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Phytochemical, Antioxidant, Anticancer and Anti-inflammatory Activities of Extracts of *Gyanoderma lucidum* from Hilly Regions of Nepal

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ABSTRACT

Gyanoderma lucidum is an oriental fungus commonly known as lingzhi or reishi mushroom in parts of China and Nepal. The fungi is well known for its numerous nutritional and ethnopharmacological properties. In the present study, the hexane, methanol and chloroform crude extracts of local Nepalese *G. lucidum* were evaluated for their phytochemical content and biological activities. The anti-oxidant activities of crude fungal extracts were studied using the standard DPPH radical scavenging assay. For anti-microbial tests, clinically important bacteria such as *Pesudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhimurium* were selected and anti-bacterial screening was conducted using the agar well diffusion technique. Anti-cancer effect of medicinal fungi was studied by evaluating its cytotoxic effects on HeLa cells. Murine (BALB/6) peritoneal macrophages were utilized as control cells. Anti-inflammatory activity was studied by evaluating the inhibitory effect of the fungal crude extracts on nitric oxide (NO) production by heat-killed *S. typhimurium* (HKST)-stimulated peritoneal macrophages. Phytochemical screening indicated that chloroform and methanol crude extracts were rich in active phytoconstituents, namely, flavonoids, glycosides and alkaloids. Anti-oxidant tests indicated that the chloroform and methanol extracts of *G. lucidum* exhibited significant ($P<0.05$) levels of anti-oxidant activity with the concentration bringing about 50% inhibition (IC_{50}) in the range of 1.62-2.21 mg/ml. Likewise, the anti-microbial assay showed that chloroform and methanol crude extracts of *G. lucidum* were most effective against *P. aeruginosa* and *S. aureus*. The methanol extract of *G. lucidum* exhibited the highest selective cytotoxicity ($P<0.05$) against HeLa cells with an IC_{50} value of 125 ± 7.27 µM. None of the extracts exhibited significant cytotoxic effect on the cell viability of peritoneal macrophages at given concentration (0-300 µg/ml). Pre-treatment of *G. lucidum* methanol extract caused significant ($P<0.05$) inhibition of NO production in HKST-stimulated peritoneal macrophages. Taken together, the results suggest that *G. lucidum* has a wide-spectrum therapeutic potential which requires further in-depth investigation.

Keywords: Cancer, Gyanoderma, Immunology, Inflammation

INTRODUCTION

Indigenous medicinal plants and fungi have been traditionally used for their anti-analgesic and anti-inflammatory properties to cure multiple diseases and disorders including fever, headache, edema, parasitic infestation, skin allergy and diarrhea [1,2]. *Gyanoderma lucidum* is the species name for edible mushroom called "Rato Chau" in Nepalese, "Lingzhi" in Chinese, "Reishi" in Japanese, "Yeongji" in Korean, "Glossy ganoderma" or "shiny polyporus" in English. The fungus has been reported to grow on logs of dead woods and tree stumps in shady places [3]. In Chinese folklore, the fruiting bodies of *G. lucidumare* perceived as a panacea for all types of illnesses including chronic hepatitis, arthritis, hypertension, hyperlipidemia, neoplasia, gastric ulcer, diabetes and atherosclerosis [4,5]. Due to the numerous medicinal properties attributed to *G. lucidum*, it is regarded as a 'mushroom of immortality' in China, Japan and Korea [4]. In these regions the fungus is cultivated and utilized as a useful source of feed supplement in animals and as food-

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in humans [6,7]. Recent studies have revealed increasing based medicine for promoting health and immune functions trend in fungus-derived drug development. Thus, *G. lucidum* being a highly significant mushroom with a rich history of ethno traditional uses makes it a luring target for researchers. Previous studies have indicated that *G. lucidum* has antioxidant properties attributed to its ability to inhibit H_2O_2-induced cell death [8]. Similarly, the anti-microbial properties of the fungal extract against pathogenic strains of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi* and *Pseudomonas aeruginosa* have been reported [9]. In addition, the fungus has also shown its promising therapeutic potentials as an effective anticancer and anti-inflammatory agent. Studies have shown that certain triterpenes from *G. lucidum* can induce apoptosis in human cancer cell lines via mitochondria-dependent pathway [5]. A Chinese herbal medicine preparation contains *G. lucidum* as the main ingredient has been reported to reduce the expression of interleukin (IL)-8, vascular endothelial growth factor and platelet-derived growth factor thus indicating potent anti-inflammatory and antitumor activities [10]. Nonetheless, the phytochemical constituents, climatic variations and genetic diversity within floral species can have substantial effect on the overall quality and efficiency of the fungal extract. The hilly regions of Nepal are known for their rich biodiversity and contain a large variety of Gyanoderma species [11]. Thus, in our present study, we investigated the local species of *G. lucidum* for its phytochemical constituents and biological activities with the main focus on antioxidant, antimicrobial, anticancer and anti-inflammation properties.

MATERIALS AND METHODS

Plant materials

The *G. lucidum* under investigation was identified and collected from the hilly region of Nepal with the help of a professional botanist. Fruiting body of the mushroom was selected for further experimentation. In brief, the fungal materials were air dried in the shade. The dried materials were crushed and ground into fine powder. The resulting powder was filtered through a sieve filter and subjected to extraction by three different solvents, namely, hexane, chloroform, and methanol using percolation with intermittent sonication. After extraction, the resulting filtrate containing phytochemicals were concentrated under reduced pressure using a rotatory vacuum evaporator. Finally, the condensed crude extract was transferred to clean, dry glass vials and allowed to dry at room temperature. The tentative yield percentage of extract fraction was calculated by dividing the weight of final extract fraction by the weight of starting fungal materials used.

Cancer cell line

The human cervical cancer cell line (HeLa ATCC CCL-2) was used for evaluation of anti-cancer effect of plant extracts. These cells were obtained from Everest Biotech™, Khumaltar, Lalitpur, Nepal. The cells were cultured and maintained using RPMI complete medium containing 90% RPMI, 10% heat-inactivated fetal bovine serum (FBS), 12.5 mM HEPES (pH 7.3) and 1x working concentration of Antibiotic-Antimycotic commercially supplied as 100x stock containing 10,000 U/ml of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of amphotericin B. All reagents used for cell culture were purchased from Gibco® (Thermofisher, USA). The cell culture condition was maintained at 37°C, 5% CO_2 using an animal cell incubator (Sheldon Labs™, USA).

Mice and generation of peritoneal macrophages

BALB/C mice were obtained from Natural Development Center, Thapathali, Kathmandu. Mice were maintained under specific pathogen-free conditions. All animal procedures were humanely performed according to the animal safety and ethics guidelines of the Institutional Animal Care and Use Committee. Peritoneal macrophages were obtained as previously described with minor modifications [12,13]. Specific-pathogen-free, typically 6 to 8 weeks old BALB/c mice were selected and injected intraperitoneally with 3 ml of 3% starch solution in PBS. On day 3, mice were euthanized using carbon dioxide. Resident peritoneal cells were obtained by washing the peritoneal cavity with sterile warm PBS (37°C). The peritoneal lavages were collected in a chilled sterile test tube since macrophages can adhere to the glass at room temperature. Macrophages were washed once with cold PBS and re-suspended in cold RPMI 1640. Cell viability was determined with the Trypan blue (Sigma-Aldrich, USA) exclusion method [14]. The number of viable macrophages in this suspension was determined using a hemocytometer chamber. The macrophages were adjusted to 4×10^5 cells/ml in complete RPMI medium containing 90% RPMI, 10% heat-inactivated fetal bovine serum (FBS) 12.5 mM HEPES (pH 7.3) and 1x working concentration of Gibco® Antibiotic-Antimycotic (Thermofisher, USA) commercially supplied as 100x stock containing 10,000 U/ml of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of amphotericin B.

Bacteria and generation of heat-killed *Salmonella typhimurium*

Standard strains of gram negative bacteria (*S. typhimurium* ATCC 14028 and *P. aeruginosa* ATCC 27853) and a single standard strain of gram positive bacterium (*Staphylococcus aureus* ATCC 25923) were used for anti-bacterial assay. These strains of bacteria were obtained from Institute of Medicine, Maharajgunj, Nepal and National Public Health Laboratory, Kathmandu, Nepal. Heat-killed *S. typhimurium* (HKST) were generated and used as previously described [15]. Briefly, *S. typhimurium* was cultured in Luria-Bertani (LB) broth and then heat-killed by incubating at 75°C for 30 min. The resulting heat-killed status of bacteria was confirmed by culturing the inoculum in LB agar. To

stimulate nitric oxide (NO) production by peritoneal macrophages the HKST with multiplicity of infection (MOI) 5 was used as an adequate source of lipopolysaccharide (LPS).

Phytochemical screening

The phytochemical screening was conducted as previously described methods with minor modifications [16-20]. The hexane, chloroform and methanol extracts were screened for the presence of alkaloids, carbohydrates, resins, phenols, flavinoids, proteins, lipids, glycosides, saponin, diterpenes, tannins and phytosterols.

Assay of inhibition of DPPH free radical scavenging activity

Antioxidant activities of the extracts were measured using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity as previously described with minor modifications [21]. The stable form of DPPH radical was utilized to evaluate the antioxidant potential of the *G. lucidum* crude hexane, chloroform, and methanol extracts. The samples were tested at various concentrations (0-10 mg/ml). In brief, 50 µl of the crude extract was added to 450 µl of Tris-HCl buffer (0.05 M, pH 7.4) and 1 ml of 0.1 mM DPPH was added to the resulting mixture. Solution in the test tubes were then shaken well and incubated in dark for an additional 30 min at the ambient temperature. Absorbance of the solutions in each tube was measured spectrophotometrically (Thermo Scientific, USA) at 517 nm. Gallic acid (Sigma-Aldrich, USA) was used as the positive control. Blank sample containing only buffer and DPPH was taken as the negative control for the experiment. The radical scavenging activity (RSA) was measured in terms of percentage for the difference in absorbance between the sample and the control divided by the absorbance of the control.

Screening for anti-bacterial activity

The antimicrobial activity of the crude extracts was studied by the agar well diffusion method with minor modifications [22]. This is the qualitative method for testing anti-bacterial efficacy. Briefly, bacteria were carpet cultured on the agar maintaining 0.5 McFarland turbidity standard. A fixed volume of extract solutions was placed in wells of equal size bored on the preset agar plates along with the positive and negative controls. Then the zone of inhibition was marked as a halo zone was measured. After 24 h incubation, the diameters of clear zones were measured to evaluate anti-bacterial efficacy of the extract being tested. Chloremphenicol (50 cg/disc) was used as a control antibiotic.

Cytotoxicity test for HeLa cells and peritoneal macrophages

The cytotoxicity test was conducted as previously described with minor modification [23]. Briefly, to determine cytotoxicity, HeLa cells or peritoneal macrophages were washed three times with PBS , re-suspended in complete RPMI medium, and then seeded on sterile 96-well tissue culture plate (0.7×10^4 cells/well) and allowed to incubate overnight. 5-flurouracil (5-FU, MW:130.07) was or *G. lucidum* extracts were dissolved in DMSO, added at a final concentration of 0-300 µg/ml and incubated for additional 48 h at 37°C, 5% CO_2. The final concentration of compound diluent (DMSO) was maintained below 0.1% in the culture medium. Cell viability was evaluated in each well by the addition of 50 µl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 2.5 mg/ml in PBS). After 4 h of incubation, the cell-free supernatants were removed and 100 µl of dimethyl sulfoxide (DMSO) was added. The optical density of formazan crystals formed was measured using a multiwall spectrophotometer (Thermo scientific, USA) reader at wavelength of 540 nm. For visual evidence of cytotoxicity, images of selected cultured cells were taken using an inverted microscope (Olympus, USA). The IC_{50} values for the growth inhibition were calculated using CalcuSyn v2.11 software (Biosoft, Cambridge, UK).

Measurement of nitric oxide

The measurement of NO was done using a previously described method with minor modification [24]. Peritoneal macrophages were seeded on 96 well plates (1.5×10^4 cells/well) and incubated for 2 h. Then the cells were pre-incubated for 1 h with various concentrations of extract and stimulated with HKST at multiplicity of infection (MOI) 5 for 24 h in complete RPMI medium. The culture supernatants were utilized for measurement of NO production. Fifty microliters of the culture supernatant were mixed with 50 µl of the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid) and the absorbance was measured at 570 nm. The NO concentrations in medium were determined by measuring the nitrite levels in the culture media supernatant. Nitrite levels in the samples were calculated from a standard curve with known concentrations of sodium nitrite.

STATISTICAL ANALYSIS

Statistical analyses were carried out using independent t-test or one way ANOVA whenever appropriate. Values were expressed as Mean ± standard error in mean (SEM). Data with $P<0.05$ were considered as statistically significant. Unless specified, all assays were performed in triplicate and all images are representative of three independent experiments.

RESULTS AND DISCUSSION

The secondary metabolites of several ethnopharmacologically important plants and fungi possess medicinal properties [6,25]. These secondary metabolites comprise a broad range of phytochemicals such as flavonoids, alkaloids, polysaccharides, saponins, glycosides, steroids, and tannins which have medically importance

bioactivities [26,27]. Such bioactive constituents can exert anti-microbial, anti-inflammatory or suppressive effects and can also stimulate the immune system to neutralize toxins or pathogens [28]. The solubility and bioactivity of different metabolites can differ depending on the nature of the solvent, thus, we investigated the crude extract of *G. lucidum* using a variety of organic solvents namely, hexane, chloroform and methanol. During solvent extraction, we observed a considerable yield from each of the solvents **(Table 1)**. Phytochemical screening indicated that proteins, carbohydrates and tannins were detected in all fractions of crude extract while fats and saponins were undetectable **(Table 2)**. The chloroform and methanol extracts of *G. lucidum* showed moderately positive results for the presence of resins. Remarkably, the methanol and chloroform extracts showed moderate to high levels of diterpenes, alkanoids, phenolic, flavonoid and glycosides in different fractions indicating the presence of these biologically active phytochemicals **(Table 2).**

Table 1. Percentage yield and physical characteristics of the *Gyanoderma lucidum* crude hexane, chloroform and methanol extracts.

Plant crude extract	Dry weight taken (g)	Weight of extract (mg)	Percentage yield (%)	Characteristics of extract	
				Color	Consistency
***G. lucidum* Hexane extract**	20	150.52 ± 6.32	7.52 ± 3.21	Yellow	Greasy (sticky)
***G. lucidum* Chloroform extract**	20	191.76 ± 5.31	9.58 ± 4.32	Dark yellow/brown	Greasy (sticky)
***G. lucidum* Methanol extract**	20	258.86 ± 8.21	17.94 ± 5.42	Light brown	Thick paste

Data are presented as Mean ± SD of results of three independent extractions

Table 2. Phytochemical screening of hexane, chloroform and methanol extract of *Gyanoderma lucidum*.

S. No.	Phytochemical Tests	Reagent Used	Sample	Intensity	Inference	Results
			GLU-HEX	+	Formation of yellow cream ppt.	Positive
		Mayer's Reagent	GLU-CHL	++	Formation of yellow cream ppt.	Positive
1	Alkaloids test		GLU-MET	+	Formation of yellow cream ppt.	Positive
			GLU-HEX	++	Formation of reddish brown ppt.	Positive
		Wagner's Reagent	GLU-CHL	+++	Formation of reddish brown ppt.	Positive
			GLU-MET	++	Formation of reddish brown ppt.	Positive
			GLU-HEX	+	Formation of violet ring	Positive
		Molish's Reagent	GLU-CHL	+	Formation of violet ring	Positive

			GLU-MET	+	Formation of violet ring	Positive
2	Carbohydrates test		GLU-HEX	+	Formation of orange red ppt.	Positive
		Benedict's Reagent	GLU-CHL	++	Formation of orange red ppt.	Positive
			GLU-MET	+	Formation of slight orange red ppt.	Positive
			GLU-HEX	-	No formation of oily stain	Negative
3	Fats and Fixed oil test	Filter Paper press Test	GLU-CHL	-	No formation of oily stain	Negative
			GLU-MET	-	No formation of oily stain	Negative
			GLU-HEX	+	Appearance of turbidity	Positive
4	Resins test	Acetone Water Test	GLU-CHL	+	Appearance of turbidity	Positive
			GLU-MET	++	Appearance of turbidity	Positive
			GLU-HEX	+	Formation of bluish black ppt.	Positive
5	Phenols test	Ferric Chloride Test	GLU-CHL	++	Formation of bluish black ppt.	Positive
			GLU-MET	++	Formation of bluish black ppt.	Positive
		Lead acetate Test	GLU-HEX	+	Formation of lite yellow colour	Positive
6	Flavonoids test		GLU-CHL	++	Formation of yellow colour	Positive
			GLU-MET	+++	Formation of intense yellow colour	Positive
			GLU-HEX	+	Formation of rose pink colour	Positive
7	Proteins and amino acids test	Xanthoproteic test	GLU-CHL	+	Formation of rose pink colour	Positive
			GLU-MET	+	Formation of rose pink colour	Positive
			GLU-HEX	+	Formation of rose pink colour	Positive
8	Glycosides test	Brontrager's test	GLU-CHL	++	Formation of rose pink colour	Positive
			GLU-MET	+++	Formation of dark rose pink colour	Positive

9			GLU-HEX	-	No Production of foam	Negative
	Saponin test	Foam Test	GLU-CHL	-	Produce foam that lasts for >10 min	Negative
			GLU-MET	-	No Production of foam	Negative
			GLU-HEX	+	Formation of bright green color	Negative
10	Diterpenes test	Copper Acetate Test	GLU-CHL	++	Formation of bright green color	Positive
			GLU-MET	+++	Formation of bright green color	Positive
			GLU-HEX	+	Formation of white ppt.	Positive
11	Tannins test	Gelatin Test	GLU-CHL	+	Formation of white ppt.	Positive
			GLU-MET	+	Formation of white ppt.	Positive
			GLU-HEX	+	Appearance of golden yellow color	Positive
12	Phytosterol test	Salkowski's Test	GLU-CHL	+	Appearance of golden yellow color	Positive
			GLU-MET	+	Appearance of golden yellow color	Positive

+++ Strongly positive:: ++: Moderately positive: + Considerably positive: : - Negative/ Not detectable

The antioxidant property enables neutralization of excess reactive free radicals and thus protects from cellular damage. This assists healthy physiology and leads to a delay of cellular senescence which is commonly termed as anti-ageing effect [29]. Similarly, free radicals can cause DNA damage and eventually lead to cancer. Many types of phenolics, diterpenes, phytosterols and flavonoids are known to possess free radical scavenging activity which makes them a potential target for the development of antioxidant and anticancer drugs [30,31]. DPPH assay is a well-established method for determining the antioxidant activity of phytochemical extracts. Thus, we utilized the percentage inhibition values for DPPH oxidation to calculate the antioxidant effect. The 50% inhibitory concentration (IC_{50}) value for the DPPH standard gallic acid was found to be 0.047 mg/ml. The chloroform extract of *G. lucidum* showed the highest anti-oxidant effects with the IC_{50} value of 1.62 ± 0.34 mg/ml **(Figure 1)**. The presence of multiple biologically active phytochemicals namely, alkanoids, phenolics, flavinoid and glycosides in the *G. lucidum* crude extract may have contributed to the observed antioxidant effect. Phytoconstituents and mainly the secondary metabolites such as flavonoids, alkaloids, tannins, polyphenols and terpenoids are known to be responsible for the antimicrobial effects of several medicinal flora [32,33]. Certain aromatic phytochemicals including quinines are known for their antimicrobial effect by rendering the substrate unusable for microbial consumption [34]. During our investigation the hexane, chloroform, and methanol extracts of *G. lucidum* exhibited significant inhibition against gram negative bacteria *S. typhimurium* **(Table 3)**. Hexane and chloroform extracts of *G. lucidum* were able to exhibit considerable inhibition toward the gram-positive bacteria *S. aureus*. Likely, the inhibitory effect of *G. lucidum* methanol extract on growth of *P. aeruginosa* was found to be comparable to that of the standard drug chloramphenicol (50 mcg), which indicates a therapeutic potential of the extract.

Screening for the cytotoxicity of the *G. lucidum* extract was conducted in the human cervical cancer cell line 'HeLa' cells using the standard MTT assay based on cell metabolic activity. These cancer cells provide a suitable *in vitro* model for preliminary screening of potential anticancer activities. The standard conventional drug for treatment of cervical cancer (5-FU) was used as the positive control [35]. Representative images from light microscopy provided visual evidence of the dose-dependent cytotoxic effect exerted by *G.*

lucidum methanol extract on HeLa cells (**Figure 2a)**. Similar dose-dependent cytotoxic effects were observed for other *G. lucidum* extracts with varying potencies of cell death induction (data not shown).

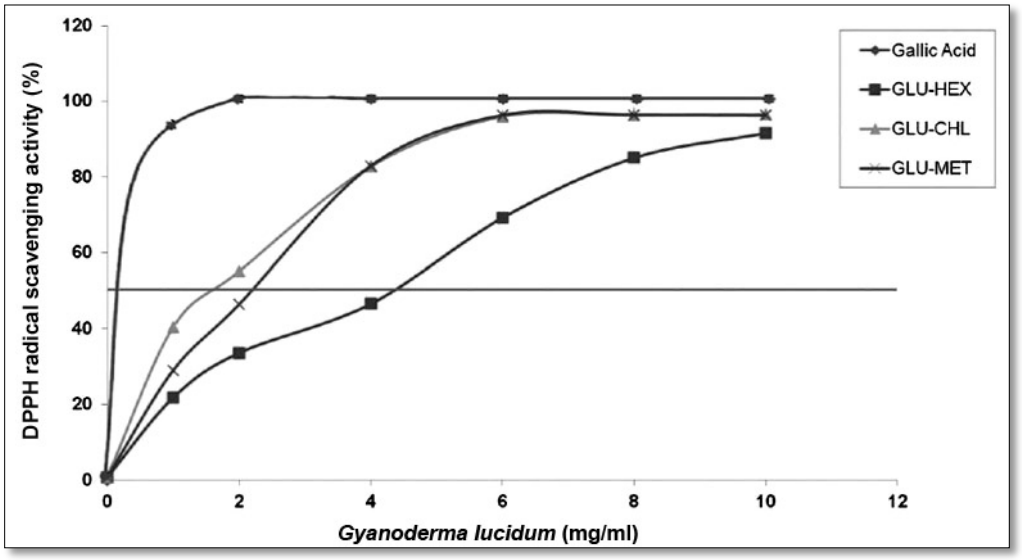

Figure 1. Antioxidant effect of *Gyanoderma lucidum* crude extract. DPPH free radical scavenging activity of *G. lucidum* crude hexane, chloroform and methanol extracts. Data are representative of three independent experiments.
GLU-HEX: G. lucidum Hexane Extract; GLU-CHL: G. lucidum Chloroform Extract; GLU-MET: G. lucidum Methanol Extract; DPPH: 2, 2-Diphenyl-1-Picrylhydrazyl

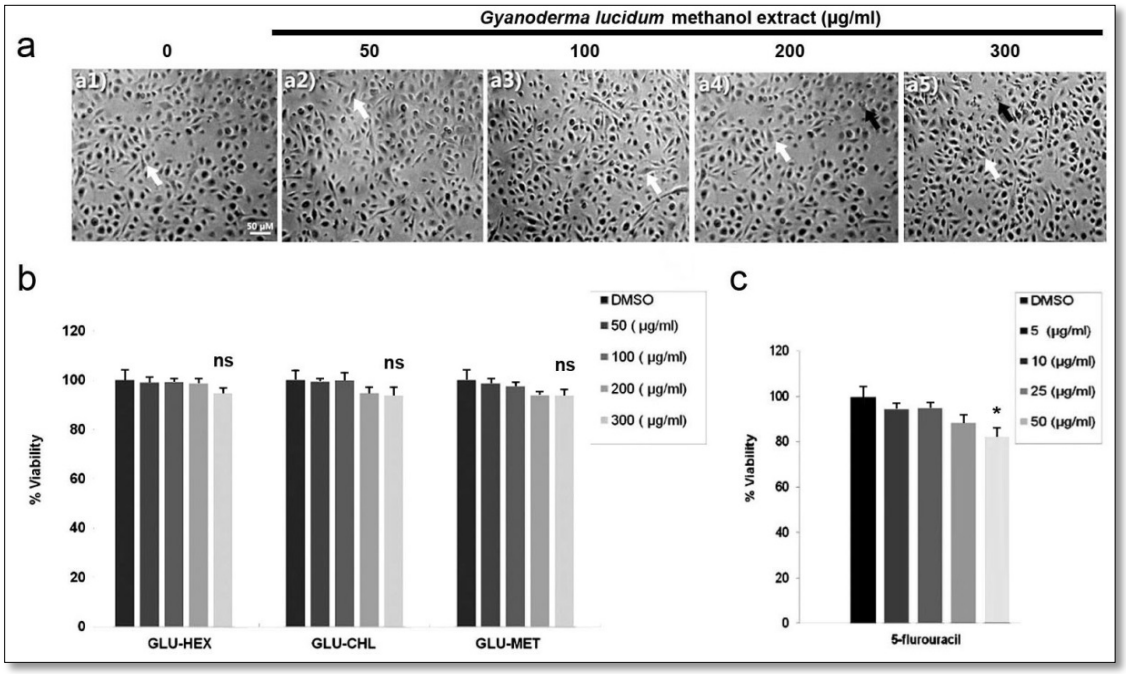

Figure 2. Cytotxic effect of *Gyanodermal lucidum* crude extract on HeLa cell culture morphology. (a) Cytotoxic effect of *G. lucidum* crude methanol extract on morphology of cultured HeLa cells after 48 h of treatment. Bar chart representing cytotoxicity of (b) *G. lucidum* crude hexane, chloroform, methanol extracts and (c) 5-flurouracil on HeLa cells after 48 h of treatment. Data are presented as Mean ± SEM of three independent experiments.
**P<0.05 v/s untreated control. White arrowhead represents live cell, black arrowhead represents dead cell*
GLU-HEX: G. lucidum Hexane Extract; GLU-CHL: G. lucidum Chloroform Extract; GLU-MET: G. lucidum Methanol Extract

Table 3. Anti-bacterial activity of crude hexane, chloroform and methanol fraction of plant extract.

Zone of inhibition (mm) with diameter of well=6 mm			
Bacterial strain	***P. aeruginosa***	***S. typhimurium***	***S. aureus***
ATCC no.	**27853**	**14028**	**25525**
G. lucidum			
GLU-HEX	12	12	14
GLU-CHL	14	14	18
GLU-MET	16	12	20
Standard			
Chloremphenicol (50 cg/disc)	18	26	28
DMSO	6	6	6

The cell viability assay indicated that treatment with DMSO alone had no significant effect on cell death **(Figure 2b)**. In contrast, 5-FU treatment exerted significant ($P<0.05$) dose-dependent cytotoxicity on HeLa cells with an IC_{50} of 12 ± 2.23 µM **(Figure 2c)**. The *G. lucidum* chloroform and hexane extracts induced moderate dose-dependent cell death of the cancer cells with the IC_{50} values 218 ± 7.45 and 190 ± 15.45 µg/ml, respectively **(Figure 2b)**. Among the crude extracts, the methanol fraction of *G. lucidum* exhibited highly significant ($P<0.05$) dose-dependent cytotoxic effect on HeLa cells with an IC_{50} value of 125 ± 7.27 µM µg/ml, which was considerable for a crude extract compared to treatment with the standard 5-FU drug **(Figures 2b and 2c)**. In contrast, the morphological evidence from light microscopy indicated little or no effect of *G. lucidum* methanol extract on the growth of peritoneal macrophages **(Figure 3a)**.

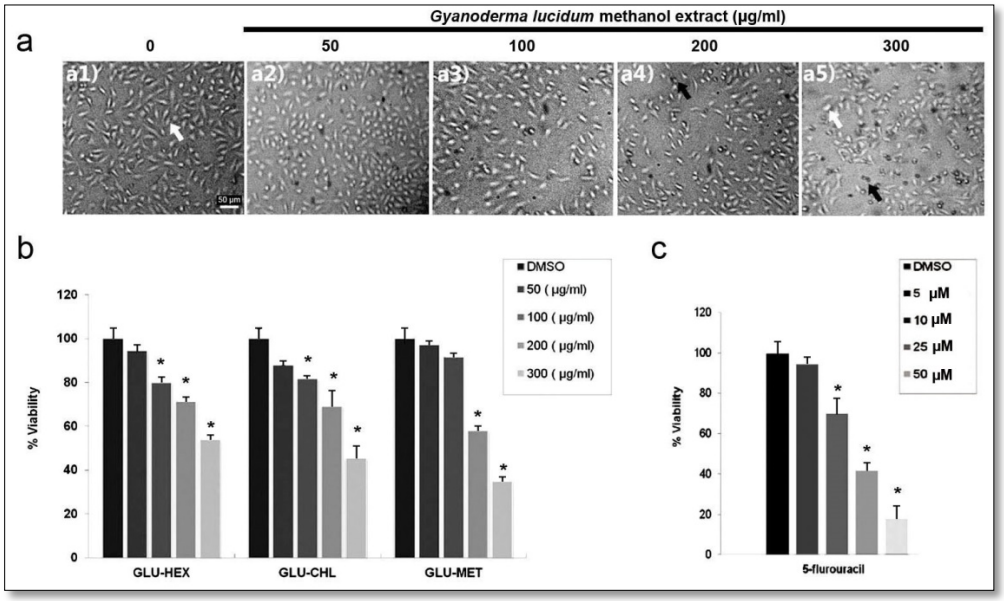

Figure 3. Cytotoxic effect of *Gyanodermal lucidum* crude extract on morphology of cultured peritoneal macrophages. (a) Cytotoxic effect of *G. lucidum* crude methanol extract on morphology of cultured peritoneal macrophages after 48 h of treatment. Bar chart representing cytotoxicity of (b) *G. lucidum* crude hexane, chloroform, methanol extracts and (c) 5-flurouracil on peritoneal macrophages cells after 48 h treatment.

Data are presented as Mean ± SEM of three independent experiments

**P<0.05 v/s untreated control, ns represents statistically non-significant. White arrowhead represents live cell, black arrowhead represents dead cell*

GLU-HEX: G. lucidum Hexane Extract; GLU-CHL: G. lucidum Chloroform Extract; GLU-MET: G. lucidum Methanol Extract

Similarly, results from the MTT assay also indicated that treatment with *G. lucidum* extract or 5-FU had little or no effect on the viability of peritoneal macrophages **(Figures 3b and 3c)**. Only the treatment with 5-FU above 50 µM exhibited considerable effect on the viability of peritoneal macrophages **(Figure 3c)**. Taken together, the results suggest that *G. lucidum* extract exerts selective cytotoxicity towards HeLa cells which is noteworthy for its therapeutic potential. Phytochemicals such as polysaccharide, triterpenoids, phenolic, flavonoids and other phytochemicals alone or in combination may be responsible for the observed effects [36,37]. Such bioactive chemicals are reported to possess anti-cancer and anti-angiogenesis properties indicating their possible contribution to the observed effects [38,39].

Macrophages are one of the principal immune cells bridging adaptive and innate immunity. Macrophages and dendritic cells stimulated with LPS can induce production of significant levels of NO *via* Toll-like receptors (TLR)-4 signaling pathways [40]. Under normal conditions, this leads to activation of immune response with secretion of pro-inflammatory cytokines and inflammatory mediators like NO to protect the host from infection or cell damage. However, excessive production of NO can be associated with tissue damage and organ dysfunction which include vasodilation, hypotension and septic shock [41,42]. In addition, over secretion of NO is known to be involved in several inflammation-associated diseases such as inflammatory bowel disease and Crohn's disease [43, 44]. Previous study has indicated that heat-killed *S. typhimurium* provides a suitable source of LPS for TLR-4-mediated immune response in macrophages and can be utilized to assess the anti-inflammatory effects of drugs [45]. In our present study, peritoneal macrophages were cultured and treated with HKST as a source of LPS to stimulate macrophages for NO production. The HKST served as a source of LPS and a ligand for TLR-4 activation and further minimized the complication of infections by live pathogens. The HKST was added at MOI 5 for maintaining the optimum amount of LPS to stimulate macrophages [15]. During our investigation, in the absence of HKST stimulation a very little amount of NO was produced by peritoneal macrophages. In contrast, HKST stimulation significantly ($P<0.05$) increased NO production by peritoneal macrophages **(Figure 4)**. Pretreatment with *G. lucidum* chloroform and methanol extracts caused significant ($P<0.05$) dose-dependent inhibition of HKST-induced NO production by peritoneal macrophages **(Figure 4)**. The results indicate that *G. lucidum* chloroform and methanol extracts have promising anti-inflammatory activity. The presence of flavonoids, phenolic acids and phenolic diterpenes may have collectively contributed to the observed anti-inflammatory effect.

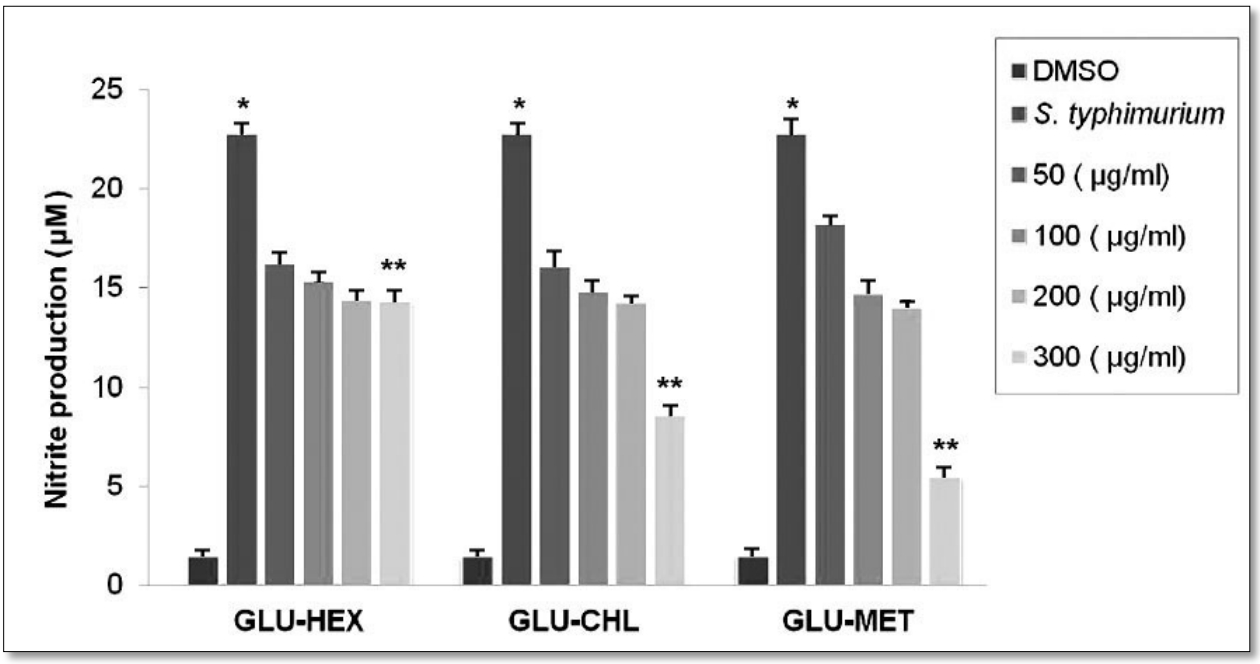

Figure 4. Inhibitory effect of *Gyanoderma lucidum* crude extract on nitric oxide production in peritoneal macrophages. Bar chart representing the effect of pretreatment with *G. lucidum* crude hexane, chloroform, and methanol extracts on nitric oxide production in HKST-stimulated murine peritoneal macrophages.
Data are presented as Mean ± SEM of three independent experiments
**P<0.05 v/s HKST-untreated control, **P<0.05 v/s HKST-stimulated control*
HKST: Heat-Killed Salmonella typhimurium; GLU-HEX: G. lucidum Hexane Extract; GLU-CHL: G. lucidum Chloroform Extract; GLU-MET: G. lucidum Methanol Extract

CONCLUSION

The results from our phytochemical screening and biological activity evaluation collectively indicate that G. lucidum extract has promising therapeutic potentials. Our study suggests that the local variety of G. lucidum can be a potent source for development of chemotherapeutic drugs and it calls for further in-depth investigation for its medicinal properties.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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