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IMMUNOMODULATORY ACTIVITY OF *ACACIA CATECHU*

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Abstract : The immunomodulatory effect of aqueous extract of *Acacia catechu* commonly known as Katha or Karangali was studied at two doses of 5 mg/kg and 50 mg/kg orally. The effect was studied in neutrophil adhesion test, mice lethality test, carbon clearance assay, cyclophosphamide induced neutropenia, serum immunoglobulin levels and the heamagglutination test. *Acacia catechu* extract showed an increase in the neutrophil adhesion to the nylon fibres, produced a significant increase in the phagocytic index and a significant protection against cyclophosphamide induced neutropenia indicating its effect on cell mediated immunity. On the other hand, *Acacia catechu* extract produced a significant increase in the serum immunoglobulin levels, increase in the haemagglutination titre values and decreased the mortality ratio in mice, suggesting its effect on the humoral arm of the immune system. From the above results, it was concluded that the aqueous extract of *Acacia catechu* has a significant effect on both cell mediated and humoral immunity.

Key words : *Acacia catechu* cell mediated immunity humoral immunity

INTRODUCTION

Immunomodulatory agents are used to either suppress or stimulate the immune responsiveness of an organism against the invading antigens. Several plant products have been reported for immunomodulatory activity and many formulations of these plant products are available to enhance the immune system.

The dried bark of *Acacia catechu* (family: leguminosae, sub family: mimosiasae) commonly known as Katha or Karangali is widely used in India for its various pharmacological effects. It is used in the treatment of passive diarrhea either alone or in combination with cinnamon or opium (1). The concentrated aqueous extract known as Khayer gum or Kutch is an astringent, cooling and digestive, beneficial in cough and diarrhea, applied externally to ulcer, boils and skin eruptions and is used extensively in Ayurvedic formulations (2). The bark in combination with other drugs is prescribed for snakebite (1). The seeds of the plant are reported to possess hypoglycemic activity in rats (3). *Acacia catechu* also shows hypotensive effect (4). The water decoction of *Acacia catechu* is widely consumed as

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health drink especially in Kerala and other south Indian states. It is believed that the water decoction can purify blood, improve skin texture and boost body's defence mechanism (personal communication). Since, the plant is widely used for treatment of various ailments and is a constituent of many formulations, apart from its long term use as health drink, the present study was undertaken to investigate its effect on cell mediated and humoral immunity in the experimental animal models.

MATERIAL AND METHODS

Experimental animals

Albino Wistar rats weighing between 180–220 g and Swiss albino mice weighing between 25–35 g was used. Institutional Animal Ethics Committee approved the experimental protocol. Animals were maintained under standard conditions in an animal house approved by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food (Lipton India Ltd., Mumbai, India) and water *ad libitum*.

Chemicals

Acacia catechu was procured from the Regional Research Institute, Bangalore, India. A voucher specimen is preserved in the Regional Research Institute for future reference (RRI/BNG/SMP drug Authentication/2007-08/56). Ethanolic extract of *Ocimum sanctum* (Phytotech extracts), Leishmann's stain (Merck, Mumbai, India), WBC diluting fluid, zinc sulphate, barium chloride (Nice Chemicals, Cochin, India), cyclophosphamide (Endoxan Injection-German Remedies, India), Indian ink, gluteraldehyde (Merck, Mumbai, India).

Organism: *Pasteurella multocida* of bovine origin and its vaccine were obtained from Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore, India.

Treatment: The animals were distributed into four groups consisting of six animals each. The first group served as control, the second group received the standard drug - *Ocimum sanctum* extract (OSE) at a dose of 100 mg/kg orally (5), the third and fourth group received low dose and high dose of aqueous extract of *Acacia catechu* at 5 mg/kg, po (ALD) and 50 mg/kg, po (AHD) respectively. These small doses were calculated from the amount of *Acacia catechu* used to prepare health drinks for human consumption. Mice lethality test had five groups, first two groups served as controls; one positive control and the other negative control.

Experimental models

1. *Neutrophil adhesion test (6, 7) :*

Albino Wistar rats were divided into different groups and were treated orally with drug or vehicle for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and the differential leukocyte count (DLC) was determined after fixing the blood smear and staining with the Leishmann's stain. After the initial counts, blood samples were incubated with 80 mg/mL of nylon fibres for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The difference in the neutrophil count before and after

incubation of blood sample with nylon fibres was determined.

2. *Carbon clearance test (8–10) :*

The four groups of Swiss albino mice were administered drug or vehicle for 5 days orally. After 48 h of the last dose of the drug, mice were injected with 0.1 mL of Indian ink via the tail vein. Blood samples were withdrawn at 0 min and 15 min. A 50 µl blood sample was mixed with 4 mL of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following equation :

$$K = (Log_e\ OD1 - Log_e\ OD2)/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively.

4. *Effect on serum immunoglobulins (11–13) :*

Albino rats were treated with the drug or vehicle orally for 21 days. Six hours after the last dose, blood samples were collected and the serum was separated by centrifugation, the collected serum was used for estimation of immunoglobulin levels. Briefly, for each serum sample to be analyzed, a control tube containing 6 mL of distilled water and a test tube containing 6 mL of zinc sulphate solution were prepared. To each, 0.1 mL of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 h at room temperature in plugged tubes. The pH of the solution was monitored through out the experimental period using pH meter. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nephelo turbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

5. *Indirect Haemagglutination Test (14, 15) :*

Rats were pretreated with the drugs for 14 days and each rat was immunized with 0.4 mL of 5×10^9 SRBC/rat through ip route, including control rats. The day of immunization was referred to as day 0. The drug treatment was continued for another 14 days and blood samples were collected from each rat on day 15 for determination of Haemagglutinating Antibody (HA) titre. The titre value was determined by titrating serum dilutions with SRBC (1.25×10^9 cell) in a microtitre plate. The plates were incubated at room temperature for 2 h and examined visually for agglutination. The minimum volume of serum required to produce haemagglutination was noted and expressed as HA titre.

3. *Cyclophosphamide induced neutropenia (16, 17) :*

Swiss albino mice received the drug or vehicle orally for 10 days. On 10th day, a neutropenic dose of cyclophosphamide (200 mg/kg, sc) was administered and this day was labeled as day zero. Blood samples were collected through retro-orbital vein. The total leucocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and DLC in treated groups were compared with

the values of the control group.

6. *Mice lethality test (14, 18):*

Swiss albino female mice were divided into five groups, the first group served as negative control and it received only vehicle and no vaccination and the second group served as an positive control and was given vehicle and vaccination. The drug or vehicle was administered orally for 21 days. On the 7th and 17th day of the treatment, the animals received haemorrhagic Septicaemic (HS) vaccine alum precipitated I.P Vet. (0.2 mL/mice, sc). On the 21st day, the animals were challenged subcutaneously with 0.2 mL of 25 LD_{50} dose of *Pasteurella multocida* organism (bovine origin) containing 10^7 cells per mL. The animals were observed for a period of 72 h and the mortality ratio was determined using the formula.

$$\text{Mortality ratio} = \frac{\text{Number of animals dead}}{\text{Total number of animals}}$$

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test. The values are expressed as mean±SEM and $P<0.05$ was considered significant.

RESULTS

Neutrophil adhesion test: When blood samples were incubated with nylon fibres, a reduction in neutrophil percentage due to the adhesion of neutrophils to the nylon fibres was observed. The percentage reduction in the neutrophil count in nylon fibre treated blood samples from the treated groups – ALD, AHD and OSE was significantly ($P<0.01$) more compared to the control group (Table I).

TABLE I: Effect on neutrophil adhesion in rats.

Treatment	*Neutrophil (%)*		*Difference*
	UB (A)	*NFTB (B)*	*A-B*
Vehicle (1 mL/kg, po)	24.50±0.85	20.65±1.08	3.85±0.47
OSE (100 mg/kg, po)	20.00±1.86	10.32±0.55	9.68±1.58**
ALD (5 mg/kg, po)	22.32±1.05	11.50±1.40	10.80±1.16**
AHD (50 mg/kg, po)	22.66±1.05	10.66±1.05	12.00±0.78**

All values are mean±SEM, n=5-6, **P<0.01 when compared to control group.

Carbon clearance assay

Administration of ALD (5 mg/kg, po) and AHD (50 mg/kg, po) produced increase in clearance of carbon particles from blood as indicated by a significant increase in phagocytic index ($P<0.01$). The OSE also produced a significant increase ($P<0.01$) in the phagocytic index (Table II).

Serum immunoglobulin levels

The administration of ALD (5 mg/kg, po) and the OSE significantly increased the serum immunoglobulin levels when compared to control. The AHD did not show any significant increase in serum immunoglobulin level when compared to the control (Table II).

Indirect haemagglutination test

The heamagglutination antibody titre value was significantly reduced by all the treatments as compared to the control (Table II).

TABLE II: Effect on phagocytic index in carbon clearance assay, serum immunoglobulin levels and HA titre in indirect heamagglutination test.

Treatment	*Phagocytic index*	*Serum immunoglobulin level (ZST units)*	*HA titre*
Vehicle (1 mL/kg, po)	0.0090±0.0025	17.076±0.308	7.083±1.357
OSE (100 mg/kg, po)	0.0390±0.0074**	27.165±0.658**	0.055±0.039**
ALD (5 mg/kg, po)	0.0385±0.0046**	24.390±1.016**	0.050±0.040**
AHD (50 mg/kg, po)	0.0638±0.0049**	14.350±1.123	0.883±0.512**

All values are mean±SEM, n=6, **P<0.01 when compared to control group, ^{ns}P>0.05; $^{++}$P<0.01, when compared to low dose.

Cyclophosphamide induced neutropenia

Administration of Cyclophosphamide (200 mg/kg, sc) produced a decrease in neutrophil count in all the groups. However, the reduction in neutrophil count was less in ALD and OSE treated groups compared to control. The ALD administration produced a 46.65% reduction in TLC and 27.28% reduction in neutrophil count and OSE treated group had 44.80% reduction in TLC and about 30.27% reduction in neutrophil count compared to 43.04% reduction in neutrohpil count and 58% reduction in TLC in control. However, the AHD did not produce any significant effect in neutrophil reduction when compared to control (Table III).

Mice lethality test

The ALD (5 mg/kg, po) and OSE (100 mg/kg, po)

TABLE III: Effect of cyclophosphamide induced neutropenia.

Treatment	*Total leucotyes count (cells/mm^3)*		*Reduction in cell number*	*% reduction*	*% neutrophils*		*Neutrophil reduction*	*% reduction*
	Before	*After*			*Before*	*After*		
Vehicle (1 mL/kg, po)	5500.00±365.95	2600.00±223.23	2900.00±227.65	58.00	13.16±1.01	7.50±0.76	5.6±0.61	43.04
OSE (100 mg/kg, po)	5041.65±296.20	2783.32±259.70	2258.32±315.80	44.80	12.65±1.30	8.83±1.35	3.8±0.30*	30.27
ALD (5 mg/kg, po)	5591.60±555.65	2983.32±235.82	2608.28±530.00	46.65	12.83±0.98	9.33±1.11	3.5±0.42*	27.28
AHD (50 mg/kg, po)	6332.30±468.80	3332.20±388.52	3000.10±358.52	52.64	16.16±0.87	9.00±1.00	7.1±0.60	44.33

All values are mean±SEM, n=5-6, *P<0.01 when compared to control group.

showed a 33.33% reduction in mortality compared to positive control and about 50% reduction as compared with the negative control. The AHD (50 mg/kg, po) produced a 16.67% reduction as compared to positive control and about 33.34% reduction as compared to negative control.

TABLE IV: Effect in mice lethality test.

Treatment	*Day 1*	*Day 2*	*Day 3*	*Mortality ratio*
Negative control	1	5	–	100
Positive control + Vaccination	–	2	3	83.33
OSE + Vaccination	–	2	1	50
ALD + Vaccination	–	1	2	50
AHD + Vaccination	1	2	1	66.66

n=6.

DISCUSSION

The results of the present study suggest that *Acacia catechu* may stimulate cell mediated immunity as shown by an increase in neutrophil adhesion to nylon fibres, increase in macrophage induced phagocytosis in carbon clearance test and reduction in cyclophosphamide induced neutropenia. It also stimulates humoral immunity as indicated by an increase in serum immunoglobulin levels, increased antibody titre in indirect heamagglutination test and reduction in mortality in mice lethality test. The ALD was more effective than AHD except in neutrophil adhesion where the later showed better effect.

The adhesion of neutrophil to nylon fibres indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of inflammation (7). *Acacia catechu* at both the doses in albino rats has showed a significant increase in the neutrophil adhesion to nylon fibres. This may be due to the upregulation of the β2 integrins that are present on the surface of the neutrophils through which, they adhere firmly to the nylon fibres. Hence, it can be inferred that *Acacia catechu* causes the stimulation of neutrophils towards the site of inflammation.

The carbon clearance test was carried out to evaluate the effect of drugs on the reticulo- endothelial system (RES). This is a diffuse system comprising of phagocytic cells, comprising of fixed tissue macrophages and mobile macrophages. The phagocytic cells in this system comprise the mononuclear phagocyte system (MPS), and the macrophage is the major differentiated cell in the MPS. Cells of the RES and MPS are known to be important in the clearance of particles from the bloodstream. When colloidal ink containing carbon particles are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (8, 10). *Acacia catechu* at both doses and OSE showed significant increase in the phagocytic index. Hence, these agents may stimulate the reticuloendothelial system.

The estimation of serum immunoglobulin level is a direct measure to detect the humoral immunity. Serum immunoglobulin refers to a group of serum molecules produced by B-lymphocytes, they are soluble and secreted form of B-cell receptors and are produced to a maximum level to counter the invasion by an antigen, hence they are

also called as antibodies. Blood contains three types of globulins-alpha, beta and gamma, based on their electrophoretic migration rate. In the present study, estimation of serum immunoglobulins was carried out using zinc sulphate turbidity test (ZST) (11). This test determines the amount of immunoglobulins present in the serum. A small amount of serum was added to a zinc sulphate solution and allowed to incubate at room temperature for 1 h. Zinc sulphate causes precipitation of the immunoglobulins, which makes the solution cloudy instead of clear. This test is fairly specific for immunoglobulins, but does not do a very good job of quantitating them and it is difficult to distinguish a borderline problem. However, this test is relatively quick and inexpensive test (12). Its drawbacks include the dependence of results on a number of factors, such as time, temperature, and particularly pH of the reaction mixture. A serious drawback is the dependence of the final turbidity on pH of the zinc sulphate solution. Prolonged storage or even a short exposure to atmospheric carbon dioxide considerably changes pH and affects the result of the reaction. The possible ways of overcoming this problem are buffering or the use of pH indicators (13). The turbidity was expressed as ZST units, which in turn indicate the amount of immunoglobulin present in the sample. *Acacia catechu* at a low dose showed a significant increase in the serum immunoglobulin level.

The indirect haemagglutination test was carried out to determine the effect of *Acacia catechu* on the humoral immunity system. This system involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting cells. These antibodies bind to antigen and neutralize it or facilitate its elimination by cross linking to form latex that is more readily ingested by phagocytic cells (14, 15). When the test animals were pretreated with *Acacia catechu* and OSE, the litre values of circulating antibodies were increased. The test involves the preparation of double dilutions of serum samples and the addition of constant amount of the SRBC. If the serum contains antibodies to the SRBC, there will be agglutination because of the formation of antibody bridges with the neighbouring erythrocytes and these settle at the bottom as latex. Unagglutinated red blood cells appear in the well bottom as a button. Sometimes, even if the sample is antibody-negative, erythrocytes do not stream but instead blanket the well bottom, indicating the haemagglutination. To avoid this false result, the SRBC were incubated with the serum in one well and without the serum but only PBS in another well. If haemagglutination was detected in the serum wells but not in control wells, the result was recorded as a titre. *Acacia catechu* at both the doses showed a very significant effect on the circulating antibody titre.

Cyclophosphamide induces myelo suppression in the experimental animals. It belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. It is also used extensively as immunosuppressant (16). *Acacia catechu* at a low dose caused a 27.28% reduction in the cyclophosphamide induced neutropenia suggesting that it may have an effect on the haemopoetic system. The

prevention of neutropenia induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin 1 (16, 17).

The mouse lethality test is one of the important tests conducted worldwide to evaluate serological responses in animals immunized with vaccines. The mouse lethality test involves injecting mice with the vaccine prior to the administration of the bacterial culture and determining the mortality ratio (14, 18). *Pasteurella multocida* is an organism, which is pathogenic to mice and acts by infecting the normal airway pathways. In this model, the mice were vaccinated with HS vaccine twice before the administration of 25 LD_{50} dose of the organism. The vaccination causes the production of antibodies. If the drug has a tendency to increase the production of antibodies to an extent that they can counter the invading pathogen, then the animals survive. *Acacia catechu* at low dose and the standard drug – OSE showed a 50% reduction in the mortality ratio.

The low dose of *Acacia catechu* was more effective compared to the high dose. The reason for exact mechanism of this can not be explained. There are many constituents present in the *Acacia catechu*, the main constituents are catechins that include catechin and epicatechin. Both these catechins are present in many other plants, and these plants are reported for different activities. The tea catechins are known to possess antibacterial, antiviral, anticancer, antiinflammatory and antioxidant activity to name few. The exact constituent(s) responsible for the immunomodulatory effect is not known. However, the catechins, by virtue of their antimicrobial (19, 20), antiinflammatory (21), antiviral (22, 23) and antioxidant (24) effect may be the main constituents responsible for their activity.

The results of the present study shows that the aqueous extract of *Acacia catechu* have significant effect on both the cell mediated and the humoral immunity.

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