's post hoc test was used for the statistical analysis.

RESULTS

Anti-proliferative activity

Both extracts of G. *lucidum* GLPS and GLTT showed selective toxicity towards cancer cell cultures. Its CTC_{50} values are 375.38 µg/ml and 354.06 µg/ml for Vero normal cells as compared to 168.54 µg/ml, 176.47 µg/ml, 121.65 µg/ml and 195.24 µg/ml for cancer cell culture MCF7, MCF-7, HEp-2, HeLa and A-549 respectively for GLPS. The extract GLTT was also specific towards cancer cells with CTC_{50} values of 176.43 µg/ml towards MCF-7, 196 µg/ml against HEp-2, 136.70 µg/ml against HeLa and 200.44 against A-549. SRB assay results for GLPS and GLTT were also found to be similar as that of MTT assay (Table 1). Both GLPS and GLTT showed better results and specificity towards HeLa cells followed by MCF-7, HEp-2 and A-549 respectively. Thus, further mechanism based studies were performed only towards HeLa cells.

Long term survival studies

HeLa cells were used for long term survival study. The cells were able to retain their regenerative capacity at the lowest concentration selected (Table 2). Numbers of colonies greater than 32 cells were considered to have retained their regenerative capacity. Both GLPS and GLTT with lowest concentration of 20 µg/ml, exhibited more than 42 colonies. Concentration of GLPS and GLTT at 100 µg/ml (below the CTC_{50} value) was selected for nuclear staining and molecular dynamic studies.

Table 2: Long term survival studies of GLPS and GLTT using HeLa cell line

	Concentration in (µg/ml)	Number of Colonies
GLPS	100	3±1.04
	80	12 ±1.51
	60	21 ±1.63
	40	31±1.51
	20	42±1.50
	Control	47±1.09
GLTT	100	6±1.03
	80	14 ±1.47
	60	24 ±1.21
	40	35±2.09
	20	45±2.09
	Control	47±1.09

Cultures were exposed to different concentration of GLPS and GLTT, below CTC_{50} value and incubated at 37°C for a period of 72 h. Colonies containing more than 32 cells were considered to have retained their regenerative capacity. Number of independent experiments =6, mean±SD

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Acridine orange staining

Modifications of the nuclear structure is one of the important aspects to study the morphological changes involved in cell. Nuclear staining with acridine orange was performed to visualize the nuclear morphology. HeLa cells were used for this study. Drug and toxicant concentrations were used based on CTC_{50}. With respect to acridine orange staining, green colour represents viable cells, yellow and orange staining represent early and late apoptotic cells respectively (Figure 1). Control cells retained their proper shape and exhibited intact nucleus without any evidence of membrane blebbing and condensation. Further, cytoplasm of the control cells were intact and without any disintegration. The cells challenged with GLPS and GLTT showed extensive damage with characteristic apoptotic and morphological changes of nuclear and cytoplasmic membrane damage with condensation, cell volume reduction and also nuclear fragmentation. The results were similar to that of the standard drug paclitaxel used at 10 µg/ml.

Microtubule dynamic study

GLPS, GLTT tested for microtubule inhibition assay against HeLa cells specifically inhibited microtubule network. The microtubule disturbance caused by GLPS, GLTT in cancer cells was similar to that of the standard drug paclitaxel used at 10 µg/ml. The disturbance caused may be due to the damage caused to microtubule networking which ultimately damage cancer cells (Figure 2).

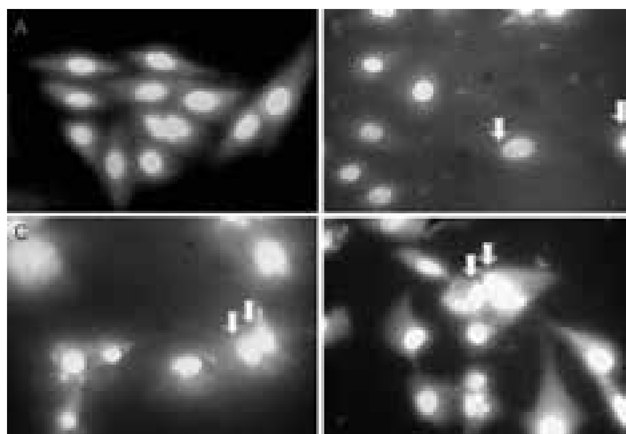

Figure 1: Acridine orange staining; A, HeLa cells untreated; B, HeLa cells Paclitaxel treated (10 µg/ml); C, HeLa cells GLPS treated (100 µg/ml); D, HeLa cells GLTT treated (100 µg/ml).

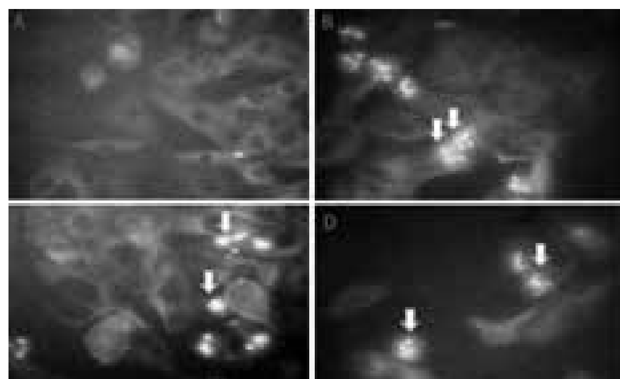

Figure 2: Microtubule inhibition studies; HeLa cells stained with fluoroscien isothiocyanate conjugated (FITC) monoclonal antibodies specific to β-tubulin. A, HeLa cells untreated; B, HeLa cells Paclitaxel treated (10 µg/ml); C, HeLa cells GLPS treated (100 µg/ml); D, HeLa cells GLTT treated (100 µg/ml).

DISCUSSION

Cultivation, collection and use of mushrooms such as *G. lucidum*, *A. auricular* and *L. edodes* for their medicinal properties has been a normal practice for hundreds of years in countries such as China, Korea and Japan[2]. Even though many mushrooms are considered to be safe, there are some reasons for the fear of mushroom poisoning caused because of the mistaken species detection or environmental and cultivation conditions of mushroom[16]. It is always necessary to evaluate the toxicity of the compounds before developing any pharmaceutical, nutraceutical and cosmeceutical product[1]. In our study, we paid more attention towards selection of Malaysian origin mushrooms based on environmental and cultivation conditions of mushrooms. It is evident from our study that *G. lucidum* total triterpenes and polysaccharides fractions were safe against normal cells without any detectable toxicity and also possessed excellent *in vitro* anticancer activity. Less toxic antioxidant compounds obtained from mushrooms can be safely and easily included in nutraceuticals and/or pharmaceuticals as therapeutic agents[6, 7]. The finding of the current studies confirms the safety and low toxicity of GLPS and GLTT towards normal cells. Further, both extracts showed specific toxicity towards cancer cells. Many natural compounds have reported apoptosis as the primary mechanism for their anticancer activity[15]. In our study, although both the fractions showed specific toxicity towards cancer cells, GLPS results were slightly better when compared with to GLTT and thus we infer GLPS to be more potent towards cancer cells.

Polysaccharides derived from mushrooms are one of the best known substances for immunomodulating and antitumor properties[17]. Various studies have confirmed that the immunomodulating and antitumor properties of polysaccharides fractions depend on factors such as

structure, site of activity, dose and mechanism[16]. Knowledge of the mechanism of action of polysaccharides involved in signaling pathway, cytokine networks and in the regulation of cancer are important to be considered. Cell growth is not only a reflection of the progression of the cell cycle but also aberrantly regulated in the majority of cancers[9]. Tumor regression potential of polysaccharides has been indicated through the cell cycle arrest and apoptosis mechanisms. There are several checkpoints involved in the regulation of cell cycle such as cdks, cdk inhibitiors and cyclins[23]. Data gained from the current study indicate that the tested polysaccharides have potent anti-proliferatory activity against four different cancer cell lines and also induced apoptosis, confirmed by nuclear staining experiments. In addition, our results indicate that the tested polysaccharides fraction has also damaged the microtubular network of the cancer cells and thus induced cell death.

The above results suggest that the tested polysaccharides have the potential to inhibit the survival of cancer cells and have promising anti-cancer activity. Anti-tumor activity of polysaccharides has direct relation with its configuration, chemical compositions and physical properties[7]. Other factors responsible for the anti-cancer activity of polysaccharides are linkage type of polysaccharide and the presence of b-glucan[15, 17].

Anti-tumor activity of b-D-glucan is well established and reported. Besides, a few researchers have reported the anti-tumor and immune stimulating properties of endo polysaccharides. In our study, we have confirmed the anti-proliferatory, nuclear staining and microtubule inhibition properties of polysaccharide fraction. Further studies such as the correlation between the anti-tumor potential and the polysaccharide structure need to be carried out.

SUMMARY AND CONCLUSION

The GLPS and GLTT extracts from *Ganoderma lucidum* demonstrate strong cytotoxic potential against all the cancerous cells. Clonogenic assay results also confirm the cytotoxic activity of the extracts. These extracts also damaged the microtubule network ultimately destructive towards the cancer cells. The present study demonstrates that GLPS and GLTT extracts have potential anti-cancer activity against cell lines at low concentrations. Overall, GLPS showed slightly better results compared to GLTT. Thus, it's worth to further investigate the mechanism of action of GLPS fraction for its anti-cancer activity.

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