



Original Research Article

Immunostimulatory effects of *Trehala manna* ethanolic extract on splenocytes and peritoneal macrophages *in vitro*

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Abstract

Background: *Trehala manna* (commonly known as Shekar tighal in Iran) is produced by the salivary glands of weevils on some *Echinops* species and has been widely used in Iranian traditional medicine for the treatment of asthma, constipation, microbial infections, etc. In this study, the effects of *Trehala manna* ethanolic extract (TMEE) on murine splenocytes and peritoneal macrophages were evaluated, *in vitro*. PHA- or LPS-stimulated splenocytes were treated with TMEE.

Materials and Methods: Then, cell proliferation and cytokine production of PHA-stimulated splenocytes were determined by MTT assay and ELISA method, respectively. We also evaluated the effect of TMEE on the viability and nitric oxide (NO) production of LPS-stimulated macrophages using MTT assay and Griess reaction, respectively.

Results: Our results showed that TMEE significantly increased PHA- and LPS-stimulated splenocytes proliferation and IFN- γ production but had no effect on the secretion of IL-4 by PHA-stimulated splenocytes. We also found that TMEE significantly increased the viability and NO production of LPS-stimulated macrophages.

Conclusion: Taken together, these results suggest that TMEE has immunostimulatory effects on splenocytes and macrophages, *in vitro*.

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Introduction

Manna is a kind of sugar material produced on the leaves and stalks of young plants by some insects or via the reaction of plant mechanical factors or temperature out of plant tissues (Mohammadi and Dini, 2003). *Trehala* manna, also known as Shekar tighal, *Echinops* manna, or insect manna, is one of the main types of manna that is produced by insects and can be found in different areas of Africa and central Asia, especially in Iran (Dorling, 2008; Mohammadi and Dini, 2003). In Iran, *Trehala* manna is produced by the salivary glands of weevils (*Larinus* spp. from Curculionidae family) on *Echinops* spp. such as *E. cephalotes* Dc, *E. endotrichus* Rech. f., *E. persicus* Stev., *E. robustus* Bunge., and *E. pungens* Trautv (Dini et al, 2002; Mohammadi and Dini, 2003).

Trehala manna contains a large variety of different components such as water-soluble polysaccharides (e.g. cellulose and trehalose), tannin, albuminoid matter, lipid, and mucilage (Karam, 2012; Hamed et al, 2015; Mohammadi and Dini, 2003). It has laxative, antitussive, anti-asthmatic, febrifuge, anti-infective, and antioxidant effects and has been widely used in Iranian traditional medicine (Dini et al, 2002; Amiri and Joharchi, 2013). Despite extensive experimental investigations on the therapeutic effects of *Trehala* manna, very

little attention has been paid to its effects on the immune system (Hamed et al, 2015).

For this purpose, in the present study, we investigated the effect of *Trehala* manna on macrophages and splenocytes, *in vitro*. Macrophages, as innate immune cells, play a central role in protecting the body against pathogens and regulating immune responses (Parihar et al, 2010). Moreover, splenocytes consist of a variety of adaptive immune cells such as B and T lymphocytes. In this study, macrophages and splenocytes were chosen as they are important parts of the innate and adaptive immune systems.

Experimental

Extract preparation

Trehala manna (Figure 1) was collected in September 2015 from plants growing in Khorasan Razavi province, Iran and authenticated by an expert pharmacognosist. For preparation of the ethanolic extract, 200 g of *Trehala* manna was macerated with 500 ml of ethanol-water mixture [EtOH: H₂O (50:50, V/V)] for 48 hr. After that, the extract was shaken and filtered, and the solvent was evaporated in a rotating evaporator (Büchi, Flawil, Switzerland) under reduced pressure until dryness. The yield of the extraction ranged from 10% to 12%.

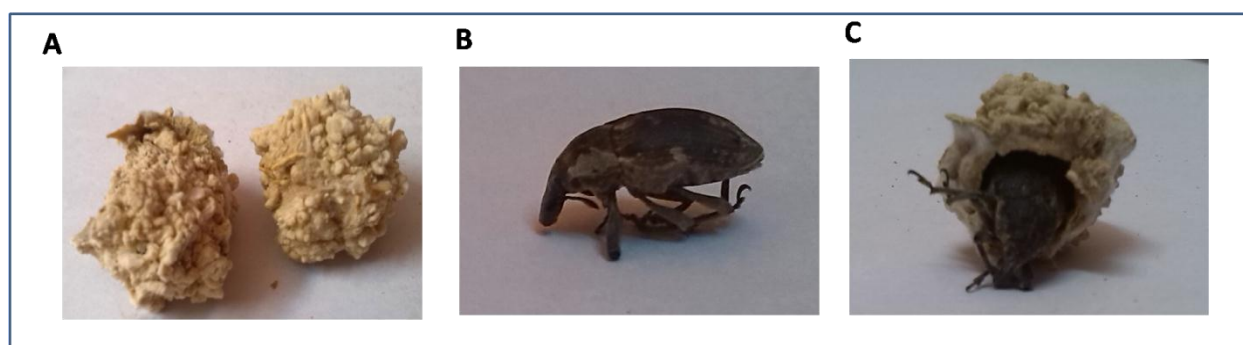


Figure 1. *Trehala* manna and Adult weevil. A) *Trehala* manna is produced by the salivary glands of weevils (*Larinus* spp. from Curculionidae family) on *Echinops* spp. B) A weevil; a type of beetle from the Curculionidae family. C) An adult weevil inside *Trehala* manna, after the larval stage.

Animals

Twenty four 6-8 week-old female BALB/c mice were purchased from Razi Institute (Mashhad, Iran). They had free access to standard mouse chow and water, *ad libitum*. The experimental protocols used in this study were approved by Animal Ethics Committee of North Khorasan University of Medical Sciences, Bojnord, Iran.

Isolation and culture of macrophages and splenocytes

Isolation of mouse peritoneal macrophages from female BALB/c mice was carried out according to the procedure described by Bibak et al. (Bibak et al, 2012). Briefly, murine peritoneal cells were harvested by lavage of the peritoneal cavity with 10 ml of RPMI 1640 (Invitrogen, Germany). The cells were centrifuged at 200g, washed and cultured in petri dishes at 37°C for 4 hr. After

removing non-adherent cells by washing, the trypsinized cells were adjusted to 1×10^6 cells/ml in complete RPMI medium (RPMI medium with 10% FCS (Invitrogen, Germany), and 50 IU/ml penicillin/streptomycin (Sigma-Aldrich, USA). The isolated cell suspensions of macrophages were also used for the next experiments.

Spleens from female BALB/c mice were removed and washed with sterile Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, USA). Spleens were finely minced with scissors, and passed through a 70 μ m mesh. After washing twice with PBS, erythrocytes were removed using 0.83% NH_4Cl (Merck, Germany), and after washing, the remaining cells were suspended in RPMI 1640 medium with 10% FCS.

Treatment of peritoneal macrophages with the ethanolic extract of *Trehala manna*

To evaluate the effects of the ethanolic extract of *Trehala manna* (TMEE) on the viability and nitric oxide (NO) production of macrophages, in each well, 2×10^5 cells (200 μ l/well in 96-well flat-bottom plates) were treated with or without lipopolysaccharide (LPS) (10 μ g/ml from *Escherichia coli* O111: B4, Sigma, USA). Next, different concentrations of TMEE (0.1, 1, 10, 20, and 50 μ g/ml) were added to wells and cells were cultured at 37°C with 5% CO₂ for 48 hr (Bibak et al., 2012). Macrophages cultured in complete RPMI medium and treated with PBS, served as the negative control group. LPS-treated macrophages cultured in complete RPMI medium, were used as positive control group. In addition, complete RPMI medium was used as the blank control group.

After 48-hr incubation (Bibak et al., 2012), cell culture supernatants were collected and evaluated for the stable end-product of NO, nitrates, and nitrites, using the Griess reaction according to the manufacturer's instruction manual (Cayman Chemical, USA). We used MTT (Merck, Germany) assay to evaluate macrophage viability after 48-hr incubation with different treatments described above.

Treatment of mitogen-stimulated splenocyte with TMEE

To investigate the immunomodulatory effect of TMEE, splenocytes were plated on a 96-well flat-bottom plates at a density of 1×10^6 /ml (200 μ l per well) and cultured in complete RPMI 1640 medium. Cells were stimulated with phytohaemagglutinin (PHA) (Baharafshan, Iran) 10 μ g/ml or LPS (Sigma-Aldrich, USA) 5 μ g/ml in the presence or absence of different concentrations of TMEE (0.1, 1, 10, 20, and 50 μ g/ml) at 37°C with 5% CO₂ for 72 hr (Bibak et al., 2012). The splenocytes cultured in complete RPMI medium and treated with PBS, served as the negative control group. After the incubation time, cell-free supernatants were collected and stored at -70°C for measurement of IL-4 and IFN- γ by ELISA. Subsequently, cell proliferation was assessed using MTT assay (Ahmadabad et al., 2011). The results were reported as Stimulation Index (SI), which is (sample OD 540 nm – blank OD 540 nm) / negative control OD 540 nm.

Cytokine assay

The concentrations of IL-4 and IFN- γ cytokines in the supernatant of cultured splenocytes were measured by sandwich ELISA according to the instructions of the manufacturer (eBioscience, USA). The sensitivity of the ELISA kits for IL-4 and

IFN- γ were 4 pg/ml and 15 pg/ml, respectively.

Statistical analysis

GraphPad Prism software version 5.0 (GraphPad Software, USA) was used for statistical analysis of the data. For each group, the mean of three experiments was calculated. In addition, one-way analysis of variance (ANOVA), followed by Tukey's tests was used for comparison of the means. Data were presented as mean \pm SEM. *p* values less than 0.05 were considered statistically significant.

Results

Effects of the ethanolic extract of *Trehala manna* on mitogen-stimulated splenocyte proliferation

In vitro analysis of PHA-stimulated splenocytes proliferation showed that TMEE significantly increased splenocytes proliferation in the concentration range of 1–50 μ g/ml (***p* < 0.01 as compared to the negative control group) (Figure 2). As shown in Figure 2, TMEE could also significantly increase LPS-stimulated splenocytes proliferation within the concentration range of 0.1–50 μ g/ml (***)*p* < 0.001 as compared to the negative control group).

IFN- γ and IL-4 levels in culture supernatants of PHA-stimulated splenocytes

To determine the effect of TMEE on Th1/Th2 cytokine pattern, we measured IL-4 and IFN- γ concentrations in culture supernatants of PHA-stimulated splenocytes using ELISA kits.

Our results showed that treatment of PHA-stimulated splenocytes with TMEE (0.1–50 μ g/ml) significantly decreased IL-4 (***)*p* < 0.001, and increased IFN- γ (***)*p* < 0.001 levels in cell culture supernatants (Figure 3).

Discussion

In this study, we investigated the immunomodulatory effect of TMEE on splenocytes and macrophages. For this purpose, we treated PHA or LPS-stimulated splenocytes and LPS-stimulated macrophages with different concentrations of TMEE, *in vitro*. We considered PHA-stimulated splenocytes as T cells, LPS-stimulated lymphocytes as B cells and macrophages as main innate immune cells.

Our results showed that TMEE significantly increased PHA- and LPS-stimulated splenocytes proliferation (Figure 2). With regard to the fact that PHA and LPS are mitogens for T and B cell activation, respectively, possibly TMEE enhances T and B cell-mediated immune responses. In other

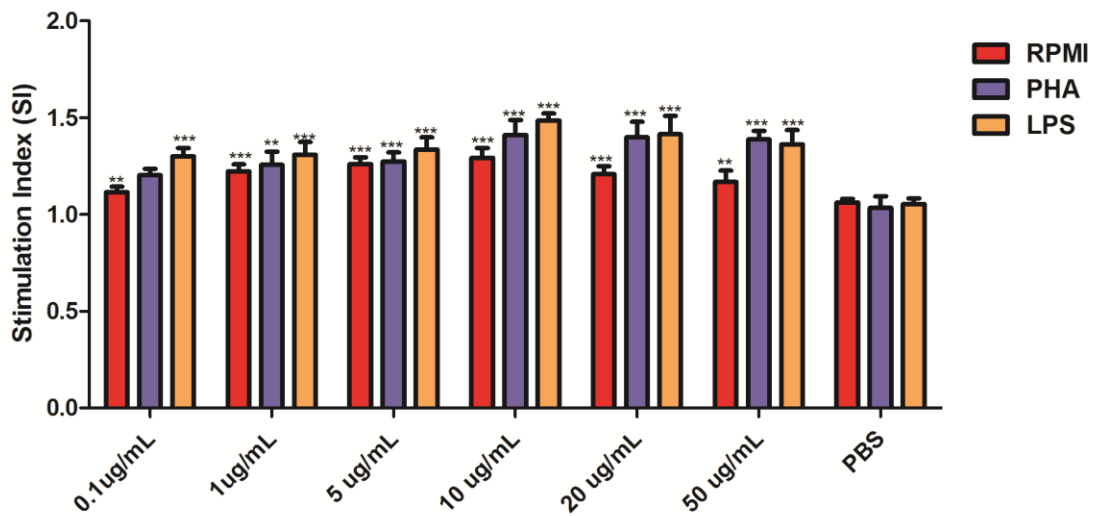


Figure 2. The effect of *Trehala manna* ethanolic extract (TMEE) on mitogen-stimulated splenocyte proliferation. Unstimulated splenocytes (RPMI group) and LPS or PHA-stimulated splenocytes were treated with different concentrations of TMEE (0.1, 1, 10, 20, and 50 µg/ml) and cell proliferation was measured by MTT assay. TMEE significantly increased LPS (0.1-50 µg/ml, ***p < 0.001) and PHA (1-50 µg/ml, **p < 0.01)-stimulated splenocytes proliferation compared to PBS group as the control group. Stimulation Index (SI): optical density of test of each group / optical density of control of each group. Results are expressed as mean ± SEM of three independent experiments.

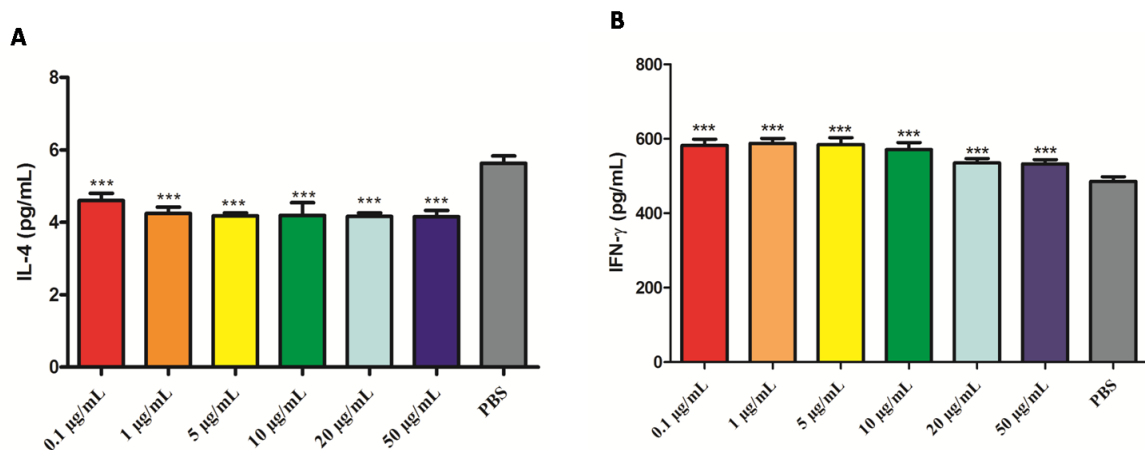


Figure 3. The effect of *Trehala manna* ethanolic extract (TMEE) on IL-4 and IFN-γ production by PHA-stimulated splenocytes. PHA-stimulated splenocytes were treated with different concentrations of TMEE (0.1, 1, 10, 20, and 50 µg/ml) and concentrations of IFN-γ and IL-4 in culture supernatants were measured by ELISA. (A and B) TMEE significantly increased IFN-γ production (***p < 0.001) and decreased IL-4 production (***p < 0.001) of PHA-stimulated splenocytes compared to control group (PHA-stimulated splenocytes treated with PBS). Results are reported as mean ± SEM of three independent experiments.

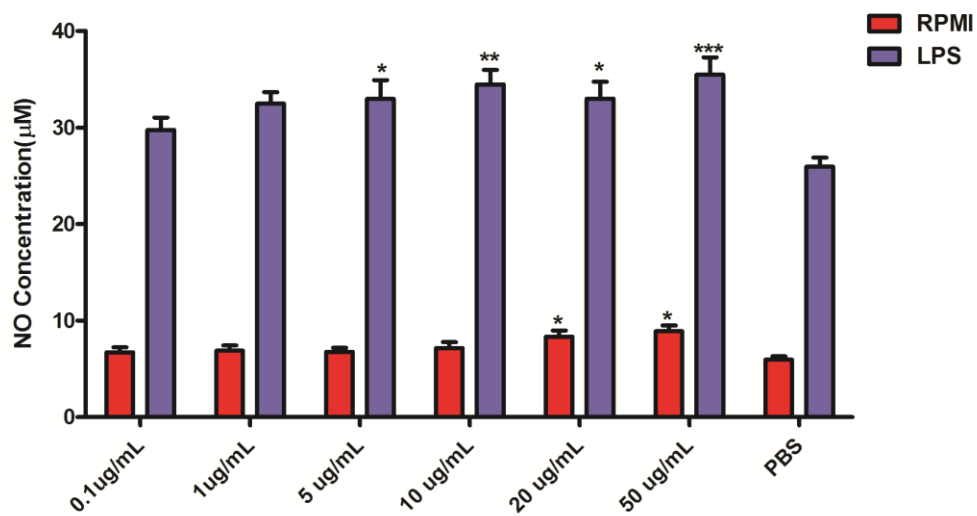


Figure 4. The effect of *Trehala manna* ethanolic extract (TMEE) on NO production by macrophages. Unstimulated macrophages and LPS stimulated-macrophages were treated with different concentrations of TMEE (0.1, 1, 10, 20, and 50 µg/ml) and NO production was measured by Griess reaction. TMEE significantly increased NO production of macrophages (at the concentrations of 20 and 50 µg/ml) and LPS stimulated-macrophages (at the concentrations of 5, 10, 20 and 50 µg/ml). * p<0.05, ** p<0.01 and *** p<0.001. Results are expressed as mean ± SEM of three independent experiments.

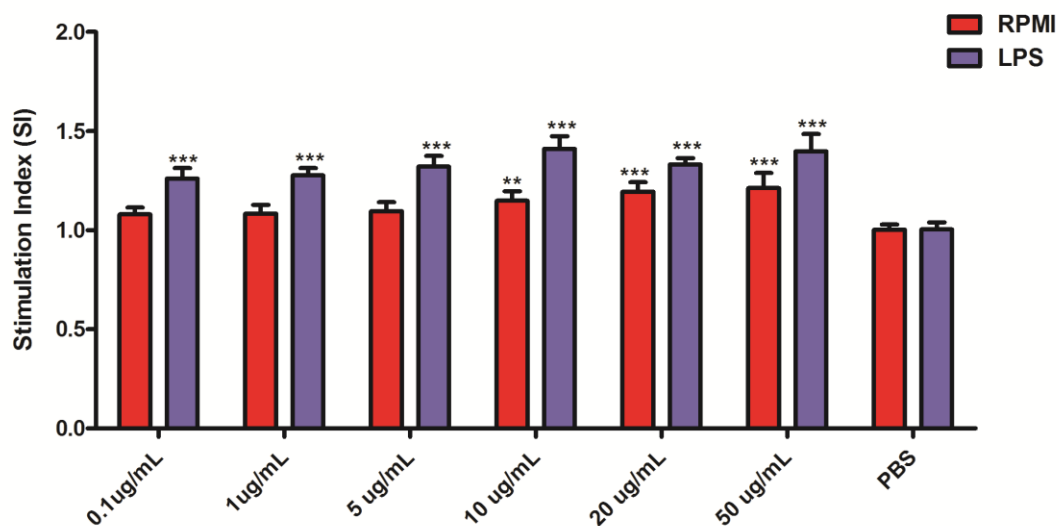


Figure 5. The effect of *Trehala manna* ethanolic extract (TMEE) on macrophages viability. Unstimulated macrophages and LPS-stimulated macrophages were treated with different concentrations of TMEE (0.1, 1, 10, 20, and 50 µg/ml) and cell viability was measured by MTT assay. TMEE significantly increased the activity of unstimulated (10 to 50 µg/ml) and LPS-stimulated macrophages (0.1 to 50 µg/ml) as compared to PBS group as the control group. **p<0.01 and ***p<0.001. Stimulation Index (SI): OD of test of each group / OD of control of each group. Results are expressed as mean ± SEM of three independent experiments.

word, this extract has immunostimulatory effects on cell-mediated and humoral immune responses. These results are in agreement with the data reported by Hamed et al., which showed that low molecular weight polysaccharides isolated from *Trehala manna* increased the proliferation of T cells (Hamed et al, 2015).

For evaluation of the effect of TMEE on the pattern of cellular immune responses, we measured the concentrations of IL-4 and IFN- γ in culture supernatants of PHA-stimulated splenocytes treated with TMEE. Our findings showed that this extract was able to decrease IL-4 and increase IFN- γ (Figure3). IL-4 and IFN- γ play key roles in generation and regulation of immune responses. IL-4 promotes Th2 differentiation and stability and inhibits Th1-cell differentiation. Conversely, IFN- γ is predominantly produced by Th1 cells and has an important role in Th1 cells differentiation, and Th2 cells suppression. Therefore, it is possible to conclude that TMEE attenuates Th2 immune response and amplifies Th1 immune response. This finding may support the usage of *Trehala manna* as an anti-asthmatic and anti-pharyngitis agent in traditional medicine (Amiri and Joharchi, 2013).

Moreover, it has been reported that several carbohydrates such as trehalose, pectin, glucogalacturonan, rhamnogalacturonan, manno-arabinogalacturonan, and

homogalacturonan exist in *Trehala manna* (Hamed et al, 2015). Ramberg et al. showed that pectin increases IFN- γ secretion by splenocytes, but decreases the secretion of IL-5, a Th2 cytokine, by splenocytes (Ramberg et al, 2010). Recent studies also showed that rhamnogalacturonan induces IL-12 and IFN- γ production from dendritic cells and macrophages (Park et al, 2013; Kim et al, 2002). Therefore, it could be suggested that carbohydrates such as pectin and rhamnogalacturonan that are present in *Trehala manna* have important roles in the secretion of IFN- γ by splenocytes.

To verify the effect of TMEE on the innate immune system, we used peritoneal macrophages. Our results showed that TMEE increases the viability and NO production of LPS-stimulated macrophages. NO is a free radical that plays an important role in a variety of biological processes (Sharma et al, 2007; Boscá et al, 2005). It boosts many aspects of inflammatory responses by releasing various inflammatory mediators from immune cells, modulating blood flow and inducing adhesion of leukocytes to the vascular endothelium cells (Boscá et al, 2005; Sharma et al, 2007). Also, nitric oxide, which is produced by immune cells such as macrophages and neutrophils, has toxic effects on bacteria and intracellular parasites (Ferreira et al, 2008). With regard to the involvement of NO in many aspects of

inflammatory responses, we suggest that *Trehala* manna has anti-inflammatory effects on macrophages. However, it has been demonstrated that trehalose, a major component of *Trehala* manna shows protective effects against macrophage infection induced by *Candida albicans* (Martínez-Esparza et al, 2007) and also has suppressive effects on inflammatory responses and lipid peroxidation (Echigo et al, 2012). Therefore, it is possible that the constituents of *Trehala* manna, except for trehalose, not only suppress the production of NO by macrophages, but also stimulate it (Figures 4 and 5).

Conclusion

In conclusion, our findings suggest that TMEE exerts immunostimulatory effects on splenocytes and macrophages and alters Th1/Th2 immune balance. These findings suggest that *Trehala* manna can be a good candidate for modifying immune system function, in cases such as tumors and allergic disease. However, further studies are required to investigate the immunomodulatory activity of polysaccharides isolated from *Trehala* manna.

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Conflict of interest

The authors have no competing interests to declare.

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