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Original Article

IMMUNOMODULATORY ACTIVITY OF AN ACETONE EXTRACT OF *TERMINALIA BELLERICA* ROXB FRUIT ON THE MOUSE IMMUNE RESPONSE *IN VITRO*

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ABSTRACT

Objectives: To investigate the immunomodulatory activity of an acetone extract of *T. bellerica* fruit.

Methods: Mitogen induced-lymphocyte proliferation using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) technique, Th1- and Th2-related cytokine production by lymphocytes using ELISA and peritoneal macrophage function in ICR mice were assayed.

Results: The results show that the extract had a mild inhibitory effect on the generation of oxidase enzyme (Phagocytic Index 0.8, 100 μg/ml) but did not influence acid phosphatase enzyme function during phagocytosis. The extract stimulated the proliferation of both T and B lymphocytes. The maximal activation (Stimulation Index 3.2, 100 μg/ml) was presented with concanavalin A induction, indicating a major effect on T lymphocyte proliferation. The extract reduced the production of IFN-γ (89%, 100 μg/ml) and IL-2 (98%, 100 μg/ml) but increased IL-10 secretion (231%, 100 μg/ml) compared to concanavalin A. Gallic acid, a pharmacological component contained in this plant, presented a similar effect as that of *T. bellerica* extract and may contribute to the immunomodulatory activity of *T. bellerica* fruits in cooperation with other phytocompounds. The decrease in the IFN-γ/IL-10 ratio indicated a shift in the Th1/Th2 balance towards a Th2-type response, which might lead to a treatment for Th1-mediated inflammatory immune diseases.

Conclusion: Our investigations show that the acetone extract of *T. bellerica* fruit possesses immunomodulatory activity, which could be used to explain its folklore applications and provide a pharmacological basis for its usefulness in immune-related disorders.

Keywords: Lymphocytes, Proliferation, Macrophages, Phagocytosis, Cytokines, Anti-inflammatory.

INTRODUCTION

Traditional, complementary, alternative, or non-conventional medicines are used by 70–95% of the global population, particularly in developing countries [1]. Traditional medicines primarily depend on the usage of plants, compared to other natural resources[2]. Herbs have been used for food and medicinal purposes for centuries. Nowadays, alternative medicine for the treatment of various diseases, including immunological disorders, is becoming more popular. Research interest has been focused on various herbs that possess immunomodulatory properties that may be useful in reducing the risk of various diseases and cancers [3]. Immune activation is an effective as well as protective approach against emerging infectious diseases [4]. Although the working mechanisms of some of the herbs are unclear and remain to be elucidated, they are worth studying further as new potential therapeutic agents for immunomodulation [3].

Terminalia bellerica Roxb (Combretaceae) is a perennial herb mainly distributed in the tropical regions and commonly found in South-East Asia, including Thailand [5]. The fruit of *T. bellerica* has been used in various ailments in the indigenous system of medicine to treat cough, asthma, colic, diarrhoea, dyspepsia, anaemia, cancer, fever and inflammation and to promote rejuvenation[5,6]. It is one of the ingredients in "triphala", an ayurvedic formulation rich in antioxidants which is believed to promote health, immunity and longevity [7] and is used to treat many diseases such as anaemia, jaundice, constipation, asthma, fever and chronic ulcers [8]. Chemically, the fruit of *T. bellerica* had been found to contain gallic acid as an active component, as well as other phytochemical compounds such as ellagic acid, ethyl gallate, chebulagic acid and β-sitosterol [9,10]. The gums of the plant have been used for the formulation of a microencapsulated drug delivery system, and a polysaccharide isolated from it is used as a pharmaceutical excipient[11]. *T. bellerica* has been scientifically shown to possess antibacterial [12], antifungal[13], antioxidant[14] and hypotensive [15] effects. The combination of three lignans and a flavan from *T. bellerica* extract showed significant anti-HIV, anti-malarial and antifungal activity *in vitro* [16]. Moreover, *T. bellerica* seed extract has therapeutic potential for the treatment of gastrointestinal diseases, especially inflammatory bowel diseases [17].

Our preliminary study of *T. bellerica* fruit extracted using various solvents on the mouse immune response showed interesting results, especially with the methanolic and acetone extract. The effect of a methanolic extract on macrophage phagocytosis and mitogen-induced lymphocyte proliferation was previously reported [18]. In the current study, we demonstrate the immunomodulatory activity of an acetone extract of *T. bellerica* fruit *in vitro*; gallic acid may contribute to this activity. Mitogen-induced lymphocyte proliferation, Th1- and Th2-related cytokine production by lymphocytes and peritoneal macrophage function in ICR mice were investigated in this study.

MATERIALS AND METHODS

Plant material

Dried fruits of *T. bellerica* were authenticated by Associate Professor Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. A specimen was prepared and deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT) dye, *p*-nitrophenyl phosphate (*p*-NPP), phytohemagglutinin (PHA), concanavalin A (con A), lipopolysaccharide (LPS), pokeweed mitogen (PWM), dimethyl sulfoxide (DMSO), zymosan A, antibiotic-antimycotic solution (100 U penicillin, 100 μg streptomycin and 0.25 μg/ml amphotericin B), phosphate buffer saline (PBS) and gallic acid (3,4,5-trihydroxy

benzoic acid) were purchased from Sigma-Aldrich (Germany). β–mercaptoethanol and Triton-X were from Fisher Scientific (UK), and foetal bovine serum (FBS) and RPMI-1640 medium were purchased from GIBCO/BRL Invitrogen (Scotland).

Animals

Female ICR mice (5-6 weeks old) were obtained from the National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand. The animals were housed under standard conditions at 25 ± 2°C and fed with standard pellets and tap water. The experiments were conducted under the surveillance of the Ethics Committee of Naresuan University, Thailand.

Preparation of extracts

The dried materials were extracted by maceration in acetone for 24 h and filtered. The filtrate was evaporated under reduced pressure until dryness and a yield of 3.7% (w/w of dried material) was obtained. The extract was dissolved in 0.1% DMSO in PBS solution. Insoluble material was centrifuged and the extract was sterilised using a 0.2 μm filter. 0.1% DMSO in PBS solution was used as the control in all experiments.

Preparation of peritoneal mouse macrophages

Peritoneal macrophages were isolated following intraperitoneal injection of FBS as a stimulant [11]. Three days later, the exudate was collected by peritoneal lavage with complete RPMI (CRPMI) 1640 medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 100 U penicillin, 100 μg streptomycin and 0.25 μg/ml amphotericin B) and centrifuged at 2000 rpm and 25°C for 10 min. The cells were washed twice and re-suspended in CRPMI medium. The cell number was adjusted to 1×10^6 cell/ml by counting in a haemocytometer and cell viability was tested by the trypan blue dye exclusion technique.

Preparation of mouse splenocytes

Mice were sacrificed and the spleens were removed aseptically. Single cells were prepared by mincing spleen fragments and pressing them through a stainless steel 200-mesh screen in CRPMI 1640 medium. After centrifugation at 2000 rpm and 25°C for 10 min, the cells were washed twice and re-suspended in CRPMI medium. The cell number was adjusted to 1×10^6 cell/ml by counting in a haemocytometer and cell viability was tested by the trypan blue dye exclusion technique.

Nitroblue tetrazolium (NBT) dye reduction assay

The NBT dye reduction assay was carried out as previously described with appropriate modifications [19,20]. Macrophages (1×10^5 cells/well) were treated with the extract (0.01, 0.1, 1, 10 and 100 μg/ml) for 24 h at 37°C in a 5% CO_2 humidified incubator. Cells were incubated with zymosan A (5×10^6 particles/well) and 1.5 mg/ml NBT dye. After incubation for 60 min, the adherent cells were rinsed vigorously with RPMI medium and washed four times with methanol. After air drying, 2 M KOH and DMSO were added and the absorbance was measured at 570 nm using a microplate reader. The phagocytic index (PI) was determined by the ratio of the optical density of the test sample to the optical density of the control.

Cellular lysosomal enzyme activity assay

The cellular lysosomal enzyme activity was used to measure acid phosphatase in phagocytes, as previously described[19,21]. Macrophages (1×10^5 cells/well) were treated with the extract (0.01, 0.1, 1, 10 and 100 μg/ml) for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The medium was removed by aspiration and 0.1% Triton X-100, 10 mM *p*-NPP solution and 0.1 M citrate buffer (pH 5.0) were added to each well. The cells were further incubated for 30 min and 0.2 M borate buffer (pH 9.8) was then added. The absorbance was measured at 405 nm using a microplate reader. The PI value was calculated as in the NBT dye reduction assay.

Mitogen-induced splenocyte proliferation assay

MTT technique was used to detect lymphocyte proliferation as previously described [19,22]. Briefly, splenocyte suspensions were treated with the extract (0.01, 0.1, 1, 10 and 100 μg/ml) alone or stimulated with the optimal dose of mitogen (PHA, con A, LPS and PWM) for 48 h at 37°C in a humidified 5% CO_2 incubator. Subsequently, 5 mg/ml MTT was added and incubation continued for a further 4 h. The culture medium was removed by aspiration, 0.04 M HCl in isopropyl alcohol and distilled water was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Tek Instrument Inc., USA). The stimulation index (SI) was determined by the ratio of the optical density of the test sample to the optical density of the mitogen.

Cytokine production assay

Production of murine IFN-γ, IL-2 and IL-10 was measured by ELISA according to the instructions of the manufacturer (eBioscience, Inc. San Diego, USA). 5 μg/ml Con A-induced splenocytes were treated with the extract (0.01, 0.1, 1, 10, 50 and 100 μg/ml) or 10 μg/ml gallic acid for 48 h at 37°C in a humidified 5% CO_2 incubator and the culture supernatants were analysed. Briefly, a 96-well microtiter plate was pre-coated overnight with capture antibody. Followed by blocking and several washings, working standards and samples were then added for incubation for 2 h. After further washing, the working detector solution containing biotinylated anti-mouse cytokine monoclonal antibody and avidin-horseradish peroxidase conjugate was added to each well and incubated for 1 h. The substrate solution was then added for incubation, followed by the addition of stop solution, and the absorbance was read within 30 min using a microtiter plate reader at 450 nm.

Determination of the gallic acid content

Gallic acid in the extract was analysed by HPLC according to the method of Xie et al. [23] with appropriate modifications. The retention time of the authentic reference sample, 0.2 mg/ml gallic acid, was 6.3 min. For the qualitative study, the retention time of the acetone extract in the chromatograms was compared to the gallic acid peaks. The amount of gallic acid in the extract was calculated using the area under the curve in the chromatograms.

Statistical analysis

All experiments were performed in triplicate or quadruplicate and the results are expressed as mean ± S. E. Statistical differences (significance level of $P < 0.05$) between groups were assessed using one-way analysis of variance, followed by multiple comparisons using Tukey's method.

RESULTS

Macrophage function assay

The enzymatic products of peritoneal mouse macrophages during phagocytosis were assayed using NBT dye reduction and lysosomal enzyme activity. The results show that *T. bellerica* extract at 1, 10 and 100 μg/ml slightly decreased the reduction of NBT dye by macrophages, achieving a similar PI value of about 0.8 times vs. the control. However, the extract did not have any effect on lysosomal enzyme activity compared to the control.

Mitogen-induced splenocyte proliferation assay

The effect of *T. bellerica* extract (0.01-100 μg/ml) on splenic lymphocyte proliferation with or without a mitogen was tested using the MTT technique. The results show that the extract alone (1-100 μg/ml) stimulated the proliferation of splenocytes in a dose-dependent manner, reaching a maximal SI value of about 2.5 (100 μg/ml) times vs. the control. In the presence of the mitogen, the extract enhanced splenocyte proliferation in a dose-dependent manner. Among the four mitogens (PHA, con A, LPS and PWM), the culture with con A induction presented the highest activation, reaching a maximum SI value of about 3.2 (100 μg/ml) times comparing to con A alone, which was more than that of PHA (SI 3.0, 100 μg/ml). Moreover, the presence of LPS and PWM led to equivalent activation of splenocyte proliferation with a maximum SI value of approximately 2.7 (100 μg/ml). The results are illustrated in Fig. 1.

Cytokine production assay

A con A-induced murine splenic lymphocyte culture was treated with *T. bellerica* extract (0.01-100 μg/ml) and the production of IFN-

γ, IL-2 and IL-10 was measured by ELISA. Gallic acid (10 μg/ml) was used as the reference substance. Con A (5 μg/ml) alone enhanced the secretion of IFN-γ, IL-2 and IL-10 to a greater extent when compared with the control. The extract at 1-100 μg/ml dose dependently decreased IFN-γ secretion with a minimal amount of about 89% compared to con A. The suppression of IL-2 production was observed at 0.1-100 μg/ml of the extract in a dose-dependent manner, with a maximal decrease of 98% compared to con A alone. The inhibition of IFN-γ and IL-2 production was also observed in the presence of gallic acid, showing a reduction of about 22% and 98%, respectively. In contrast, the extract at 100 μg/ml markedly increased IL-10 production by approximately 231%, while gallic acid augmentation IL-10 production by about 30% compared to con A. The results are shown in Fig. 2.

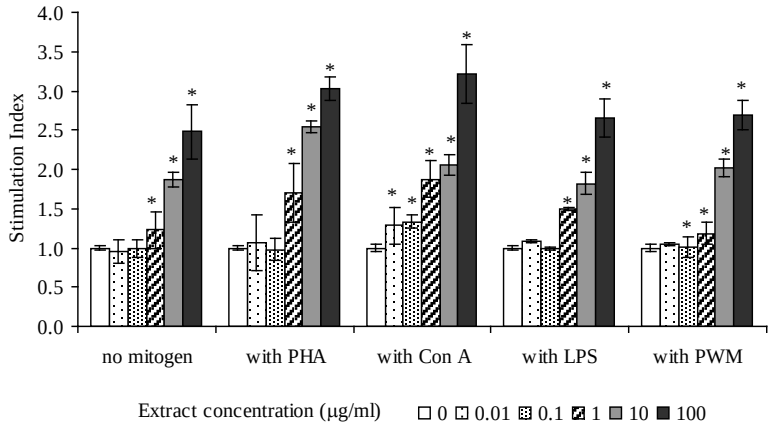

Fig. 1: Effects of *T. bellerica* acetone extract on *in vitro* proliferation response of mouse splenocytes without or with 5 μg/ml of a mitogen (PHA, con A, LPS and PWM). Each value represents the mean ±S. E. of triplicates compared to the control (0.1% DMSO in PBS solution) or mitogen alone; *$P < 0.05$.

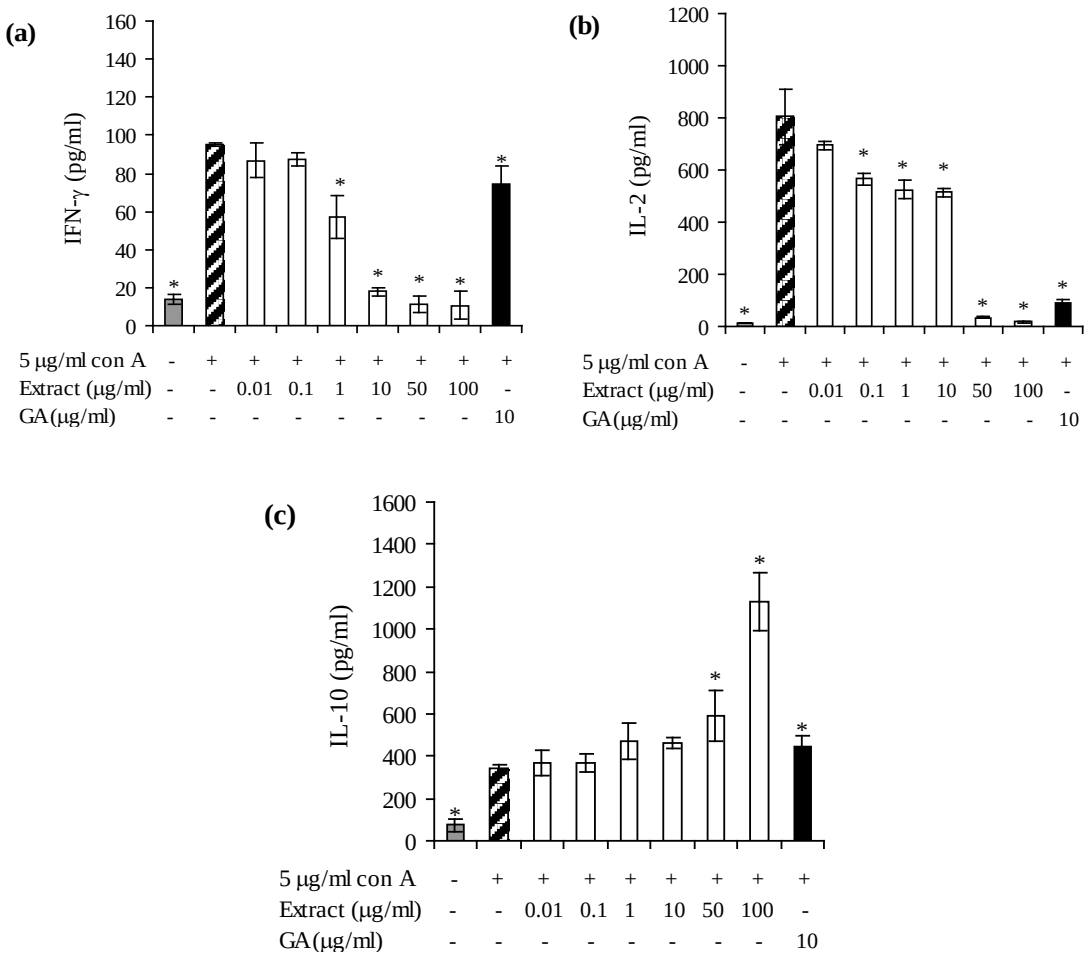

Fig. 2: Effects of *T. bellerica* acetone extract on production of (a) IFN–γ; (b) IL-2 and (c) IL-10 response of mouse lymphocytes with the induction of 5 μg/ml con A, using 10 μg/ml gallic acid (GA) as reference substance. Each value represents the mean ± S. E. of quadruplicates comparing to con A; *P <0.05.

DISCUSSION

Despite the traditional medical use of *T. bellerica* to treat a variety of ailments including inflammation and immune-related disorders such as asthma, cancer and fever, there is little scientific evidence to support its therapeutic efficacy. The present investigation aimed to demonstrate its immunomodulatory activity *in vitro*. To assess the possible mechanisms of the immunological effect of this plant, an acetone extract of *T. bellerica* fruit was tested regarding peritoneal macrophage function, mitogen induced-lymphocyte proliferation and cytokine production by lymphocytes from ICR mice.

It is well-known that macrophages play a significant role in the defence mechanism against host infection and in killing tumour cells. The modulation of the antitumor properties of macrophages by various biological response modifiers is an area of interest for cancer chemotherapy [24]. Our results show that *T. bellerica* extract slightly decreased the NBT dye reduction response of macrophages in a dose-dependent manner but did not affect lysosomal enzyme activity. These results indicate that the extract had a mild inhibitory effect on the generation of the oxidase enzyme during intracellular killing by macrophage but did not affect acid phosphatase enzyme function.

Previous reports have shown that gallic acid, a pharmacological mediator present in this plant, has a suppressant effect on macrophage chemiluminescence[25] but has no effect on zymosan A-induced peroxynitrite production in macrophages[26]. Thus, the weak reduction in superoxide anion production by macrophages mediated by *T. bellerica* extract in the current study might be due to gallic acid or other polyphenol compounds. Our investigations suggest that *T. bellerica* extract may be useful in the supportive treatment of immune disorders involving reactive oxygen species (ROS) production by phagocytic cells.

Lymphocyte transformation is an *in vitro* technique commonly used to assess cellular immunity in humans and other animals; this response is an *in vitro* correlate of an *in vivo* immune response [27]. In this study, the proliferative response of lymphocytes in terms of metabolic activity augmented by *T. bellerica* extract was assessed by a colorimetric MTT assay. Since the presence of a mitogen in the system can enhance the possible activation pathway of an extract[28], PHA and con A were used for the activation of different T cell subtypes whereas LPS and PWM were used to stimulate B cell proliferation through T cell-independent and T cell-dependent pathways, respectively[19].

Our investigation shows that *T. bellerica* extract alone at high concentrations had a stimulatory effect on lymphocyte proliferation. An increase in both T and B lymphocyte proliferation was observed in the presence of stimulants. The extract at 100 μg/ml with con A gave the maximum activation which was more than that of PHA, suggesting a stimulatory effect of the extract on T cell proliferation, mainly through the same mechanism as con A. Moreover, the extract with LPS increased lymphocyte proliferation in a similar manner as that with PWM. This indicates that the extract also affected B cell proliferation, through both T cell-independent (LPS) and T cell-dependent (PWM) pathways. The results suggest that the extract affected both cell-mediated immunity (CMI) and humoral immunity (HI), but the response inclined to CMI dominance since the major effect of the extract was on T cell proliferation.

To investigate the possible mechanisms by which the extract affected lymphocyte proliferation, possibly related to cytokine regulation, Th1 and Th2 cytokines produced by mouse lymphocytes were analysed. Con A was again used as the stimulant since the extract with the induction of con A gave the maximum activation in the MTT assay among the four mitogens. Gallic acid was used as the reference substance to assess the possibility of it being the active ingredient responsible for the immunomodulatory activity of *T. bellerica* extract.

Cytokines are the main regulators of the immune response and play an important role in regulating the proliferation and differentiation of lymphocytes [29]. Th1 cells produce pro-inflammatory cytokines such as IFN-γ and IL-2 which activate phagocytes, natural killer cells and cytotoxic T cells and are associated with host defence against bacteria, viruses and fungi [30]. In contrast, Th2 cells produce anti-inflammatory cytokines such as IL-4 and IL-10 and are associated with allergic reactions [30,31]. Our results show that *T. bellerica* extract led to the inhibition of IFN-γ and IL-2 production by con A-induced lymphocytes while a considerable increase in IL-10 expression compared to con A treatment alone was observed.

The Th1/Th2 cytokine balance is one of the most important regulatory mechanisms in the immune system and can be evaluated by the measurement of certain cytokine patterns, i. e. Th1 and Th2 profiles[32]. The decrease in the IFN-γ/IL-10 ratio observed in the present study suggests a shift in the Th1/Th2 balance towards a Th2 type response, which might be helpful in the treatment of Th1-mediated inflammatory immune diseases such as multiple sclerosis, rheumatoid arthritis and type 1 insulin-dependent diabetes mellitus[33].

During our investigations, we considered the possibility that the effect of *T. bellerica* extract on lymphocyte proliferation was related to cytokine regulation. This may affect the proliferative stimulation of Th2 lymphocytes. The reduction in IFN-γ and IL-2 production could be partially attributed to an increase in the expression of IL-10, a potent anti-inflammatory cytokine, which suppresses Th1 lymphocyte activation[30,34]. Additionally, since IL-10 can promote the HI response[30], the effect of the extract in terms of the enhancement in IL-10 may stimulate B-lymphocyte proliferation, which could be observed in this study. The anti-inflammatory activity of *T. bellerica* is supported by its effects on inhibiting NF-κB/DNA interactions as shown by the EMSA assay and a reduction in pro-inflammatory IL- 8 expression in cystic fibrosis cells [35].

The fruit of *T. bellerica* had been found to contain gallic acid as an active component, along with other phytochemical compounds such as ellagic acid, ethyl gallate, chebulagic acid, β-sitosterol[9,10], lignans and flavan [16]. Under our HPLC analytical conditions, gallic acid was detected in the acetone extract of *T. bellerica* at a level of approximately 0.3% (w/w of the dried material). Gallic acid has a wide range of biological activities, including anti-oxidant, anti-inflammatory, anti-microbial and anti-cancer activities [36,37] and was used as a reference reagent in the current study. Gallic acid has been reported to enhance mouse splenic B cell proliferation [38] and inhibit PHA-induced human lymphocyte proliferation[39]. The effect of gallic acid on the inhibition of mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine (TNF-α, IL-6) expression[40] has also been demonstrated. Moreover, gallic acid and it derivatives have suppressant effects on IFN-γ and IL-2 (Th1) as well as IL-4 and IL-5 (Th2 cytokines) expression by anti-CD3-stimulated splenocytes[41]. Based on previous reports along with our finding that gallic acid inhibited IFN-γ and IL-2 and stimulated IL-10 production by con A-stimulated lymphocytes to a similar degree to that of *T. bellerica* extract suggests that gallic acid may contribute to the immunomodulatory activity of *T. bellerica* fruits, or may cooperate with other phytocompounds. The concentration range of the extract used in this study did not cause cytotoxicity in either macrophages or splenocytes, as more than 85% viable cells were detected after treatment.

CONCLUSION

The present study confirms the traditional uses of *T. bellerica* fruits relating to immunomodulatory activity on both CMI and HI responses. The anti-inflammatory properties of *T. bellerica* extract could be a therapeutic of choice in the future. Detailed insight into the molecular mode of action of *T. bellerica* extract leading to the observed immunomodulatory activity is lacking and further study is required.

CONFLICT OF INTERESTS

Declared None

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