Original Article

Immunomodulatory Activity of Polysaccharide from *Trametes gibbosa* (Pers.) Fr (Basidiomycota, Fungi) Mediated by TLR4 Signaling Pathway

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Abstract

Background: *Trametes* species possess remarkable immunomodulatory and anticancer effects which are mainly related to the activation of innate immune receptors by their polysaccharide constituents. In this study, we investigate the effect of *Trametes gibbosa* (Pers.) Fr. polysaccharide fraction (TGP) on activation of TLR-4 receptor and subsequent release of IL-8 in HEK-Blue™ hTLR4 cells.

Materials and Methods: The polysaccharide fraction was purified using ethanol precipitation and dialysis methods. The total sugar content and monosaccharide composition were analyzed by phenol–sulfuric acid and chromatographic methods. FT-IR spectroscopy was also performed for structure characterization of the polysaccharide. The activation of TLR4 was determined by measuring the secreted embryonic alkaline phosphatase in the culture media.

Results: The results indicated that the total sugar content of TGP was about 90%, which glucose was the major constituents. FT-IR analysis showed the characteristic bands of polysaccharides. TGP was able to activate the TLR-4 signaling pathway in a dose-dependent manner. Moreover, the significant increase of IL-8 was observed in cells treating with TGP. The HEK-Blue Null2™ reporter cells lacking TLR4, did not respond to LPS and TGP.

Conclusion: The results suggest that TLR4 signaling cascade serve as targets for immunomodulatory activity of *T. gibbosa* which could address the anticancer properties of *Trametes* species.

Keywords: Immunomodulation, polysaccharides, toll-like receptors, *Trametes gibbosa*

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Introduction

Polysaccharides (PSs) are biomolecules made up of long chain monosaccharides. These compounds have been used as food and medicine for centuries and have been reported naturally from various sources such as fungi, plants and bacteria.[1] Recent studies demonstrated that PSs are the major active ingredients in various types of edible and medicinal mushrooms. Bioactivities of these compounds are related to their structure, molecular weight, size, composition and stereochemistry. Mushroom PSs are mainly glucan type that are present in the cell walls. Various type of glucan with different glycoside linkage such as (1→3), (1→6)-β-glucan, and (1→3)-α-glucan, have been detected in medicinal mushrooms.[2]

Much attention has been paid to mushroom PSs, due to their potential biological activities including immunomodulatory,

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antitumor, and antiinflammatory properties. Mushroom-derived PSs have the ability to reinforce immune function via enhancement of different types of innate immune cells activation such as macrophage, dendritic cells, cytotoxic T cells, and natural killer cells.[3]

The role of the immune system in cancer management has been a subject of great interest during centuries. Several studies have been conducted to investigate the immune modulating responses in cancer prevention and treatment. In cancer pathogenesis, several components of the innate immunity are activated to restrict the tumor cells.[4]

Polysaccharide-mediated immune cell stimulation can occur via binding to pattern recognition receptors (PRR) on the cells surface such as toll-like receptors (TLRs) and Dectin-1 which triggers a series of signal transduction pathways including protein kinases and nuclear factor (NF)-κB transcription factors.[5,6]

TLRs expression by innate immune system cells, are stimulated by pathogen-associated molecular patterns (PAMPs). The TLRs family plays an important role in the recognition of a wide variety of bacterial [such as lipopolysaccharide (LPS) of gram-negative bacteria cell wall], fungal, and viral components in the host defense system.[7] Triggering the TLR4 via PSs leads to the activation of NF-κB signaling pathway with subsequent release of pro-inflammatory cytokines.[8] These cytokines commonly include TNF, IL1-β, and IL-8.[7]

Trametes gibbosa (Pers.) Fr, the Lumpy bracket, is one of the common polypore mushrooms at the phylum Basidiomycota and Polyporaceae family that distributed in different parts of Asia and Europe. It grows rapidly on the trunks of dead hardwoods and cause them to rot.[9,10] Such decomposition and decay are vital processes, playing an important role for the ecology of forest. Traditionally, *Trametes* species were used for thousands of years due to therapeutic potentials.[10] Based on Chinese traditional medicine, *T. gibbosa* possess anticancer properties.[11] Several bioactive PSs such as krestin, lentinan, and schizophyllan have been isolated from *Trametes versicolor* (L) Lloyd*, Lentinus edodes* (Berk.) Singer, *Schizophyllum commune* Fr. and etc., for investigation of their promising anticancer and immune-stimulating properties.[2]

It has been reported that the polysaccharide fraction from *T. gibbosa* fruit body possess several pharmacological activities such as antioxidant, anti-inflammatory,[12,13] genoprotective, antineurodegenerative, antiviral (against type A influenza), hypoglycemic, and hypolipidemic[11] properties. Also, the alcoholic extract from this mushroom can significantly induces the immune system cells activity. Further study indicates that TLR4 is the innate immune receptor of PSs from *T. versicolor* (Berk.) Singer (Krestin), *Ganoderma lucidum* (Curtis) P. Karst. and *Phellinus linteus* (Berk. & M.A. Curtis) Teng which triggers the activation of immune cells for suppression of tumor cells and enhance the immune responses.[14-16] Thus, the PSs of medicinal mushrooms with potential immunomodulatory activities, are suitable alternative for developing a new immune-adjuvant drug for the immunotherapy of cancer and infectious diseases caused by viral agents, gram-positive and negative bacteria, and parasite.[17]

Despite the immune system stimulating activity and anticancer properties of *Trametes* species, there is not a comprehensive study about the immunomodulatory activity of *T. gibbosa* PSs. In this study, we investigated the immunomodulatory activity of polysaccharide rich fraction from *T. gibbosa* (TGP) through the activation of NF-kB pathway by TLR4 targeting and assessment of IL-8 level as a pro-inflammatory cytokine using HEK-Blue™ hTLR4 cells. The HEK-Blue™ Null2 Cells was also used to show the dependency on TLR4 expression.

Materials and Methods

Materials

Dialysis tubing D0405-100FT, DPPH, and MTT were purchased from Sigma Aldrich (USA). Monosaccharide reference standards (glucose, galactose, fructose, arabinose, xylose, and mannose), trifluoroacetic acid and TLC silica gel G plates were obtained from Merck (Germany). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories (Grand Island, NY, USA). The HEK-Blue™ hTLR4, HEK-Blue™ Null2 cells, HEK-Blue™ Detection, and LPS-EB (Standard LPS, *E. coli* 0111: B4) were obtained from InvivoGen. Human IL-8 ELISA kit was provided by Invitrogen eBioscience (San Diego, CA, USA). The other chemicals and solvent used were in analytical grade.

Preparation and purification of polysaccharide fraction

T. gibbosa polysaccharide fraction was prepared as described previously with minor modifications.[18] The mushroom fruit bodies were collected from the Caspian Hyrcanian forests, Mazandaran, Iran. Saeed Ali Mousazadeh (Agriculture and resource research center, Mazandaran, Iran) identified the mushroom. The voucher specimen (MAZ-B_3-0101-001) was deposited in the herbarium of Mazandaran University of medical sciences. The amount of 250 g of dried mushroom material were extracted with methanol to remove the nonpolar compounds. Then, submitted to cold and hot water extraction, subsequently, for 6 hours in triplicate. The cold-water extraction is necessary to remove irrelevant polar compounds such as storage PSs and phenolic compounds. The hot aqueous extract was concentrated by vacuum evaporation. The filtrate was mixed with 96% ethanol (1:3 v/v) and allowed to stand at 4°C overnight to precipitate the PSs and finally centrifuged (5,000 rpm, 10°C) for 20 min. The sediment washed severally with methanol for more purification and finally dialyzed against tap water for 24 h (14 kDa cut-off). The purified polysaccharide fraction was dried by freeze dryer.

Total carbohydrate determination

Phenol-sulfuric acid method was used for determination of total carbohydrate content of polysaccharide.[19] 150 µL of sulfuric acid (96%) was added to 50 µL of sample (100 µg/mL)

and standard glucose solution (5-100 µg/mL). Immediately, 30 µL of phenol 5% aqueous solution was added to each tube. At the end, the mixture was kept in water bath (90°C) for 5 min. Different concentrations of D-glucose were used for preparation of standard curve.

Monosaccharide composition analysis

For the monosaccharide composition analysis, TGP (5 mg) was hydrolyzed by trifluoroacetic acid (TFA) 2 M in boiling water bath for 8 h. Then evaporated to dryness under reduced pressure to remove excess TFA. The hydrolyzed product was analyzed using thin layer chromatography (TLC).

TLC method: The TLC plate (silica gel G, aluminum sheet, 250 µm thickness) was impregnated with NaH_2PO_4 solution (0.3 mol/L) and left overnight. Then, the plate activated at 115°C for 1h in oven before use. TGP hydrolysate and six standard monosaccharides solution (glucose, galactose, fructose, mannose, xylose and arabinose) were applied on TLC plate. Two distinctive solvent system used for mobile phase, at first the plate was developed with solvent A [4:3:1 (v: v: v) n-butanol-acetone-water] to a distance of 9.0 cm from origin in a TLC chamber. The plate was dried using a hair drier. For the second time, the TLC plate were developed with solvent B [8:4:7:3 (v:v:v:v) n-butanol-ethyl acetate-isopropanol-water]. After drying, aniline-phthalate reagent was used for spraying the plate. Finally the plate was heated to 105°C for 10 minutes.[20]

Infrared spectral analysis of the TGP

Infrared spectrum of the sample was recorded with a Cary 630 FTIR spectrophotometer, and the data collection was achieved by Cary 630 MicroLab PC software (Agilent Technologies, USA). Spectrum was recorded in the wavenumber range between 400–4000 cm^{-1}.

DPPH-free radical scavenging activity

The DPPH free-radical scavenging activity of *T. gibbosa* extract was determined as described before.[21] Different concentration of extract and vitamin C (standard compound) (1.5 mL each) were mixed with 1.5 mL of DPPH in methanol (0.15 mM). The absorbance at 517 nm was determined after 30 min against blank (methanol). The following formula was used for measuring the DPPH radical inhibition.

$$\text{Scavenging rate} = [(A_0 - A_s)/A_0] \times 100$$

Where A_0 and A_s are the absorbance of DPPH solution without a sample and the absorbance of sample with DPPH, respectively.

In vitro immunomodulatory assessment of TGP in HEK-Blue™ hTLR4 Cells

HEK-Blue™ cell culture

The HEK-Blue™ hTLR4 and HEK-Blue™ Null2 cells were obtained from InvivoGen, USA. Briefly, cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL Normocin (InvivoGen).

Determination of cell viability

MTT assay was used for determination of cell viability. The cells were incubated for 24 h. Then, 20 µL of MTT solution (5 mg/mL) was added into each well, and the plates were incubated at 37°C for 4h. Next, the MTT solution was removed, and 100µL of dimethyl sulfoxide was added and shaken for 5 min. The absorbance at 570 nm was determined after 30 min.[22]

TLR4 activation assay

HEK-Blue™ hTLR4 cells are engineered HEK293 cells that stably co-express the human TLR4 and NF-κB-inducible-secreted embryonic alkaline phosphatase (SEAP) reporter gene. The activation of NF-κB in HEK-Blue hTLR4 cells leading to secretion of SEAP in culture media. Chromogenic substrate in specific media converted by SEAP into product. SEAP activity can be monitored by a colorimetric assay.

HEK-Blue™ hTLR4 cells and HEK-Blue™ Null2 cells (as control), were cultured as described before. When the cells reach about 80% confluence, they were subcultured. Then, the cells were seeded in multiwell plate at 25,000 cells per well. After 24 h, the supernatant was removed, and the cells incubated in HEK-Blue™ cell culture medium. Next, the cells were treated with TGP at concentrations of 1, 10, 50, and 100 µg/mL, LPS-EB (Standard LPS, *E. coli* 0111:B4) (100 ng/mL), LPS+TGP (combination of LPS and various concentration of TGP), and PBS as negative control. SEAP production was determined using the spectrophotometric analysis of supernatant at 630 nm with Microplate Reader (BioTek, USA).[23]

Determination of IL-8 concentration by ELISA

The ELISA kit (eBioscience) was used for determination of IL-8 levels in supernatant of HEK-Blue™ hTLR4 cultured cells. The cells (2×10^5 cells/well) were cultured with TGP (final concentration of 1, 10, 50, 100 µg/mL) for 24 h. LPS (100 ng/mL) and PBS were used as positive and negative controls, respectively.

Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA). The *P* value less than 0.05 was considered statistically significant.

Results

Structural assignment of dialyzed TGP extract

The *T. gibbosa* polysaccharide fraction was prepared using hot water extraction, ethanol precipitation, and dialysis methods. The fraction yield was calculated 7.2 g/100 g dry mushroom weight.

Based on the calibration curve prepared with standard solutions of glucose, the total polysaccharide content of the fraction was calculated 92 g/100 g dry weight of the fraction.

The TLC analysis of hydrolysate from TGP [Figure 1] showed the presence of glucose.

FT-IR analysis

The infrared spectra of TGP is illustrated in Figure 2. The spectra exhibited a broad intensive hydroxyl group stretching vibration band (3400 cm^{-1}), along with C-O stretching vibration band (1025 cm^{-1}) which are characteristic of PSs. Additionally, the peak in 2920 cm^{-1} is due to CH_2 asymmetric stretching vibration. The bands at 1637 cm^{-1} was NH bending vibration to the amide group.

DPPH-free radical scavenging activity

The EC_{50} value of TGP and vitamin C was determined 2346.31 ± 0.001 µg/mL and 12.8 ± 1.4 µg/mL, respectively. This indicated the poor antioxidant activity of TGP.

In vitro immunomodulatory assessment of TGP in HEK-Blue™ hTLR4 cells

Cell viability

To evaluate the effect of TGP on cell survival of HEK-Blue ™hTLR4 cells, the MTT assay was performed. Different concentrations of TGP (5, 10, 25, 50, 75, and 100 µg/mL) were used to assay the toxicity. Based on the results, TGP had no toxicity and did not affect cells viability at various concentration on HEK-Blue™ hTLR4 compared with untreated cells [Figure 3].

The activation of TLR4 by TGP in HEK-Blue™ hTLR4 cells

The SEAP secretion in the culture media was used as a marker for evaluation of TLR4 activation by TGP. Based on the results, different concentrations of TGP (1, 10, 50, 100, µg/mL) significantly increased the secretion of SEAP via stimulation of TLR-4 receptor in HEK-Blue hTLR4 ™ cells in a dose-dependent manner compared to control (cells treated with PBS). The combination of LPS and different concentrations of TGP (which was employed for evaluation of the synergistic activity) did not show any significant effect on TLR-4 activation compared to LPS-treated cells [Figure 4].

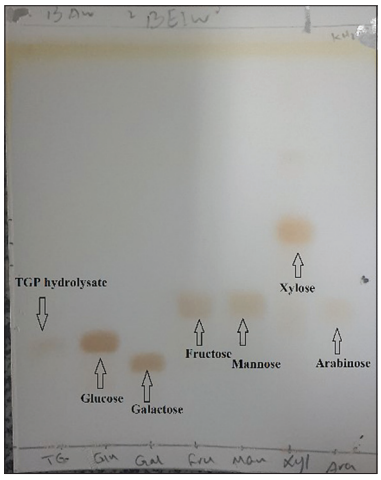

Figure 1: Monosaccharide composition analysis of *T. gibbosa* Polysaccharide fraction by TLC. Lane 1 corresponds to hydrolysate of TGP. Lane 2 to 7 corresponds to six standard monosaccharides

To show the dependency on TLR4 expression, the HEK-Blue™ Null2 cells (which are nonresponsive to TLR4 ligands) was used. We observed that HEKBlue-null2 cells did not respond to LPS and different concentrations of TGP. No significant difference was observed between PBS as negative control and TGP-treated HEK-Blue™ Null2 cells [Figure 5].

The present results showed that the secretion of SEAP in HEK-blue hTLR4 cells is mediated by TLR-4 activation.

Effect of TGP on IL-8 secretion in HEK- Blue hTLR4 cells

To evaluate the effect of TGP on the production of IL-8, the HEK-Blue hTLR4 cells were treated with different concentrations of TGP (1, 10, 50, and 100 µg/mL). ELISA was used for quantitative determination of IL-8 in cell culture supernatant. As expected, IL-8 secretion was efficiently increased by TGP compared to control ($p < 0.0001$). Based on the result, the TGP was effective as LPS to induce the secretion of IL-8 cytokine in HEK- Blue hTLR4 cells [Figure 6].

Discussion

LPS, N-acetyl glucosamine and beta glucan are the common PRR ligands which are constituents of bacterial or fungal cell walls.[14] The activation of innate immune system as first line defense against pathogen invasion, induces the secretion of inflammatory cytokines and the subsequent activation of adaptive immunity. TLRs as innate immune system receptors can be activated through a wide range of innate immune cells and non-immune cells, such as dendritic cells, macrophages, human retinal pigment, gingival, bronchial, endometrial, and uterine epithelial cells.[24] It is known that mushroom derived PSs possess immune-modulatory and antitumor activities.[2]

It was reported that the binding of mushroom derived PSs to TLR4 receptor, can activate the immune responses by improving the secretion of TNF-alpha, IL-8, and other inflammatory cytokines which restricted the tumor cells activities and proliferation.[8,14]

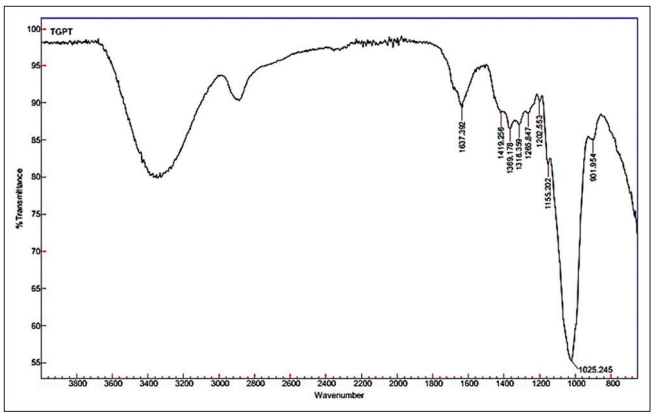

Figure 2: Infrared spectrum of *T. gibbosa* Polysaccharide fraction in the range of 4000-400 cm-1

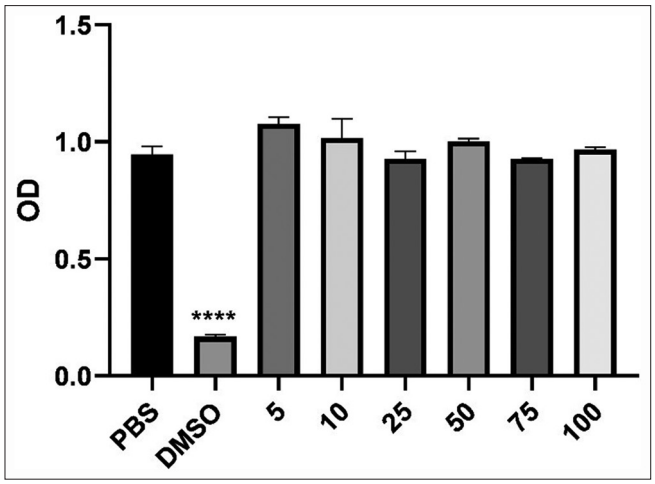

Figure 3: Effects of *T. gibbosa* polysaccharide fraction on cell viability of HEK-Blue hTLR4 cell line. The results were compared with control (cells treated with PBS), while 10% DMSO is used as a positive control inducing cell lethality. Data are expressed as Mean ± SD. (****) $P < 0.0001$ versus the control group (PBS)

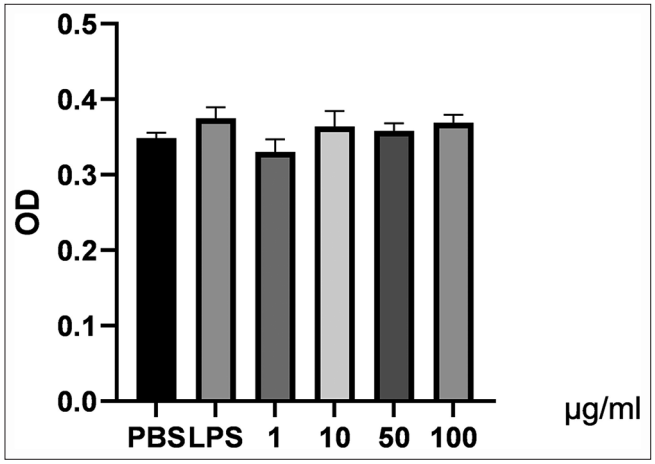

Figure 5: Effect of *T. gibbosa* polysaccharide fraction on SEAP production in HEK-Blue™ Null2 cells. LPS and PBS were used as positive and negative controls, respectively. Data are expressed as Mean ± SD

We hypothesized that TGP may bind to TLR-4 receptor, resulting in increase of the proinflammatory cytokine.

The present study showed that different concentration of TGP activated the TLR4 receptor in HEK-Blue™ hTLR4 cells in a dose dependent manner. Additionally, the secretion of IL-8 was induced in this cell line.

The TGP was prepared using the method of hot water-extraction, ethanol precipitation and dialysis. Spectrophotometric analysis of TGP revealed the presence of high carbohydrate content. Based on FTIR spectra, the bands at 3400 cm^{-1} and 1025 cm^{-1}, represent the hydroxyl and C-O stretching vibration bands, respectively, which are characteristic of PSs. Absorptions at 1025 cm^{-1}, 1070 cm^{-1} (overlapped by 1025 cm^{-1}) and 1155 cm^{-1} in the range of 1200-1000 cm^{-1} indicating the monosaccharide in TGP had a pyranose ring. The band at 901 cm^{-1} due to

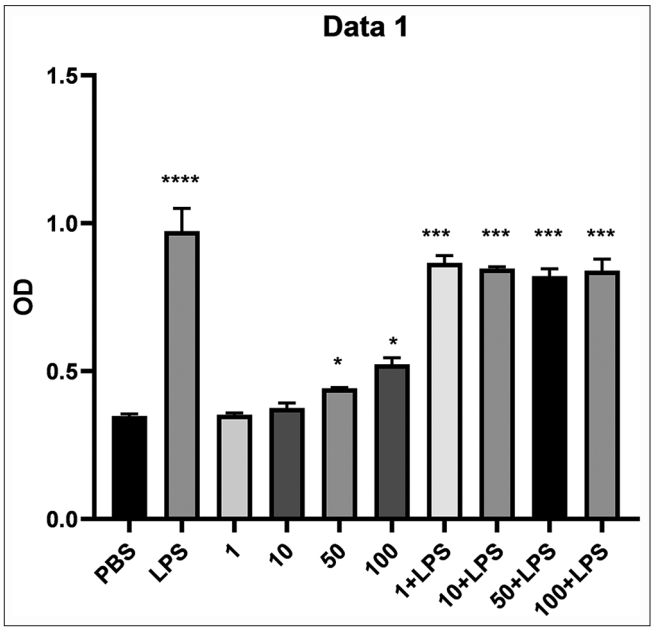

Figure 4: TLR4 activation in HEK-Blue hTLR4 cells by *T. gibbosa* polysaccharide fraction and its synergistic effect with LPS. Data are expressed as Mean ± SD. (*) $P < 0.05$, (***)

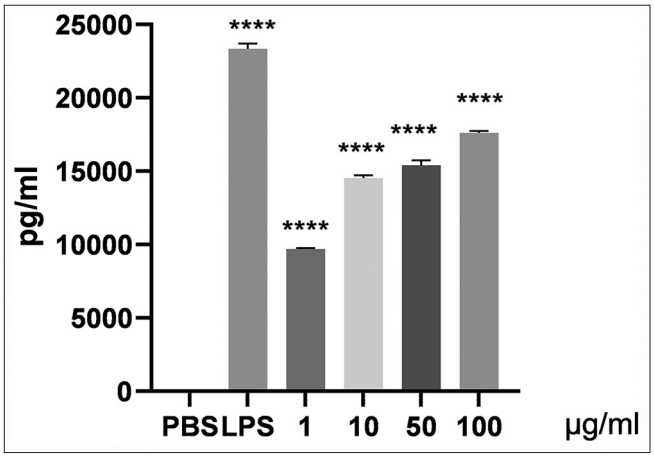

Figure 6: The concentration of IL-8 in HEK-Blue hTLR4 cells treated with *T. gibbosa* polysaccharide fraction and LPS. Data are expressed as Mean ± SD. (****) $P < 0.0001$ versus the control group (PBS)

C_1-H_1 stretching vibration in the FTIR spectrum indicated β configuration. The amide band at 1637 cm^{-1}, might be related to some residual protein in TGP.[25]

Recent investigations showed that the PSs isolated from various fungal species including *Trametes*, possess the potent immunomodulatory activity via TLR4 activation and the subsequent release of some interleukins. Based on Price *et al*.,[14] who investigated the immunomodulatory activities of PSs isolated from *T. versicolor* and a *G. lucidum* in J774A.1 macrophage cell line and primary peritoneal macrophages, the release of inflammatory cytokines increase dose dependently in response to the PSs or mushroom preparations. They reported that the TLR4 receptor is

involved in the activation of innate immune system by mushroom PSs. Similarly, Kim *et al*.[16] indicated that the functional maturation of dendritic cell by proteoglycan from *P. linteus* is mediated via TLR2 and/or TLR4. It seems that the activation of the innate immune system by mushroom PSs is mediated via TLRs.

This finding is consistent with the results of the present study that TGP activated the TLR4 receptor in HEK-Blue™ hTLR4 cells. Wang *et al*.,[8] further demonstrated that the activation of TLR4 polysaccharo-peptide from *T. versicolor* (PSP) in peritoneal macrophages from C57BL/10J (TLR4+/+) mice can increases the production of proinflammatory cytokines such as TNF-α and IL-6 in culture media. Based on recent studies, some PSs from the other fungal sources such as *Polyporus umbellatus* (Pers.) Fr. and *Poria cocos* F.A. Wolf can also increase the TNF-α, IL-1β, and nitric oxide production and activate the phagocytosis in macrophage cell lines.[26,27] Similarly, we demonstrate here that the TGP-treated HEK-Blue™ hTLR4 cells, induce the release of IL-8 as a proinflammatory cytokine.

In agreement with the above findings, the observed immunomodulatory effects of *T. gibbosa* could be attributed to activation of the innate immune system cells via the polysaccharide constituents of this mushroom.

The expression of IL-8 as a potent neutrophil chemotactic factor, is mediated by activation of TLR receptors.[28] Neutrophils are considered as potent antitumor effector cells. Neutrophils can release different antimicrobial and cytotoxic compounds for destroying malignant cells. Moreover, the neutrophil chemotaxis causes the attraction of other cells with antitumor activity.[29]

The role of TLRs in the development of potential target for cancer immune therapies have recently become the focus of several investigations.

Glycan-based drugs possess broad spectrum of therapeutic activities with low toxicity and costs. These drugs have potential immunoregulatory effects which can be combined with other chemotherapeutics. Lentinan and Krestin, two mushroom derived PSs, were approved as an adjuvant for some cancer therapy in Japan. These two PSs have been widely used for cancer combination therapy in several types of cancers such as lung, breast, esophageal, hepatic and gastric cancer.[30,31] Based on the present results, the activation of TLR4 receptor and secretion of IL-8 in HEK-Blue™ hTLR4 cells via TGP could represent the anticancer properties of this mushroom.

Conclusion

The present results showed that the polysaccharide fraction from *T. gibbosa* (a higher basidiomycetes) interact with TLR-4 to induce IL-8 secretion in HEK-Blue™ hTLR4 cells. This line of research will help future research to investigate PRR binding, and immune modulating activities of novel therapeutic agents that may promote antitumor immunity.

Declarations

Ethics Approval

This study was approved by Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Approval Code IR.MAZUMS.REC.1397.67) in 2018/6/27.

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Conflicts of interest

There are no conflicts of interest.

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