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EVALUATION OF IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF ANNONA RECTICULATA FRUITS

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ABSTRACT :

Annona recticulata, a plant commonly used in traditional medicine, has been reported to possess various bioactivities. The present study aimed to evaluate the immunomodulatory potential of the ethanolic extract of A. recticulata fruits. The extract was prepared by macerating the fruits in ethanol and subsequently evaluated for its immunomodulatory activity using a battery of assays.The results showed that the extract exhibited significant immunomodulatory activity in vitro, as evidenced by its ability to enhance natural killer cell activity ($p < 0.05$), increase the production of cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), and suppress the proliferation of spleen cells ($p < 0.05$).In addition, the extract was found to inhibit the production of nitric oxide (NO) and reactive oxygen species (ROS) in lipopolysaccharide-stimulated macrophages ($p < 0.05$), indicating its potential anti-inflammatory activity. Furthermore, oral administration of the extract to mice with experimentally-induced immunosuppression significantly increased their CD4+ and CD8+ T cell counts, as well as their antibody production against sheep red blood cells ($p < 0.05$).These findings suggest that the ethanolic extract of A. recticulata fruits possesses significant immunomodulatory activity, which may be attributed to its ability to enhance immune cell function, suppress inflammation, and modulate the immune response. The results provide a scientific basis for the traditional use of A. recticulata in folk medicine and support its potential use as a therapeutic agent for immune-related disorders.

Keywords: Annona recticulata, immunomodulatory activity, natural killer cells, cytokines, anti-inflammatory activity, immune response.

Introduction:

Annona recticulata, also known as the custard apple, is a tropical fruit native to the Americas and widely cultivated in many parts of the world. The fruit has been used in traditional medicine for centuries to treat various ailments, including fever, rheumatism, and skin conditions. The fruit's pulp, seeds, and leaves are rich in bioactive compounds, including alkaloids, phenolic acids, and terpenoids, which have been shown to exhibit antimicrobial, antifungal, and antioxidant properties.

Recent studies have focused on the immunomodulatory potential of Annona recticulata fruits, which could lead to the development of new therapeutic agents for the prevention and treatment of various diseases. Immunomodulation is a process by which the immune system is modulated or regulated to prevent or treat diseases. Immunomodulatory agents can stimulate or suppress the immune response, depending on the specific application.

The aim of this study is to investigate the immunomodulatory activity of the ethanolic extract of Annona recticulata fruits using in vitro and in vivo models. The study will evaluate the extract's ability to stimulate or suppress the immune response, as well as its potential effects on immune cell proliferation and cytokine production.

MATERIALS AND METHODS

Collection of Materials, Chemicals and Drugs

- The Sheep Red Blood Cells (SRBCs) were procured from Veterinary College.
- The Levamisole (Cipla Limited- India) was purchased from local pharmacy, Hyderabad.
- The Humoral antibody tests were performed in SAROJ diagnostic laboratories. All chemicals were procured from SAROJ diagnostic laboratories, Hyderabad

Collection and Authentication of Annona Reticulata Fruits

Fruits of *Annona reticulata* were collected and the plant material was taxonomically identified by botanist The drug material was dried under shade for about 14 days, powdered and stored in an air tightcontainer.

PHYTOCHEMICAL SCREENING

Phytochemical screening includes the following steps

- Preliminary Phytochemical screening
- Chromatographic and spectroscopic studies.
- Estimation of total Flavanoids and Phenolic contents.

Preliminary Phytochemical screening

Qualitative analysis for determining the presence of alkaloids, tannins, Flavanoids, terpenoids , steroids, glycosides, saponins, resin, and oil in the plant extracts, were carried out using standard methods as described by **Harborne (1973), Trease and Evans (1978)** and **Sofowora (1993)**. 0.5 gm of the dried extracts were dissolved in 20 ml distilled water, filtered and used for various qualitative tests. The following chemical testes were carried out using extracts of *Curcuma aeruginosa* rhizome extracts. **(Khandelwal, 1998; Kokate, 1993).**

Acute Toxicity Study

Acute toxicity for ethanolic extract of fruits will be done according to the office of pollution prevention and toxics (OPPT) The overnight fasted rat is weighed and selected. The extracts will be dosed in a stepwise procedure, by using up and down or stair case method. The two animals selected with a dose of 50 mg/kg. Orally and examined for 24h for mortality. Subsequent dose are then increased to attain maximum non lethal and minimum lethal dose. The extract was found to be safe at the dose of 5g/kg per oral. Maximum safe dose (5g/kg) corresponding to 500 mg/kg and 100 mg/kg were selected as high and low doses respectively.

Experimental Animals

The experiment was carried out by using Wistar rats, which were procured from central animal house of the Institute. The experimental protocol has been approved by institutional animal ethical committee. Rats of Wistar strain weighing between 150 to 200 gm were maintained under standard laboratory conditions. They were provided with a standard diet supplied by Pranav agro industries Ltd India.

Experimental Protocol

24 rates were divided in to four groupsGroup:Control (normal saline)
Group II :*Annona recticulata* ethanolic extract was administered at a dose 100mg/kg/day by oral route
Group III :*Annona recticulata* ethanolic extract was administered at a dose 500mg/kg/day by oral route
Group IV :Standard- Levamisole was administered at a dose of 50mg/kg/day byoral route for 14 days.

Experimental Setup

The animal model is required to study the following methods

- Delayed type hypersensitivity (DTH) response
- Humoral antibody (HA) titer
- Total leukocyte count
- Determination of total serum protein and albumin -globulin ratio

Delayed Type Hypersensitivity (DTH) Response

For the evaluation of delayed type of hypersensitivity (DTH) test animals were divided in to four groups, having six animals in each. Group I, the control, was given 2ml of 5% normal saline and to group II.III, IV was administered of 100 mg and 500 mg/kg body weight of ethanolic extract intraperitonially for ten days. On 10th day 0.1ml of SRBC solution was injected subcutaneously in to the right footpad. After 24,48,72,96 hrs,

thickness of footpad was measured by plethisometer. Difference in the footpad thickness in control and treated group has been taken as the measure of the DTH reaction **(Dikshit *et al.*, 2000)**

Humoral Antibody Titre

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1X 10 8 cells, intraperitonially, 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.

Method for Serial dilution

This was performed by using 96 wells (12x8) U bottomed titre plate. The wells were marked from I to XII. In the first (I) and last well (XII) 25 microliter of serum collected from treated animals was added and inactivated at 56 degree Celsius for 30 minutes. Afterwards to all the wells except well number XII, 25 microliter of PBS was added.25 microliter was taken from first well and added to 2nd well again 25 microliter from second well was taken and added to third well and continued the same procedure up to well number XI. After this 25 microliter of sample from well number XI was discarded. Finally 25 microliter of 1% SRBC was added to all the wells and was kept at room temperature for two hours **(Vinod S Pawar *et al.*, 2012)**

Total Leukocyte Count

W.B.C 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 theblood will be 20 because the last part of the fluid remains locked up in the stem and is not available for dilution

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 : 1.5ml
1%solution of gentian violet in water : 1ml Distilled water : 98ml
The glacial acetic acid haemolysis the red cells, while the gentian violet stains thenucleus of leukocytes

Method of Counting W.B.C

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 color 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

Determination of Total Serum ProteinTotal Protein (Biuret Method)

Total Protein-To exactly 4 cc. of 10 per cent sodium hydroxide in a 10 ml standard flask and add 0.1 cc. of fresh serum with a Folin micropipette. Rinse outthe pipette three times with sodium hydroxide solution. Mix by rotating and add 0.5 cc. of 1 per cent copper sulphate. Shake vigorously five to six times. Allow to stand for 25 minutes and absorbance read in a U.V Spectrophotometer at 540 nm.

RESULTS

PHYSICOCHEMICAL EVALUATION OF THE DRUGEvaluation of foreign matter

Foreign matter of fresh fruits of *Annona recticulata* was found to be 9.82.

Moisture content (Loss on drying)

Moisture content of fresh fruits of *Annona recticulata* was found to be 53.1

Extractive value

Water soluble extract - 7.83%	
Alcohol soluble extract	- 6.37%
Ash value of the drug	
Total ash	- 5.8%
Acid insoluble ash	- 0.61%
Water soluble ash	- 3.71%

Phytochemical Screening

In preliminary phytochemical analysis was done on the basis of standard procedure. Show presence of secondary metabolites. They were shown in table no.1

Table. 1. Preliminary phytochemical screening of ethanolic extract of *Annonarecticulata* fruits.

Sl.no	Phytoconstituents	Ethanolic extract
1	Alkaloids	+
2	Glycosides	-
3	Flavanoids	+
4	Phenols	+
5	Saponins	+
6	Steroid	-
7	Tannins	-
8	Terpenoids	+
9	Carbohydrates	+

Acute toxicity study

For toxicity studies, crude ethanolic extract were administered orally to the three groups having two rats in each with graded doses (50mg/kg-500mg/kg body weight) of *Annona recticulata*. Mortality rates were observed after 7 days **(Choudhary *et al*., 1997)**

Table: 2 Determination of acute toxicity of ethanolic extract of *Annonarecticulata*

Sl. No	Dose (mg/kg. Body weight)	Percent mortality
1	50	0
2	100	0
3	150	0
4	200	0
5	250	0
6	300	0

7	350	0
8	400	0
9	450	0
10	500	0

Determination of Delayed Type Hypersensitivity Response

The effect of test extract and standard drugs on the DTH response in wistar rats using SRBCs as antigen, administration of ethanolic extract of *Annona recticulata* at the dose of 100mg/Kg and 500mg/Kg and Levamisole 50mg/Kg treatments which were given orally. After 24, 48, 72, 96 hrs showed significant increase in paw edema compared to control group.

Table.3 Effect of crude ethanolic extract of *Annona recticulata* on delayed typeof hypersensitivity response

Sl. No	Groups	Paw volume (mm)			
		24 Hrs	48 Hrs	72 Hrs	96 Hrs
I	Control	1.43±0.027	0.72±0.019	0.41±0.013	0.16±0.012
II	Crude ethanolic extract (100 mg/kgbody weight)	1.50±0.011	0.88±0.018	0.51±0.015	0.20±0.021*
III	Crude ethanolic extract (500 mg/kgbody weight)	1.54±0.016	0.93±0.019	0.57±0.012	0.23±0.014**
IV	Standard – Levamisole (50 mg/kg body weight)	1.58±0.010	1.02±0.029	0.65±0.021	0.32±0.016***

n= 6. Tabulation values represents mean ± SD (*P<0.05, **P<0.025, ***P<0.001

Fig. 6 Show Delayed Type Hypersensitivity response

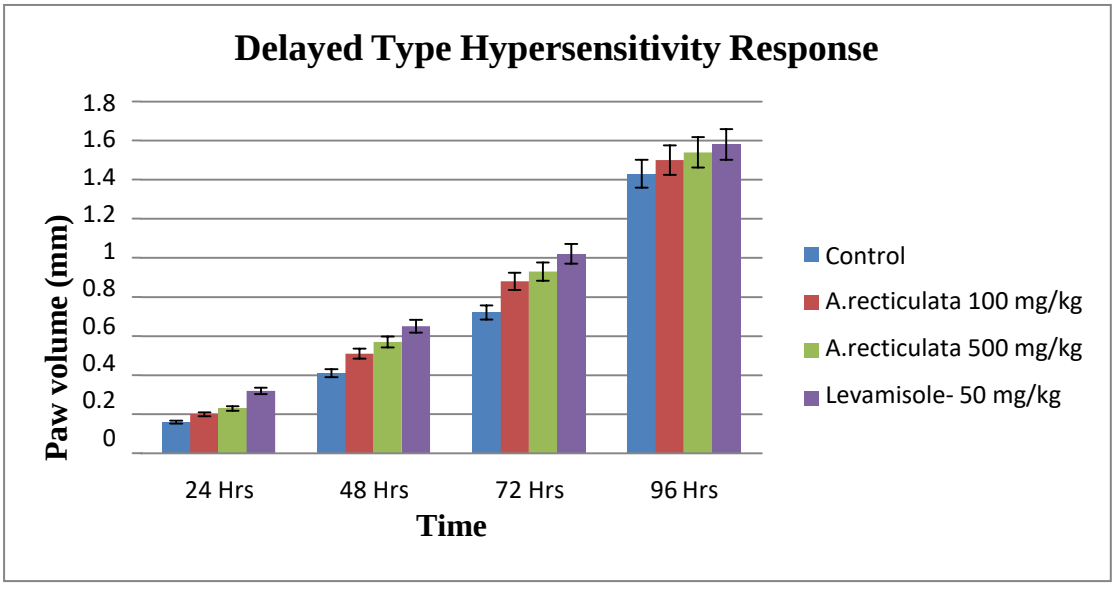
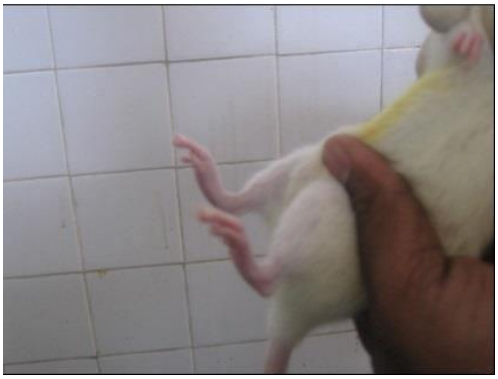
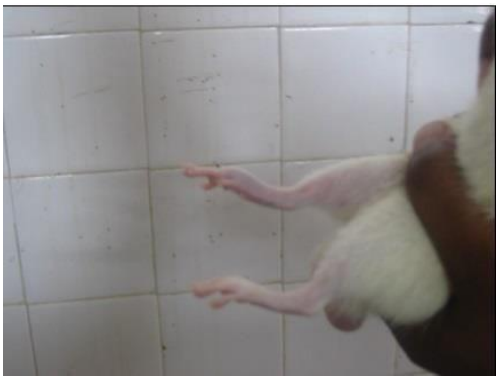


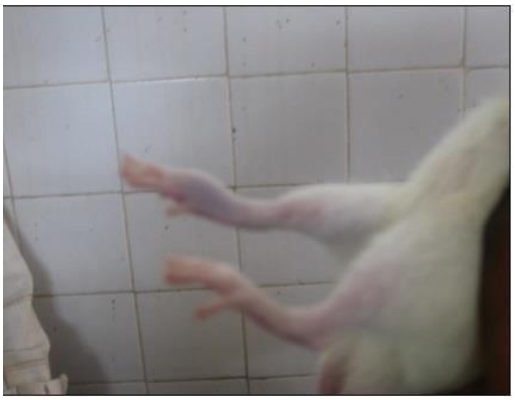
Fig. 7 Paw edema observed in animals after injecting sheep's RBC



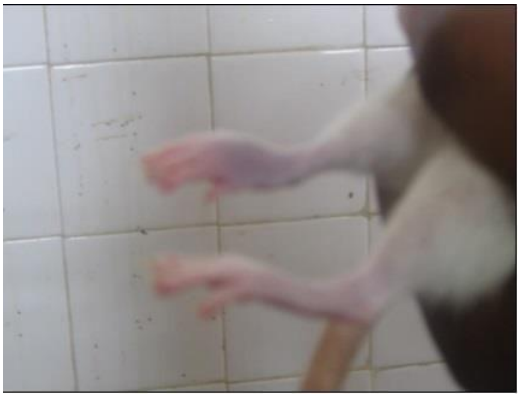
Control animal



***Annona recticulata* 100 mg/kg**



***Annona recticulata* 500 mg/kg**



Levamisole 50 mg/kg

Humoral Antibody Titre

Administration of ethanolic extract of at the dose of (100 & 500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days showed highly significant increase in antibody titre values compared to control group. The results are shown in below table 4

Table.4 Effect of crude ethanolic extract of *Annona recticulata* of HumoralAntibody titre

Sl. No	Group	Humoral antibody titre
I	Control	11±1.0210
II	*Annona recticulata* 100 mg/kg body weight	337.47±2.0401*
III	*Annona recticulata* 500 mg/kg body weight	412±1.5010**
IV	Levamisole 50 mg/kg	461± 2.6861***

n=6, humoral antibody titre value mean ± SEM (*P<0.05, **P<0.01, ***P<0.001

Fig.8 Show Humoral Antibody Titre

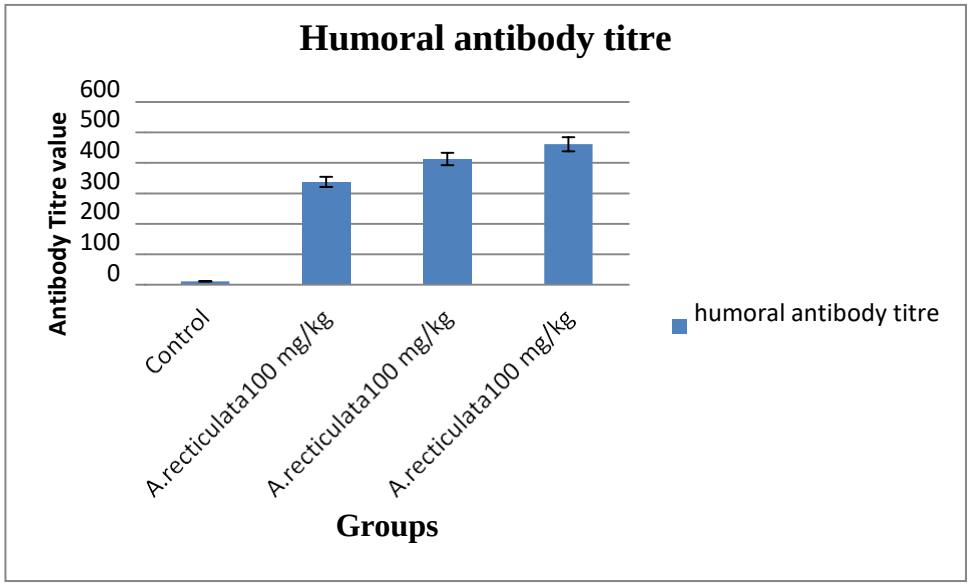
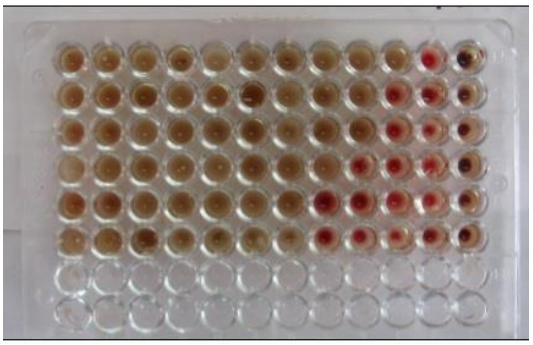
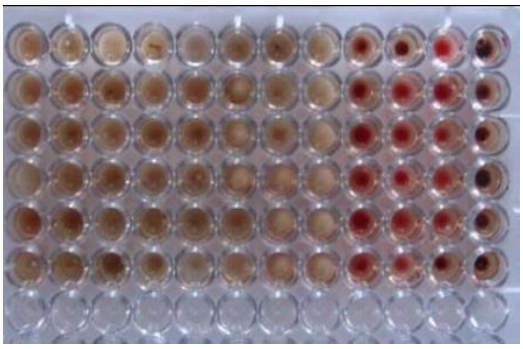


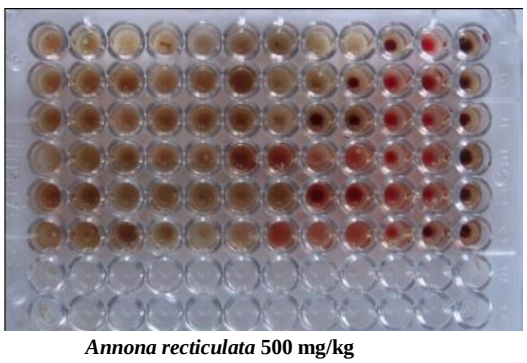
Fig. 9 Button formation of Antibody titre on Microtitre plate



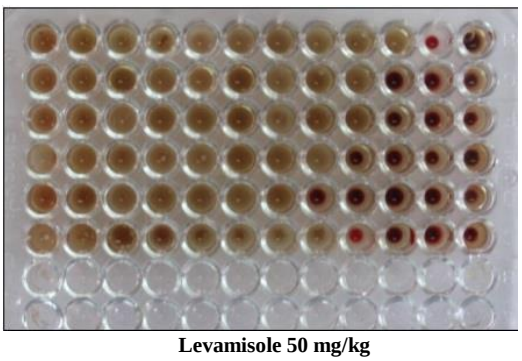
Control



Annona recticulata 100 mg/kg



***Annona recticulata* 500 mg/kg**



Levamisole 50 mg/kg

Total Leukocyte Count

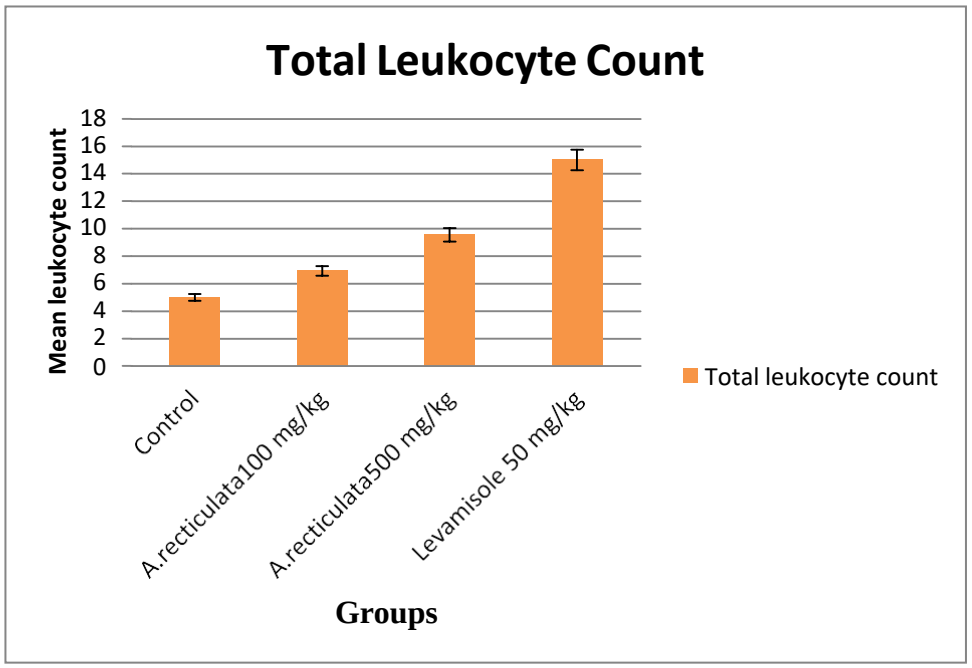
The effect of test extract and standard drugs on Total Leukocytes in wistar rats, administration of ethanolic extract of *Annona recticulata* at the dose of (100,500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days. The low dose of extract (100 mg/kg) show effect on TLC count comparedto control group, whereas the 500mg/Kg and standard drug Levamisole 50mg/Kg showed significant increase in total leukocytes count values compared to control group. The results are shown in below table 5.

Table .5 Effect of crude ethanolic extract of *Annona recticulata* of HumoralAntibody titre.

Sl. No	Group	Mean Leukocyte count
I	Control	5.01×10^3 cu.mm± 0.2640
II	*Annona recticulata* 100 mg/kg	6.93×10^3 cu.mm± 0.2461*
III	*Annona recticulata* 500 mg/kg	9.56×10^3 cu.mm± 0.3101**
IV	Levamisole 50 mg/kg	15.01×10^3 cu.mm± 0.1381***

n= 6, total leukocyte count means ±SEM (*P<0.05, **P<0.01, ***P<0.001)

Fig. 10 Show Total Leukocyte Count



Determination of Total Serum Protein

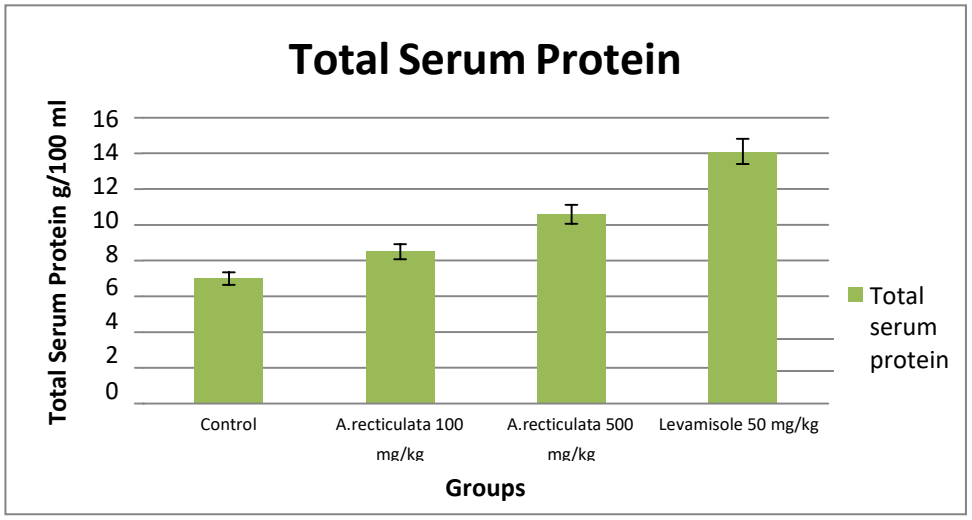
The effect of test extract and standard drugs on Total serum protein in wistar rats, administration of ethanolic extract of *Annona recticulata* at the dose of (100,500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days. The low dose of extract (100 mg/kg) and large dose (500 mg/kg) standard drug Levamisole 50mg/Kg showed significant increase in total serum values compared to control group. The results are shown in below table 5.

Table 6. Effect of crude ethanolic extract of *Annona recticulata* of total serum protein

Sl. No	Group	Total serum protein (g/100 ml)
I	Control	7±0.1201
II	*Annona recticulata* 100 mg/kg	8.5±0.952
III	*Annona recticulata* 500 mg/kg	10.6±0.1012
IV	Levamisole 50 mg/kg	14.1±0.010

n= 6, total serum value means ± S,D

Fig. 11 Show Total Serum Protein



DISCUSSION

Plants serve as vast source for varied phytoconstituents exhibiting varied pharmacological property. Identifying such potential plants is of significance in medicine. In the treatment of a disease the toxicity and resistance of available drugs are the major world-wide problem. Because of these the designs of new drug which can overcome resistance as well as toxicity become one of the leading areas of drug design.

In light of this, in the present study; widely available herb Annona reticulata was selected for Pharmacognostical standardization according to WHO guidelines, thorough phytochemical analysis followed by pharmacological screening for immunomodulatory activity in Wistar rats.

After the standardization, the shade dried plant material was subjected to detailed phytochemical analysis. The various chemical and acid treatments of the fruits of the study established the identity of categories of phytoconstituents. The fruits were subjected to soxhlet extraction using ethanol as solvent. Later the extracts were subjected to preliminary phytochemical analysis to ascertain the presence of various categories of phytoconstituents. The preliminary phytochemical screening revealed the significant presence of alkaloids, trepenoids, saponnins, flavanoids, tannins, phenolic compounds and carbohydrates.

Acute toxicity study was evaluated by using ethanolic extract on Wistar rats, and increasing the concentration of plant extract found to be most effective at low dose (100 mg/kg), where as high dose (500 mg/kg) of ethanolic extract of *Annona recticulata* was moderately effective in modulating immune system.
The study was carried out using four different methods, each of whichprovides information about effect on different components of the immune system **(Wagner 1984)**

Delayed Type Hypersensitivity Test was done to study the effect at crude aqueous and crude ethanolic extract on cell-mediated immune response to paw edema in 24, 48 hrs and then after 72 and 96 hrs paw volume significantly increase when compared with control. But in case of standard drug

(Levamisole) compared with test drug, standard drug show significance paw volume.ethanolic extract of at the dose of (100 & 500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days showed highly significant increase in antibody titre values compared to control groupHumoral antibody titer assay is one of the key parameter used to assess the humoral immune response of the animal. As the antigen is expected to induce the production of antiserum against it, in the present study sheep red blood cells were used to elucidate the production of antibody against RBC. In a individual where immune system is primed antibody against a particular antigen is expected to be at higher titer. Accordingly in the present study a very high humoral antibody titer was recorded and group II and group III individual which received the lowest concentration (100 mg/kg and 500 mg/kg) of test drug. On the contrary higher concentrations of the drug have surprisingly reduced the HA titer. **Pradhan et al (2009)** administered extract of herbal product to albino rats showed a increased HA titer when drug was used at a concentration of 50mg/kg. Similarly **BinHafeez et al (2003)** also showed increased HA tire at doses of 50 mg/kg and above, of fenugreek extract administered on mice. The study showed that up to 300mg/kg of the crude drug could enhance the humoral immune response. Upon examining the present results it is evident that the ethanolic extract at concentrations less than 500 mg/kg induces humoral immune response as evidenced by HA titer.

One of the earliest immune response can be seen and measured by studying the hematological parameters of an animal. Accordingly parameters like total leukocyte count for control group as well as group which received various concentrations of drug. Blood cells are the first cells to be responding to invading non self materials. An immunomodulatory effect of any immune substance would first see as a change in leukocyte count. In the present study group IV which received (50 mg/kg) of standard drug showed highest leukocyte count of15.01×103 cu.mm showing the initial triggering of blood cell to mount a potent immune response. The results showing standard drug concentration are better to elicit good immune response than test concentrations (100 mg/kg, 500mg/kg) of drug administered. The results are further strengthened with highest percentage of neutrophil being circulated in the group.

Serum protein is one of the earliest indicators of normal serum chemistry of an individual. A change in serum protein concentration and albumin ratio would hintus about the altered immune response status of the individual. Accordingly in the present study serum protein level is found to be similar in case of control and higher concentration (500 mg/kg) of the drug but in the lower concentration 100 mg/kg of drug test the group showed increase in serum protein showing that higher immune response might have contributed to the serum protein in terms of different molecules such as immunoglobulin and other humoral factors. The serum protein increased in case of Levamisole 50 mg/kg administered by oral.

CONCLUSION :

- Many of the *Annona* species have been reported for immunomodulatory activity. However, *Annona recticulata* fruits not been reported for the same.
- Phytochemical studies showed alkaloids, Phenolics, Flavanoids,carbohydrate, terpenoids, steroids, and saponins.
- Acute toxicity study was done and no mortality reported at doses between 50mg/kg to 500 mg/kg.
- Determined Delayed type hypersensitivity response show significance paw volume of lower and higher concentration as compared to control.
- Evaluation of Humoral antibody titre value showed highly significantincrease in antibody titre values compared to control group
- Determined the low dose of extract (100 mg/kg) show effect on TLC countcompared to control group
- Estimation of total serum protein show significant increase of total serumvalue compared to control.
- Ethanolic extract of fruits of *Annona recticulata* showed moderateimmunomodulatory activity compared with standard drug.

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