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Immunopotentiating properties of extracellular polysaccharide from *Trametes hirsuta* strain VKESR

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ABSTRACT

The effectiveness of extracellular polysaccharides isolated from *Trametes hirsuta* strain VKESR was evaluated for their immunostimulating properties by *in vitro* lymphocyte proliferation, phagocytic assay, NK cell activity and cytokine quantification. The splenocyte proliferation assay showed that the extracellular glucans found to have good stimulation index and enhances the NK cell mediated tumor killing. It promotes phagocytosis in treated macrophages in a dose dependent manner. Furthermore, the polysaccharides exhibit significant stimulatory effect on cytokines IL-2 and IFN-γ whereas the polysaccharide has moderate stimulatory effect pro-inflammatory cytokine TNF-α. These immunostimulating properties help in combating various diseases and could be a promising beginning for further research to study the role of extracellular polysaccharide on the host immune systems.

1. Introduction

The host immune system plays a major role in the prevention of dreadful diseases. The immune system is a system of biological structures and processes within an organism that protects against disease (Solomon et al., 1974). To function properly, an immune system must detect a wide variety of agents from viruses to parasitic worms and distinguish them from the host healthy tissue (Röllinghoff, 1997). Although immune system possesses a wide array of microbial detection and host defense mechanisms, pathogen evasion of the immune surveillance and destruction is a frequent occurrence. The impairment of immune system by autoimmune disorders, malnutrition and immunodeficiency diseases and pathogenic evolution causes escape of immune surveillance, an important characteristic of several diseases including malignancies (Wajchman et al., 2004). These diseases could be effectively suppressed by elevating the activity of immune system (Lake & Robinson, 2005).

Elevation and activation of immunity using drugs or nutrient substances are otherwise called as immunostimulation. The bioactive compounds are used to stimulate or activate the immunity are called as immunomodulators or biological response modifiers (BRM). Immunostimulation is an effective as well as protective approach against emerging infectious diseases (Hackett, 2003). Many polysaccharides isolated from mushroom are considered, to be BRMs and it enhances various type of immune effector cells such as macrophages, cytotoxic T cells and natural killer (NK) cells both *in vivo* and *in vitro* (Adachi et al., 1989; Kim et al., 2006). In these processes, various cytokines released from macrophages/monocytes may play an important role in the initial activation (Takeyama et al., 1987). Moreover, they are known to play critical roles in the induction, regulation and expression of both humoral and cellular immune responses (Ohno et al., 1993). Polysaccharides mainly present as glucans with different types of glycosidic linkages such as (1 → 3), (1 → 6)-β-glucans and (1 → 3)-α-glucans and as true heteroglycans, while others mostly bind to protein residues as polysaccharides protein complexes. Many reports suggest that polysaccharides lentinan, schizophyllan and PSK (Krestin) were made of β-glucans, isolated from *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor* respectively. It has been widely used as an anti-cancer and immunomodulatory agent (Ooi & Liu, 2000).

Polysaccharides were reported to be the active immunomodulators that potentiates both innate and adaptive immunity. Polysaccharides can directly act on the membrane receptors found in various immune cells such as macrophages, neutrophils, monocytes, NK cells and dendritic cells (Wasser, 2002). A possible mechanism for their action is by binding to β-glucan-specific

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receptors on the innate immune cells, such as monocytes or macrophages, which may lead to subsequent activation of adaptive immunity (Akramiene, Kondrotas, Didziapetriene, & Kevelaitis, 2007; Rop, Mlcek, & Jurikova, 2009). Reports also suggest that it can boost up the opsonic and non-opsonic phagocytosis and can trigger a cascade of cytokines release, such as tumor necrosis factor (TNF)-α and various types of interleukins (Zhang et al., 2007).

This study was aimed to evaluate the effectiveness of extracellular polysaccharides on immunostimulating properties. Therefore, in the present study, the immunopotentiating ability of the polysaccharides was studied by *in vitro* lymphocyte proliferation, phagocytic assay, NK cell activity and cytokine production.

2. Material and methods

2.1. Microorganism

A wood rotting fungus was isolated from a forest region near by Chennai, India and identified as *Trametes hirsuta* by morphological methods. The mushroom genomic ribosomal DNA was sequenced and submitted to the gene bank NCBI as *T. hirsuta*, strain VKESR with the accession number HQ849552 VERSION HQ849552.1 GI: 323522325. The culture was deposited in the Culture Collection Centre, CAS in botany, University of Madras, Chennai as MUBL No. 4251.

2.2. Isolation and purification of the extracellular polysaccharides

T. hirsuta was inoculated in a liquid medium containing 15% glucose, 1% peptone, 1% yeast extract, 0.05% KH2PO4, 0.05% K2HPO4, 0.05% MgSO4. The submerged cultures were grown in 4 L Haffkine flask containing 2 L infusion medium. The flasks were incubated on a rotary shaker under the conditions of 100 rpm at 26 °C for 15 days. After removal of the mycelium by filtration, the culture filtrate was subjected to the Sevag method (Staub, 1965). The supernatant was added with four volumes of 95% (v/v) ethanol to precipitate the extracellular polysaccharides (EPS). The EPS was recovered by centrifugation (2100 × *g*, 20 min) and freeze-dried to obtain crude extracellular polysaccharides. EPS were quantified by the phenol/sulphuric acid method (DuBois et al., 1956) using glucose as a standard curve. The crude EPS was dialyzed against double distilled water for three days and then injected to a column (50 × 2.0 cm^2) of DEAE-52 cellulose CL-6B equilibrated with distilled water. Then, the column was eluted with distilled water and different concentrations of NaCl aqueous solution (0–2 M NaCl) stepwise at 45 mL/h and 2.5 mL/tube. Each tube was assayed by phenol–sulphuric acid method for sugar contents (DuBois et al., 1956). Fractions eluted were collected, dialyzed, concentrated and lyophilized and subjected to immunological assays.

2.3. Phagocytic assay

Peritoneal macrophages were isolated from Wistar rats by intra peritoneal injection of 10 ml of ice-cold PBS (10 mM, pH 7.4). The resident macrophages were harvested by peritoneal lavage and the cells were subsequently cultured in RPMI 1640 complete media. The purity of macrophages was tested by adherence. Peritoneal macrophages (10^6 cells/ml) after harvest were cultured in complete RPMI 1640 media in 96-well plate. Polysaccharides were added to wells in different concentrations. The cells were cultured for 24 h at 37 °C in humidified 5% CO_2 incubator. Briefly, 1×10^7 cells of *Staphylococcus aureus* (ATCC 29213) in 0.1 ml of phosphate buffer saline (PBS) were added to 0.1 ml of samples in a micro-plate and incubated for 30 min at 37 °C, after thorough mixing. After incubation, the plate was mixed gently and 0.05 ml of this suspension smeared on the glass slide. After air-drying, the smears fixed in ethanol, stained with Giemsa, the peritoneal macrophages with and without phagocytized bacteria were counted. Assay was performed in three replicates.

2.4. Splenocyte proliferation assay

Spleen collected from sacrificed rat under aseptic conditions in Hank's balanced salt solution (HBSS), was minced using a pair of scissors and passed through a fine steel mesh to get a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × *g* at 4 °C for 10 min) the pelleted cells were washed many times in PBS and re-suspended in RPMI-1640 complete medium. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocytes were seeded into four wells of a 96-well flat-bottom microtitre plate at 5×10^6 cell/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), or RPMI1640 medium containing various concentrations of extracellular polysaccharides were added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 44 h, 50 μl of MTT solution (2 mg/ml) were added to each well and incubated for 4 h. The plates were centrifuged (1400 × *g*, 5 min) and the untransformed MTT was carefully removed by pipetting. To each well 150 μl of a DMSO working solution (180 μl DMSO with 20 μl 1 N HCl) was added, and the absorbance was evaluated in an ELISA reader (Bio-Rad, USA) at 570 nm after 15 min. The stimulation index (SI) was calculated based on the following formula: SI, the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.5. Assay of natural killer (NK) cell activity

The Wistar rats were sacrificed by cervical dislocation, and spleens were collected under aseptic conditions in RPMI-1640 medium. Spleen cells of Wistar rats were prepared by gently mincing and grinding the spleen fragment in RPMI-1640 medium on a fine steel mesh and centrifuged at 1500 rpm/min at 4 °C for 10 min and then removed the supernatant. The residue dissolved in 10 mL Tris–NH_4Cl for breaking red blood cells and kept it at room temperature for 5–10 min with shaking and centrifuged at 1500 rpm/min at 4 °C for 10 min again. The supernatant was gently removed and the precipitate was transferred to a sterile container and washed with RPMI-1640. At last, the cells were resuspended in 5 mL RPMI-1640 medium and cells viability exceeded 95%. Briefly, an aliquot of 100 μL of splenocytes named effector cells at 5×10^6 cells/mL was seeded into each well of a 96-well flat-bottom microtitre plate, thereafter samples were added giving a final volume of 200 μL and then incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. The target cells of K562 were resuspended in the RPMI-1640 medium with the final concentration of 5×10^5 cell/mL. A 100 μL of K562 target cells and 100 μL of effector cells were added to 96-well plates to give the effector to target cell (E:T) ratio of 10:1. After incubation for 4 h at 37 °C, 5% CO_2 and saturated humidity, 50 μl of MTT solution (2 mg/ml) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay. Three kinds of control measurements were performed: target cells control, blank control and effector cells control.

NK cell activity was calculated as following equation:

$$\text{NK activity}(\%) = \frac{(\text{ODT} - (\text{ODS} - \text{ODE}))}{\text{ODT}} \times 100\%$$

where, ODT = optical density value of target cells control; ODS = optical density value of test samples; ODE = optical density value of effector cells control.

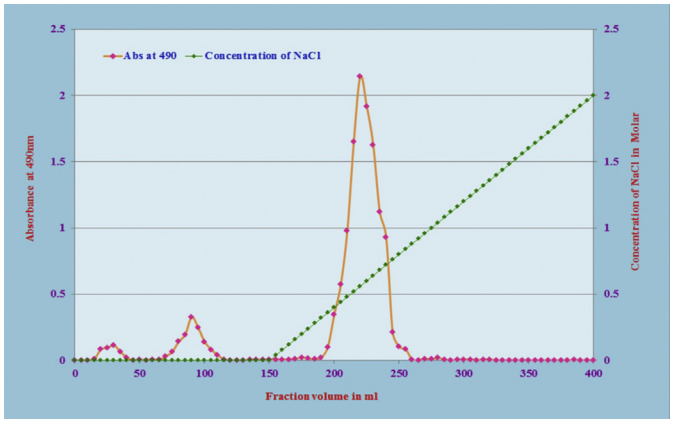

Fig. 1. Purification of extracellular polysaccharide by DEAE cellulose chromatography.

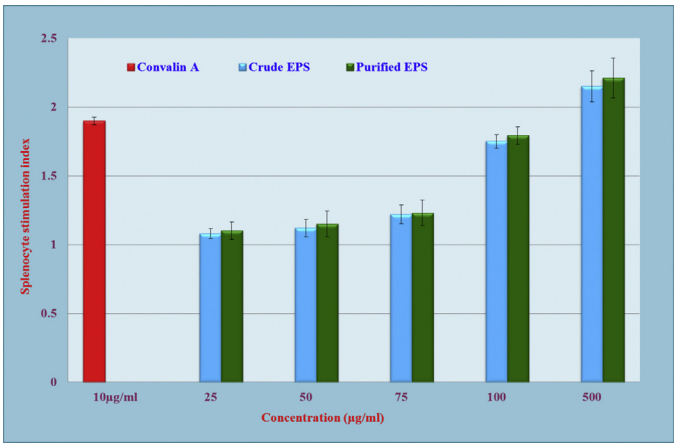

Fig. 2. Effect of extracellular polysaccharide of *T. hirsuta* on splenocyte proliferation.

2.6. Estimation of cytokine levels in the supernatants of cultured splenocytes

Splenocytes (5×10^5 cells/well) from normal rat prepared as described before were incubated with Concavalin A (final concentration 5 μg/ml), various concentration of purified polysaccharides in 24-well culture plates at 37 °C in 5% CO_2. After 48 h, the plate was centrifuged at $1400 \times g$ for 5 min and the supernatant were collected for the detection of IL-2, TNF-α and IFN-γ levels using commercial ELISA kits.

2.7. Statistical analysis

Data were analyzed by SPSS and expressed as mean ± standard deviation (SD) for at least three replicates. Significance was determined at $p < 0.05$ by one-way analysis of variance (ANOVA tests).

3. Results

3.1. Extraction and purification of polysaccharide from T. hirsuta

The crude polysaccharide was extracted with 85% ethanol from submerged culture of *T. hirsuta* and then subjected to dialysis against double distilled water. The dialyzed polysaccharide was further subjected to DEAE-cellulose chromatography. The purification with DEAE chromatography, leading to the isolation of two neutral fractions (eluted with water) and one anionic fraction (eluted with 0.1–2 M NaCl fractions). The neutral fractions are labeled as fraction 1 (F1) and fraction 2 (F2) and the anionic fractions were labeled as AF (Fig. 1). The results showed that the F1 and F2 correspond to 2.3% and 8.2% of the total eluted polysaccharide fractions. Anionic fraction was found to be the major fraction and corresponds to 89.5% of the total eluted polysaccharides fractions. The major AF was the purified polysaccharide chosen for analysis.

3.2. Effect of extracellular polysaccharide of T. hirsuta on Splenocyte proliferation

Proliferation of splenocytes is an indicator of immunoactivation. The splenocyte proliferation index (SPI) of PBS control was close to 1, above which indicates stimulatory effect on immune system. The results show that both crude and purified extracellular polysaccharides were able to induce proliferation splenocytes as shown in Fig. 2. The proliferation activity was measured using the splenocytes proliferation index. The stimulation index shows that the extracellular polysaccharide of *T. hirsuta* significantly enhances the proliferation at a dose dependent manner. The stimulation index was found to be minimum (SPI = 1.08, 1.1) at a concentration of 25 μg/mL and found to be maximum (SPI = 2.15, 2.21) at a concentration 500 μg/mL for both crude and purified EPS respectively. At the lower concentration, the stimulatory index was lower than that of the Concavalin A (1.898) but at higher concentration the results were higher and stimulation index was comparable with the Concavalin A. The results of SPI of both crude and purified polysaccharide found to be similar hence the further work was carried out in purified EPS.

3.3. Effect of extracellular polysaccharide of T. hirsuta on NK cell mediated cytotoxicity

Tumor cell elimination is known to be mediated in part by the cytotoxic activity of NK cells. Therefore, in this study effect of the extracellular polysaccharide from mushroom *T. hirsuta* on NK cell cytotoxic activity against NK cell sensitive K562 cells was evaluated and the results were shown in Fig. 3. The results show that a gradual increase in the NK cell mediated cytotoxicity was observed with increase in the concentration of the extracellular polysaccharide of *T. hirsuta*. At lower doses there was negligible amount of activity was found ie only 4.3 ± 0.2% at the concentration 25 μg/mL which was very closer to the control (4.1%). The concentration above 50 μg/mL shows a moderate NK cell mediated cytotoxicity and was maximum at the concentration of 500 μg/mL with killing percentage of 28.9 ± 0.8%. The result shows that the extracellular

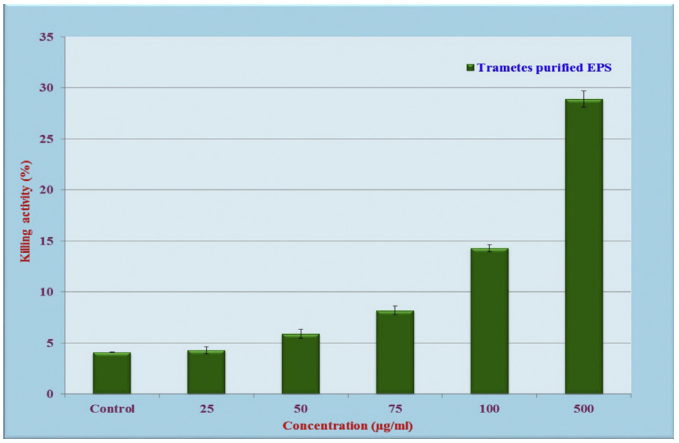

Fig. 3. Effect of extracellular polysaccharide of *T. hirsuta* on NK cell mediated cytotoxicity.

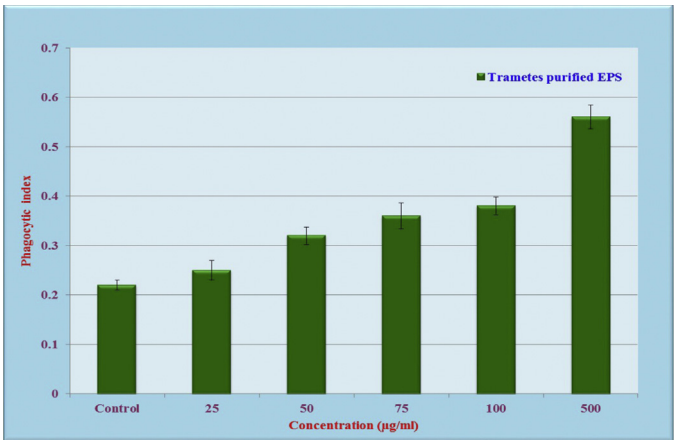

Fig. 4. Effect of extracellular polysaccharide of *T. hirsuta* on phagocytic assay.

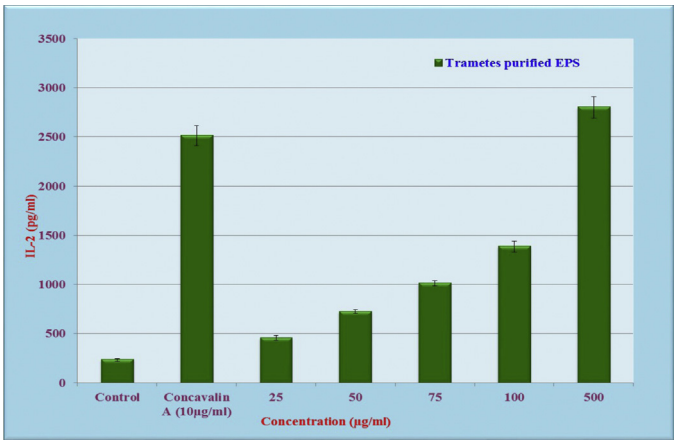

Fig. 5. Effect of extracellular polysaccharide of *T. hirsuta* on production of cytokine IL-2 by rat splenocytes.

polysaccharide from *T. hirsuta* could significantly increase the NK cell mediated cytotoxicity.

3.4. Effect of extracellular polysaccharide of T. hirsuta on the phagocytic activity

Polysaccharides are good stimulators of macrophage owing to the presence of various receptors on the macrophage membrane. To check the ability of the extracellular polysaccharide from *T. hirsuta* to stimulate macrophage, number of phagocytes containing *Staphylococcus aureus* was counted against the total number of phagocytes counted after stimulating the macrophages in presence of extracellular polysaccharide *in vitro*. As shown in Fig. 4 the results show that phagocytic indexes of the extracellular polysaccharide were significantly higher with increasing concentration of extracellular polysaccharide. A maximum phagocytic index of 0.56 ± 0.03 was found at the concentration of 500 μg/mL of extracellular polysaccharide. At lower concentration (25 μg/mL) the phagocytic index was 0.25 ± 0.02 and found to be minimal. However, the 100 μg/mL of the extracellular polysaccharide shows moderate amount of phagocytic activity with phagocytic index of 0.38 ± 0.01.

3.5. Effect of extracellular polysaccharide of T. hirsuta on the cytokine production of the rat splenocytes

In order to elucidate the stimulatory activities of purified EPS, the concentrations of three cytokines, namely IL-2, TNF-α and IFN-γ, in the culture medium of lymphocytes were determined by ELISA. The IL-2, IFN-γ and TNF-α production by polysaccharide activated spleen lymphocytes of rat were shown in Figs. 5–7. The results show that the extracellular polysaccharides have stimulatory effect on the production of cytokines IL-2, IFN-γ and TNF-α by the rat splenocytes in a dose dependent manner. The extracellular polysaccharides at a lower concentration was found less active, but stimulate very lower level of cytokines i.e., at a concentration of 25 μg/mL the level of cytokines were 456 ± 11 pg/mL, 189 ± 14 pg/mL, 4286 ± 124 pg/mL for IL-2, TNF-α and IFN-γ respectively. The IL-2, TNF-α and IFN-γ cytokines level of the control splenocytes were 232 ± 12 pg/mL, 159 ± 8 pg/mL, 3418 ± 121 pg/mL. The Concavalin A is known mitogen showed strong stimulation of cytokines. The level of IL-2, TNF-α and IFN-γ cytokines in case of Concavalin A were 2512 ± 31 pg/mL, 968 ± 17 pg/mL and 24232 ± 254 pg/mL respectively. However, the results were comparable with Concavalin A at a higher concentration of extracellular polysaccharides for IL-2 and IFN-γ. The level of IL-2, TNF-α and IFN-γ cytokines at the concentration of 500 μg/mL of polysaccharides were 2800 ± 22 pg/mL,

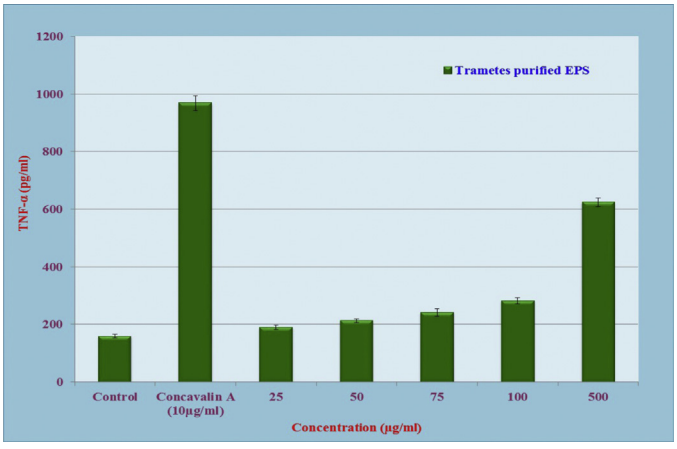

Fig. 6. Effect of extracellular polysaccharide of *T. hirsuta* on production of cytokine TNF-α by rat splenocytes.

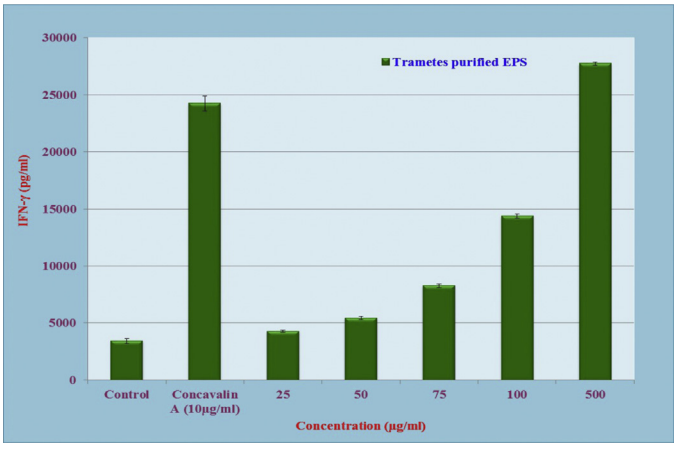

Fig. 7. Effect of extracellular polysaccharide of *T. hirsuta* on production of cytokine IFN-γ by rat splenocytes.

624 ± 24 pg/mL, 27722 ± 412 pg/mL respectively. These results show the polysaccharide a significant simulator of cytokines except for TNF-α where the moderate stimulation was achieved.

4. Discussion

Cell mediated immune system plays an important role in antitumor defense. In addition to killing the tumor cells directly, T-cells can produce many lymphocyte factors which could promote the proliferation and differentiation of immune cells, macrophage phagocytosis and the capacity of killing target cells, so that they play a role in preventing tumor (Kim et al., 2001). Many reports suggested that the antitumor activity of the polysaccharides from several traditional mushrooms was mediated through augmentation of the immune response (Cho & Leung, 2007; Lee & Jeon, 2005; Schepetkin & Quinn, 2006; Wasser, 2002).

Lymphocytes are the key effector cells of mammalian immune system. The capacity to elicit an effective T and B-cell immunity can be shown by the stimulation of lymphocyte proliferation response (Marciani et al., 2000). The evaluation of substances that either promote or inhibit immunocyte proliferation is crucial to the study of immunomodulation and drug discovery. It is generally known that Con A stimulates T-cells and LPS stimulates B-cell proliferation. There are a number of studies reported that polysaccharides from various sources such as plants, mushrooms and animals can enhance the immunocyte proliferation (Kurashige, Akuzawa, & Endo, 1997). This study showed that extracellular polysaccharide from *T. hirsuta* could significantly promote the splenocyte proliferation. Similar proliferating activity was observed in splenocytes treated with polysaccharides from *Trametes versicolor* (Cui et al., 2007), *Trametes robiniophila* (Jia, Dong, Lu, Guo, & Wei, 2009), *Polyporus albicans* (Sun et al., 2008), *Phellinus linteus* (Kim et al., 2006) and *Pleurotus ostreatus* (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). The results indicated that the extracellular polysaccharides from *T. hirsuta* should significantly increase the activation potential of splenocytes.

NK cells make up 10–15% of peripheral blood lymphocytes (Campbell and Colonna, 2001) and represent the first line of defense against many pathogens, especially viruses. Upon activation, NK cells begin to proliferate and secrete cytokines as a means of communication with other components of the immune system, in particular T cells. NK cells are best known for their capacity to kill tumor cells and there is evidence for their role in controlling infection in the earliest phases of body's immune responses. One of the primary objectives of immunomodulation is to enhance the number and vitality of NK cells. Generally, polysaccharide inducing NK cells activation increases with increasing concentration of polysaccharide. The results obtained from this study shows that it significantly increases the NK cell mediated cytotoxicity in a dose dependent manner. Similar result was also observed by Chien et al., (2004) on the Polysaccharides of *Ganoderma lucidum*, which increased NK cell cytotoxicity in cord blood. Moreover, the polysaccharide from *Pleurotus ostreatus* (Sarangi et al., 2006) and *Trametes robiniophila* (Jia et al., 2009) also enhanced the NK cell mediated cytotoxicity, which is comparable with NK cell activity induced by *T. hirsuta*. The results suggest that the polysaccharide of *T. hirsuta* found to have good antitumor activity by activating the NK cell mediated cytotoxicity.

Macrophages, which are the part of innate immune system, play an essential in protecting our body from invading cells includes cancerous cells (Kurashige et al., 1997). It is well documented that polysaccharides like $\beta(1 \rightarrow 3)$ glucans bind to toll-like receptors on macrophage and trigger activation processes (Taylor et al., 2002). Glucan is the most widely and commonly observed macrophage activator in nature. In the present study, the phagocyte activity of extracellular polysaccharide was significantly higher than that of the control. Similar results were observed with polysaccharide–peptide complex from cultured mycelia of mushroom *Tricholoma mongolicum*, which can activate macrophage significantly (Wang et al., 1996). A series of studies using purified polysaccharide showed a stimulatory effect on the macrophages (Benson et al., 2010; Percival, 2000). These effects include an increase in phagocytosis, chemotaxis and oxidative burst of either neutrophils or macrophages (Sadigh-Eteghad et al., 2011). Many polysaccharides have been reported for their immunomodulatory activities in mammals as they modify the activity of macrophages both *in vitro* and *in vivo* (Yoshizawa et al., 1993). The results showed that extracellular polysaccharides from *T. hirsuta* enhanced the macrophage phagocytosis, consistent with the previously reported findings on polysaccharides of *Morchella esculenta* (Cui, Guo, & Xiao, 2011).

Cytokines are central mediators of pathological processes and they are involved in necrosis, inflammation, apoptosis and fibrosis. Cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions. IL-2 has many immunopotentiating effects, such as proliferation of T-cells, B-cells, NK cells and monocytes, augmentation of cytotoxicites of T-cells and NK cells and *in vivo* generation of lymphokine-activated killer (LAK) cells, which exhibit high cytolytic activities against autologous tumor cells (Asano et al., 1997). IFN-γ is one of the major important immunoregulatory molecule with antitumor and immunomodulatory properties (Blankenstein & Qin, 2003). IFN-γ could inhibit cell proliferation and angiogenesis in the tumor microenvironment (Boehm et al., 1997). TNF-α is the most important proinflammatory cytokine, a peptide mediator released by monocytes and macrophages in response to various stimuli including bacterial LPS (Chamulitrat et al., 1994). In the present study, the levels of IL-2, TNF-α and IFN-γ were increased significantly with the concentration of polysaccharide. In general, the immunostimulatory effects of mushroom polysaccharides are attributed to macrophages and T-cells. Several studies reported changes in cytokine production of macrophages or monocytes in mice and in humans, e.g., enhanced production of TNF-α after *in vivo* polysaccharide administration (Lull et al., 2005; XuJie et al., 2008). Overproduction of TNF-α is associated with a wide range of pathologic conditions and has therefore led to much recent effort to find ways to down regulate its production or inhibit its effects *in vivo* (Marriott et al., 1998). Thus, our results show that the extracellular polysaccharides are moderate stimulator of proinflammatory cytokines TNF-α and suggested that the extracellular polysaccharides might modulate the production of cytokines by splenocytes as mentioned in previous studies (Kohguchi et al., 2004; Ning et al., 2003).

The results of the above study show that the polysaccharides from *T. hirsuta* possess a significant immunostimulatory property, although the exact underlying mechanism of extracellular polysaccharide is yet unknown, based on the results presented above, can conclude that extracellular polysaccharides have immunomodulatory activity by proliferating splenocyte, by stimulating macrophages and NK cells involved in nonspecific immunity.

5. Conclusion

This study showed immunostimulating potentials of both crude and purified polysaccharide from *T. hirsuta*. On the whole the extracellular polysaccharide has good *in vitro* immunostimulating properties and help in combating various diseases. This research could be a promising beginning for further research on role of extracellular polysaccharide the host immune systems. Further studies

required to establish these properties under *in vivo* condition may be important in developing potent, safer drug.

References

Adachi, Y., Ohno, N., Ohsawa, M., Sato, K., Oikawa, S., & Yadomae, T. (1989). Physiochemical properties and antitumor activities of chemically modified derivatives of antitumor glucan "grifolan {LE"} from Grifola frondosa. *Chemical and Pharmaceutical Bulletin*, *37*(7), 1838–1843.

Akramiene, D., Kondrotas, A., Didziapetriene, J., & Kevelaitis, E. (2007). Effects of beta-glucans on the immune system. *Medicina (Kaunas)*, *43*(8), 597–606.

Asano, Y., Kaneda, K., Hiragushi, J., Tsuchida, T., & Higashino, K. (1997). The tumor-bearing state induces augmented responses of organ-associated lymphocytes to high-dose interleukin-2 therapy in mice. *Cancer Immunology, Immunotherapy*, *45*(2), 63–70.

Benson, J. M., Pokorny, A. J., Rhule, A., Wenner, C. A., Kandhi, V., Cech, N. B., & Shepherd, D. M. (2010). Echinacea purpurea extracts modulate murine dendritic cell fate and function. *Food and Chemical Toxicology*, *48*(5), 1170–1177. http://dx.doi.org/10.1016/j.fct.2010.02.007.

Blankenstein, T., & Qin, Z. (2003). The role of {IFN-gamma} in tumor transplantation immunity and inhibition of chemical carcinogenesis. *Current Opinion in Immunology*, *15*(2), 148–154.

Boehm, T., Folkman, J., Browder, T., & O'Reilly, M. S. (1997). Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, *390*(6658), 404–407. http://dx.doi.org/10.1038/37126.

Chamulitrat, W., Jordan, S. J., & Mason, R. P. (1994). Nitric oxide production during endotoxic shock in carbon tetrachloride-treated rats. *Molecular Pharmacology*, *46*(2), 391–397.

Cho, W. C. S., & Leung, K. N. (2007). In vitro and in vivo anti-tumor effects of Astragalus membranaceus. *Cancer Letters*, *252*(1), 43–54. http://dx.doi.org/10.1016/j.canlet.2006.12.001.

Cui, J., Goh, K. K. T., Archer, R., & Singh, H. (2007). Characterisation and bioactivity of protein-bound polysaccharides from submerged-culture fermentation of Coriolus versicolor Wr-74 and ATCC-20545 strains. *Journal of Industrial Microbiology & Biotechnology*, *34*(5), 393–402. http://dx.doi.org/10.1007/s10295-007-0209-5.

Cui, Jin-long, Guo, S., & Xiao, P. (2011). Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of Aquilaria sinensis. *Journal of Zhejiang University Science B:Biomedicine & Biotechnology*, *12*(5), 385–392. http://dx.doi.org/10.1631/jzus.B1000330.

Hackett, C. J. (2003). Innate immune activation as a broad-spectrum biodefense strategy: Prospects and research challenges. *Journal of Allergy and Clinical Immunology*, *112*(4), 686–694. http://dx.doi.org/10.1016/S0091.

Jia, X.-N., Dong, W., Lu, W.-D., Guo, L.-Z., & Wei, Y.-X. (2009). In vivo immunostimulatory and tumor-inhibitory activities of polysaccharides isolated from solid-state-cultured Trametes robiniophila Murrill. *World Journal of Microbiology and Biotechnology*, *25*(11), 2057–2063. http://dx.doi.org/10.1007/s11274-009-0109-0.

Kim, G.-Y., Lee, J.-Y. J.-O., Ryu, C.-H., Choi, B. T., Jeong, Y.-K., Lee, K.-W., Jeong, S.-C., et al. (2006). Partial characterization and immunostimulatory effect of a novel polysaccharide-protein complex extracted from Phellinus linteus. *Bioscience, Biotechnology, and Biochemistry*, *70*(5), 1218–1226.

Kim, K. D., Choi, S. C., Kim, A., Choe, Y. K., Choe, I. S., & Lim, J. S. (2001). Dendritic cell-tumor coculturing vaccine can induce antitumor immunity through both {NK} and {CTL} interaction. *International Immunopharmacology*, *1*(12), 2117–2129.

Kohguchi, M., Kunikata, T., Watanabe, H., Kudo, N., Shibuya, T., Ishihara, T., Iwaki, K., et al. (2004). Immuno-potentiating effects of the antler-shaped fruiting body of Ganoderma lucidum (Rokkaku-Reishi). *Bioscience, Biotechnology, and Biochemistry*, *68*(4), 881–887.

Kurashige, S., Akuzawa, Y., & Endo, F. (1997). Effects of Lentinus edodes, Grifola frondosa and Pleurotus ostreatus administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, *N*-butyl-*N*-butanolnitrosoamine. *Immunopharmacology and Immunotoxicology*, *19*(2), 175–183. http://dx.doi.org/10.3109/08923979709007657.

Lake, R. A., & Robinson, B. W. S. (2005). Immunotherapy and chemotherapy – A practical partnership. *Nature Reviews Cancer*, *5*(5), 397–405. http://dx.doi.org/10.1038/nrc1613.

Lee, K. Y., & Jeon, Y. J. (2005). Macrophage activation by polysaccharide isolated from Astragalus membranaceus. *International Immunopharmacology*, *5*(7–8), 1225–1233. http://dx.doi.org/10.1016/j.intimp.2005.02.020.

Lull, C., Wichers, H. J., & Savelkoul, H. F. J. (2005). Antiinflammatory and immunomodulating properties of fungal metabolites. *Mediators of Inflammation*, *2005*(2), 63–80. http://dx.doi.org/10.1155/MI.2005.63.

Marciani, D. J., Press, J. B., Reynolds, R. C., Pathak, A. K., Pathak, V., Gundy, L. E., Farmer, J. T., et al. (2000). Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine*, *18*(27), 3141–3151.

Marriott, J. B., Westby, M., Cookson, S., Guckian, M., Goodbourn, S., Muller, G., Shire, M. G., et al. (1998). {CC-3052:} a water-soluble analog of thalidomide and potent inhibitor of activation-induced {TNF-alpha} production. *The Journal of Immunology*, *161*(8), 4236–4243.

Ning, J., Zhang, W., Yi, Y., Yang, G., Wu, Z., Yi, J., & Kong, F. (2003). Synthesis of beta-(1 → 6)-branched beta-(1 → 3) glucohexaose and its analogues containing an alpha-(1 → 3) linked bond with antitumor activity. *Bioorganic and Medicinal Chemistry*, *11*(10), 2193–2203.

Ohno, N., Saito, K., Nemoto, J., Kaneko, S., Adachi, Y., Nishijima, M., Miyazaki, T., et al. (1993). Immunopharmacological characterization of a highly branched fungal (1 → 3)-beta-D-glucan, {OL-2}, isolated from Omphalia lapidescens. *Biological and Pharmaceutical Bulletin*, *16*(4), 414–419.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*(7), 715–729.

Percival, S. S. (2000). Use of echinacea in medicine. *Biochemical Pharmacology*, *60*(2), 155–158.

Rop, O., Mlcek, J., & Jurikova, T. (2009). Beta-glucans in higher fungi and their health effects. *Nutrition Reviews*, *67*(11), 624–631. http://dx.doi.org/10.1111/j.1753-4887.2009.00230.

Röllinghoff, M. (1997). Immunity, components of the immune system and immune response. *Biologicals*, *25*(2), 165–168. http://dx.doi.org/10.1006/biol.1997.0078.

Sadigh-Eteghad, S., khayat-Nuri, H., Abadi, N., Ghavami, S., Golabi, M., & Shanebandi, D. (2011). Synergetic effects of oral administration of levamisole and Echinacea purpurea on immune response in Wistar rat. *Research in Veterinary Science*, *91*(1), 82–85. http://dx.doi.org/10.1016/j.rvsc.2010.07.027.

Sarangi, I., Ghosh, D., Bhutia, S. K., Mallick, S. K., & Maiti, T. K. (2006). Anti-tumor and immunomodulating effects of Pleurotus ostreatus mycelia-derived proteoglycans. *International Immunopharmacology*, *6*(8), 1287–1297. http://dx.doi.org/10.1016/j.intimp.2006.04.002.

Schepetkin a, I., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*(3), 317–333. http://dx.doi.org/10.1016/j.intimp.2005.10.005.

Solomon, G. F., Amkraut, A. A., & Kasper, P. (1974). Immunity, emotions and stress. With special reference to the mechanisms of stress effects on the immune system. *Annals of Clinical Research*, *6*(6), 313–322.

Sun, Y., Wang, S., Li, T., Li, X., Jiao, L., & Zhang, L. (2008). Purification, structure and immunobiological activity of a new water-soluble polysaccharide from the mycelium of Polyporus albicans (Imaz.) teng. *Bioresource Technology*, *99*(4), 900–904. http://dx.doi.org/10.1016/j.biortech.2007.01.029.

Takeyama, T., Suzuki, I., Ohno, N., Oikawa, S., Sato, K., Ohsawa, M., & Yadomae, T. (1987). Host-mediated antitumor effect of grifolan {NMF-5N}, a polysaccharide obtained from Grifola frondosa. *Journal of Pharmacobio-dynamics*, *10*(11), 644–651.

Taylor, P. R., Brown, G. D., Reid, D. M., Willment, J. A., Martinez-Pomares, L., Gordon, S., & Wong, S. Y. C. (2002). The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *The Journal of Immunology*, *169*(7), 3876–3882.

Wajchman, H. J., Pierce, C. W., Varma, V. A., Issa, M. M., Petros, J., & Dombrowski, K. E. (2004). Ex vivo expansion of CD8 + CD56+ and CD8 + CD56– natural killer T cells specific for MUC1 mucin. *Cancer Research*, *64*(3), 1171–1180. http://dx.doi.org/10.1158/0008-5472.

Wang, Z. T., Ng, T. B., Yeung, H. W., & Xu, G. J. (1996). Immunomodulatory effect of a polysaccharide-enriched preparation of Codonopsis pilosula roots. *General Pharmacology*, *27*(8), 1347–1350.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*(3), 258–274. http://dx.doi.org/10.1007/s00253-002-1076-7.

XuJie, H., Na, Z., SuYing, X., ShuGang, L., & BaoQiu, Y. (2008). Extraction of BaChu mushroom polysaccharides and preparation of a compound beverage. *Carbohydrate Polymers*, *73*(2), 289–294. http://dx.doi.org/10.1016/j.carbpol.2007.11.033.

Yoshizawa, Y., Enomoto, A., Todoh, H., Ametani, A., & Kaminogawa, S. (1993). Activation of murine macrophages by polysaccharide fractions from marine algae (Porphyra yezoensis). *Bioscience, Biotechnology, and Biochemistry*, *57*(11), 1862–1866.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*(1), 4–19. http://dx.doi.org/10.1016/j.tifs.2006.07.013.