

Immunological Role of *Annona muricata* (Dietary Supplement of gravula) Against Cypermethrin's Toxic Effects in Female Rats

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Abstract

The current research was conducted to investigate the toxic effects of Cypermethrin (CYP) on female rats and the immunological response with or without the oral administration of Graviola (Dietary Supplement) by the use gavager. Sixteen adults female rats aged 60-65 days divided equally into four groups; the first group was orally gavaged with 1 ml of sterile normal saline as a control group, the second group was orally gavaged with 14.5mg/kg B.W. of cypermethrin, while the third group was orally gavaged with 2.5mg/ml of graviola. Whereas the fourth group was gavaged with 14.5mg/kg of B.W. of cypermethrine plus 2.5mg/ml of graviola. On day 30 the cellular and humoral immune responses were examined for all groups, blood samples were gathered for phagocytic activity measurement and serum was separated by ELISA method for measuring IgG titer. The mean value of phagocytic indices in First, Second, Third and Fourth groups as follow: the first group was (22±3.83) as a control group, the second group was (13.6±2.54) that treated with cypermethrin, the third group was (89±1.82) that treated with graviola and the fourth group that treated with cypermethrin+graviola was (68.6±2.21); The current study showed that the graviola had a marked increase in the cellular immune response through the high increase of phagocytic indices due to Toll-like receptor 4 (TLR4) activation. The result of this study showed that the mean value of phagocytic indices in rats that gavaged with CYP, decreased significantly ($p < 0.05$) to 13.6±2.54 as compared to that gavaged with normal saline (control group), which was 22±3.83. The mean value of phagocytic indices in rats that gavaged with both CYP and Graviola. Increased significantly ($p < 0.05$) to 68.6±2.21 as compared to that gavaged with normal saline (control group), which was 22±3.83. And the group that gavaged with Graviola increased significantly ($p < 0.05$) to (89±1.82) as compared with the control group. Whereas rats that gavaged with Graviola. Resulted in a further significant ($p < 0.05$) increase to 89±1.82. The result of ELISA test expressed high value of antibody titers against Graviola 4.86±0.68 g/ml as compared with values of 1.96±0.09 g/ml against cypermethrin.

Key Words: Cypermethrin, Graviola, Rats, humoral immune immunity

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Introduction

Cypermethrin (CYP) has been approved as the common name for (RS)—cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate by the International Organization for Standardization CYP is a synthetic pyrethroid that is used topically to manage ticks, fleas, moth and blowflies (EMA,2004). Alfacypermethrine (α -CYP) (two of the four cypermethrine cisomers) is the most powerful CYP and is widely used in farming, cattle and domestic ectoparasitese to safeguard human health (Gilbert et al, 1989; Mueller et al., 2006).

CYP isn't free from side effects, signs such as muscle tremors, ataxia, limb weakness, seizures, coma and death from respiratory depression have been recorded in rabbits following ingestion of a large dose of CYP, while its dermal contact in the facial region may cause tingling or subjective numbness sensation (Sandhu and Brar, 2000). CYP is extremely toxic to fish, bees and water insects but low toxic to birds and when used according to the label instructions apps around the home or other housing locations pose little danger to aquatic life (US Environmental Protection Agency, 1989). The absorption and excretion of CYP from the digestive tract requires a swift course (Kumar *et al.*, 2004). Within 48 hours, both male and female rats excreted 50-65% of CYP in their urine. Rats excreted 30% of the CYP within 3 days in their feces (WHO, 2009). Significantly decreased fertility in male rats at a dose rate of 1 mg / kg / day following intermediate oral exposure to CYP; additional reproductive toxicity studies may be intended to support or refute these outcomes (Abd-El-Aziz *et al.*, 1994). *Annona muricata* is a miraculous evergreen tree discovered in South and North America's rainforest. This tiny tree in herbal medicine is well known and has several names: Sour-sop, Brazilian Paw Paw, Guanabana. The plant can grow to a height of 5-6 metres. Graviola has big, shiny, dark-green leaves and delicious fruits frequently referred to as paw paw. The latter are cardiac-shaped and have a diameter of about 15-20 cm (Chang, 2003). Thanks to the novel alkaloids discovered in its plants and roots, Graviola acts as an antidepressant. While undergoing therapy with this plant, however, one should be very cautious because these alkaloids may be poisonous to the nervous system. Scientists indicate they could lead to Parkinson's disease. Another very significant chemical compound found in fruits is the serotonin inhibitor that gives pleasure (Rottscholl *et al.*, 2016). Immunology is the body's protection against infection research. We live with microorganisms, many of which cause illness. Yet despite this ongoing exposure, we rarely get sick. How is the body defending itself? How does the body eliminate the invader and heal itself when infection occurs? And why are we developing long-lasting immunity and overcoming infectious disasters to many once experienced? These are the issues addressed by immunology, which we are studying to comprehend the cellular and molecular level defenses of our body against infection. Immunology is a science that is comparatively new. Its origin is frequently credited to Edward Jenner (Murphy and Weaver, 2009). An immune response can be split conveniently into two components: (1) a particular reaction to a specified antigen and (2) a more unspecific increase to that reaction. A significant characteristic of the particular reaction is that during a second exposure to that antigen there is a faster reaction to the antigen. The booster impact is the memory of the original reaction. The specific immune response can be split into two parts for comfort: (1) the humoral response and (2) the cellular reaction to a specified antigen. However, the lymphocyte mediates both reactions. Humoral reactions are antibodies that are generated in reaction to a specified antigen, and they are proteins, have comparable structures, and can be split into different groups of immunoglobulins. Cellular reactions are determined by cells and can be transmitted only by cells. The general dogma held that the immune response involved only antibodies until the 1940s. Dr. Merrill Chase, who started his experiments in a laboratory dedicated mainly to humoral responses, showed obviously in a series of elegant experiments that immunity is not only humoral, but that a cellular reaction by lymphocytes can also generate immunity (Zabriskie, 2009).

Materials and methods

Drug

Graviola (*Annona muricata*) was obtained from the Ebay company (U.S.A).

Methods

Experimental Animals

16 adult female rats were purchased from Pharmaceutical Control Laboratory/ Baghdad. Animal's care and management was following the animal care policy of Al-Qasim Green University. Animals ages between 60-65 days, weights ranged 165-185g, placed in plastic cages especially designed for this purpose and strung with metal hoods, equipped singly to drink water system and furnished sawdust and has clean cages and sterilized with disinfectant care. Experimental animals underwent to the laboratory conditions and suitable temperatures and water has been provided. Rats were kept under controlled temperatures (18–22 °C) and relative moisture (60[±10] percent) at 7:00 AM to 7:00 PM light / dark cycle and had ad libitum access to conventional rodent chow (pellets) and filtered water.

Experimental design (Study design)

The current study was performed in the animal's house / at College of Veterinary Medicine / Al-Qassim Green University, for a period of one month and half started from the first of January to the mid of February 2019. The rats were fed on commercial assorted pellets, green fodder twice daily and clean water for one week before beginning the experiment by rearing in separate, cleaned and disinfected cages. They were divided into four equal groups and treated as following:

- * The first group of 4 rats was gavaged with 1 ml of normal saline as a control group.
- * The second group of 4 rats was orally gavaged with 0.2ml of (14.5 mg/kg B.W. cypermethrine) (10% CYP completed to 0.5 ml distilled water (according to dose recommended by (Manna *et al.*,2006)) for one month.
- * The third group of 4 rats was orally gavaged with 1ml of (2.5 mg /kg. b.w Graviola according to dose recommended by(Taylor, 2002)). for one month.
- * The fourth group of 4 rats was orally gavaged with cypermethrin +Graviola for one month.

The blood samples were collected for serum separation from all the above groups after thirty days. Sera were stored individually at 4°C for humeral immune response by measuring Abs titers (IgG) through ELISA technique.

Sample collection

At the end of the study and After 24 hours from last exposure , we fastened the animals for (4 hours from 8 o'clock a.m. till midday), all animals were anaesthetized by using xylazine 0.1 ml & ketamine 0.1 ml. Blood sample was collected from each rat directly from the heart by using the heart puncture (cardio-puncture), by using (5ml) sterile disposable syringe in order to draw (5ml) of blood. Then putting 3 ml of blood in test tube containing on the EDTA anticoagulant substance to prevent coagulation of the blood and all tubes kept in minus (80°C) waiting for conducting analyzes of the blood for hematological parameters. And putting 2 ml of blood in gel tubes for serum separation from all the above groups. Sera were stored individually at (4°C) for humeral immune response by measuring Abs titers (IgG) through ELISA technique.

Phagocytosis:

The ability of phagocytic cell for engulfing the Nitro blue tetra zolium (NBT) was determined according to (Park et al.,1968) that modified by (Matula and Paterson ,1971) preparation of NBTdye:1mg of NBT dye was dissolved in 1ml of PBS PH 7.2 and let to stand for a few minutes and vigorously shaken. Then 0.1 ml of NBT solution was transferred to a vial, containing heparinized blood of immunized or control group of animals and the mixture was mixed incubate at 37°C for 15-30 minutes, then removed and let to stand for 15 minutes at room temperature again and the mixture was mixed to clean glass slide for preparing a smear (thick) to avoid distraction of erythrocytes & leukocytes, finally the smear was treated with Gimsa stain & examined finally microscopically with oil immersion (100x) (at least 200 cells randomly may be enumerated).

$$\text{Phagocytic Index} = \text{No. of engulfing cells} / \text{Total No. of phagocytic cells} \times 200$$

Statistical Analysis

The statistical results of the data were analyzed according to Complete Randomized Design (C.R.D.) . The mean differences between the averages of the studied traits were determined at the probability level of (0.01) using the Duncan test (Duncan, 1995). Statistical data were analyzed using the (SAS, 2010).

Results and Discussions

ELISA test for detection levels of humeral immunity (Ab titers):

In this study sixteen serum samples of the four groups of rats(4 samples/group) were analysed for the antibodies (IgG) titers with the ELISA technique. Comparison of the mean values of the Abs titers between rats groups that gavaged orally with normal saline alone or with either CYP. or Graviola only or with both CYP. And Graviola are shown in table (1).

The results of the ELISA test revealed that there were significant ($p < 0.05$) differences in the mean values of the Abs titers between all the four groups.

The mean value of Abs titer in rats that gavaged with CYP. alone decreased significantly ($p < 0.05$) to 1.96 ± 0.09 as compared to dose that gavaged with normal saline (control group) which was 2.97 ± 0.58 . The mean value of the Abs titer in rats that gavaged with both CYP. and Graviola increased significantly ($p < 0.05$) to 3.92 ± 0.42 , whereas rats that gavaged with Graviola only resulted in a farther significant ($p < 0.05$) increase of the mean value of Abs titer to 4.86 ± 0.68 .

Table (1): Mean values and standard error of antibody (IgG) titers of the immunized groups and non-immunized groups after 30 days:

group	time	24 hours Mean \pm SE g/ml
1 st group. (control)		2.97 \pm 0.58 C
2 nd group Cyp.		1.96 \pm 0.09 D
3 rd group Grav.		4.86 \pm 0.68 A
4 th group Cyp.+Grav.		3.92 \pm 0.42 B

In this study a significant decline in antibody titer in CYP treated group suggesting CYP induced immune suppression, similar observation recorded by (Nishal *et al.*,2012) reported a decrease *Salmonella typhimurium* Ab. titer of rats with CYP treated. (Stelzer and Gordon ,1984; Desi *et al.*,1986) also showed inhibited the proliferation of mouse T and B cells. According to results mentioned above, the study suggested that graviola stimulated the humoral immune response and such high IgG titer was similar to those observed by (De Sousa *et al.*,2010) who indicated that the GDS Fruit Extract was used as an humoral immunity enhancement and this could be attributed to its ingredients such as annonaceous acetogenins, which cause an increase in IgG titer. other researchers (Adeyemi *et al.*, 2010) showed that oral immunization of rats with gravula induced the production of a highly important level of total IgG (especially IgG1) this agree with current results, Primary antibody responses to immunization by Grav. and the production of IgM and IgG3 in early infection appear to be stimulated mainly by a T-independent mechanism (Dai *et al.*, 2011). The results are contract with Paul *et al.*,(2013) Kinetic analysis of immunoglobulin subclasses also may persist and these confirmed by (Rottscholl *et al.*, 2016) study that showed persistently high levels of IgG1 and IgG2a subclasses in immunized animals with Grav. until 6 weeks of immunization, whereas IgG3 existing a significant decline after 1 month of immunization. In infected non-immunized control mice, all IgG subclasses showed a gradual increase after the first month of infection until the experiment termination.

Phagocytic assay to determine cellular immunity.

The mean values of phagocytic indices in both treated groups and control are shown in table (2). The mean value of phagocytic indices in rats that gavaged with CYP, decreased significantly ($p < 0.05$) to 13.6 ± 2.54 as compared to that gavaged with normal saline (control group), which was 22 ± 3.83 . The mean value of phagocytic indices in rats that gavaged with both CYP. And Graviola. Increased significantly ($p < 0.05$) to

68.6±2.21 as compared to that gavaged with normal saline (control group), which was 22±3.83. Whereas rats that gavaged with Graviola. Resulted in a further significant ($p<0.05$) increase to 89±1.82.

Table(2):The mean values of **phagocytic indices** in immunized and non-immunized groups:

group	Time
	24 hours
1st group Control group	22±3.83 D
2nd group treated with Cyp.	13.6±2.54 C
3rd group immunized Grav.	89±1.82 A
4th group treated with CYP.+Grav.	68.6±2.21 B

The current study showed that the graviola had a marked increase in the cellular immune response through the high increase of phagocytic indices due to the activation of Toll-like The current study showed that the graviola had a marked increase in the cellular immune response through the high increase of phagocytic indices due to the activation of Toll-like receptor 4 (TLR4) by damaging the associated molecular pattern molecules that released from the dead hepatocytes and triggered an inflammatory response, this result was in agreement with (Goon *et al.*,2016) who mentioned that the graviola components and TLR4-mediated an activation of innate immune signaling pathways. It is worth noticing that the degree of up-regulation and increased cytokine production was less than those induced by Lipopolysaccharides (LPS). The major component in graviola which is annonaceous acetogenins that induces macrophage activation and activates innate immune responses requires a further study. Several researches also provided new information on the productive immune response, indicating that Grav. can skew the type 1-type 2 cytokine towards a preferentiallyTh2 polarization (Yuan , 2003) mentioned Grav. caused high IL-4 and IL-13, low IFN-g, and no IL-12 production, yet Th1and Th2 responses depend not on the quantity of cytokines shaped but also on the Th1/Th2 ratio. The IFN-g/IL-4 ratio is related to the lymphocyte phenotype. It is notable that the amplified production of IL-4 and IL-10 in Grav. immunization (Rady *et al.*, 2018).

Conclusions

The results of statistical analysis of immunological teste of the four groups indicated that Gravula (5 mg\kg) had antitoxic effects can protect CYP.-induced toxicity effects in rats

Conflict of interest

None of the authors have any conflicts of interest to declare.

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The research was performed independently, there is no funding, influence over study design, analyses, manuscript preparation, or scientific publication.

Ethical clearance

The project was approved by the local ethical committee (College of Veterinary Medicine/ Al-Qasim Green University.(C413/12) .

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