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Potentiation of macrophage activity by thymol through augmenting phagocytosis

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ABSTRACT

The potent role of thymol, a natural compound, in modulation of macrophage activity was evaluated by determining all the sequential steps involved during phagocytosis. We found a significant increase in the proliferation of splenocytes in the presence of thymol and it proved to be a good mitogen. Uptake capacity of macrophages was enhanced due to increased membrane fluidity after treatment with thymol and it also increases lysosomal activity of macrophages. Data of superoxide anion generation revealed the involvement of thymol in the generation of respiratory burst as it potentiated this property of macrophages at a concentration of 150 μM. In the case of TNF-α, IL-1ß and PGE_2 a decreased level of secretion was observed 154 ρg/ml, 736.1 ρg/ml, and 151 ρg/ml respectively when compared with lipopolysaccharide treated cells, where the level of these cytokines was significantly high. We also determined the anti-complementary activity of thymol which showed to be more effective than rosmarinic acid. Thus, the results obtained from the study suggest the potential role of thymol as a natural immunostimulatory drug which can be used in the treatment of various immunological disorders.

1. Introduction

Immune system is an intricate meshwork of the cells and tissues that act together to defend an individual from extraneous invaders such as microorganism. The crucial thing about the immune system is its proper modulation. Any kind of alteration in the immune system either by activating or inhibiting its function is termed as immunomodulation. Those substances which have the potential to interact with immune system and are involved in the modulation of immune response i.e. stimulation or suppression are coined as immunomodulators [1,2]. There are vast varieties of immunomodulators which includes natural compounds, some bacterial products, lymphokines and synthetic drugs. There are three targeting areas for a potent immunomodulator i.e. stimulation, suppression and restoration of the immune system. Unlike vaccine, most of the immunomodulatory agents are not real antigens but antigenomimetics or so called mitogens. Production of memory cells is not observed by immunomodulator because of its non-specific and non-antigenic properties. Due to this reason, the effect of immunomodulator agents is reduced after a short period of time [3].

Nowadays plant and animal originated natural products seem to be the potent medicinal agent against clinical disorders [4]. Some of these are supposed to encourage the health by providing nutrition to tissues and interfering in the drug resistance mechanism in various infections [5]. Plumbagin, aristolochic acid, barberin, cichoric acid and boswellic acid are some of plant oriented compounds which have been reported for their immunomodulatory activity [6].

Thymol (2-isopropyl-5-methylphenol) is a phenolic compound found in many medicinal plants derived essential oils. A plant *Thymus ciliates* is found to have immense amount of thymol [7]. Most of the food and cosmetic industries use thymol as a preservative and antioxidant compound. Its protective nature against caries and plaques attracts the field of dental medicine. Thymol is a potent compound used to elicit the release of calcium from skeletal and smooth muscles to activate ryanodine receptors and suppress the transient outward K^+ and the L-type Ca^{2+} current in mammalian ventricular cardiomyocytes. Release of the stored intracellular calcium in neural cells and potentiation of GABAA receptor properties of thymol has been reported. Recent studies reported on thymol demonstrate its activity as a α1-adrenergic agonist in circular smooth muscle cells isolated from guinea pig stomach, which activates the transient receptor potential channel TRPV3 of the tongue and nasal epithelium and also inhibition of acetylcholinesterase activity in vitro. Its strong property of neutralizing COX1 activity or elastase released by neutrophils suggests its anti-inflammatory potential too [8].

The present study was designed to investigate the possible role of thymol in modulation of macrophages activity, by determining the all steps of phagocytosis involved during antigen processing.

2. Materials and methods

2.1. Chemical and biological reagents

All the chemicals, unless otherwise stated, were of the highest quality and were used as supplied. Thymol, lipopolysaccharide (*Escherichia coli*

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055: B5), Concanavalin A, Griess reagent and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all the solvents were HPLC grade supplied by J.T. Baker (Phillipsburg, NJ, USA).

2.2. Cells and cell culture

RAW 264·7, a murine macrophage cell line, was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal calf serum (Invitrogen, Carlsbad, CA) and 1% penicillin–streptomycin cocktail at 37 °C in a 5% CO_2 incubator.

2.3. Preparation of mouse splenocytes

Pathogen free mice were sacrificed by cervical dislocation, after removal, the spleen was rinsed with Phosphate Buffered Saline (PBS). Single cell suspensions were prepared by mechanical disruption of tissue in 1 ml of ice cold RPMI containing 10% (v/v) fetal calf serum, with the help of a cell strainer remaining clumps were removed. An amount of 1×10^7 cells were seeded in a 16-mm well and incubated in 5% CO_2 at 37 °C for 3 h. The supernatant together with the non-adherent cells were collected by centrifugation at 180 ×*g* at 37 °C for 10 min. The cell pellets were re-suspended in RPMI medium containing 10% (v/v) heat-inactivated FCS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), the concentration was adjusted to 1×10^6 cells/ml. Hematocytometer and trypan-blue dye exclusion technique were used for counting of viable cells.

2.4. Splenocyte proliferation assay

Splenocyte proliferation assay was done by a reported method [9], with some minor modifications. Splenocyte suspension was prepared as described above and distributed (100 μl per well) on 96-well plates and cultured with different concentrations of thymol (50,100 and 150 μM) for 24 h at 37 °C in a humidified 5% CO_2 atmosphere incubator. A standard drug concanavalin A (2 μg/ml) was used as a positive control. After completion of the incubation period splenocyte proliferation activity was tested by MTT assay. The optical density was determined at 570 nm by microplate reader (Bio-Tek, WA, USA) and the splenocyte proliferation was expressed as OD values.

2.5. Mitogenic and co-mitogenic activity assay

A previously reported method was used for this assay [10], with some minor modifications. Briefly, the mouse splenocytes were suspended in RPMI-1640 medium supplemented with 10% fetal calf serum at a concentration of 1.5×10^6 cells/ml. An amount of 200 μl per well of cell suspension was inoculated in a 96 well plate and stimulated by thymol at different concentrations (50, 100 and 150 μM) to determine the mitogenic potential of thymol. In the co-mitogenic test, all concentrations of thymol were added in the presence of concanavalin A (2 μg/ml). Thereafter, cells were incubated for 48 h at 37 °C in a humidified 5% CO_2 atmosphere, after the incubation period 20 μl of MTT per well was added and further incubated for 4 h. A total of 100 μl acidified isopropyl alcohol was added to the culture and homogenized for at least 10 min to fully dissolve the colored material. The absorbance at 570 nm was measured in an ELISA reader. The direct mitogenic effect of the compounds tested was expressed as Stimulation Index of mitogenic (SImit) = mean A570 test compound/mean A570 control, and the comitogenic effect was expressed as Stimulation Index of co-mitogenic (SIco-mit) = mean A570 (conA + thymol)/mean A570 con A. Each substance was tested minimally in at least three independent experiments.

2.6. Determination of membrane fluidity of macrophages for total phagocytosis activity

To examine the effect of the thymol on membrane fluidity of macrophages we used the RAW264.7 cell line. Assay was based on the neutral red uptake capacity of cells as described previously [9], with some minor modifications. Briefly, RAW 264.7 cells (1×10^5 cells/well) with various concentrations of thymol as well as with PBS for control group were incubated in 96-well plates for 24 h at 37 °C in a humidified 5% CO_2 incubator. After the incubation period, supernatant was removed thoroughly, and 200 μl of 0.075% neutral red was added to each well and co-incubated with macrophages for 30 min at 37 °C. Next the wells were aspirated and rinsed with 200 μl PBS three times. Cells were lyzed in 200 μl per well of 100 mM acetic acid: dehydrated alcohol = 1:1 (v/v), and the plates were incubated for 8 h at 4 °C for sufficient schizolysis of cells and release of phagotrophic neutral red. The optical density was determined at 490 nm by microplate reader and the phagocytosis was expressed as OD values.

2.7. Assessment of cellular lysosomal enzyme activity during phagocytosis

The cellular lysosomal enzyme was evaluated by measuring the acid phosphatase activity in macrophages as previously described [11]. Briefly, 20 μl of the adherent macrophages (1×10^6 cells/ml), 40 μl of DMEM medium and 20 μl of the thymol (50, 100 and 150 μM) were added in a flat bottom 96-well cell culture plate and incubated at 37 °C in humidified 5% CO_2 atmosphere for 24 h. The medium was removed by aspiration and 20 μl of 0.1% Triton X-100 (Sigma, MA) was added in each well. Subsequently, an amount of 100 μl of 10 mM p-nitrophenyl phosphate (p-NPP) solution (Sigma, Germany) and 50 μl of 0.1 M citrate buffer (pH 5.0) were added, the plate was further incubated for 30 min. After incubation period, 150 μl of 0.2 M borate buffer (pH 9.8) was added and the absorbance at 405 nm was measured. The stimulation index (SI) was calculated as the absorbance ratio of the treated system and the control system.

2.8. Nitroblue tetrazolium (NBT) dye reduction assay

The NBT dye reduction assay was carried out as previously described with some minor modifications [12]. Briefly, 20 μl of the adherent macrophages (1×10^6 cells/ml) and 40 μl of DMEM were put in a flat bottom 96-well plate. An amount of 20 μl of the thymol was added in each well at a concentration of 50, 100 and 150 μM. After incubation for 24 h at 37 °C in humidified 5% CO_2, 20 μl of phorbol myristate acetate (10 ng/ml) and 20 μl of NBT solution in PBS (1.5 mg/ml) were added and the mixture was further incubated at the same condition. After incubation for 1 h, the adherent macrophages were rinsed vigorously with DMEM medium, and washed four times with 200 μl methanol (Sigma, USA). After air-dried, 120 μl of 2 M KOH, and 140 μl of DMSO were added. The absorbance was measured at 570 nm by a microplate reader. Stimulation index (SI) was calculated as the absorbance ratio of the treated and control macrophages.

2.9. Estimation of cytokine release by ELISA

The RAW 264.7 cells were cultured with various concentration of thymol (50,100 and 150 μM) for 12 h. After the incubation period a further treatment with LPS (100 ng/ml) and PBS as a control was done and cells were incubated for 6 h. The supernatant was collected by centrifugation and release of cytokines level were estimated by sandwich ELISA using polystyrene micro-ELISA plates and TNF-α and IL-1ß ELISA kit (Invitrogen, Frederick, MD, USA). Primary antibodies used in this assay were monoclonal anti mouse TNF-α/IL-1ß and secondary antibodies were specific biotin conjugates. In the case of protein standard, recombinant mice TNF-α/IL-1ß were used. Assays were performed as per

manufacturer's instructions. Absorbance was measured using ELISA plate reader.

2.10. Prostaglandin E2 determination

Prostaglandin levels in cell supernatants were measured by a sandwich ELISA using polystyrene micro ELISA plates (ENZO, USA). Monoclonal anti-mouse PGE2 antibodies were used as capture; and specific biotin conjugates were added as secondary antibodies. Recombinant mouse prostaglandin was used as protein standard. Assays were performed according to manufacturer's instructions. Absorbance was measured in ELISA plate reader.

2.11. Anti-complement assay through the classical pathway

The assay system, based on the complement fixation test was done utilizing a reported method [13] with minor modifications using antibody sensitized sheep RBCs (SRBC) as indicator cells. In detail, sheep erythrocytes were washed twice with VBS (Veronal buffered saline) and the cell count was adjusted to 5×10^8 cells/ml, by diluting with VBS. Human serum was diluted with VBS to a concentration giving about 50% hemolysis. Two volumes of hemolysin solution was added to one volume of adjusted sheep erythrocyte suspension, which was incubated at 37 °C for 30 min and excessive antibody was eliminated by washing with VBS. 50 μl of diluted human serum (complement source), and 50 μl of different concentrations of thymol were added to a round bottomed 96-well microplate and pre-incubated at 37 °C for 30 min followed by an addition of 50 μl sensitized SRBC and incubation at the same condition. Rosmarinic acid was used as a positive control. The microplate was centrifuged at 900 ×*g* for 5 min and to determine the degree of hemolysis, 50 μl samples of the supernatants were mixed with 200 μl H_2O in 96-well flat-bottom microtiter plates, and absorbance was measured at 405 nm. 100% lysis was obtained by adding distilled H_2O to sensitized SRBC. Complement fixing activity of the thymol was plotted as initial sample concentrations when added to the wells versus % inhibition of hemolysis.

2.12. Statistical analysis

Experimental results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

3. Results

3.1. An enhanced proliferation of splenocytes was observed by thymol in a dose dependent manner

To evaluate the potency of thymol as an immunomodulator, we evaluated its effect on spleen cell proliferation as they play a crucial role during infection and inflammation. All concentrations of thymol used in this study were significantly effective in proliferating splenocytes with optical density of 0.202 ± 0.008, 0.219 ± 0.008 and 0.244 ± 0.01 ($P < 0.001$) at the concentration of 50, 100 and 150 μM respectively when compared with untreated control cells. The data proved that presence of thymol can boost the proliferation of splenocytes (Fig. 1). We further compared the activity of thymol on splenocyte proliferation with a commercially available standard drug i.e. Con A, and at a higher concentration of 150 μM, thymol expressed significant activity ($P < 0.05$) than Con A.

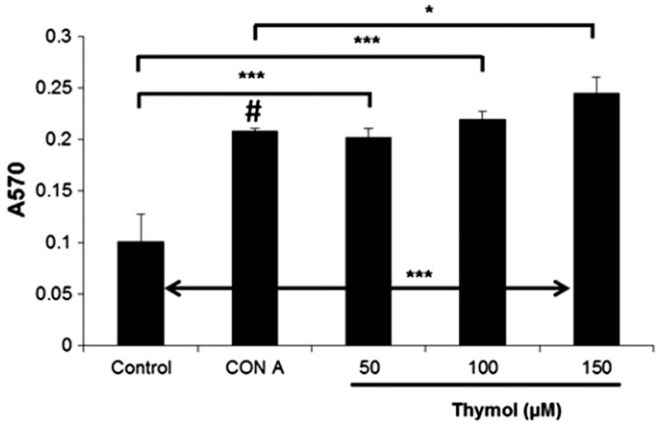

Fig. 1. Effect of thymol on splenocyte proliferation. An amount of 1×10^6 cells were seeded in each well and stimulated by different concentration of thymol (50, 100 and 150 μM) for 72 h. PBS was treated as a negetive control and concanavalin A used was positive control for the test. Proliferation of cell was determined by MTT assay. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-values <0.05, *** represents P-values <0.001 and # represents P-values <0.01.

3.2. Thymol manifested an influential mitogenic property during mitogen and co-mitogen assessment

To identify the possible role of thymol on the mitosis of the cell which is an essential requirement to be a good immunomodulator, the mitogenic property was determined by calculating the stimulation index of splenocyte at various concentrations of thymol. As shown in Fig. 2, all three concentrations of thymol (50, 100 and 150 μM) encouraged the cells to trigger mitosis with a stimulation index of 0.947 ± 0.009, 1.033 ± 0.009 and 1.140 ± 0.01 respectively. However, the significant result was recorded at a concentration of 150 μM ($P < 0.01$). To determine the co-mitogenic role of thymol, we treated splenocytes with thymol as well as a standard mitogen Con A (2 μg/ml). A slightly increased activity of thymol was observed during this assessment with a stimulation index of 0.917 ± 0.004, 1.147 ± 0.002 and 1.211 ± 0.009 in accordance with the concentrations.

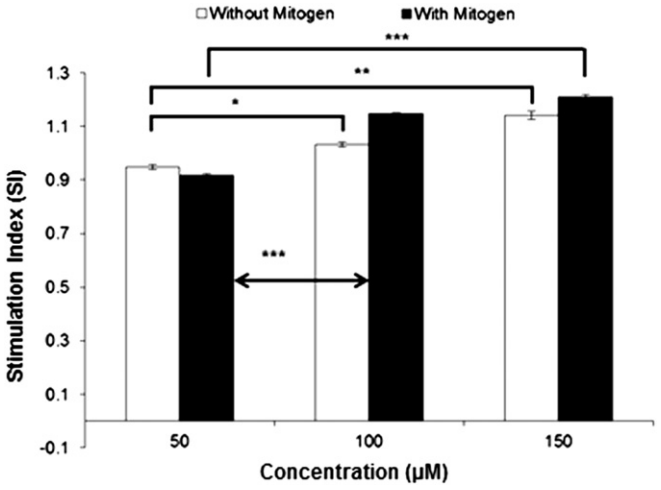

Fig. 2. Mitogenic and co-mitogenic potential of thymol. Cell suspension of splenocytes was inoculated in 96 well plate and different concentration of thymol (50, 100 and 150 μM) was added for the mitogenic assay. In co-mitogenic test splenocytes were co-incubated with different concentration of thymol and concanavalin A (2 μg/ml) for 48 h. MTT assay was done to determine the mitogenic and co-mitogenic activity. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-values <0.05, ** represents P-values <0.01, and *** represents P-values <0.001.

3.3. Membrane fluidity of macrophages was enhanced after treatment with thymol which indicates a rapid phagocytosis

Membrane fluidity is very important in the uptake of particle by phagocytic cells. Keeping this in mind we analyzed this function of macrophages by neutral red dye uptake assay. Cells were treated with PBS as a control and various concentration of thymol. After addition of neutral red solution (0.075%) optical density at 490 nm was recorded and results were expressed by calculating stimulation index. Though all the concentrations were found to be effective ($P < 0.001$), the concentration of 150 μM was recorded to have the best activity giving an optical density of 0.053 ± 0.001 comparing 0.037 ± 0.0001, 0.042 ± 0.0004 of 50 μM and 100 μM respectively. In control untreated cells an optical density of 0.010 ± 0.001 was noticed (Fig. 3).

3.4. p-Nitrophenyl phosphate test showed an increased production of lysosomal enzymes, suggesting accretion in phago-lysosomal action

Secretion of lysosomal enzyme is a requisite process involved in phagocytosis, which ultimately degrades the antigen by phago-lysosome fusion. We analyzed this step by measuring the acid phosphatase activity based on the ability of phosphatases to catalyze the hydrolysis of p-NPP (p-nitrophenyl phosphate) to p-nitrophenol, which can be measured at the absorbance of 405 nm. An increased lysosomal activity was recorded in accordance with the concentration (Fig. 4). 100 and 150 μM was found to have significant activity ($P < 0.005$) when compared with concentration of 50 μM.

3.5. An increase in respiratory burst was observed due to enhanced production of the superoxide anions, determined by nitroblue tetrazolium assay

A major event during phagocytosis is the production of superoxide anion to create oxidative stress for antigen. To determine the influence of thymol in this ability of macrophages, we carried out nitroblue tetrazolium (NBT) assay. The ability of nitroblue tetrazolium to absorb superoxides and producing a purple blue color from original yellow one helps to detect the superoxide anions. The data obtained from this test suggests that, thymol potentially provoked the production of superoxide anions in a concentration dependent manner. At initial concentration (50 μM) stimulation index of 0.518 ± 0.01 was calculated which increased to 0.775 ± 0.05 at the concentration of 100 μM, and the highest

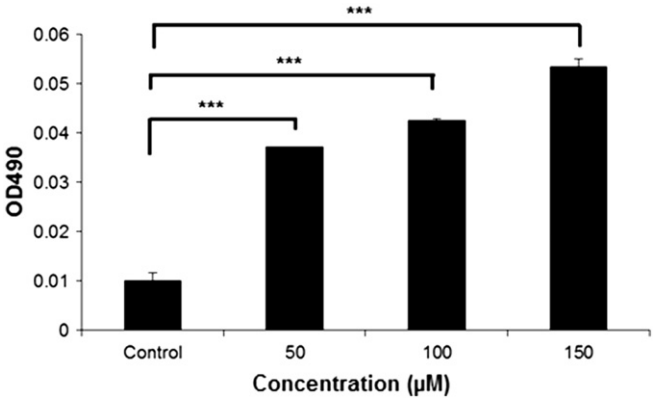

Fig. 3. Effect of thymol on membrane fluidity of murine macrophages. RAW264.7 cells with various concentrations of thymol (50, 100 and 150 μM) as well as with PBS for control group were incubated in 96-well plates for 24 h at 37 °C. Neutral red uptake assay was done to determine the total phagocytosis. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-values <0.05, ** represents P-values <0.01, and *** represents P-values <0.001.

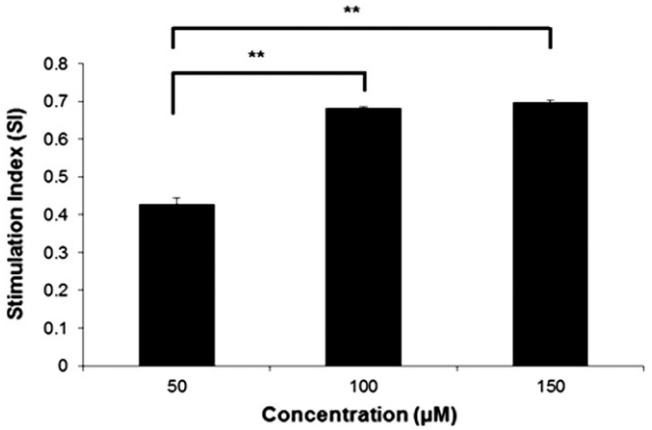

Fig. 4. Assessment of cellular lysosomal enzyme activity during phagocytosis. RAW264.7 cells with various concentrations of thymol (50, 100 and 150 μM) as well as with PBS for control group were incubated in 96-well plates for 24 h at 37 °C. Measurement of acid phosphatase activity was done by using p-nitrophenyl phosphate (p-NPP) solution. The phagocytic stimulation index (SI) was calculated as the absorbance ratio of the treated system and the control system. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. ** represents P-value <0.01.

stimulation index (1.26 ± 0.11) was observed at a concentration of 150 μM (Fig. 5).

3.6. Level of cytokines was decreased after treatment with thymol

Pro-inflammatory cytokines such as TNF-α, IL-1ß and PGE_2 play a major role in the development of inflammation. So, we determined the potency of thymol on release of these cytokines and found a significant decrease in the level of all three cytokines. A significant increase in TNF-α was observed in LPS (100 ng/ml) treated cells (201.5 ρg/ml) when compared to control untreated cells having an amount of 26.5 ρg/ml ($P < 0.001$). However, thymol significantly decreased the level of TNF-α in a dose dependent manner ($P < 0.005$ and $P < 0.001$). As depicted in Fig. 6A, a decrease in level of TNF-α 186.3, 172 and 154 ρg/ml was observed at a range of 50, 100 and 150 μM concentration of thymol respectively. In the case of IL1-ß, 50 μM concentration of thymol was observed to decrease the level significantly ($P < 0.001$). Though at a concentration of 100 μM an increase in the IL1-ß level

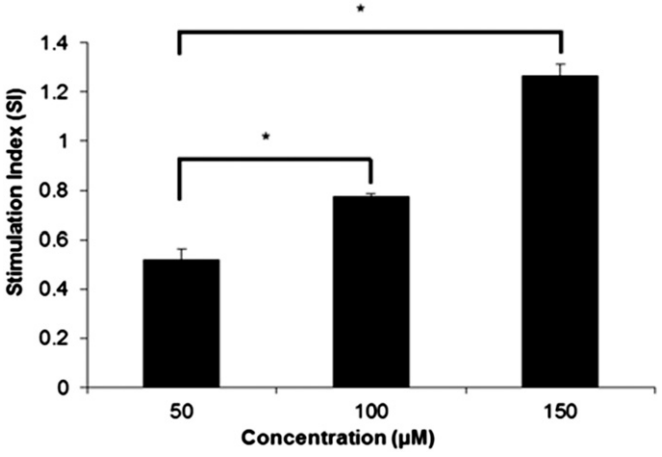

Fig. 5. Effect of thymol on superoxide anions generation. Cells with various concentrations of thymol (50, 100 and 150 μM) were incubated in flat bottom microtiter plates for 24 h. After incubation period nitroblue tetrazolium dye reduction assay was done. Stimulation index was calculated. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-value <0.05.

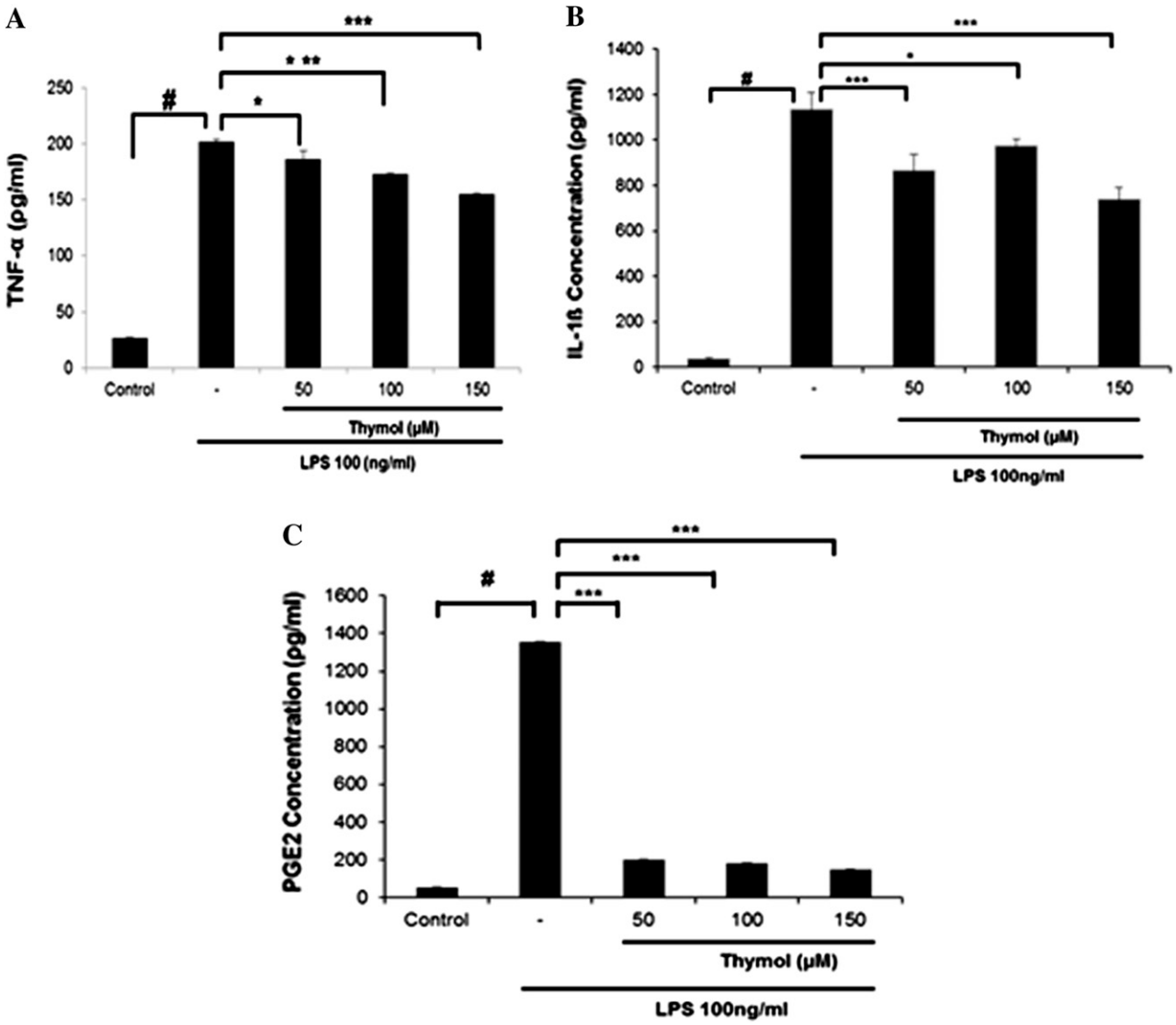

Fig. 6. Effect of thymol on cytokines level. The RAW264.7 cells were cultured with various concentration of thymol (50,100 and 150 μM) for 12 h after the incubation period a further treatment with LPS (100 ng/ml) and PBS as a control was done and cells were incubated for 6 h of period. Amount of various cytokines in supernatant was measured by ELISA. (A) TNF α, (B) IL-1ß and (C) Prostaglandin E2. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-value <0.05, *** represents P-value <0.001 and # represents P-value <0.001.

was observed yet it was significantly less ($P < 0.05$) than those treated with LPS (Fig. 6B). Lowest level of IL1-ß was observed at 150 μM concentration of thymol (736.1 ρg/ml) which significantly differ from cells treated with LPS only ($P < 0.01$).

A decrease in secretion of PGE_2 was also observed at a concentration dependent manner (Fig. 6 C). At a concentration of 50 μM thymol significantly decreased the level of PGE_2 ($P < 0.001$) which was continued to decrease at 100 and 150 μM concentrations ($P < 0.001$).

3.7. Anti-complement property of thymol was observed through inhibition of the classical pathway

We determined the potential of thymol to inhibit the classical complement pathway. Sensitized sheep erythrocytes were used as an indicator of hemolysis to calculate percentage of inhibition. Thymol at a concentration of 50 μM showed a significant inhibition of complement activation by 45.05% ($P < 0.05$). 45.11% ± 0.07 and 48.13% ± 0.05 inhibitions were observed at a concentration of 100 μM and 150 μM respectively (Fig. 7). We also compared thymol with a standard anti-complement compound i.e. rosmarinic acid. All concentration of thymol found to have significant anti-complement activity when compared with 100 μM concentration of rosmarinic acid (43.89% ± 0.001).

4. Discussion

The first line of defense against extraneous invaders is provided by cells of the innate immune system. The major factor which makes innate immunity stronger is its speedy response towards the antigen. Phagocytic cells are the important players of innate immunity which includes neutrophils, monocytes and macrophages. Macrophages perform intracellular killing of antigen by their specialized mechanism and are able to recruit other cells like neutrophils after producing various cytokines [14]. An impaired functioning of macrophages can alter the innate immune response, leading to chronic granulomatous and other various disorders. So, modulation of macrophage activity seems to be very important in regulation and proper functioning of the innate immunity. Here, in this present investigation we thoroughly examined all the steps involved during phagocytosis and uncovered the role of thymol in the potentiation of macrophage activity.

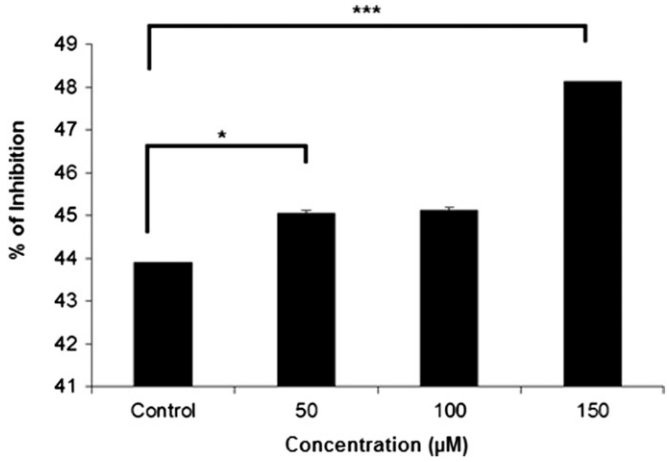

Fig. 7. Anticomplementary activity of thymol. Antibody sensitized sheep red blood cells (SRBC) were used as an indicator to determine anticomplementary activity of thymol. Diluted complement source, sensitized sheep red blood cells and different concentration of thymol were added in a round bottom microtiter plate and were incubated for 30 min 37 °C. Degree of hemolysis was determined and percentage of inhibition was calculated. Rosmarinic acid (100 μM) was used as a positive control. Results are depicted as the mean ± standard error of the mean (SEM). Statistical significance was calculated using the Students t-tests. * represents P-value <0.05 and *** represents P-value <0.001.

The very first step of phagocytosis is engulfment of the antigen which is accompanied by an increase in membrane fluidity and the activation of some membrane proteins. There is a high correlation between phagocytosis by phagocytic cell and the membrane fluidity [15]. In our study we detected a significant increase in phagocytosis at all the concentration used, but at a higher concentration (150 μM) thymol showed the better activity than other two concentrations used (50 and 100 μM). The result suggests that thymol enhanced the fluidity of membrane thus increasing in the capacity of engulfment.

After engulfment, there are several mechanisms involved to deal with antigen intracellularly, like secretory organelles. These organelles are intended to secrete proteins and deliver integral proteins to the cell surface and phagosomal membranes [16,17]. These secretions include proteases, myeloperoxidase, lactoferrin, vitamin B_{12}-binding protein and lysozymes [17,18]. Macrophages possessing an acid phospholipase A_2 were reported earlier which was secreted in response to phagocytic stimuli such as zymogen [19]. We evaluated this crucial step in our experiment by using p-nitrophenyl phosphate (p-NPP) assay, and found a concentration dependent stimulation in lysosomal activity. At a low concentration of thymol (50 μM) significant stimulation index was recorded which increased by gradual increase in concentration.

Respiratory burst is the third major event in the process of phagocytosis to respond the antigen, which is achieved by generation of superoxide anions through phagocyte nicotinamide dinucleotide phosphate (NADPH) oxidase which is also referred as respiratory burst oxidase. This phagosomal and plasma membrane-associated enzyme complex is normally in dormant state but rapidly get in action when cells are in contact with antigen or inflammatory stimuli [19,20] to generate superoxide which is a precursor to numerous microbicidal oxidants, including hydrogen peroxide and myeloperoxidase-catalyzed formation of hypochlorous acid [21]. An alteration of superoxide generation or absence of these enzymes leads to formation of inflammatory granulomas which is a distinctive feature of chronic granulomatous diseases [21]. We determined this activity with the help of nitroblue tetrazolium (NBT) chemiluminescence assay. As some of reactive oxygen intermediates have a very short time existence at high energy state, but the release of this energy is in the form of chemiluminescence which can be easily determine spectrophotometrically [22]. The results obtained from NBT test evidenced that even at a low concentration; thymol can potentiates this mechanism of macrophages which further we observed was increased in a concentration dependent manner. We hypothesized, that thymol is probably involved in the small GTP-binding protein i.e. Rac, which act as molecular switches through conformational changes between the GDP and GTP-bound forms. Rac-GTP binds to the membrane and to p67phox, and is required for NADPH oxidase activity [21].

As we mentioned above are the key factors involved to potentiate phagocytosis thus increasing macrophage activity, but there are some critical issues too, which when initiated respond against these activities. They can impair the potency of macrophages which ultimately cause inflammatory disorders and among these factors cytokines are critical one. The network of cytokines and their signaling pathways have been well recognized in regulation of both innate and acquired immune responses [23]. One of the important cytokine which is involved in inflammation is tumor necrosis factor α (TNF-α). TNF-α can induce fever, either directly via stimulation of PGE_2 synthesis by the vascular endothelium of the hypothalamus, or indirectly by inducing release of IL-1 [23]. We determined that thymol possess a potency to alter the level of TNF-α in a dose dependent pattern. A significantly decreased level of TNF-α by thymol was observed at a concentration dependent manner when compared with LPS treated cells which indicates its anti-inflammatory property. Another important cytokines which is either stimulated by TNF-α or directly activated and involved in inflammatory responses is IL-1ß a potent pro inflammatory cytokine which is engaged in various type of cellular function including proliferation, activation and differentiation [24]. Through induction of IL-8 and activation of neutrophils IL-1ß induces the chemotaxis of leukocytes [25]. Thus in the present study we determined the effect of thymol on release of IL-1ß, and found a decrease in its level as the concentration was increased of thymol, suggesting its involvement in signal transduction pathway essential for the production of IL-1ß.

Another cytokine of importance is Prostaglandin E_2 (PGE_2) which is involved in the generation of inflammation. Synthesis of PGE_2 is dependent on two cyclooxygenase (COX) isoforms, COX-1 or COX-2, through conversion of arachidonic acid to PGH_2 [26,27]. Expression of COX-1 is observed in most of the tissues to maintain homeostasis by producing PGE_2 [28]. An up-regulation of COX-2 is seen in the case of inflammatory condition, so it is believed to have a role on production of PGE_2 [29]. This was the reason why we concentrated to identify the possible role of thymol against PGE_2. As we were expecting, we found a decrease in the production of PGE_2 by thymol in accordance with concentration. The reduction in the PGE_2 level by thymol is supposed to be due to its inhibitory effect on COX-2 [30].

Our next target of study was complement system, which when activated by any of the classical, alternative or lectin pathway eliminates the extraneous invaders to provide infection free environment. On the other hand, there are numerous data demonstrating that its undesired activation induces inflammation and finally tissue damage. Severe acute respiratory syndrome (SARS) an infectious disease which is responsible for many deaths is reported to be because of excessive activation of complement system [31]. We decided to evaluate anti complementary activity (classical pathway) of thymol which works on a principle of hemolysis of erythrocytes after binding with free complement. There was no significant difference between two initial concentrations but a significant percentage of complement inhibition was observed at highest concentration used. Data obtained from this experiment demonstrated the involvement of thymol in classical complement pathway which potentially inhibits its activation.

Beside of all these potentials, an immunomodulator should have potency to be a good mitogen and must enhance the proliferation of immune cells. Keeping this in mind, we also determined this property of thymol against splenocytes proliferation, mitogen and co-mitogen assay. Data acquired from these assays attested that thymol is a potent immunostimulator as it increases the proliferation of splenocytes and was found to involve in mitosis also in concentration dependent manner.

In conclusion, thymol effectively enhanced phagocytosis by interacting intracellular mechanism of macrophages, which suggests its potency to be a good immunostimulatory drug to deal with various immunological diseases such as chronic granulomatous diseases and lazy leukocyte

syndrome, which alters the macrophage function and leads to impair the innate immune response. Inhibitory effect of thymol against various inflammatory cytokines proposes its potential use as an anti-inflammatory drug, especially in the case of lipopolysaccharide induced inflammation. Availability of thymol in natural spices used in routine diet supports that it is safe to use thymol by both immunocompromised patients and healthy individuals as well, for proper regulation of immune response. Further researches, on involvement of thymol in other immunological cells and immunity can insight its other beneficial aspects in biological system.

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