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Research Article

Phytochemical Screening and Immunomodulatory Study on Hydroalcholic Extract of *Annona squamosa* Leaves on Albino Rats

Chandkamadhu*, Sale Shashikanth, Y Bala Madhuri, Rosna Benny, V Swathi and B Srilatha

Department of Pharmacology, MLR Institute of Pharmacy, Jawaharlal Nehru Technological University Hyderabad, India

***Corresponding Author:** Chandakamadhu, Department of Pharmacology, MLR Institute of Pharmacy, Jawaharlal Nehru Technological University-Hyderabad, Hyderabad 500043, India.

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Abstract

The aim of the present study was to investigate the immunomodulatory activity of hydroalcholic extract of *Annona squamosa* leaves in experimental model of immunity i.e., Delayed Type Hypersensitivity (DTH), Humoral Antibody Titre (HA), Total Leukocyte Count (TLC), and Differential Leukocyte Count (DLC). The hydroalcholic extract of *Annona squamosa* in two different doses 200 mg/kg and 400 mg/kg was tested for their immunomodulatory action, out of which the higher dose of 400 mg/kg showed statistically significant immunomodulatory activity.

Keywords: *Immunity; Delay Type Hypersensitivity; Humoral Antibody Titre; Total Leukocyte; Differential Leukocyte Count; Hydroalcholic Extract*

Introduction

Immunology is the study of the way in which the body defends itself against invading organisms or internal invaders (Tumors) [1] has developed rapidly over the last 40 years, and particularly during the last 10 years with the advent of molecular techniques. It is now a rapidly moving field that contributing critical tools for research and diagnosing, and therapeutics for treatment of a wide range of human disease [2].

Humoral Immunity: In which circulating immunoglobulins (antibodies) are produced by the plasma cells, derived from lymphocytes arising from the lymphoid tissue of the gut, known as B cells.

Cellular Immunity: It is medicated by thymus-derived cells known as T cells [3], which interact with the antigen to reduce lymphocytes [4]. The immune response is an essential defense mechanism against the invasion of the body by bacteria. Sometimes the immune reaction to an antigen does not only produce antibodies which damage the antigen, but may also damage body tissue causing hypersensitivity reactions [5]. These reactions are of four types: Type (1g E or regain Dependant) reaction, Type II (cytotoxic tissue-specific antibody) reaction, Type III (Immune-complex disease, Arthus reaction), Type IV (cell-mediated delayed hypersensitivity reactions) [6]. A study of the immune systems in vertebrates requires suitable animal models. The choice of an animal depends on its suitability for attaining a particular research goal. If large amounts of antiserum are sought, a rabbit, goat, sheep, or horse might be an appropriate experimental animal [7]. If the goal is development of a protective vaccine, the animals chose meet be susceptible to the infectious agent so that the efficacy of the vaccine can be assessed. Mice of rabbits can be used for vaccine development if they are susceptible to the pathogen. For most basic research in immunology, mice and Rats have been the experimental animals of choice. They are easy to handle, are genetically well characterized and have a rapid breeding cycle. The immune system of Rats and mice has been characterized more extensively than that of any other species [8]. There is no effective drug for treatment of certain infections like AIDS, hepatitis, and other viral infections.

For other infections the drug (mainly antibiotics) being used are becoming ineffective due to development of microbial resistance, necessitating the search for newer drugs [9]. Any such new drug will be available only at an exorbitant cost due to the product patent norms under WHO agreement. In Siddha, Ayurveda and other ancient systems of medicine, many plants and plant preparations are reported to be useful in the treatment of infections [10,11]. When screened by modern scientific methods these preparations did not show any anti-microbial activity [8]. These drugs may not probably act directly upon the microbes. Instead may stimulate the body's defense mechanism (immune system) and thereby help to cure the infection. Hence by screening herbal drugs and their extracts for their immunostimulant property [12] it may be possible to get effective, cheaper new molecular entity for the treatment of various infections. It may be hoped that such type of drugs will not produce microbial resistance, since they do not act on the microbes and will not have adverse side effects since they are from (natural) plant origin [13]. This work is to prove the immunomodulatory property of in wistar rats by studying the Delayed Type Hypersensitivity (DTH), Humoral Antibody titre (HA), Total Leukocyte Count(TLC), and Differential Leukocyte Count (DLC) in the hydroalcholic extract of *Annona squamosa* leaves [14,15].

Material and Methods

Collection of plant materials

The leaves of *Annona squamosa* were collected near dundigal village and shade dried until the color turns to brownies. Then the leaves are powered and the resultant powder was taken for extraction.

Preparation of aqueous extract

The leaves were extracted with sufficient quantity of distilled water and ethanol in ratio of 70:30. Total 400 gm of leaves was subjected to extraction. The leaves were soaked in the beaker with ethanol and water ratio (30:70) for 72 hrs with regular stirring [16]. Then it was filtered through a thin muslin cloth, the resultant extract was subjected to freeze drying at the Center of Advanced Studies (CAS) at the Marine Biology department, of Annamalai University at Parangipettai. The yield was 30 gm and perform the phytochemical screening [17].

Experimental Animals

The experimental protocol has been approved by institutional animal ethics committee. MLR Institute of Pharmacy, JNTUH, Reg. No: 1567/po/a/11/CPCSEA, Rats of wistar strain weighing between 150 to 250 gm were maintained under standard laboratory conditions. They were provided with a standard diet supplied by Pranav Agro Industries Ltd. India and water ad libitum at central animal house

Experimental Protocol

24 rats were divided into four groups of six animals each.

Group I: Control

Group II: Extract was *Annona squamosa* administered at a dose 200 mg/kg/day by oral route for 14 days.

Group III: Extract was *Annona squamosa* administered at a dose 400 mg/kg/day by oral route for 14 days.

Group IV: Standard-Levamisole was administered at a dose of 50 mg/kg/day by oral route for 14 days.

Experimental Setup

The animal model is required to study the following [18-25]

a. Delayed type hypersensitivity (DTH) response
b. Humoral antibody (HA) titer
c. Total leukocyte count
d. Differential leukocyte count

Determination of Delayed Type Hypersensitivity Response (DTH)

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1X 10^8 cells, intraperitoneally, on day 0. On Day 8, after immunization the thickness of the right hind footpad was measured using a Vernier caliper. The rats were then challenged by injection of 1 X 10^8 sub SRBCs in the left hind footpad. The footpad thickness was measured again after 24h of challenge. The difference between the pre and post challenge footpad thickness, expressed in mm was taken as a measure of the DTH response. The following formula to be used to measure the DTH response [26].

$$\frac{\text{Left foot pad challenged with antigen} - \text{Right foot pad control}}{\text{Left foot pad challenged with antigen}} \text{ X } 100$$

Humoral Antibody Titre

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1X 10^8 cells, intraperitoneally, on day 0. Blood samples were collected in micro centrifuge tubes from individual animals of all the groups by retro orbital vein puncture on day 10. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the hemagglutination technique [26].

Method for Serial Dilution

This was performed by using 96 wells (12 x 8) U bottomed titre plate. The wells were marked from I to XII. In the first (I) and last well (XII) 25 µl of serum collected from treated animals was added and inactivated at 56 °C for 30 minutes. Afterwards to all the wells except well number XII, 25 µl of PBS was added.25 µl was taken from first well and added to 2nd well again 25 µl from second well was taken and added to third well and continued the same procedure up to well number XI. After this 25 µl of sample from well number XI was discarded. Finally 25 µl of 1% SRBC was added to all the wells and was kept at room temperature for two hours [26].

Total Leukocyte Count

WBC Diluting Pipette

It has got three graduations. Two graduations 0.5 and 1 are present on the stem of the pipette and the third mark 11 is placed just above the bulb. Blood is drawn up to mark 0.5 and the rest of the bulb is filled by sucking up diluting solution up to the mark 11, the bulb of the pipette is so constructed that it holds exactly 20 times the volume of fluid contained in the stem of the pipette up to mark 1. Although fluid is drawn up to 11, the dilution of the blood will be 20 because the last part of the fluid remains locked up in the stem and is not available for dilution [26].

The counting chamber

The ruling area consists of 9 square millimeters. The central of the smallest squares are separated by triple lines in which RBC will be counted. The side of each square for counting WBC is ¼ mm.

Diluting fluid for WBC (Turks fluid)

Commonly the fluid is made up as follows: Glacial acetic acid (1.5 ml); 1% solution of gentian violet in water (1 ml); Distilled water (98 ml). The glacial acetic acid haemolysis the red cells, while the gentian violet stains the nucleus of leukocytes.

Method of counting WBC: The white cells are counted in four corners of 1 square millimeter ruled area on both sides. The white cells are recognized by the retractile appearance and by the slight colour given to them by the stain contained in the diluting fluid. The cells touching the left side and upper side of boundary line are not counted [26].

Differential Leukocyte Counts

A thin blood film was made on a clean, dry, glass slide. It was dried fixed and stained to differentiate the different types of leukocytes. Hundred leukocytes were counted and percentage of different leukocytes was calculated [26].

Composition of leishman's stain

It contains a mixture of methylene blue and eosin dissolved in acetone free methanol

Procedure

A thin blood film was made on a clean dried glass slide. It was dried and stained with leishman's stain solution. The drop of leishman's stain was counted & 2 minutes was allowed to fix the blood film. Fixation means nucleus and various cellular organs will be fixed without any damage to the cells or cellular organs. After 2 minutes double the quantity of distilled water was added over the slide and waited for 7 minutes. In the mean time the stain will initiate the chemical reaction. The acidic dye eosin will initiate various acidophilus structures and some neutrophyllic granules and basic dye will stain structure like nucleus, basophilic granules, and cytoplasm of the lymphocyte and monocytes. After 7 minutes the slide was washed in a slow stream of water later it was dried in air. One drop of cidar wood oil was placed over the film. The cells were identified and entered into 100 squares. This gives the % of different types of leukocytes present in rat blood.

Results

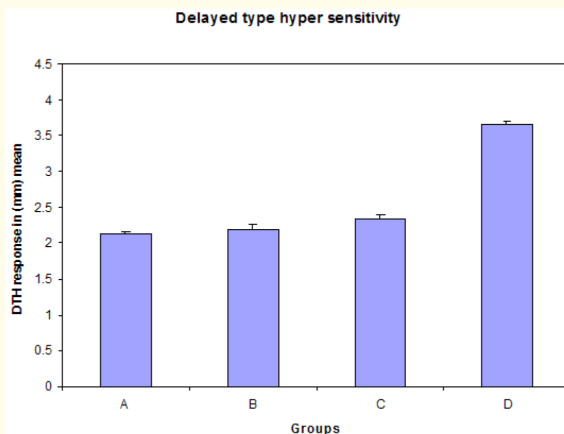
Name of the Plant	Alk	Carb	Gly	Tan	Phytos	Flav	sapo	Pro	muci
Annona squamosa Hydroalcholic extract	+	+	-	+	+	+	+	--	+

Table 1: *Phytochemical Screening.*
Alk: Alkaloids; Carb: Carbohydrates; Gly: Glycosides; Tan: Tannins; Phtos: Phytosterol; Flav: Flavonoids; Sapo: Saponins, Pro: Proteins; Muci: Mucilages.

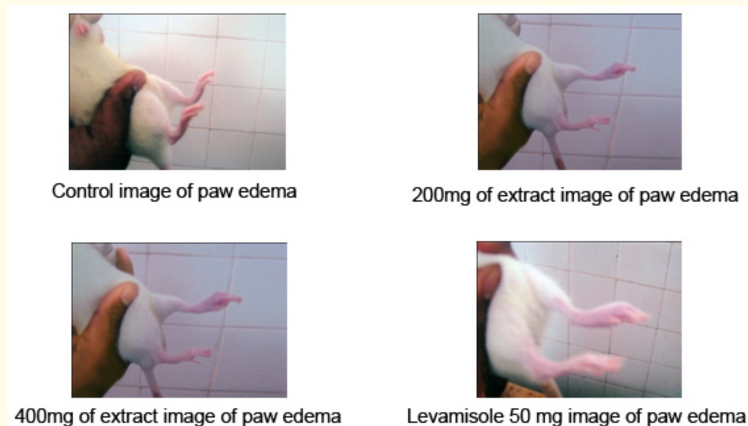
Group	Treatment	Dose	DTH response in (mm) mean paw edema ± SEM (n = 3)
1	Control	-	2.162 ± 0.033
2	Test extract-I of *Annona squamosa*	200 mg/kg	2.200 ± 0.058
3	Test extract II of *Annona squamosa*	400 mg/kg	2.319 ± 0.067
4	Standard levamisole	50 mg/kg	3.754 ± 0.033**

Table 2: *Delayed type hyper sensitivity. Effects of Test Extracts and Standard Drug on DTH Response in Rats Using Sheep's RBCs as Antigen. DUNNETT t test and p values as significant.*
**if $p < 0.05$, highly significant **if $p < 0.01$, as compared to control.*

Graph 1



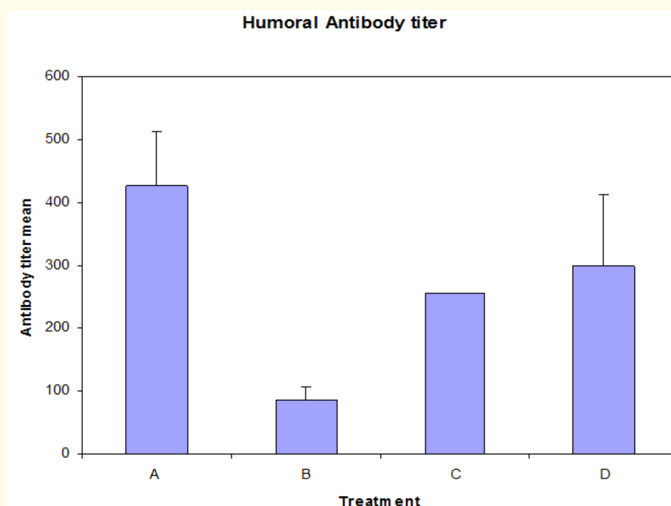
A: Control; B: Annona squamosa-200 mg/kg; C: Annona squamosa-400 mg/kg; D-Levamisole-50 mg/kg



Group	Treatment	Dose	Antibody titer mean ± SEM (n = 3)
1	Control		437.67 ± 85.33
2	Test extract-I of *Annona squamosa*	200 mg/kg	86.44 ± 21.33
3	Test extract II of *Annona squamosa*	400 mg/kg	267.00 ± 0.00
4	Standard levamisole	50 mg/kg	309.67 ± 112.89

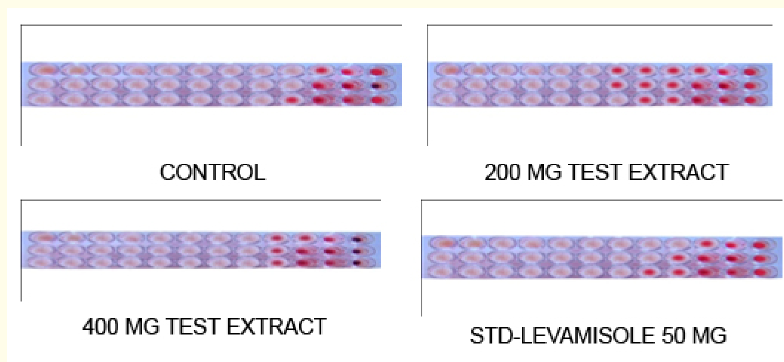
Table 3: *Humoral Antibody Titer.*

*DUNNETT t test and p values as significant * if p < 0.05, as compared to control*



A: Control; B: Annona squamosa 200 mg/kg; C: Annona squamosa 400 mg/kg; D-Levamisole 50 mg/kg

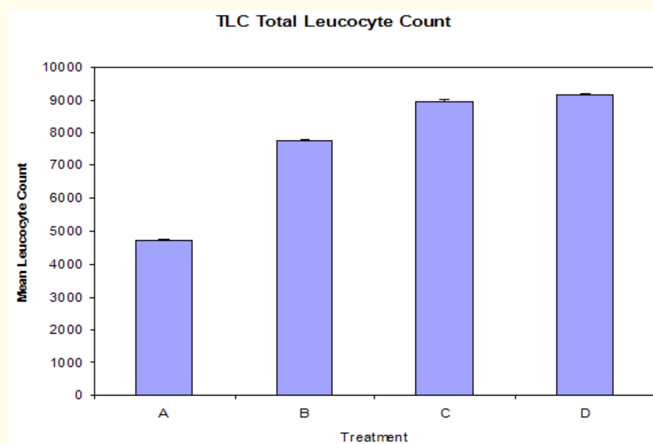
Micro Titer Plate



Group	Treatment	Dose	Mean Leucocyte Count (n = 3)
1	Control		4847.67 ± 31.80
2	Test extract-I of *Annona squamosa*	200 mg/kg	7837.67 ± 67.41**
3	Test extract II of *Annona squamosa*	400 mg/kg	9073.33 ± 63.60**
4	Standard Levamisole	50 mg/kg	9284.33 ± 14.53**

Table 4: *Total Leukocyte Count.*

*DUNNETT t test and p values as significant *if p < 0.05, highly significant **if p < 0.01, as compared to control.*

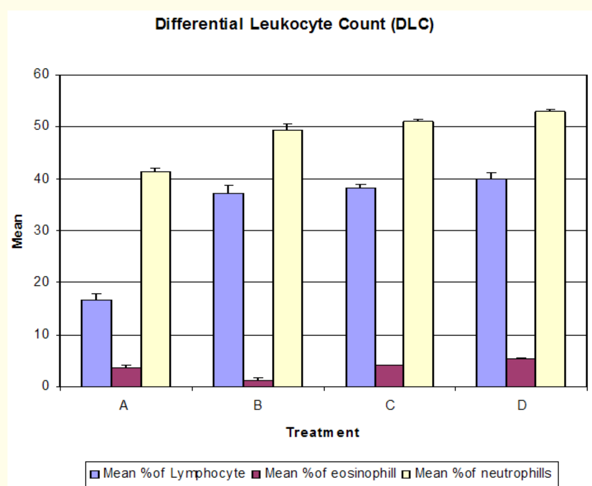


A: Control; B: Annona squamosa 200 mg/kg; C: Annona squamosa 400 mg/kg; D: Levamisole 50 mg/kg

Group	Treatment	Dose	Mean % of Lymphocyte	Mean % of eosinophill	Mean % of neutrophils
1	Control		17.67 ± 1.33	3.77 ± 0.33	43.33 ± 0.88
2	Test extract-I of *Annona squamosa*	200 mg/kg	38.43 ± 1.45**	1.44 ± 0.33*	50.33 ± 1.20*
3	Test extract II of *Annona squamosa*	400 mg/kg	39.44 ± 0.67**	4.50 ± 0.00	52.00 ± 0.58**
4	Standard Levamisole	50 mg/kg	41.00 ± 1.15**	5.44 ± 0.33	54.00 ± 0.58*

Table 5: *Differential Leukocyte Count.*

*DUNNETT t test and p values as significant *if p < 0.05, highly significant. **if p < 0.01, as compared to control .*



A: Control; B: Annona squamosa 200 mg/kg; C: Annona squamosa 400 mg/kg; D: Levamisole 50 mg/kg

Two doses of the aqueous extract were used for the pharmacological investigation. The doses were administered in the form of oral solution.

Discussion

Delayed Type Hypersensitivity Response

Oral administration of *Annona squamosa* extracts (200-400 mg/kg p.o) for 14 days caused the following DTH reactivity in rats. The results obtained in table 2 indicate that the control animals did not show any characteristics increase in paw edema. The animals treated with lower dose 200 mg/kg showed no significant increase in paw edema as compared with the control. The animals treated with higher doses 400 mg/kg showed no significant increase in paw edema when compared with the control. The wistar rats treated with standard drug levamisole 50 mg/kg showed highly significant increase in paw edema as compared to control. It is based on the stimulatory effect of the standard drug on chemotaxis dependent leukocyte migration, the antigen antibody formed immune complexes, which are known to induce local inflammation [27] with increased vascular permeability, edema and infiltration of PMN leucocytes.

Humoral Antibody Titer

Oral administration of *Annona squamosa* extract (200-400 mg/kg) for 14 days showed the following reaction in rats. The control animals did not show any characteristic humoral antibody titer. The results obtained indicate that animals treated with lower dose (200 mg/kg) as well as higher dose (400 mg/kg) of *Annona squamosa* and standard drug Levamisole (50 mg/kg) showed no significant increase humoral antibody titer when compared with the control group. The animals treated with the standard drug Levamisole (50mg/kg) also showed no significant increase in the titre value. The antigen antibody reaction results in agglutination. The relative strength of an antibody titre is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The antibody titre is useful to measure the changes in the amount of the antibody in the course of an immune response.

Total Leukocyte Count

Oral administration of *Annona squamosa* for 14 days showed the following changes. The results obtained indicate that there was not a significant increase in mean total leukocyte count in the control animals. The animals treated with lower dose of aqueous extract of *Annona squamosa* (200 mg/kg) showed highly significant increase in mean total leukocyte when compared to control. The animal treated with higher dose of aqueous extract of *Annona squamosa* (400 mg/kg) also showed highly significant increase in mean total leukocyte count as compared to control. The standard drug levamisole (50 mg/kg) showed a highly significant increase in the mean total leukocyte count as compared to control.

Differential Leukocyte Count

Oral administration of *Annona squamosa* for 14 days showed the following count in rats. The results obtained indicates that the animals treated with lower dose aqueous extract of *Annona squamosa* (200 mg/kg) showed a significant increase in mean percentage of lymphocytes, eosinophils and neutrophils respectively as compared to control. The animals treated with higher dose of aqueous extract of *Annona squamosa* (400 mg/kg) showed a highly significant increase in mean percentage of lymphocytes & neutrophils respectively as compared to control. The standard drug levamisole (50 mg/kg) also showed a highly significant increase in mean percentage of differential leukocyte count.

Conclusion

The study was undertaken to carry out the Immunomodulatory activity of aqueous extract of Annona squamosa. For the experimental work the dried leaves was powdered and were extracted with distilled water and was freeze dried. The preliminary phytochemical tests of the extract indicated the presence of carbohydrate, glycosides, and flavonoids. The hydroalcholic extract of Annona squamosa in two different doses 200 mg/kg and 400 mg/kg was tested for their Immunomodulatory action, out of which the higher dose of 400 mg/kg showed statistically significant Immunomodulatory activity.

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