Isolation of the intracellular and extracellular polysaccharides of *Ganoderma neojaponicum* (Imazeki) and characterization of their immunomodulatory properties

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ABSTRACT

Background: The role of polysaccharides isolated from the *Ganoderma* species of fungi in innate immunity has recently become a topic of research. Although some work has been conducted concerning *Ganoderma lucidum*, the characteristics of polysaccharides isolated from *Ganoderma neojaponicum* (Imazeki) as immunomodulatory agents are largely unknown. The aims for this study were to isolate and characterize the intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPSs) of *G. neojaponicum* from STR reactor.
Results: The production of EPS and IPS was optimized on day 4 of the cultivation time in 2 L STR reactor based on the amount of biomass yield, total carbohydrate, β-glucan and α-glucan content. Further analysis, both the EPSs and IPSs showed the enhancement on proliferation and increment of phagocytosis activities of macrophage (RAW264.7) cell lines. Using an oral toxicity test, we also observed that 2000 mg/kg body weight/day dosage of dried *G. neojaponicum* mycelium does not cause any significant toxic effects on Sprague–Dawley rats in 14 d of administration.
Conclusion: The findings of this study indicate that the IPSs and EPSs of *G. neojaponicum* have the potential to be used as immunomodulating agents to stimulate the innate immune system for fighting infectious diseases. The polysaccharides from *G. neojaponicum* have to be further commercially explored as an alternative for medicinal Ganoderma variety of *G. lucidum* production.

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1. Introduction

Ganoderma is a genus of basidiomycetes fungi belonging to the order Polyporales and family Ganodermataceae. One member of this family, *Ganoderma neojaponicum* (Imazeki), has also been reported to contain β-1,3-linked and β-1-6-linked D-glucose polysaccharides that have been identified as potential anti-tumor agents and may function to enhance the immune system [1]. Further, *G. neojaponicum* has been used for medicinal purposes in China and as a traditional food ingredient in Taiwan [2,3]. In Malaysia, *G. neojaponicum* is used by the indigenous population for the external treatment of stomach pains and as a strong herbal treatment in natural healing and alternative medicine practices under the name 'cendawan senduk'. To date, the extraction and functional characterization of polysaccharides isolated from *G. neojaponicum* (Imazeki) are far from complete.

Crude polysaccharides existing in both the dried mycelium (DM) which contains intracellular polysaccharides (IPSs) and the cultivation medium (DB) which contains secreted extracellular polysaccharides (EPSs) can be extracted using a hot water process. This hot water extraction method involves a boiling procedure used previously to concentrate bioactive compounds to the desired amount or to levels suitable for therapeutic use [4]. Following extraction, the partial purification of the β-glucans can be achieved using ethanol precipitation.

Phagocytosis plays a critical role in triggering the adaptive immune response and functions to defend the host against harmful pathogens. According to Laskin [5], macrophages serve as antigen-presenting cells and act as the primary control switch during the immune response. Although the polysaccharide fraction of *Ganoderma lucidum* has been widely discussed for its immunomodulatory, anti-inflammatory, and anti-cancer properties, as far as we know, there are limited reports on the immunomodulatory properties of the IPSs and EPSs of *G. neojaponicum* (Imazeki).

2. Materials and methods

2.1. Inoculum

G. neojaponicum (KLUM61076) was collected from Bahau Reserve Forest, Negeri Sembilan, Malaysia and deposited at the

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University of Malaya Culture Collection, Mushroom Research Centre (Kuala Lumpur, Malaysia). The stock culture was maintained on malt extract agar (MEA, Oxoid). Cultures were inoculated in petri dishes and incubated at $26 \pm 2°C$ for 7 d. After this initial period, the cultures were used for seed culture inoculation.

2.2. Cultivation medium and chemicals used

Potassium dihydrogen phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4), magnesium sulfate heptahydrate ($MgSO_4 \times 7H_2O$) and Tween 80 were purchased from Sigma Chemical Co., USA. Malt extract agar (MEA) was obtained from Oxoid, UK. Spent yeast, a by-product of the brewery industry was obtained from Carlsberg Brewery (Malaysia) Berhad, Petaling Jaya, Selangor, Malaysia, while the brown sugar, a product of the cane industry was purchased from Malayan Sugar Manufacturing Co. (Berhad), Malaysia. The Mushroom and Yeast β-glucan kit (K-YBGL) was obtained from, Megazyme Int, Ireland.

2.3. Submerged cultivation

Culture fermentations were performed in a 2-L STR reactor (Biostat®A plus, B. Braun International, Germany), whereby 100 mL of seed culture was inoculated to a 1-L working volume in a stirred tank reactor (STR). The cultivation medium was composed of 5.74% (w/v) brown sugar, 0.06% (w/v) spent yeast, 0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4, and 0.05% (w/v) $MgSO_4 \times 7H_2O$ with addition of 0.46% (v/v) Tween 80. The cultivation medium was added to the STR and allowed to run at 27°C, with aeration of 1.3 vvm for 4 d prior to the seed culture inoculation. The reactor was then set at a constant agitation speed of 160 rpm and pH 6.

2.4. Extraction of EPSs and IPSs

EPSs and IPSs from *G. neojaponicum* were extracted using cultivation broth (B) and the mycelium (M), respectively. This procedure was carried out as previously described by Hsieh et al. [6]. The mycelium was separated from the broth by centrifugation (Beckman Coulter, Germany) at 10,000 rpm for 10 min. The dried broth (DB) was prepared by drying the broth in a freeze dryer for 2 d. The EPSs were extracted by adding the broth to double volumes of 95% (v/v) ethanol and incubating overnight at 4°C. Subsequently, the EPSs were dried to remove the residual ethanol with a freeze dryer for an additional 2 d. The DM was prepared by washing the filtered mycelium with the same volume of distilled water, followed by freeze drying. The IPSs were prepared by extracting the DM with distilled water at 1:10 w/v and then sterilizing the mixture at 121°C for 30 min. Double volumes of 95% (v/v) ethanol were then added to aliquots of the DM extract solution and incubated overnight at 4°C in order to precipitate the IPSs. Four *G. neojaponicum* extracts (EPSs, IPSs, DM and DB) were evaluated for their immunomodulatory properties.

2.5. Analysis of β-glucan and total polysaccharides assay

The Mushroom and Yeast β-glucan kit (K-YBGL) was used in determining the β-glucan content. The β-glucan content was calculated by subtraction of total glucan and α-glucan. For total glucan, 0.1 mL of prepared extract was mixed with 0.1 mL of a mixture of exo-1,3,β-glucanase (20 U/mL) and β-glucosidase (4 U/mL) for 60 min in 40°C water bath. Then, 3 mL of glucose oxidase was added to each tube and incubated for another 20 min. The aliquots were measured at an absorbance of 510 nm. For α-glucan, 0.1 mL of prepared aliquots was added to 0.1 mL sodium acetate buffer followed by the addition of 3 mL of glucose oxidase to each tube and incubation at 40°C for another 20 min. The absorbance of the α-glucan solution was read at 510 nm against a reagent blank. The content of total polysaccharides was determined by phenol-sulphuric acid assay according to Dubois et al. [7]. 0.1 g of DM, DB, IPSs and EPSs was diluted into 1 mL distilled water in test tube. Then, 1 mL of 5% (v/v) phenol solution and 5 mL of concentrated sulphuric acid were added to each tube. The mixtures were allowed to stand at room temperature for 10 min. The prepared aliquots were read using spectrophotometer (Cary WinUV, Agilent, USA) at 490 nm absorbance.

2.6. Cell culture

The macrophage cells (RAW264.7) were cultured in order to assess the immunomodulatory capabilities of the polysaccharides in this study. The RAW264.7 cells were obtained from the ATCC (USA) and were maintained in DMEM medium (Invitrogen, Carlsbad, USA) containing 100 U/mL penicillin (PAA, Austria), 100 U/mL streptomycin (PAA, Austria), and 10% v/v (PAA, Austria) fetal bovine serum. The cells were grown in a 37°C incubator under a humidified atmosphere containing 5% CO_2 (Shel Lab, USA). In the present study, the DM, DB, IPSs, and EPSs of the *G. neojaponicum* extracts were tested further for their in vitro immune response activity.

2.7. Proliferation effect

The changes in proliferation activity of the RAW264.7 macrophages were monitored after the addition of *G. neojaponicum* extract. Cells were seeded into 96-well microplates at a concentration of 1×10^6 cells/well for 24 h at 37°C in CO_2 incubator. The cells were treated for 24 h with 100–1000 μg/mL of *G. neojaponicum* extract. After incubation, 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) (Sigma Aldrich, USA) was mixed with 170 μL of fresh medium and added to each well. The cells were then incubated for 4 h at 37°C. Thereafter, the medium was removed and 100 μL of DMSO extraction buffer (Merck, Germany) was added to each well in order to extract and solubilize the formazan crystals. The cells were then incubated for an additional 20 min at 37°C. Finally, the 570 nm wavelength was read for each well on an ELISA Reader (Versamax with SoftMax Pro 5.3, USA). The calculation used to determine proliferation is shown below:

$$\text{proliferation rate} (\%) = \left(\frac{\text{sample absorbance–control absorbance}}{\text{control absorbance}} \right) \times 100\%.$$

2.8. Phagocytotic ability

The levels of phagocytosis were assessed by neutral red dye uptake as previously reported [8]. RAW264.7 cells were seeded at a density of 1×10^6 cells per well in 96-well microplates, followed by treatment with 100–1000 μg/mL of *G. neojaponicum* extract for 24 h. Then, 0.075% (w/v) of neutral red was added and the cells were incubated for an additional 30 min. Next, the cells were rinsed to remove leftover dye and then blotted dry. The cells were then resuspended in ethanol (50% v/v) containing glacial acetic acid (1% v/v), and the absorbance was measured at 540 nm in a microplate reader (Versamax with SoftMax Pro 5.3, USA). The calculation of absorbance was translated into phagocytotic ability using the calculation given below:

$$\text{phagocytotic ability} = \frac{\text{sample absorbance–control absorbance}}{\text{control absorbance}} \times 100\%.$$

2.9. Acute oral toxicity of DM of G. neojaponicum

The DM of *G. neojaponicum* was selected as a representative sample of the *G. neojaponicum* extracts produced in this study for safety

evaluation with regard to its in vivo acute oral toxicity. To perform this testing, the DM was sent to an independent specialist services laboratory at SIRIM, Selangor, Malaysia. This study was approved by the Animal Care and Use Committee of SIRIM Berhad and performed in compliance with Testing Guidelines for Safety Evaluation of Drugs (Notification No: SIRIM-IACUC/IBRC/B19-27/0002). In this analysis, 2000 mg/kg of the DM was solubilized and administered orally to Sprague Dawley rats at 10 mL/kg. The rats were then observed over a 14-d period to monitor mortality and physical/behavioral changes as described by OECD [9]. Two groups, a control group (normal diet without DM; n = 5) and a test group (normal diet with the addition of DM; n = 5), were used to determine the acute oral toxicity.

2.10. Statistical analysis

Statistical data obtained during the profiling of G. neojaponicum were analyzed using the Duncan multiple range test, while the in vitro and in vivo data were analyzed with the t-test. All statistical analysis was conducted using SAS Version 9.3 (SAS Institute, Cary, USA) (SAS, 1990). Statistical differences were considered significant for P-values less than 0.05.

3. Results and discussion

3.1. Isolation of IPSs and EPSs from G. neojaponicum in submerged culture using a 2 L STR reactor

Cultivation of G. neojaponicum (Imazeki) was conducted in a low-cost medium consisting of 37.25 g/L brewery spent yeast, 91.3 g/L brown sugar, 4.6 mL/L, Tween 80, 0.5 g/L $MgSO_4 \times 7H_2O$, 0.5 g/L KH_2PO_4, and 0.5 g/L K_2HPO_4. G. neojaponicum was allowed to grow in a 2 L stirred tank reactor containing this medium for 6 d. Time profiles of dry mycelial biomass, pH and reducing sugar according to cultivation day for the DM, DB, EPS, and IPS samples of G. neojaponicum in 2 L STR reactor are shown in Fig. 1.

The maximum yield of dry mycelial biomass (25.32 g/L) was obtained on the fourth day of G. neojaponicum cultivation. Yang and Liau [10] and Tang and Zhong [11] also indicated maximum yield of dry mycelia biomass G. lucidum at 22 g/L and 21.9 g/L in their studies. A similar trend was observed for the β-glucan content and total polysaccharide levels, which were also found to peak (23.56 and 115.89% (w/v), respectively) on day 4 during cultivation in the 2 L STR reactor (Fig. 2). Meanwhile, high values that were obtained for total polysaccharides with phenol-sulphuric acid assay implied possible interference of proteins or other compounds [12]. The different polysaccharide extracts showed varying glucan contents. Total glucan contents of G. neojaponicum extracts (DM, DB, EPSs, and IPSs) were 27.46, 26.1, 5.83 and 10.45%, respectively on day 4 of cultivation time.

While, α-Glucan content of G. neojaponicum extracts (DM, DB, EPSs, and IPSs) ranged from 0.22 to 4.14%.

The same trends were also observed for total glucan and α-glucan contents with the highest values (27.46 and 4.14% (w/v), respectively) in DM on day 4 in STR reactor (Fig. 3). Components of the fungal cell wall are α and β-glucan. The function of β-glucan is to support the fungal cell shape while, α-glucan can contribute to cell wall integrity [11]. Thus, the decreasing of the β and α-glucan contents might be due to the decreasing of the cell wall level when cell lysis occurred towards the end of the fermentation [13].

The levels of β-glucan and total carbohydrate production for the EPS and IPS samples were also found to be the highest on day 4, which put the production in parallel with the overall mycelial biomass. Thus, we can conclude that the production of polysaccharides and β-glucan is associated with the overall level of G. neojaponicum cell growth. The IPS extract had lower amounts of β-glucan (3.57%) and total carbohydrate (7.65%) on day 4 compared to the EPS extract at 9.41 and 80.81%, respectively. Meanwhile, the DB had the highest amounts of β-glucan (23.56%) and total carbohydrate (115.89%), followed by the DM at 23.32 and 98.19%, respectively. Similar precipitation performance was observed using the ratio of 1:1 and 1:4 (v/v) ethanol concentrations in G. neojaponicum polysaccharide extraction method (data not shown). The time needed for purifying the EPSs and IPSs was decreased by utilizing ethanol precipitations at a 1:1 (v/v) broth to polysaccharide ratio, a faster and more economical method compared to those methods that utilized 1:4 (v/v) ratio [14].

The pH profile of G. neojaponicum cultivation showed a decreasing pattern, dropping from a pH of 6.02 to 4.6. This decrease may be caused by the production of organic acid from various carbon sources in the media and biomass during consumption and cell growth [15]. Furthermore, based on the growth curve, the logarithmic phase appeared to require high levels of nutrients and sufficient oxygen, with the highest levels utilized between day 2 and day 4. This is indicated by the reduced levels of dissolved oxygen inside the STR on day 4. The oxygen deficiency observed at this stage could explain why cell growth diminished after day 4. We also observed that almost all of the reduced sugar was consumed by G. neojaponicum during cultivation, from 7.30 to 5.21 g/L between day 2 and day 6. Thus, our data indicate that glucose consumption by G. neojaponicum is also largely dependent on cell growth.

From the preceding results and discussion it could be noted that G. neojaponicum can be cultivated using submerged fermentation for mycelium and polysaccharide production. The polysaccharides in submerged fermentation can be produced in large quantities by upscaling production, an approach which offers convenient control and easy downstream processing. Submerged culture can greatly decrease the cultivation period, and enhance polysaccharide production compared with extracting polysaccharides from basidiocarps. The bioreactor can easily manipulate environmental conditions like
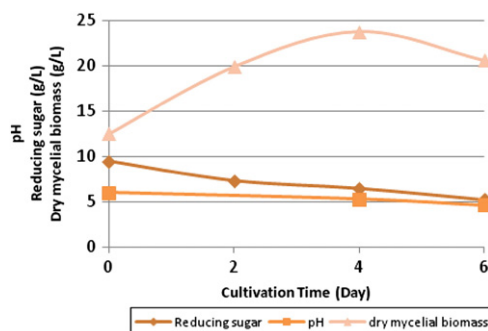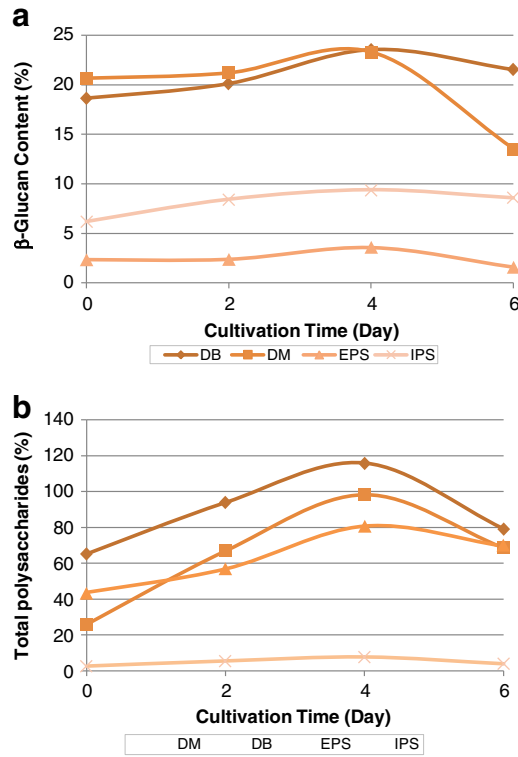

Fig. 1. Time profiles of dry mycelial biomass, pH and reducing sugar according to cultivation day for G. neojaponicum in 2 L STR reactor.

Fig. 2. Time profiles of the total polysaccharide (TPS) levels (a) and β-glucan content (b) according to cultivation day for the DM, DB, EPSs, and IPSs of *G. neojaponicum* in 2 L STR reactor.

temperature, agitation, dissolved oxygen and pH to obtain maximum yield of polysaccharide. Polysaccharide produced can be purified and fractionated through various methods such as ethanol concentration, fractional precipitation, ion-exchange chromatography, gel filtration and affinity chromatography. The purified polysaccharides are significant for the evaluation of immunomodulatory properties of *G. neojaponicum*.
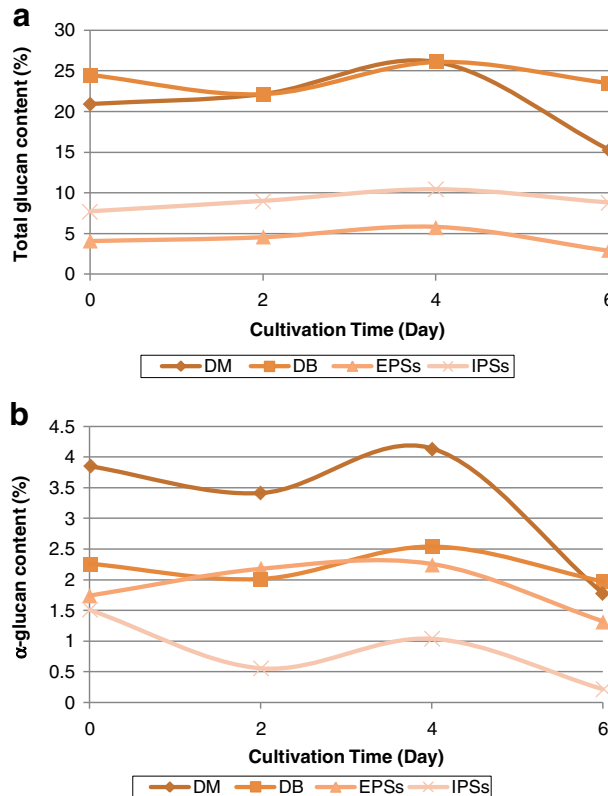
Fig. 3. Time profiles of the total glucan (a) and α-glucan content (b) according to cultivation day for the DM, DB, EPSs, and IPSs of *G. neojaponicum* in 2 L STR reactor.

3.2. Evaluation of the immunomodulatory properties of DM, DB, and the polysaccharides from G. neojaponicum

3.2.1. Effect of G. neojaponicum extracts on RAW264.7 macrophage proliferation

The DM and DB as well as the polysaccharide extracts (IPS and EPS) of *G. neojaponicum* were evaluated to determine their effect on the proliferative activities on RAW264.7 macrophages. As a positive control, β-glucan from yeast glucan (YG) extract was also introduced to the macrophages and showed a significant proliferation index of 0.81, while the negative control CTL was observed to have a proliferation index of 0.09. As indicated in Fig. 4, the highest increase in macrophage proliferation was found in the 1000 μg/mL concentration of IPSs, which increased by 226% compared to the negative control samples. From these data, we can conclude that no significant cytotoxic effects are apparent in the macrophages treated with *G. neojaponicum* extract compared to the negative control. Further, the IPS and EPS extracts showed higher levels of proliferation compared to the DM or DB. *G. neojaponicum* extracts and these high levels were found to be dose-dependent. The *G. lucidum* is also dose-dependent associated with cytotoxic effect on RAW264.7 cell proliferation [16]. Polysaccharide extract of *Lentinus edodes* also has a similar stimulating effect on proliferation of cultured macrophages [17]. An analysis of *G. neojaponicum* extracts on phagocytic ability using macrophages RAW264.7 has resulted in the following data on immunomodulatory properties.

3.2.2. Effect of the G. neojaponicum extracts on the phagocytotic ability of RAW264.7 macrophages

The DM, DB, IPSs, and EPSs of *G. neojaponicum* were evaluated with regard to their effect on the phagocytotic ability of RAW264.7 macrophages using a neutral red uptake assay. Fig. 5 shows the macrophage phagocytotic ability 24 h after being treated with 100–1000 μg/mL of the *G. neojaponicum* extracts. The RAW264.7 macrophages demonstrated dose-dependent, incremental increases in their phagocytotic ability compared to the negative control. Further, the highest levels of phagocytosis, and therefore the highest immune response, were observed in the IPSs and EPSs, with IPSs showing the most potent increase (460%) at the 1000 μg/mL concentration.

The polysaccharide fraction of *G. neojaponicum* is a strong stimulator to the macrophages. The preceding analysis highlighted that macrophages function by eliminating pathogenic microorganisms, decomposed parts of cells and foreign particles and also granulocytes. The macrophages in turn produce mediators utilized by cells in communicating with one another within the immune system. Phagocytosis is an initial and critical part in host defense against pathogen particularly in its role in triggering the adaptive immune response. Many studies suggested that phagocytotic activities are
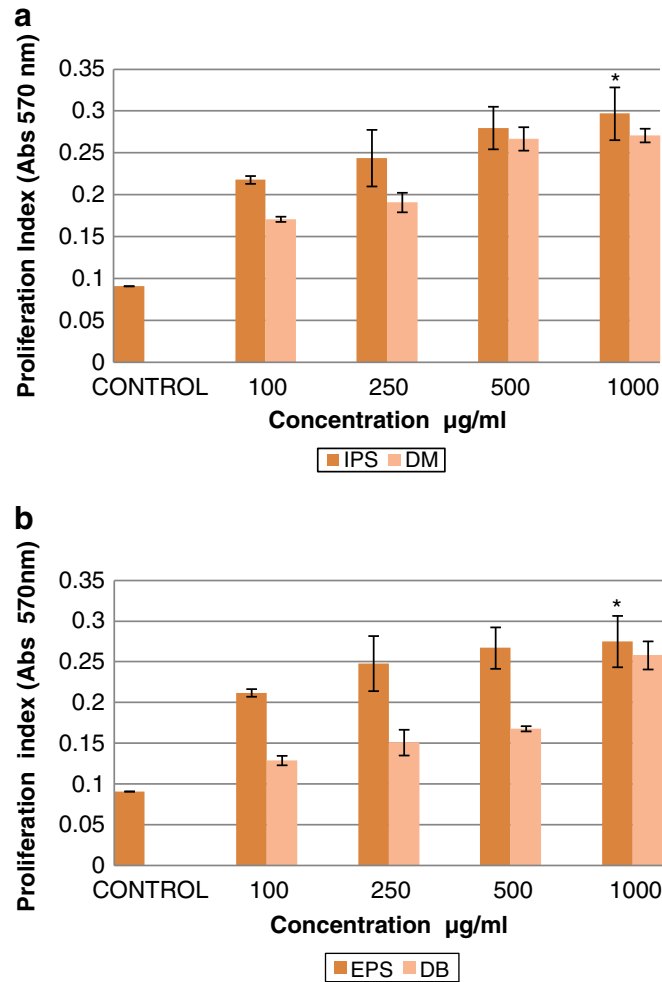

Fig. 4. Effects of the *G. neojaponicum* extracts on the proliferation of RAW264.7 macrophages. Means ± S.E. (n = 5) for (a) DM and IPSs and (b) DB and EPSs. Results were calculated by dividing experimental data by control values. Cells treated with DMEM medium were used as a negative control. * Statistically significant (P < 0.05) difference in comparison with the control. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean.
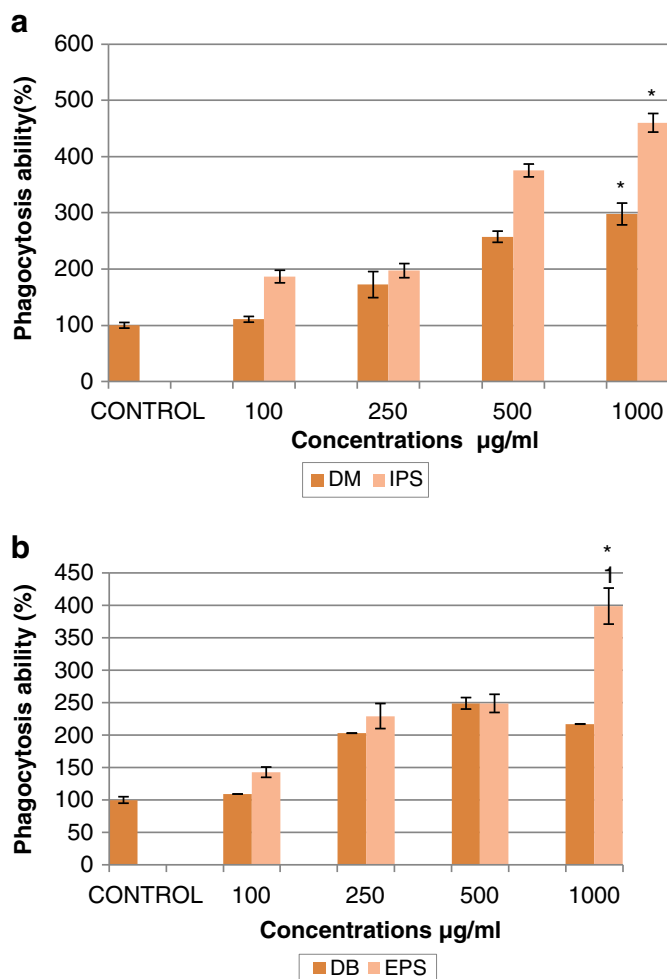
Fig. 5. Changes in the phagocytotic ability of RAW264.7 cells after treatment with (a) DM and IPSs and (b) DB and EPSs of *G. neojaponicum*. Results are expressed as the mean ± S.E. (n = 5). Cells treated with DMEM were used as a negative control. * Statistically significant (P < 0.05) difference in comparison with the control. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean.

stimulated in the macrophage cell and its mediators, these are vital for tumor rejection and destruction of cancer cells.

3.3. Oral acute toxicity of DM of G. neojaponicum

3.3.1. Evaluation of acute toxicity test of G. neojaponicum mycelium

The dried *G. neojaponicum* mycelium was chosen to be evaluated for its safety in vivo to ascertain potential clinical applications and uses involving the consumption of *G. neojaponicum* polysaccharide extracts. In this study, a single dose of DM was administered to Sprague–Dawley rats to determine acute oral toxicity. The effects of

Table 1
Summary of body and organ weights of treated Sprague–Dawley rat treated with dried mycelium of *G. neojaponicum*.

Organ/body	Treated group (g)	Control group (g)
Heart	0.37 ± 0.01	0.375 ± 0.12
Liver	2.69 ± 0.07	2.601 ± 0.05
Spleen	0.20 ± 0.00	0.188 ± 0.01
Kidneys	0.71 ± 0.01	0.713 ± 0.01
Stomach	0.30 ± 0.01	0.304 ± 0.01
Body weight (g)	210.00 ± 4.60	211.60 ± 9.60

Note: Organ body index = (organ weight × 100) / body weight; dried mycelium of *G. neojaponicum* was given to rats at a dosage of 2000 mg/kg. (Mean ± S.E., n = 5).

this oral administration were then monitored over a 14-d period according to Chan and Hayes [18].

Overall, following DM treatment, the animals in both the control group and treatment group were found normal. Individual body and organ weights of both groups are summarized in Table 1. These results show that following a single dose, the polysaccharide extract has no adverse effects, indicating that the medium lethal dose (LD50) could be greater than 2000 mg/kg body weight in rats. In comparison with the current literature, oral administration of a hot water extract of *G. lucidum* (5000 mg/kg) to mice for 30 d also resulted in no significant changes in body weight, hematological features, or organ weight [19]. Furthermore, an alcoholic extract of *G. lucidum* given to young rats (1.2 and 12 g/kg daily over 30 d) also showed no signs of toxicity with regard to major organ size or overall growth and development of the rats [19]. Thus, it was not altogether surprising that there were no signs of toxicity or death observed in our animals upon administration of 2000 mg/kg of DM of *G. neojaponicum*.

3.3.2. Hematology analysis

A hematology test was carried out on the animals treated by the dried *G. neojaponicum* mycelium, which included a full blood count, renal function test, liver function test, and glucose metabolism test. Table 2 shows the blood profile (red blood cell count (RBC), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular

Table 2
Hematology and biochemical analysis of untreated group (control) and treated group (dried mycelium) for *G. neojaponicum*.

Biochemical test	Abbreviation	Unit	Untreated group (control)	*G. neojaponicum* extract
Full blood count				
Red blood count	RBC	$\times 10^{12}$/L	7.2 ± 0.3	6.9 ± 0.2
White blood count	WBC	$\times 10^{5}$/L	7.5 ± 1.6	7.2 ± 1.8
Hemoglobin	Hb	g/L	140.4 ± 4.8	135.4 ± 4.3
Hematocrit (packed cell volume)	PCV	L/L	0.4 ± 0.0	0.4 ± 0.0
Mean corpuscular volume	MCV	fL	48.0 ± 12.0	61.4 ± 0.5**
Mean corpuscular hemoglobin concentration	MCHC	g/L	322.6 ± 6.1	319.2 ± 1.5
Thrombocytes-platelets	Thromb	$\times 10^{9}$/L	622.8 ± 30.0	818.6 ± 61.0**
Lysis index	I. Index	Unit	Lysed	Lysed
Renal function test				
Total protein	T. Prot	g/L	66.2 ± 1.6	62.5 ± 1.7
Albumin	Alb	g/L	40.6 ± 1.2	41.4 ± 0.9
Creatinine	Crea	umoL/L	72.4 ± 1.5	69.6 ± 2.8
Urea (blood urea nitrogen)	Urea	mmoL/L	8.2 ± 0.7	6.4 ± 0.9
Phorus	Phos	mmoL/L	3.7 ± 0.3	3.7 ± 0.4
Calcium	Ca	mmoL/L	2.8 ± 0.0	2.8 ± 0.0
Sodium	Na	mmoL/L	146.8 ± 0.8	146.5 ± 0.9
Potassium	K	mmoL/L	6.3 ± 0.4	6.0 ± 0.6
Chloride	Cl	mmoL/L	97.0 ± 0.3	96.4 ± 0.8
Liver function test				
Alanine aminotransferase	ALT	U/L	55.9 ± 2.8	49.4 ± 5.2
Alkaline phosphatise	ALP	U/L	105.5 ± 11.2	108.8 ± 4.
Aspartate aminotransferase	AST	U/L	140.0 ± 12.5	117.9 ± 8.5**
Cholesterol	Cholt	mmoL/L	2.2 ± 0.1	2.1 ± 0.1
Triglyceride	Trig	mmoL/L	0.3 ± 0.1	0.2 ± 0.0
Gamma glutamyltranspeptidase	GGT	U/L	<0.3	<0.3
Total bilirubin	T Bil	umoL/L	0.6 ± 0.2	0.9 ± 0.1
Glucose metabolism				
Glucose	Gluc	mmoL/L	2.8 ± 1.0	2.5 ± 0.4
Urinanalysis	U.A.	umoL/L	99.7 ± 19.2	80.5 ± 19.0

Note: Values are mean ± SE (n = 5) at 5% level of significance ($P < 0.05$).
** Significant compared with untreated group (control).

hemoglobin concentration (MCHC)) of the rats following oral administration of the DM of *G. neojaponicum*. Of these, at dosage of 2000 mg/kg of *G. neojaponicum*, only the MCV and a number of thrombocyte-platelets were significantly altered compared to the untreated control group. Further, in this study, no significant effects were observed on total protein and albumin levels. This indicated that protein catabolism was not affected. Therefore, the polysaccharide extracts used in this study did not result in any adverse effects on protein degradation. Further, no differences were found in the serum urea nitrogen levels between the test and control groups after being treated with *G. neojaponicum*.

Liver function tests (aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, gamma GT (gamma-glutamyltranspeptidase) and a renal function test (serum creatinine)) were also conducted to observe any abnormalities in these organs caused by the DM extract. Serum creatinine was not affected by the oral administration of the extract, indicating that the kidney function was not affected. Further, oral administration of *G. neojaponicum* did not cause any significant differences in the levels of ALT and ALP compared with the control group. However, the levels of AST were significantly altered from 140.0 ± 12.5 U/L in the untreated control group to 117.9 ± 8.5 U/L in the DM treated group.

Regarding total cholesterol and triglyceride levels, there was no significant difference observed between the treatment and control groups, indicating that there was no change in the level of blood fats following the consumption of the dried *G. neojaponicum* mycelium. A similar observation noted previously, indicated no change in the blood profile following administration of *G. lucidum* [20]. Thus, we conclude that a preliminary dose of 2000 mg/kg body weight/day of dried *G. neojaponicum* mycelium is indicating its present safety level of

toxicity. However, safety consumption of polysaccharides extract of *G. neojaponicum* either as a drug or functional food requires further toxicology testing. Further testing should focus on the sub-acute, sub-chronic, and chronic systemic toxicity tests. At this point the findings do indicate that it is safe for *G. neojaponicum* to be used as a functional food or for medicinal treatment.

4. Conclusion

This preliminary study shows that *G. neojaponicum* can enhance RAW264 proliferation and increase of phagocytosis. These results indicated that *G. neojaponicum* could potentially be used as an immunomodulating agent for stimulating the innate immune system for infectious disease treatment. Further, the DM, administered at doses up to 2000 mg/kg body weight/d for 14 d had not caused any changes in mortality or result in any toxic effects on the rats. Although this study has revealed certain effects on immunomodulatory properties, however further research still need to be conducted. The focus can be on additional purification and identification of the specific polysaccharide(s) of *G. neojaponicum* to better understand its immunomodulatory properties.

Conflict of interest

No conflict of interest for both organizations.

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