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Full Length Research Paper

Immunomodulatory effects of orally administered aqueous extract from *Eupolyphaga sinensis* Walker

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This paper reported the immunnodulation tests of *Eupolyphaga sinensis* Walker in immunity. Mice were fed with *E. sinensis* water-decoction at a dose of 1.89, 3.78 and 7.56 g/kg/d for 4 weeks, then their immune function was studied. The results of the effects of *E. sinensis* water-decoction on spleen index, thymus index and carbon expurgation experiments of mice, indicated that *E. sinensis* did not affect visceral index, but enhanced observably carbon expurgatory index and phagocytic index. *E. sinensis* water-decoction could stimulate response of delayed hypersensitivity. The proliferation of concanavalin A (ConA)-induced mitogenic reponse for spleen lymphocyte was also increased. The amount of hemolytic antibody in mice serum increased when compared with those of the control group. *E. sinensis* can enhance immunce function of mice. Therefore *E. sinensis* has immunnodulation function which could be developed and utilized as health food resource.

Key words: *Eupolyphaga sinensis* Walker, mice, immunoregulation, immunological function.

INTRODUCTION

Insects have proven to be very important sources of drugs for modern medicine (Ahn et al., 2002). Chemical screening applied to some insects has confirmed the presence of various types of bioactive substances (Costa-Neto, 2002). *Eupolyphaga sinensis* Walker belongs to the family Polyphagidae and the order Blattaria. This species is widely distributed in China, but there are few reports on them in other countries. *E. sinensis* is the main specie bred and it is used in Chinese traditional medicine as component of some traumatic or vulnerary medicines (Tang et al., 2006; Zhang et al., 2008). Studies showed that it plays an important role in dealing with thrombi. *E. sinensis* has proven to be a very important source of drugs for modern medicine (Zhou et al., 1994; He et al., 2004).

E. sinensis is generally regarded as a rich source of protein, vitamins, essential amino acids, minerals and essential fatty acids like linolenic acid. The contents of toxic heavy metals were lower than national standards (Tang et al., 2005b). *E. sinensis* recently have been used in health product production. The cockroaches are also sometimes bred, together with scorpion which has medicinal importance, and their nymphs serve as food for the latter (Tang et al., 2005a).

Modulation of immune responses to alleviate diseases has been of interest for many years. There is growing interest in identifying and characterizing insects with immunomodulatory activity. A number of insects used in

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Abbreviations: CRBC, Chicken red blood cells; **PR,** phagocytic rate; **PI,** phagocytic index; **DNFB,** dinitrofluorobenzene; **DTH,** delayed-type hypersensitivity reaction; **PBS,** phosphate-buffered saline; **RPMI 1640,** Roswell park memorial institute medium 1640; **Con A,** concanavalin A; **MTT,** methylthiazoletetrazolium; **O.D,** optical density; **SRBC,** sheep red blood cells.

Chinese traditional medicines and food systems for rejuvenation therapy have been demonstrated to modulate immune responses (Zhang and Xu, 1990). Despite the fact that a lot of studies have been reported, they were limited to the researches on morphological, physiological and biological features (Tang et al., 2004); few rese-arches on the effect of *E. sinensis* on immune responses have been done yet. In the present study, we reported immunomodulatory activity of *E. sinensis* in normal mice. The objective of this paper is to provide the scientific basis for the comprehensive utilization of the insect, improve the insect utilization efficiency and evaluate the potential for future applications.

MATERIALS AND METHODS

Insect material

E. sinensis were subsequently cultured in the insect rearing room at the Anhui Agricultural University on a diet of wheat bran at 26 ± 1°C, 55 ± 5% relative humidity and a photoperiodic regime of 12 h light and 12 h darkness. *E. sinensis* used were obtained from the laboratory stocks. The insects were burnt to death by boiling water and dried to constant weight at 80°C. Lastly, the powder of dried *E. sinensis* was gotten by smashing.

Two hundred and fifty gram powdered samples were weighted and extracted with distilled water by stirring at room temperature for 2 h. The resultant suspension was filtered through a two-fold layer of muslin. Then the filtrate was co

ncentrated to 400 ml with rotary evaporator and the liquid was 0.625 g/ml (equivalent to raw material) and diluted before use. The extract was prepared just before the experiments.

Animals

Normal KunMing mice were purchased from the Laboratory Animal Center of Anhui Institute of Medical Science and the animals were kept in air controlled rooms. The experiments were conducted on female KunMing mice weighing 20 ± 2 g maintained at 25 ± 2°C with normal mouse chow and water *ad libitum*. The animals were housed, five per cage and maintained on 12 h day and night cycle. The animals were divided into four groups comprising ten rats each.

Group I: Control animals (distilled water control)
Group II: Animals fed with larvae powder extract (low-dosage group 1.89 g · kg^{-1} · d^{-1})
Group III: Animals fed with larvae powder extract (moderate-dosage group 3.78 g · kg^{-1} · d^{-1})
Group IV: Animals fed with larvae powder extract (high-dosage group 7.56 g · kg^{-1} · d^{-1})

Mice were orally administered a dose of 0.025 ml · g^{-1} body weight of larvae powder extract once daily for four consecutive weeks. The control mice were given distilled water only by the same method. After four weeks of oral administration, the immunomodulatory effect of the *E. sinensis* was obtained from each experimental group by the method described previously.

Immune organ weight of mice

Mice were sacrificed 24 h after last administration, and then the body, thymus and spleen were collected and weighed. The weight index of thymus and spleen can be described by mg · g^{-1} body weight:

$$\text{Thymus Index} = \frac{Weight\ of\ thymus\ (mg)}{Body\ weight\ (g)}$$

$$\text{Spleen Index} = \frac{Weight\ of\ spleen\ (mg)}{Body\ weight\ (g)}$$

Phagocytosis of peritoneal macrophages

Chicken blood was put into a sterile flask containing crystal ball and shaken to remove the fiber. The solution was rinsed three times with saline before centrifugation at 2000 rpm for 10 min. The supernatant was abandoned and the chicken red blood cells (CRBC) were prepared.

Phagocytosis of mice was detected using the method described by Lin et al. (1995) with slight modification. Briefly, on the last administration day, 1 ml of 20% (v/v) CRBC was intraperitoneally injected into each mouse, and the mice were euthanized 30 min later. Two milliliters saline was injected into the abdominal cavity and 1 ml fluid was then collected to make a smear for each mouse. The smears were incubated at 37°C for 30 min in a wet box, fixed with acetone-methanol (1/1, v/v) solution, and then stained by 4% (v/v) Giemsa-phosphoric acid dye. The number of macrophage ingesting CRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytic rate (PR) and phagocytic index (PI) were calculated using the following formula:

$$\text{PR (\%)} = \frac{Number\ of\ macrophage\ ingesting\ CRBC}{Number\ of\ total\ macrophage} \times 100\%$$

$$\text{PI} = \frac{Number\ of\ total\ ingested\ CRBC}{Number\ of\ total\ macrophage}$$

Delayed-type hypersensitivity reaction to dinitrofluorobenzene

After 24 days of oral administration, mice were sensitized to dinitrofluorobenzene (DNFB) by placing 100 µl 1% (w/v) DNFB in acetone-castor oil on the shaved abdomen of recipients with the area of 3 X 3 cm. Five days later, 20 µl 1% (w/v) DNFB solution was used to challenge the right ear of the mice. The antigen challenge was evaluated by the methods described with some modification. Twenty-four hours later, a piece of left and right ear with the diameter of 8 mm was obtained with a stiletto. The delayed-type hypersensitivity reaction (DTH) was calculated using the following formula:

DTH = Weight of right ear (diameter 8 mm)
- Weight of left ear (diameter 8 mm)

Lymphocyte-proliferation assays

After four consecutive weeks of oral administration, spleens were removed aseptically from mice and teased in cold (4°C) phosphate-buffered saline (PBS, pH 7.2) and pressed through a nylon sieve to liberate the cells. The suspension was allowed to sediment for

Table 1. Effect of *E. sinensis* on the immune organ weights of mice.

Group	Dosage ($g \cdot kg^{-1} \cdot d^{-1}$)	Thymus index ($mg \cdot g^{-1}$ body weight)	Spleen index ($mg \cdot g^{-1}$ body weight)
Negative control	0	3.19±0.14a	5.87±0.16a
Low-dosage group	1.89	2.95±0.17a	6.02±0.20a
Moderate-dosage group	3.78	3.08±0.13a	5.94±0.18a
High-dosage group	7.56	2.85±0.08a	5.81±0.21a

Values in the same column followed by different letters are different ($P < 0.05$) from one another.

Table 2. Effect of *E. sinensis* on the nonspecific immune response assessed by phagocytic rate and phagocytic index.

Group	Dosage ($g \cdot kg^{-1} \cdot d^{-1}$)	Phagocytic rate (%)	Phagocytic index
Negative control	0	16.87±2.14c	0.15±0.04d
Low-dosage group	1.89	17.27±1.98c	0.21±0.06c
Moderate-dosage group	3.78	22.12±2.97b	0.27±0.05b
High-dosage group	7.56	26.44±2.41a	0.37±0.06a

Values in the same column followed by different letters are different ($P < 0.05$) from one another.

several minutes and the supernatant was centrifuged (400 g, 10 min, 4°C). The pellet was suspended in 2 ml 0.17 M ammonium chloride to disintegrate erythrocytes (2 min). The remaining cells were washed three times with cold PBS. The last pellet was suspended in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, Sigma USA). Under sterile conditions, the spleen cells were adjusted to a concentration of 3×10^6 cells/ml in 2 ml RPMI 1640 and dispensed in 24-well tissue-culture plates (1 ml/well). The cell viability was measured by using the trypan blue dye exclusion method. Spleen cells were incubated at 37°C in an atmosphere comprising 5% CO_2 / 95% air in 24-well tissue-culture plates with 50 µl concanavalin A (Con A, Sigma USA) solution or pure medium for 72 h. Lymphocyte proliferation was determined using a methylthiazoletetrazolium (MTT) assay. At 4 h before the end of the incubation period, the 0.7 ml of supernatant was removed and 0.7 ml of RPMI 1640 medium was added to all wells of an assay. Subsequently, 50 µL of MTT solution (Sigma USA) was added to all wells of an assay, and plates were incubated for another 4 h under the same conditions. 1 ml isopropanol was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes, the plates were read on a Universal Microplate Spectrophotometer, using a test wavelength of 570 nm. The results are presented as optical density (O.D.) ± SD.

Antibody producing cells

The number of plaque forming cells from the spleen was determined by the Jerne's Plaque assay (Jerne et al., 1963). After 24 days of oral administration, 0.2 ml of 2 % (v/v) sheep red blood cells (SRBC) was intraperitoneally injected into each mouse. Five days later, spleens were removed aseptically from mice and teased in cold (4°C) PBS (pH 7.2) and pressed through a nylon sieve to liberate the cells. The suspension was allowed to sediment for several minutes and the supernatant was centrifuged (400 g, 10 min, 4°C). The pellet was suspended in 2 ml 0.17 M ammonium chloride to disintegrate erythrocytes (2 min). The remaining cells were washed three times with cold PBS. The last pellet was suspended in RPMI 1640 medium. Under sterile conditions, the spleen cells were adjusted to a concentration of 5×10^6 cells/ml in 5 ml RPMI 1640. 25 µl of spleen cell suspension and 50 µl of 10% SRBC (v/v) were mixed with equal volumes of Hank's solution (pH 7.2), and incubated at the slides which were spread by molten agarose in an atmosphere comprising 5% CO_2/95% air for 1.5 h. Subsequently, number of plaques was counted using a colony counter.

Statistical analysis

The results are reported as mean ± standard deviations. Statistical evaluation of the data was done using student's t-test. A probability value of <0.05 was considered significant.

RESULTS

Effect of *E. sinensis* on immune organ weights

Effect of *E. sinensis* administration on immune organ weight of mice is given in Table 1. No significant difference on weights was found in groups supplemented with different dosages of *E. sinensis* when compared with distilled water treated group.

Effect of *E. sinensis* on phagocytosis of peritoneal macrophages

Phagocytic rate was significantly increased from 16.87% in negative control to 22.21 and 26.44%, respectively, in group treated with 3.78 g and 7.56 $g \cdot kg^{-1}$ body weight *E. sinensis* (Table 2). The phagocytic index was 0.15 in negative control, which was significantly increased to 0.21, 0.27 and 0.37, respectively, in groups treated with 1.89, 3.78 g and 7.56 $g \cdot kg^{-1}$ body weight *E. sinensis*.

Table 3. Effect of *E. sinensis* on the cellular immune response assessed by DTH reaction and lymphocyte proliferation.

Group	Dosage (g · kg^{-1} · d^{-1})	Difference in ear thickness (mg)	The O.D. value of lymphocyte proliferation induced by ConA
Negative control	0	2.98±0.56 b	0.208±0.013 b
Low-dosage group	1.89	3.05±1.28 b	0.214±0.017 b
Moderate-dosage group	3.78	4.58±1.17 a	0.231±0.021 b
High-dosage group	7.56	5.05±1.54 a	0.274±0.024 a

Values in the same column followed by different letters are different ($P < 0.05$) from one another.

Table 4. Effect of *E. sinensis* on the humoral immune response assessed by antibody producing cells.

Group	Dosage (g · kg^{-1} · d^{-1})	Number of plaque (×10^3/ spleen cells)
Negative control	0	68.14±7.21 c
Low-dosage group	1.89	74.12±9.95 c
Moderate-dosage group	3.78	85.45±9.43 b
High-dosage group	7.56	96.12±9.91 a

Values in the same column followed by different letters are different ($P < 0.05$) from one another.

Therefore, it was dose-dependent to potentiate the nonspecific immune response in mice.

Evaluation of *E. sinensis* on cellular immune response

The effect of *E. sinensis* on the DTH reaction in mice was tested. As shown in Table 3, difference in ear thickness was significantly increased from 2.98 in negative control to 4.58 and 5.05, in group treated with 3.78 and 7.56 g· kg^{-1} body weight *E. sinensis*.

The O.D. value of lymphocyte proliferation induced by Con A was significantly increased from 0.208 in negative control to 0.274, in group treated with 7.56 g · kg^{-1} body weight *E. sinensis* (Table 3). However, no significant differences were found in the dosage of 1.89 g and 3.78 g. kg^{-1} body weight when compared to negative control.

Evaluation of *E. sinensis* on humoral immune response

E. sinensis extract administration was found to significantly enhance the number of antibody producing cells in spleen. As shown in Table 4, the number of plaques was significantly increased from 68.14 × 10^3 in negative control to 85.45 × 10^3 and 96.21 × 10^3, respectively in group treated with 3.78 g and 7.56 g · kg^{-1} body weight *E. sinensis*. It was dose-dependent to potentiate the humoral immune response in mice.

DISCUSSION

The insects are thought to be one of the biggest biological resources that have not been fully exploited by human being. China is one of the earliest countries to exploit insect resources in the world and has been the top producer for over one thousand years of many insect-related industrial products, such as silk, insect wax and Chinese gallnuts (Zhang et al., 2008). *E. sinensis*, as an important drug in the traditional Chinese herbs, contains abundant active components, such as proteins, amino acids, unsaturated fatty acids, flavones, alkaloids, etc. The insect is considered safe and used as traditional Chinese medicine and edible insect (Tang et al., 2006). In fact, essential nutrients and an array of phytonutrients have been shown to affect almost every aspect of the immune system (Geetha et al., 2005). In view of this, the present study was carried out to investigate the effect of *E. sinensis* on some immunological parameters.

Administration of *E. sinensis* was found to increase phagocytic rate and phagocytic index significantly, indicating that the extract could stimulate the nonspecific immune response system. Moreover, the extract was not found to stimulate the weight of spleen and thymus, indicating that the extract was not harmful to the immune organs. The extract from *E. sinensis* could stimulate T cell-mediated immunity through delayed-type hypersensitivity. DNFB that acted as the antigen could induce the activation of the T cell, which would subsequently release different kinds of cytokine and attack the target cell (Yang et al., 2006). It is well known that T cells respond to

stimulation by Con A and enter into cell division (Rasool and Sabina, 2009). To understand the immunomodulatory mechanism of the extract, *in vitro* studies were carried out using rat lymphocytes. Our studies have demonstrated that the extract alone significantly stimulated the lymphocyte proliferation.

In conclusion, nonspecific, cellular and humoral immune responses are three dominant indexes to evaluate the immunomodulatory activity of the test samples, which were determined to be efficient with the positive results. Therefore, *E. sinensis* enhanced all three indexes when compared with negative control. The results indicated that *E. sinensis* had a potency to potentiate immune responses in mice. At present, we do not know which compounds are responsible for the immunostimulatory activity produced by this extract. Further studies using isolated compounds are in progress.

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