Optimization extraction of *Ganoderma lucidum* polysaccharides and its immunity and antioxidant activities

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ABSTRACT

Extraction of *Ganoderma lucidum* polysaccharides (GLP) was optimized by response surface method (RSM). By running the optimization program with design expert within the experimental range investigated, the following optimum values were obtained: extraction time 230 min; extraction temperature 95 °C, and extraction number 5. The predicted polysaccharides production was 1.45%. Results showed that GLP significantly reduced the levels of serum IL-6 and TNF-α levels and increased the levels of serum IL-2, IL-4 and IL-10 in GLP-treated rats compared to gastric cancer model rats. In addition, administration of *Ganoderma lucidum* polysaccharides to GLP-treated group of rats improved the levels of serum and gastric tissue SOD, CAT and GSH-Px toward the control values in a dose-dependent manner. These findings show that GLP can enhance immunity and antioxidant activities in gastric cancer rats.

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1. Introduction

Ganoderma lucidum is a medicinal mushroom that has been used as a Chinese traditional folk remedy for centuries. In the regions of China and other Asian countries, *G. lucidum* is used to treat many diseases, including hepatitis, hypertension, hypercholesterolemia and gastric cancer [1]. Its bioactive substances are mainly triterpenes and polysaccharides which have been isolated from its fruiting body, mycelia and spores [2–5]. Triterpenoids extracted or isolated from *G. lucidum* have been reported to be responsible for many of the pharmaceutical activities of *G. lucidum* [6].

Gastric cancer is the second commonest cause of death from malignant disease worldwide [7]. The relationship between gastric carcinoma with nutrition is one of the oldest known but perhaps one of the most poorly understood. Dietary factors seem to be involved in the development of gastric cancer, and it has been suggested by researchers that high salt concentration in the diet increases the risk of gastric cancer [8,9]. Sodium chloride has also been reported to enhance both the initiation and promotion of gastric carcinoma in experimental animals [10–12]. Antioxidant compounds, such as vitamin C and E, have a key role for prevention and termination of development of gastric cancer [13].

Experimental stomach cancers induced by the administration of N-methyl-N9-nitro-Nnitrosoguanidine (MNNG) in Wistar rats which shows similarities to human gastric tumors have been extensively used to test a wide variety of plant extracts and phytochemicals for chemopreventive potential [14]. The mechanism of action of MNNG is thought to be because of its decomposition to short-lived highly reactive electrophiles, of which the alkonium ion is probably the ultimate mutagen, and electrophilic attack on nucleophilic sites of DNA bases leads to altered bases [15].

To further explore the gastricoprotective action, we studied the effect of *G. lucidum* polysaccharides on gastric tissue damage in the MNNG-treated rats.

2. Material and method

2.1. Preparation of polysaccharides extract

The *G. lucidum*, which were purchased locally (Taizhou Herb-Market, Taizhou city, China), was identified by Professor F.L. Tian (College of Life Sciences and Biotechnology, Wenzhou Medical University). A voucher specimen (No. 20110913) is maintained in the institute. The dried *G. lucidum* were ground in a mortar, and then soaked in distilled water (300 g/5000 ml), followed by refluxing for 3 h and cooling. The undissolved materials were removed by filtration though a Whatman 41 filter paper (Clifton, NJ, USA) followed by a membrane filter with a 0.45 μm pore size (Millipore, Billerica, MA, USA). The filtrate was freeze-dried, yielding a final amount of *G. lucidum* polysaccharides of approximately 30 g.

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2.2. Optimization of extraction conditions

The present work involves optimization of different parameters governing the extraction process. The general practice of determining these optima is by varying one parameter while keeping the others at an unspecified constant level. The major disadvantage of this single variable optimization is that it does not take into consideration the interactive effects among the variables; thus it does not depict the net effects of various parameters on the reaction rate. In order to overcome this problem, optimization studies have been done using Response surface methodology (RSM). RSM is an effective statistical technique for optimizing a complex processes. RSM reduces the number of experimental trials required to evaluate multiple parameters and their interactions. It is less laborious and less time-consuming than other approaches. In this study, the optimization of polysaccharides extraction process was carried out by three chosen independent process variables including extraction time, temperature and extraction number used in the extraction. The ranges and levels of variables investigated in the research are given. The total amount of polysaccharides extracted was taken as response of the system. The response variable, R (extraction yield %) can be expressed as a function of the independent process variables according to the following response surface quadratic model:

$$R = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j + \varepsilon \qquad (1)$$

where, β_0 is the constant coefficient, β_i, β_{ii}, β_{ij} are the coefficients for the linear, quadratic and interaction effect, x_i and x_j are the independent variables and ε is the error.

The experimental data were analyzed by the software, Design Expert Version 6.0.6 (Stat-Ease, USA). The adequacy of the developed model and statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA). The interaction among the different independent variables and their corresponding effect on the response was studied by analyzing the response surface contour plots.

2.3. Animals and chemicals

Fifty 6-week-old male Wistar rats were supplied by the experiment animal Institute of Taizhou hospital. They were housed in polycarbonate cage with hard wood chips in an air-conditioned room ($23 \pm 2\,^\circ$C, $55 + 10\%$ R.H.) with a 12 h light/dark cycle. MNNG was obtained from Aldrich Chem Company, USA.

2.4. Experimental design

The rats received a basal diet (energy, 15.1591 kJ/kg; crude protein, 22.08%; crude fat, 4.10%; crude fiber, 3.15%; ash, 5.14%; sand silica, 1.13%) and were divided into 5 groups of 10 animals each. Group I served as the control and was given saline orally for 20 weeks. Group II were induced with MNNG 200 mg/kg body weight by oral gavage at days 0 and 14 and saturated NaCl (1 ml per rat) was given 3 days for 4 weeks and maintained till the end of the experimental period. Group III were induced with MNNG + NaCl (as in group II) and treated with *G. lucidum* polysaccharides (400 mg/kg body weight, dissolved in saline) simultaneously for 20 weeks from the first dose of MNNG + NaCl. Group IV was treated with *G. lucidum* polysaccharides (800 mg/kg body weight, dissolved in saline) from the sixth week of MNNG (as in group II) induction up to end of the experimental period. The experiment was terminated in the 21st week, and all rats were killed by cervical dislocation after an overnight fast. Blood was collected, and the plasma separated

was used for analysis. Stomaches were excised to prepare a 10% homogenate for biochemical measurements.

2.5. Biochemical analysis

Serum IL-2, IL-4, IL-10, IL-6 and TNF-α levels were measured with commercially available kits. All tests followed the manufacturer's instructions.

Malondialdehyde (MDA), a marker for lipid per-oxidation was estimated according to the method of Dousset et al. [16]. The reaction mixture in a total volume of 1.0 ml contained 0.25 ml of sample, 0.375 ml of working thiobarbituric acid (TBA) solution. This mixture was boiled for 15 min in water bath, cooled and 0.75 ml of n-butanol was added. The tubes were centrifuged at $2500 \times g$ for 15 min. The pink color extracted in the supernatant was read at 531 nm to measure the amount of MDA formed in each sample. A standard curve using 1,1,3,3-tetraethoxy propane was prepared and MDA concentrations in the experimental samples were extrapolated from the standard curve. The MDA results were expressed as μmol/mg or ml.

Reduced glutathione (GSH) was measured by the method of Beutler et al. [17]. Briefly, to 0.1 ml of sample was added 0.9 ml distilled water and 1.5 ml of precipitating reagent (3.34 g metaphosphoric acid, 0.4 g EDTA and 60.0 g sodium chloride). Tubes were shaken and allowed to stand for 5 min at room temperature ($25 \pm 1\,^\circ$C). The mixture was centrifuged for 15 min at 4000 rpm at $4\,^\circ$C. In 1.0 ml supernatant, 4.0 ml of phosphate solution (0.3 M disodium hydrogen phosphate) and 0.5 ml 5–50-dithiobis-(2-nitrobenzoic acid) (DTNB) (80 mg in 1% sodium citrate) were added. The development of yellow color complex was read immediately at 412 nm on a spectrophotometer (Bio-Rad spectrophotometer, smart spec-3000). A standard curve using GSH was prepared and GSH concentrations in the experimental samples were extrapolated from the standard curve. GSH concentrations were calculated and expressed as l mol of GSH/mg or ml.

The activity of superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 ml reaction mixture contained 100 mM Tris/HCl (pH 7.8), 75 mM NBT, 2 μM riboflavin, 6 mM EDTA, and 200 μl of supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% as described by Winterbourn et al. [18].

The catalase (CAT) activity was determined according to the Aebi method [19]. The rate of H_2O_2 decomposition was followed by monitoring absorption at 240 nm. One unit of CAT activity is defined as the amount of enzymes required to decompose 1 μmol of hydrogen peroxide in 1 min.

Glutathione peroxidase (GSH-Px) activity was analyzed by a spectrophotometric assay. A reaction mixture consisting of 1 ml of 0.4 M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 1 ml of 5 mM NaN$_3$, 1 ml of 4 mM GSH, and 0.2 ml of supernatant was preincubated at $37\,^\circ$C for 5 min. Then 1 ml of 4 mM H_2O_2 was added and incubated at $37\,^\circ$C for further 5 min. The excess amount of GSH was quantified by the DTNB method as described by Sharma and Gupta [20]. One unit of GSH-Px is defined as the amount of enzyme required to oxidize 1 nmol GSH/min.

2.6. Statistical analysis

All values are expressed as means $\pm$ standard deviation (SD) of ten samples. ANOVA followed by student Newmans–Keuls test was used to evaluate the significance differences of the results obtained. All computations were performed using SPSS software.

Table 1
Box-Behnkex design of *Ganoderma lucidum* polysaccharides.

No.	A (extraction time, min)	B (extraction temperature, °C)	C (extraction number)	R (extraction yield (%))
1	−1.00 (190 min)	−1.00 (90 °C)	0.00 (5)	0.96
2	1.00 (230 min)	−1.00	0.00	1.15
3	−1.00	1.00 (100 °C)	0.00	0.96
4	1.00	1.00	0.00	1.16
5	−1.00	0.00 (95 °C)	−1.00 (4)	0.87
6	1.00	0.00	−1.00	1.07
7	−1.00	0.00	1.00 (6)	1.05
8	1.00	0.00	1.00	1.47
9	0.00 (210 min)	−1.00	−1.00	0.99
10	0.00	1.00	−1.00	0.99
11	0.00	−1.00	1.00	1.12
12	0.00	1.00	1.00	1.18
13	0.00	0.00	0.00	1.24
14	0.00	0.00	0.00	1.19
15	0.00	0.00	0.00	1.2
16	0.00	0.00	0.00	1.21
17	0.00	0.00	0.00	1.21

3. Results and discussion

Experimental data for extraction of *G. lucidum* polysaccharides are given in Table 1. The predicted values were obtained from model fitting technique using the software design expert version 6.06 and were seen to be sufficiently correlated to the observed values.

The computed model *F*-value of 14.31 was higher then tabular value of $F_{0.01(14,15)} = 3.56$, implying the model are significant at 1% confidence level. The model also showed statistically insignificant lack of fit, as is evident from the lower computed *F* value (*14.64*) than the tabular $F_{0.05(10,5)}$ value (10.1) at 1% level. And the pure error was very low, indicating good reproducibility of the data obtained. With very small *p*-value (0.0001) from the analysis of ANOVA and a suitable coefficient of determination ($R^2 = 0.9484$), the quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response (% yield) and the significant variables (Table 2).

The overlaid contour plot, response surface plot of yield for certain pairs of variables was shown at Fig. 1(a), (b) and (c). By running the optimization program with design expert within the experimental range investigated, the following optimum values were obtained: extraction time, 230 min; extraction temperature 95 °C, and extraction number 5. The predicted polysaccharides production was 1.45% (Fig. 1).

IL-2 is produced by the activated T cells and has a powerful immunoregulatory effect on a variety of immune cells [21]. IL-4 plays an important role in IgE synthesis by activating the pre-T helper cells to the Th2 cells that trigger the isotype switching form, IgM/IgG to IgE, in B cells [22]. IL-4 has also been demonstrated to inhibit the production of interferon-γ (IFN-γ) and downregulates the differentiation of Th1 cells [23]. Interleukin-10 (IL-10) is an important immunoregulatory cytokine. It is involved in the regulation of inflammatory responses through direct influence over tumor necrosis factor (TNF)-α production. Also, IL-10 plays an important role in the course of infectious diseases, for instance, the severity to which meningococcal meningitis progresses is associated with serum IL-10 concentrations, such that a high serum IL-10 level was observed in patients with a poor or fatal outcome, whereas patients with mild disease and a good prognosis had lower serum IL-10 levels [24–26].

In this study, when compared with the normal control rats, serum IL-2, IL-4 and IL-10 levels were significantly decreased in the model control groups. In GLP-treated groups, the concentrations in serum IL-2, IL-4 and IL-10 were significantly higher than those of the model control group (Table 3). Moreover, the effect was dose-dependent.

The inflammation is caused by the overproduction of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-6. These cytokines mediate the activation of inflammatory
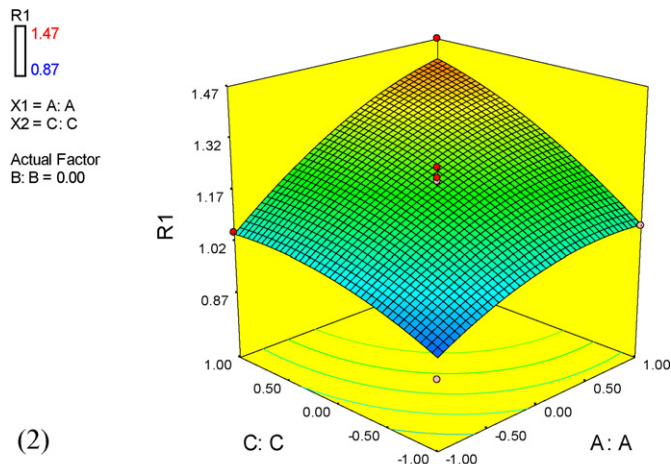
Table 2
ANOVA for response surface quadratic model analysis of variance.

| Source	Sum of squares	df	Mean square	F value	p value Prob. > F	
Source	0.31	9	0.034	14.31	0.0010	Significant
A–A	0.13	1	0.13	53.21	0.0002	
B–B	6.125E-004	1	6.125E-004	0.26	0.6287	
C–C	0.10	1	0.10	42.25	0.0003	
AB	2.500E-005	1	2.500E-005	0.010	0.9215	
AC	0.012	1	0.012	5.05	0.0595	
BC	9.000E-004	1	9.000E-004	0.38	0.5594	
A2	0.012	1	0.012	5.08	0.0589	
B2	0.041	1	0.041	17.13	0.0044	
C2	7.164E-003	1	7.164E-003	2.99	0.1274	
Residual	0.017	7	2.396E-00			
Lack of fit	0.015	3	5.125E-003	14.64	0.0127	Significant
Pure error	1.400E-003	4	3.500E-004			
Cor. total	0.33	16				
SD	0.049		R^2	0.9484		
Mean	1.12		Adj. R^2	0.8822		
C.V. %	4.38		Pred. R^2	0.2372		
Press	0.25		Adeq. precision	12.884		

(1)

(2)

(3)
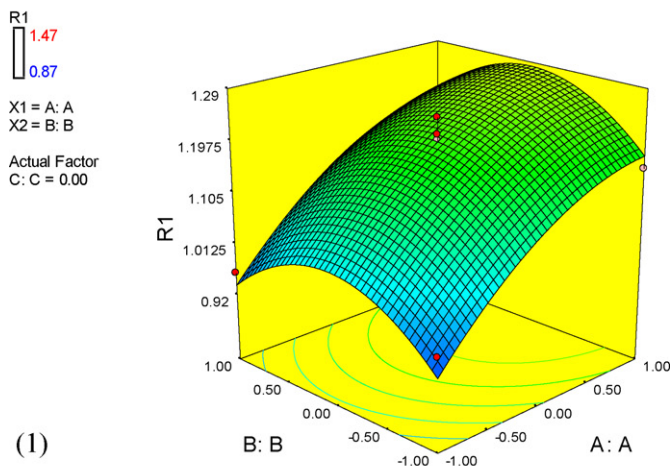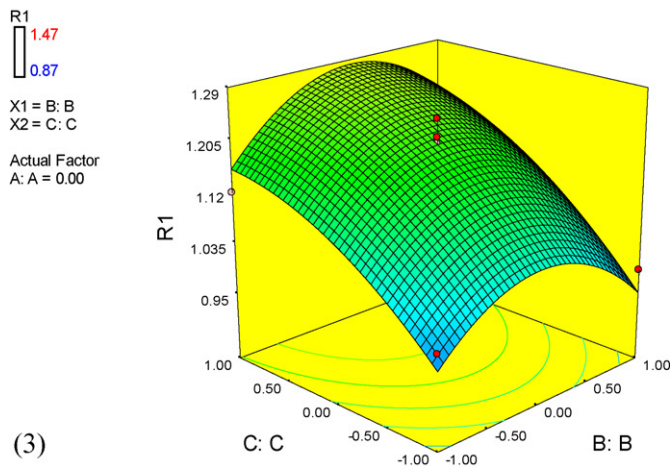
Fig. 1. Response surface plots of extraction parameters.

Table 3
Effect of GLP treatment on serum IL-2, IL-4 and IL-10 levels.

Group	IL-2 (ng/mL)	IL-4 (ng/mL)	IL-10 (ng/mL)
NC	7.391 ± 0.528	24.91 ± 1.91	106.62 ± 8.42
MC	3.273 ± 0.254[a]	15.99 ± 1.45[a]	70.82 ± 4.82[a]
GLP (400 ml/kg b.w.)	4.705 ± 0.343	18.05 ± 1.52[c]	80.54 ± 7.58[b]
GLP (800 ml/kg b.w.)	7.053 ± 0.582[c]	22.08 ± 2.31[c]	97.41 ± 6.63[c]

[a] $p < 0.01$, compared with group NC.
[b] $p < 0.05$.
[c] $p < 0.01$, compared with group MC.

cells and synovial proliferation, finally leading to joint destruction. The cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which are mainly produced by activated monocytes or macrophages, stimulate bone resorption and also enhance the production of PGE2 in several type of cells including calvarial osteoblasts [27]. IL-6, a potent mitogenic polypeptide, stimulates cell proliferation in a variety of cell types [28]. It has also been reported that TNF-α cooperatively potentiates IL-1β-stimulated PGE2 production [29]. The mechanism by which cytokines, such as TNF-α, and IL-6 act in concert to stimulate prostaglandin production is, however, unclear.

Our work showed that when compared with the normal control rats, serum IL-6 and TNF-α levels were significantly higher in the model control groups. In GLP-treated groups, the concentrations in serum IL-6 and TNF-α were significantly lower than those of the model control group (Table 4). Moreover, the effect was dose-dependent.

When oxidative stress causes damage in organs, a variety of cytokines and enzymes located in the cytosol are released into the blood, thereby causing changes in cytokine and enzyme levels in the plasma. The assays of cytokines and enzymes in the plasma are useful quantitative indicators for the extent and types of organ damage. In the present study, MNNG activated inflammatory cells, leading to the synthesis and release of certain pro-inflammatory cytokines, such as TNF-α and IL-6. By analyzing the level of those inflammatory mediators in plasma, a significant increase was found which verified that MNNG toxicity was closely related with inflammatory mechanisms when oxidative damage occurred. However, when animals were treated with *G. lucidum* polysaccharides, these cytokine levels were comparatively lower than the model control group in a dose-dependent manner, which is likely to relate to the anti-inflammatory activity of *G. lucidum* polysaccharides previously reported [30]. *G. lucidum* polysaccharides suppressed the production of TNF-α and IL-6 inflammatory cytokines and stimulated the production of IL-2, IL-4 and IL-10, which suggested *G. lucidum* polysaccharides had protective capabilities due to its anti-inflammatory activity.

Gastric cell and tissue injury associated with acute and chronic inflammation is due to the toxicity of ROS generated in stomach [31]. It has been widely accepted that a large number of free radicals is generated in the peptic ulcer and gastritis, but its mechanism is unclear. Oxygen derived free radicals play an important role in the pathogenesis of the injury of the digestive system [32]. In addition, the involvement of oxygen derived free radicals are well

Table 4
Effect of GLP treatment on serum IL-6 and TNF-α levels.

Group	IL-6 (ng/mL)	TNF-α (ng/mL)
NC	83.62 ± 5.11	2.03 ± 0.14
MC	97.04 ± 5.94[a]	4.51 ± 0.32[a]
GLP (400 ml/kg b.w.)	94.42 ± 5.21[b]	3.98 ± 0.26
GLP (800 ml/kg b.w.)	86.28 ± 4.03[c]	2.69 ± 0.16[c]

[a] $p < 0.01$, compared with group NC.
[b] $p < 0.05$.
[c] $p < 0.01$, compared with group MC.

Table 5
Effect of GLP on serum, gastric MDA and GSH level.

Group	MDA		GSH	
	Serum	Gastric tissue	Serum	Gastric tissue
NC	3.82 ± 0.22	4.61 ± 0.27	270.52 ± 17.83	292.15 ± 22.18
MC	7.31 ± 0.41^a	7.75 ± 0.41^a	175.9 ± 12.05^a	180.63 ± 10.73^a
GLP (400 ml/kg b.w.)	6.03 ± 0.29^b	6.62 ± 0.28^c	224.15 ± 20.14^c	243.18 ± 18.39^c
GLP (800 ml/kg b.w.)	4.92 ± 0.21^c	5.49 ± 0.18^c	190.48 ± 15.29^c	197.06 ± 15.02^c

[a] $p < 0.01$, compared with group NC.
[b] $p < 0.05$.
[c] $p < 0.01$, compared with group MC.

Table 6
Effect of GLP on serum SOD, CAT and GSH-Px activities.

Group	SOD	CAT	GSH-Px
NC	205.71 ± 18.94	39.75 ± 2.17	28.75 ± 1.83
MC	137.89 ± 11.27^a	61.52 ± 4.31^a	58.05 ± 4.01^a
GLP (400 ml/kg b.w.)	189.33 ± 16.08^b	53.29 ± 2.95^b	49.05 ± 3.28^b
GLP (800 ml/kg b.w.)	216.48 ± 18.49^b	45.06 ± 2.22^b	32.17 ± 2.17^b

[a] $p < 0.01$, compared with group NC.
[b] $p < 0.01$, compared with group MC.

established in the pathogenesis of ischemic injury of gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol, and *Helicobacter pylori* [33]. The benefits of reducing power play an important role for the redox system in stomach with gastritis and gastric cancer. Carcinogenic chemicals including MNNG are known to cause oxidative stress by inducing the formation of toxic OFR such as the hydroxyl radical (OH.). Of particular concern is the ability of OH$^\bullet$ radicals to traverse cell membranes causing deleterious effects at sites far from the target tissue [34]. Oxidative stress associated with increased levels of LPO and other thiobarbituric acid reactive intermediates are linked to cancer progression [35,36].

Our present results clearly indicated that MNNG resulted in a significant decrease in GSH level in serum and gastric tissue as compared to normal control animals. This indicated that oxidative injury had happened in gastric cancer animals. *G. lucidum* polysaccharides dose-dependently significantly decreased the MDA and increased GSH levels as compared to model control animals (Table 5).

Present results also revealed that MNNG produced a statistically significant decrease ($p < 0.01$) in serum and gastric tissue SOD, CAT and GSH-Px activities in model control rats compared to normal control animals (Tables 6 and 7). Superoxide dismutase is believed to protect cells, notably erythrocytes against superoxide- and hydrogen peroxide-mediated damage [37]. Catalase is widely distributed in all tissues and catalyzes the breakdown of H_2O_2. The source of H_2O_2 is mainly SOD-mediated dismutation of SOD radicals generated by enzymatic and nonenzymatic systems. Several reports have cited decreased activities of SOD and catalase in various carcinogenic conditions [38,39]. The observed decline in SOD and catalase activities in our study might be because of the increase in circulating lipid peroxides, which reportedly results in the accumulation of superoxide anions that are capable of

traversing membranes causing deleterious effects at sites beyond the tumor [40]. Glutathione peroxidase is reported to react with H_2O_2 to prevent the intracellular damage caused. In the present study, a decrease (group II) in the activity of SOD, CAT and GSH-Px could be because of free radical-mediated alterations in the antioxidant defense system [41–43]. Administration of *G. lucidum* polysaccharides to GLP-treated group of rats improved the levels of serum and gastric tissue SOD, CAT and GSH-Px toward the control values in a dose-dependent manner. This suggested that *G. lucidum* polysaccharides could reduce free radical formation and oxidative damage in gastric carcinogenesis.

4. Conclusion

In conclusion, the present study demonstrated that GLP treatment decreased serum and stomach lipid peroxidation level, enhance immunity and antioxidant activities in MNNG-induced gastric cancer rats. These results suggest that GLP treatment may be beneficial for therapy of gastric cancer diseases.

Table 7
Effect of GLP on gastric SOD, CAT and GSH-Px activities.

Group	SOD	CAT	GSH-Px
NC	298.37 ± 24.09	50.27 ± 4.16	49.03 ± 3.68
MC	138.15 ± 11.52^a	24.18 ± 1.92^a	21.59 ± 1.54^a
GLP (400 ml/kg b.w.)	174.29 ± 14.08^b	38.02 ± 3.05^b	34.72 ± 2.63^b
GLP (800 ml/kg b.w.)	254.01 ± 23.16^b	45.18 ± 3.28^b	45.18 ± 3.11^b

[a] $p < 0.01$, compared with group NC.
[b] $p < 0.01$, compared with group MC.

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