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Immunomodulatory effect of aqueous extract of *Piper nigrum* L. in mice model

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ABSTRACT

The study was carried out to evaluate the effects of water extracts of *Piper nigrum* L. fruitsat two doses (1 and 5) mg/ kg body weight daily for 30 days on the immune response of BALB/c mice by estimating of serum concentration of IL-2, IL-4, IL-10 andINF-γ using ELISA test.

Oral administration of the extract at the two doses did not produce signs of toxicity, behavioral changes or animal death.

The highest serum concentration values of IL-2 and INF-γ were founded in mice group treated with 5 mg/kg of the aqueous extract (182.40 pg/ml and 1547.00 pg/ml ,respectively), while the lowest concentration were founded in the group treated at dose 1 mg/kg of the aqueous extract (97.60 pg/ml and 945.20 pg/ml) respectively. On the other hand the highest concentrations of IL-4 and IL-10 (78.30 pg/ml and 51.90 pg/m respectively) were founded in mice group treated with 1 mg/kg of *P.nigrum* aqueous extract while the lowest concentration of IL-4 and IL-10 (55.70 pg/ml and 37.50 pg/ml respectively) were founded in group treatedcat dose 5 mg/kg.

Keywords: aqueous, black pepper, extract, *Pipernigrum* L., cytokine

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الملخص باللغة العربية

أجريت هذه الدراسة لتحديد تأثير تركيزين (1و5 ملغ/ كغم من وزن الجسم) للمستخلص المائي لثمرة نبات الفلفل الأسود (*Piper nigrum* L) المعطى يوميا لمدة 30 يوما على الاستجابة المناعية للفئران المختبرية نوع BALB/c من خلال تحديد تراكيز IL-2، IL-4، IL-10، INF-y، في المصل باستخدام اختبار ELISA.

أظهرت النتائج أن التجريع الفموي للمستخلص المائي بالتركيزين لم يحدث أية أعراض للتسمم أو تغييرا في السلوك أو موت الفئران المختبرية خلال مدة التجربة، وقد اعطت المجموعة المعاملة بالمستخلص المائي لنبات الفلفل الأسود بتركيز 5 ملغ/كغم من وزن الجسم أعلى تراكيز IL-2، INF-y (182.40 pg/ml ، 15.47 pg/ml) على التوالي، بينما كانت أقل النتائج في المجموعة المعاملة بتركيز 1 ملغ/ كغم من وزن الجسم (97.60 pg/ml و 945.20 pg/ml) على التوالي.

من ناحية أخرى، وجدت أعلى تراكيز IL-4 و IL-10 في مجموعة الفئران المعاملة بالمستخلص المائي بتركيز 1 ملغ/كغم من وزن الجسم (78.30 pg/ml و 51.90 pg/ml) على التوالي، فيما وجدت التراكيز الأقل في المجموعة المعاملة بالمستخلص المائي بتركيز 5 ملغ/كغم من وزن الجسم (55.70 pg/ml و 37.50 pg/ml) على التوالي.

INTRODUCTION

The black pepper, *Piper nigrum* (Piperaceae) has traditionally been used as both spice and medicine. It contains small quantities of chemopreventive compounds such as β-carotene, piperine, tannic acid and capsaicin (1). It stimulates the digestive enzymes of pancreas, protects against oxidative damage, lowers lipid peroxidation, and enhances the bioavailability of a number of therapeutic drugs (2). In addition, its anti-inflammatory activities have been demonstrated in rat models of carrageenan-induced rat paw edema, cotton pellet-induced granuloma, and a croton oil-induced granuloma pouch (3).Black pepper and cardamom extracts act as potent modulators of the macrophages (4). Macrophages function as antigen-presenting cells (APCs) and participate in the activation of the adaptive arm of the immune response (5). These inflammatory cells produce large amounts of tumor necrosis factor (TNF), interleukin (IL)-12 and interleukin (IL)-23 and therefore are important drivers of antigen specific type I helper T cell responses (6). T cell activation is therefore the hallmark of the initiation of the adaptive immune response (7). Indeed it is now well known that APC maturation via CD40 ligation (8) and notch stimulation in T cells is the connecting link between innate and adaptive immunity (9).

T helper (Th) lymphocytes differentiate into Th1, Th2 and regulatory T (Treg) cells, and play an important role in the serial adaptive immune response to various infectious agents through the production of specific cytokines. Th1 cells secrete interferon gamma (IFN-γ) and protect their host against intracellular pathogens and viruses (10). Th2 cells produce interleukin 4 (IL-4), IL-5 and IL-13, and support the role of B cells in removing parasites (11). Additionally, Treg cells play a critical role in the regulation of immune cellhomeostasis by producing IL-10 and transforming growth factor-beta (TGF-β) (12, 13).

The aims of the present study were to evaluate the effect of the aqueous extract of *Piper nigrum* L. on adaptive immune response in BALB/c mice.

MATERIALS AND METHODS

Preparation of aqueous extract of *Piper nigrum*

Water extraction was prepared by boiling 100 gram of *Piper nigrum* in 1000 ml distilled water for 15 minutes. The flask was plugged and removed from the heat and allowed to cool at room temperature.

After cooling, the content of the flask was filtered and dried to prepare the required concentrations (14).

Experimental animals

Thirty BALB/c mice 4-5 weeks old weighting 15-28 grams were obtained from the animals unit, college of medicine, university of Baghdad, Iraq. The animals were divided into three groups, each group consists of 10 mice, and the animals were bred in standard mice cages and fed with a suitable quantity of water and complete diet.

The first and second groups were given 0.1 ml as an oral dose of 1 mg/kg b.w. and 5 mg/kg b.w. respectively of *Piper nigrum* aqueous extract daily for 4 weeks. While third mice group were given 0.1 normal saline daily for the same period.

The animals were monitored for apparent signs of toxicity for 30 days. On the 31st day after treatment, the animals were scarified and the serum was separated after the blood to measure the levels of IL-2, IL-4, IL-10 and INF-γ.

Estimation of IL-2, IL-4, IL-10 and INF-γ value in serum

Immunomodulatory effect of the aqueous extract *P. nigrum* were evaluated by estimation of serum IL-2, IL-4, IL-10 and INF-γ.

These interleukins were measured in serum by using ELISA according to the instructions of eBioscience company, USA.

Briefly, microtiter plate was coated with 100 µl/well of capture antibody (pre-titrate purified anti- IL-2, IL-4, IL-10 or INF-γ antibody). The plate was sealed and incubated overnight at 4 °C. Cover film was removed and the plate was washed with 250 µl/well washing solution (1xPBS, 0.05 Tween-20) this procedure was repeated five times. Wells were blocked with 200 µl/well of 1x Assay Diluent and incubated at room temperature for 1 hour. Washing step was as mentioned above. 1x Assay Diluent was used to perform 2-fold serial dilutions of standards to make the standard curve. 100 µl/well of 1x Assay Diluent was added to the blank well. One hundred µl/well of standards and serum samples were loaded to appropriate wells and the wells were covered and incubated at room temperature for 2 hours. Plate was washed as mentioned above. 100 µl/well of detection antibody (pre-titrated biotin-conjugated antibody) was added to each well. The plate was sealed and incubated at room temperature for 1 hour. Cover film was removed and the plate was washed as described previously. 100 µl/well of Avidin-HRP was added to each well and the plate was sealed and incubated for 30 minutes at room temperature. Plate was washed as in step 2 and repeated for total seven washes. 100 µl/well of substrate solution, tetramethylbenzidine (TMB), to each well and incubated for 15 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution to each well. The absorbance of each well was read at 450 nm using microplate reader. The sample concentrations were determined depending on a standard curve.

Statistical analysis

Data are expressed as the mean values ± standard deviation (SD) of samples. The statistical significance of the differences between various groups was determined by PostHoc test (LSD alpha 0.05) and one-way analysis of variance (ANOVA) using SPSS version 18.0 software. Differences were considered statistically significant for $p<0.05$.

RESULTS

Enzyme linked immune-sorbent assay test were done to estimate immune responses after oral inoculation of *Piper nigrum* to determine the titers of IL-2, INF-γ, IL-4 and IL-10 in mice sera.
Tables (1-4) show the mean and standard deviation values of serum concentration of IL-2, INF-γ, IL-4 and IL-10 respectively in mice sera.

Table (1): The ELISA results of IL-2 concentration in serum expressed as pg/ml

Mice groups	No.	Mean	S.D	S.E
1 mg/Kg of *Piper*	10	97.60	±9.070	2.868
5 mg/Kg of *Piper*	10	182.40	±4.648	1.470
Control	10	21.30	±6.533	2.066

Table (2): The ELISA results of INF-γ concentration in serum expressed as pg/ml

Mice groups	No.	Mean	S.D	S.E
1 mg/Kg of *Piper*	10	945.20	±11.282	3.568
5 mg/Kg of *Piper*	10	1547.0	±5.538	1.751
Control	10	321.30	±7.660	2.422

Table (3): The ELISA results of IL-4 concentration in serum expressed as pg/ml

Mice groups	No.	Mean	S.D	S.E
1 mg/Kg of *Piper*	10	78.30	±11.624	3.676
5 mg/Kg of *Piper*	10	55.70	±7.379	2.334
Control	10	22.20	±6.374	2.015

Table (4): The ELISA results of serum IL-10 concentration expressed as pg/ml

Mice groups	No.	Mean	S.D	S.E
1 mg/Kg of *Piper*	10	51.90	±4.954	1.567
5 mg/Kg of *Piper*	10	37.50	±13.770	4.354
Control	10	21.20	±6.070	1.919

Tables (1-4) showed serum concentration values of IL-2, INF-γ, IL-4 and IL-10, respectively. Serum concentration values of IL-2 and INF-γ were 97.60 pg/ml and 945.20 pg/ml , respectively at dose 1 mg/kg and 182.40 pg/ml and 1547.00 pg/ml ,respectively at dose 5 mg/kg. On the other hand the concentrations of IL-4 were 78.30 pg/ml and 55.70 pg/ml at dose 1 and 5 mg/kg respectively, while the concentrations of IL-10 were 51.90 pg/ml and 37.50 pg/ml at dose 1 and 5 mg/kg respectively.
There was significant difference ($p<0.05$) between treated and control groups (figures 1-4) of serum interleukins concentration IL-2, INF-γ, IL-4 and IL-10, respectively. On other hand Figures 1 and 2 show that serum concentrations of IL-2 and INF-γ in group 2 were highest than the concentration of group 1, while the highest values of IL-4 and IL-10 were in serum concentration of group 1 rather than group 2.

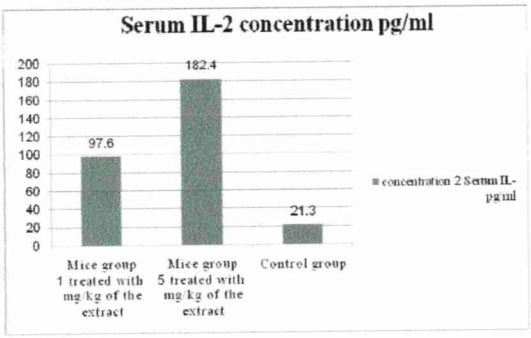

Figure (1): The ELISA results of IL-2 concentration in serum expressed as pg/ml

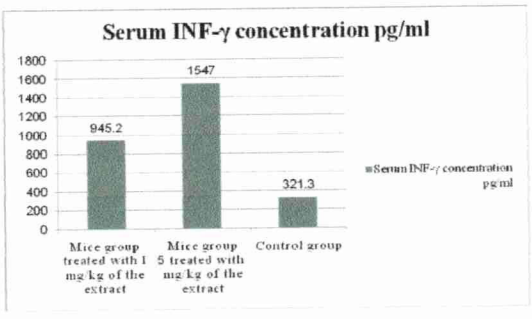

Figure (2): The ELISA results of INF-γ concentration in serum expressed as pg/ml

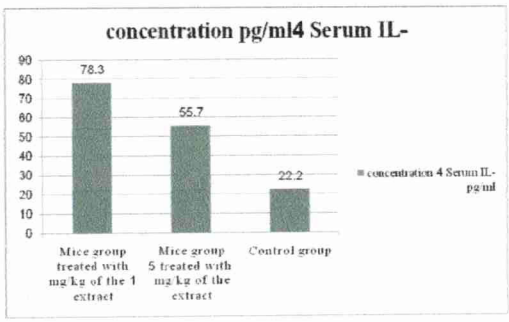

Figure (3): The ELISA results of IL-4 concentration in serum expressed as pg/ml

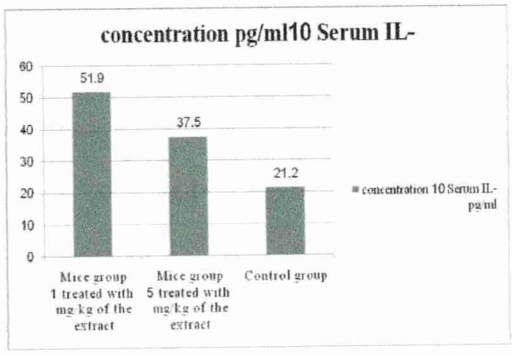

Figure (4): The ELISA results of IL-10 concentration in serum expressed as pg/ml

DISCUSSION

After the mice were orally given daily doses 1, 5mg/kg of the water extract from the dried fruits of *P. nigrum*, neither signs of toxicity nor death of mice were observed during the 30 days experimental period, similar results were also obtained by studying the same doses on mice in which the pepper did not cause toxicity at the acute toxicity study (15).

In this study the concentration of IL-2 and INF-γ in mice sera was significantly increased ($p>0.5$) by the increase of the concentration of aqueous extract (5 mg/kg) as shown in figures 1 and 2. IL-2 and INF-γ concentration in mice sera were estimated to reflect TH1 response. These result are in agreement with Vaidya and Rathod, (4), who found that aqueous extracts of black pepper is potentially capable of modulating the function of macrophages. Exposure of P388D1 cells to high concentrations of black pepper extract led to enhanced proliferation of these cells, whereas when exposed to low concentrations of extract of black pepper, the cells displayed greatly reduced proliferative activity (4).

On the other hand, IL-4 and IL-10 concentration in mice sera were estimated to reflect TH2 response. From the presented results (figures 3 and 4) IL-4 and IL-10 concentration in mice sera were significantly ($p>0.5$) decreased with the increase of the aqueous extract of *P. nigrim*. This finding were in line of Kim and Lee, (16), who founded that piperine from *P.nigrum* has been observed to exert a suppressive effect on OVA-induced asthma in mice (16).

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