Tropical Journal of Pharmaceutical Research August 2018; 17 (8): 1515-1521
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i8.8

Original Research Article

Immunomodulatory effect of anise (*Pimpinella anisum*) in BALB/c mice

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Sent for review: *Revised accepted: 19 March 2018*

Abstract

Purpose: *The aim of this work was to investigate the effect of anise decoction consumption on lymphocytes activity, complete blood count (CBC) and nitric oxide (NO) production in BALB/c mice.*
Methods: *BALB/c mice were given anise tea instead of drinking water and the effect on selected immune parameters was analyzed after 1 and 2 weeks of treatment.*
Results: *Cell activity of anise treated mice was significantly higher than control group at week 2 as revealed by mixed lymphocyte reaction (MLR2. The spleen cells of anise treated mice showed a significant higher lymphocyte proliferative response to in vitro challenge with phytohaemagglutinin (PHA).compared to controls at week two of treatment. The increase in mouse foot thickness as indicator of delayed type of hypersensitivity (DTH) was less in anise treated mice compared to control group. Nitric oxide production by peritoneal macrophages in response to activation with lipopolysaccharides (LPS) was reduced by anise treatment after 1 and 2 weeks of treatment andno significant changes in CD4 and CD8 were noticed either at week 1 or 2 of treatment.*
Conclusion: *This study provides preclinical evidence that anise possesses immunomodulatory activity when administered orally in mice and selectively activates cell-mediated immune mechanisms*

Keywords: *Immunomodulatory, Anise, Traditional medicine*

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INTRODUCTION

Anise (*Pimpinella anisum*) is an annual aromatic plant that has been used for many centuries in folk medicine [1]. Historically, anise has been used because of its flavor, as an aid for digestion, for colic and combat nausea. Early English herbalists recommended the herb for promoting milk production for nursing mother [1], for treatment of headache, asthma bronchitis and infant colic. Moreover, anise extracts has anti-fungal actions [2] and as selective estrogen receptor modulators, is expected to prevent of osteoporosis in old ages in rats [3,4].

The main component that gives the characteristic sweet flavor to anise is the volatile oil (1-4%), of which 72-90% is trans-anethole [5] which has been documented to have phytoestrogen activity in animals [6]. Antimicrobial effect of anethole has been proven against bacteria, yeast, and fungi

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[7,8]. Moreover anethole has been shown to inhibit tumor necrosis factor (TNF)-induced NF-kappaB activation and IkappaB alpha phosphorylation and degradation when added to cell cultures [9]. Eugnol, estragol, p-anis aldehyde, anise alcohol, acetophenone, pinene and limone are also important compounds found in anise seed extracts [1]. Macrophages function as the primary line of defense against invading pathogens. Their ability to kill and phagocytize microorganisms has been reported [10]. They possess several substances, including nitric oxide (NO), with which they can kill bacteria or inhibit the growth of invading microorganisms. NO regulates the cellular immune response by the inhibition of T cell adhesions and migration [11] as well as T cell proliferation [12,13].

T lymphocyte is a cornerstone in the generation of acquired immune response. There are 2 main classes of lymphocytes. These are the B lymphocytes (B cells) and T lymphocytes (T cells) which mediate humoral and cellular immune responses, respectively [14]. T cells respond to antigen stimulation by proliferation and formation of armed effector cells. *In vitro* stimulation of T-lymphocytes to proliferate can be achieved using polyclonal mitogens such as phytohaemagglutinin (PHA) and Concavalin A (Con A) [15] which gives the opportunity for the exploration of modulation in cell activity. Lymphocyte proliferation assay is reliable, simple and can be performed easily and reflect the activation status of these cells. In this study LPS is used to activate macrophages and B-Lymphocytes to proliferate, whereas PHA is used to stimulate T-lymphocyte proliferation in treated and non-treated Balb/c mice.

To our knowledge no trials have been conducted to verify and elucidate the effect of anise on the immune system, though it is approved for use for relieving cough and indigestion by the German Commission E, equivalent to The U.S. Food and Drug Administration, (commission website). The aim of the present study is to evaluate the effect of anise on parameters of the immune response of BALB/c mice.

EXPERIMENTAL

Animals

Following ethical approval, 6 to 8 weeks female old BALB/c (8mice/group) weighing 20 to 25 g were used in this study. Animals were bred and housed at the Yarmouk University animal house facilities under standard conditions of temperature (23-25C), 12 h light/dark cycle and given food and water *ad libitum*.

Anise decoction *preparation and treatment*

Anise seeds (12.5gm) purchased from local markets were added to 250 ml boiled water and left to stand covered for 1 hour. Filtered anise tea was poured fresh in the mouse drinking bottles, wrapped with aluminum foil and changed daily. Control mice drinking bottles were filled with water and the drinking volumes for both groups were recorded daily.

Antigens

Fresh blood was collected from sheep sacrificed in the local slaughter-house. Sheep red blood cells (SRBCs) collected in Alsever's solution were washed three times in PBS (pH 7.2) and adjusted to a count of 12.5×10^8/ml. Cell suspension of 40µl were used for the purpose of delayed type hypersensitivity (DTH) testing. Sensitized mouse footpad of both anise treated and control mice were inoculated after 4 days with sheep RBC and the foot thickness was measured as an indicator of DTH.

Complete blood count (CBC)

The total white blood cell count, differential analysis of white blood cells (WBCs), red blood cell count and hemoglobin (Hb) concentration were determined using an 18 parametric automated cell counter (Orphée, France).

DTH testing

A group of mice pretreated with anise extract and a control group were sensitized with 1×10^8 washed SRBC per mouse in a volume of 40 µl by foot pad inoculation as described by Lagrance *et al* [16]. DTH was measured as the increase in footpad thickness 24 h after injecting the other foot of each mouse with an eliciting dose of SRBC using the same cell count per dose.

Spleen cell culture and cell proliferation assay

At the time the experiment was terminated (1 and 2 weeks post treatment), spleens were excised and ground to cell suspension. The erythrocytes were removed by treatment of the cell suspension with ammonium chloride and then cultured in 96-well plates (Linbro, USA) at 5×10^6 cells/ml in RPMI 1640 complete media containing 10% FCS (PAA, Austria), 200mM L-glutamine, 100 IU of penicillin, and 10µg of streptomycin per

ml (GIBCO laboratories, USA) at 5% CO_2. PHA (5µg/mL) and LPS (20 µg/mL) (Sigma, USA) or RPMI media alone were used for cell stimulation. Cell proliferation assay using the methyltetrazolium test (MTT) was carried out as previously described by AlQaud *et al* [17]. Four hours before culture termination, 10 µl of 5mg/ml solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, USA) was added to each well. Each plate was centrifuged, the supernatant discarded, a mixture of DMSO and ethanol (1:1) was added and then mixed until the blue formazan crystals were completely dissolved. The plate was read at 570 nm in a microplate reader (Asys Hitech, Austria).

Flow cytometry (FCM)

Two million cells per sample (both anise treated and control groups) were counted and characterized by flow cytometry (BD Biosciences, NJ, USA). Cells were incubated with 1 µl of FITC conjugated goat anti mouse CD4or PE conjugated goat anti mouse CD8 (PharMingen, San Diego, CA) for 30 min. on ice and in a dark place. Cells were washed 2 times with PBS and resuspended in 100 µl PBS containing 2% FCS. When samples were not run immediately, cells were fixed with 3% paraformaldehyde in PBS for 15 min at 4ºC The results were analyzed with Cell Quest software (BD Biosciences).

Mixed lymphocyte reaction (MLR)

Freshly prepared mouse spleen cells were washed and combined with Mitomycin C-treated human lymphocytes (in triplicate) at a cell density of $1X10^5$ cells /well in 96-well flat-bottom plates and co-cultivated for 5 days in RPMI-1640 medium containing 10% FCS (PAA, Austria), 200mM L-glutamine, 100 IU of penicillin and 10µg of streptomycin per ml (GIBCO laboratories, USA) at 5%CO_2. Control wells were used in which either mouse lymphocyte or human inactivated lymphocytes were cultured alone. Four hours before culture termination, 10 µl of 5mg/ml solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) were added to each well. Color development and measurement was carried out as mentioned previously.

Nitric oxide (NO) assay

The concentration of NO in peritoneal cell culture supernatant was determined using Greiss reagent as described in Gomez-Flores [18]. Mouse peritoneal cells collection and treatment was done according to Al-Qashi *et al* [19] with minor modifications. Cells were collected by washing with 10mL RPMI and left to adhere to a culture plate for 3 hours at 37C. Floating non-adherent cells were decanted and adherent cells were washed and incubated with a culture media containing 20µg/ml LPS or media alone. After 48 hours, 50µl of cell culture supernatant was mixed with equal volume of sulfanilamide solution. After incubation at room temperature, in dark, for 5-10 min, another 50µl of 10% N-1-naphthylethylenediamine dichloride (sigma, USA) in distilled water were added to each well. The plates were incubated for further 5-10 min, and the absorbance was measured within 30 minutes using a plate reader at 570nm. The concentration of nitrite in all samples (experimental and controls) was compared to the nitrite standard reference curve.

Statistical analysis

Statistical analysis for differences among groups was done using the non-parametric (Mann-Whitney) t-test. Results were considered significant at $P < 0.05$.

RESULTS

Increase in blood granulocytes after anise treatment

Analysis of the complete blood count data (Table 1) showed that higher values of blood granulocytes were recorded in anise treated mice when compared to control group ($P = 0.038$). On the splenic level, no significant differences in CD4 and CD8 percentages were found among anise treated and control mice at week 1 and 2 of treatment. Moreover, neither the number of red blood cells nor the concentration of hemoglobin was affected by treating mice with anise extract.

Lymphocytes activity and anise consumption

The immunostimulatory capacity of anise was assessed using the mixed lymphocyte reaction (MLR) (Figure. 1). Lymphocytes obtained from mice that have been treated with anise for 2 weeks showed significantly higher activity in response to xenogeneic cells when compared to control mice ($p = 0.004$). Moreover, Anise consumption resulted in a significantly higher lymphocyte proliferative response of spleen cells after *in vitro* challenge with PHA compared to control group ($p = 0.002$) at week two of treatment (Figure 2). However, higher but no significant response of splenic cells to LPS was revealed after 2 weeks of treatment ($p = 0.059$).

Table 1: Effect of anise treatment on mouse whole blood cells and splenic CD4 and CD8 lymphocytes (n = 8 per group)

CBC	Mouse group			
	Treated (W1)	***Control (W1)***	***Treated (W2)***	***Control (W2)***
TWBC	5.7±2.8	6.78±3.20	7.2±2.2	7.5±2.2
Lym	4.77±2.16	5.93±2.8	5.2±1.5	6.3±1.8
Mon	0.41±0.23	0.49±0.2	0.45±0.15	0.5±0.18
Gran*	0.51±0.43	0.36±0.2	1.5±0.9	0.7±0.3
% Splenic CD4			28.0±2.9	24.7±3.8
% Splenic CD8			4.7±2.5	4.38±2.5

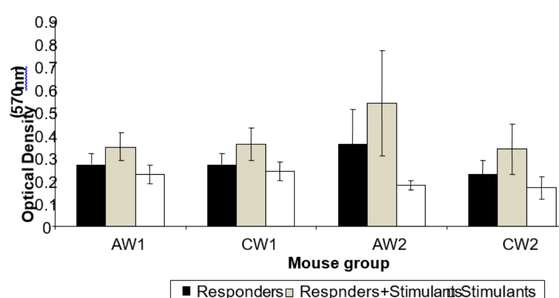

Figure 1: Effect of anise treatment on the cell activity (mixed lymphocyte reaction) of *BALB/c mice. Splenic cell suspension was prepared from each mouse and adjusted to 5x10^6 cells/ml. 100 µl of cell suspension (responders) were mixed with 100 µl of human lymphocytes (5x106 cells/ml) treated for 4 hr with mitomycin C and incubated for 72 hrs. The color of reaction product was detected at 570 nm wavelength using (MTT) test. Data are means ± SD. Experiments were performed by triplicates, * (P<0.05) when compared to control wells.*

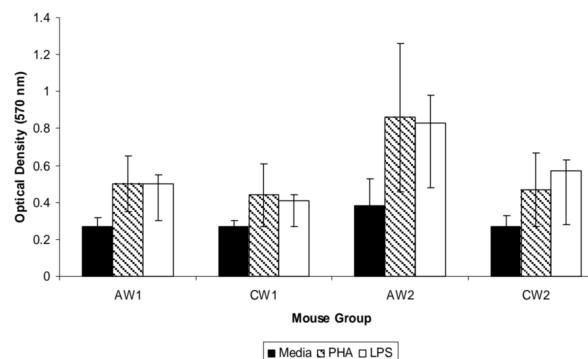

Figure 2: Splenocyte proliferation and viability assay of anise treated and control BALB/c mice after 1 and 2 weeks of treatment. *A total of 100 µl of 5x10^6 cells/ml cultured cells were stimulated with PHA or LPS diluted in RPMI 1640 or RPMI 1640 alone for 72 h at 37°C under 5% CO_2. Four hours before culture termination, 10 µl of 5 mg/ml MTT solution was added to each well. Optical density was measured at 570-nm wavelength. Experiments were performed by triplicates, * (p <0.05) when compared to control wells; PHA -AW2 vs CW2, p = 0.002; LPS -AW2 vs CW2, p < 0.001*

Effect of anise treatment on DTH response in mice.

The difference between the pre- and post-challenge foot thickness expressed in mm was taken as a measure of DTH. In anise treated group the foot-pad diameter before SRBS challenge was 1.88+0.24mm and after SRBS challenge 2.24+0.26mm, while in water group the footpad diameter before SRBS challenge was 1.90+0.25 mm and after SRBS challenge was 2.47+0.17. An increase of 0.36 and 0.57 mm in foot-pad thickness in anise treated and a control animal was recorded.

Anise treatment effect on NO production by stimulated macrophages

Measurement of the levels of NO in the supernatants of LPS stimulated peritoneal macrophages taken from anise treated mice revealed a reduction in secreted NO compared to the NO levels in untreated mice macrophages (41.9 vs 34.5 µM for week 1 and 38.6 vs 23.6 µM for week 2 respectively) (Figure.3). This reduction was statistically significant at both 1 and 2 weeks of treatment ($p < 0.05$).

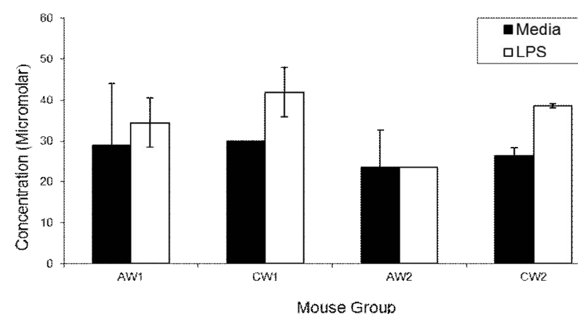

Figure 3: Average nitric oxide (NO) secreted by peritoneal macrophages harvested from anise treated and control mice. *The concentration of NO in all samples (experimental and controls) was compared to the NO standard reference curve.* LPS CW1 vs LPS AW1, $p = 0.04$

DISCUSSION

The present study demonstrates that anise significantly increased the cell-mediated immune response as indicated by MLR and cell proliferation assay in BALB/c mice. In addition, anise treatment increased the absolute granulocyte count in peripheral blood whereas splenic CD4 and CD8 were not affected.

Peritoneal macrophages of treated mice secreted lower nitric oxide.

Activated macrophages transcriptionally express inducible NOS responsible for prolonged and profound production of NO [20]. The release of NO can lead to amplification of inflammation as well as tissue injury [21]. Therefore, inhibition of NO production is a very important target in the development of anti-inflammatory agents. In this present study, the production of NO by macrophages was altered by the treatment of mice with anise. This was accompanied by an elevation of the lymphocyte activity and neutrophil count observed *in vitro*. Usually, NO decreases the proliferation of Th1 cells and the production of IL-2 while increasing the IL-4 synthesis by Th2 cells [22-24]. Th1 type response is generated by viral and some bacterial pathogens and is associated with aggressive cellular immune response [14,25]. In contrast, the Th2 response is characteristic for parasitic chronic infection (immunoregulatory response) [14]. Moreover, NO regulates the adhesion and migration of leukocytes to the site of inflammation [26]. The previously mentioned studies may explore the inverse correlation between the high lymphocyte proliferation, lymphocyte activity against xengeneic stimuli and the levels of NO produced by the peritoneal macrophages [22,23,26]. Although the high levels of NO may have deleterious effect on immune response, NO inhibits the growth of wide variety of pathogens including viruses, bacteria, parasites and fungi [27].

The immunostimulant activity of anise may be related to the presence of a number of constituents such as Eugenol, anethol, in the essential oil. Eugenol, one of the anise ingredients, has been investigated intensively due to its use in root canal sealers[28]. A dose dependent enhancement of the plaque-forming cell response to sheep erythrocytes and the natural killer cell activity was seen in C57BL/6 treated mice[29]. Moreover, the inhibition of NO levels secreted by stimulated macrophages is in accordance with results revealed by De Oliveira Mendes *et al* [28]. They found an inhibitory effect of eugenol preparations on the phagocytic activity of macrophages and nitric oxide production in response to interferon-gamma. Moreover, previous study from Li *et al* [30]reported that eugenol can suppress NO release and inducible NO synthase expression in LPS-treated murine macrophages. One other major component of anise that may have immunomodulatory effect is anethol. Anethol has been considered to be an active estrogenic agent [6] that reduces the levels of NO and prostaglandins (PGE_2) in the inflammatory exudates in acute inflammation [31]. The effect of estrogen on the generation of NO in different tissues is well documented [32,33]. It has been found that estrogen reduces the production of NO by non-immune cells [34,35] but elevates that secreted by immune cells only when activated by mitogen [32] However, numerous studies confirmed the immunoregulatory rather than immunostimulatory effect of estrogen [36,37]. In our present study we favor the regulation of NO by estrogen like component of anise that in turn leads to the removal of its suppressive effect on lymphocyte proliferation and leukocyte migration.

Previous study by Esteva˜o-Silva *et al* [38] showed that anethole and eugenol have inhibitory effects on *in vitro* leukocyte chemotaxis induced by fMLP and LTB4. Additionally, anethole and eugenol exert inhibitory effects on rolling and adherent cells, and on the number of cells that migrated to perivascular tissue. These finding explain why we have high neutrophils number in total blood count.

It is known that DTH reaction is associated with cell mediated immunity and this plays a role in many inflammatory disorders [39]. When there is DTH reaction, there is large influx of non-specific inflammatory cells. This happens when antigens activate sensitized T-cells (generally Th1 subsets), and promote the secretion of cytokines such as IFN-γ [14]. The main effect of these cytokines is to recruit and activate macrophages, leading to an increase in vascular permeability, vasodilatation, macrophage accumulation, and activation of phagocytic activity. Although a reduction of DTH response pad thickness in anise treated and control animals was insignificant, inhibition of DTH response could indicate that the anise extract exerted immunosuppressive activity on particular cytokines responsible for cell recruitment to site of inflammation. More studies are needed to reveal the mechanisms involved in immunosuppressive activity of anise in DTH on humeral and cellular level.

CONCLUSION

This study provides preclinical evidence that anise possesses immunomodulatory activity when administered orally in mice. This activity can be recognized, at least in some measure, by the inhibitory effect on production of nitric oxide and increased cell mediated immune response. Further experiments need to evaluate additional properties and mechanisms involved in the immunomodulatory effects of anise.

DECLARATIONS

Acknowledgement

The authors are grateful to Yarmouk University for providing the facilities to conduct this study.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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