

**IMMUNOMODULATORY ACTIVITY OF METHANOLIC EXTRACT OF
SYZYGIUM CUMINI SEEDS**

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Summary

The study was conducted to investigate the immunomodulatory activity of methanolic extract of *Syzygium cumini* seeds (SME) in mice and rats at doses of 100, 200, 300, 400 and 500 mg/kg orally. Immunomodulatory activities on humoral and cellular immunity were studied by carbon clearance method in mice and hemagglutination (HA) titre, delayed type hypersensitivity (DTH) reaction in rats. SME enhanced the carbon clearance, HA titre and DTH reaction in a dose dependent manner. SME also significantly increased the white blood cells and lymphocytes count. The effects were compared to the standard drug Levamisole. The results suggest that the methanolic extract of *Syzygium cumini* seeds possesses promising immunomodulatory activity.

Key words: *Syzygium cumini*, carbon clearance, delayed type hypersensitivity, hemagglutination titre, immunomodulation

Introduction

In recent times, focus on plant research has been intensified all over the world and a large amount of evidence has been collected to show immense potential of medicinal plants used in various traditional systems (1). In Indian system of medicine, a large number of herbal drugs have been advocated for various types of diseases/stress related disorders (2). The term immunomodulatory means regulation of the immune system by suppression and stimulation of the cells and organs of the immune system (3, 4).

The modulation of immune responses to alleviate disease has been of interest for many years and the concept of rasayana in Ayurveda is based on related principles (5). The immunomodulatory agents from plant origin which are claimed to induce para immunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (6). It is now being recognized that immunomodulatory therapy could provide an alternative to conventional chemotherapy to a variety of diseased conditions (2).

In India, many Ayurvedic practitioners are using various indigenous plants for the treatment of different types of immune disorders. Although the applications of these medicaments have sound tradition and a rational background according to the Indian system of medicine, perhaps it is essential to investigate the rationality of their use in modern scientific terms.

Syzygium cumini (Syn. *Eugenia cumini*, *Eugenia jambolana*, jambul, black plum) is a tree of the family Myrtaceae distributed in Asia. The barks, leaves and seed extracts of *Syzygium cumini* have been reported to possess anti-inflammatory (7), hypoglycemic (8), antibacterial (9) and anti HIV activity (10). In the indigenous system of medicine, the seed extract of *Syzygium cumini* reported to be useful in the treatment of various inflammatory disorders. In recent years, the extract of seeds, leaves and bark of *Syzygium cumini* has been extensively studied for anti-inflammatory activity (8, 11, 12).

In view of the importance of this herbal plant the present study was undertaken to investigate the immunomodulatory effects of the methanolic extract of *Syzygium cumini* seeds in mice and rats.

Material and Methods

Plant Material

The fully mature *Syzygium cumini* seeds were collected locally during the month of June of 2008. The plant was botanically identified and authenticated by Prof. K. Srinivasa Rao (Department of Pharmacognosy, Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa, India) and voucher specimen was deposited in the department herbarium.

Preparation of Plant Extract

The *Syzygium cumini* fruits were first washed well and pulp was removed from the seeds. Seeds were washed several times with distilled water to remove the traces of pulp from the seeds. The seeds were dried at room temperature and coarsely powdered. The powder was extracted with hexane to remove lipids. It was then filtered and the filtrate was discarded. The residue was successively extracted with methanol using cold percolation method (8). The percentage yield was 10.38% in methanol. The extract was stored at -70°C

until further use. Henceforth, the methanolic extract of *Syzygium cumini* seeds will be called as SME. The extract and standard drug were administered in the form of suspension in water with 1% Sodium carboxy methyl cellulose (SCMC) as suspending agent.

Preliminary Phytochemical Screening

One gram of the methanol extract of *Syzygium cumini* was dissolved in 100 ml of its own mother solvent to obtain a stock of concentration 1% (v/v). The extract thus obtained was subjected to preliminary phytochemical screening (13, 14).

Drugs/Chemicals

Levamisole was supplied by Khandelwal Laboratories, Mumbai, India. All other chemicals used for this study were of analytical grade.

Animals

Albino rats (175-200 g) and albino mice (18-25 g) were procured from Mahaveer Enterprises, Hyderabad, India were used in the study. They were maintained under standard laboratory conditions at ambient temperature of $25\pm 2^{\circ}\text{C}$ and $50\pm 15\%$ relative humidity with a 12-h light/12-h dark cycle. Animals were fed with a commercial pellet diet (Rayans Biotechnologies Pvt Ltd., Hyderabad) and water *ad libitum*. The experiments were performed after prior approval of the study protocol by the institutional animal ethics committee of Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa, India. The study was conducted in accordance with the guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Pharmacological Experiment

***In vivo* Carbon Clearance Method**

The mice were divided into 7 groups. Group-I act as control group, Groups- II to VI treated with SME at different doses (100, 200, 300, 400 and 500 mg/kg b.wt) and Group-VII was treated with Levamisole-50 mg/kg orally. At the end of 7 days, the mice were injected with carbon ink suspension-0.1 ml/10 g (1.6% w/v in 1% gelatin, in saline) via tail vein (15). Blood samples were drawn (in 0.15% w/v disodium EDTA solution, 50 μl) from the retro orbital plexus at intervals of 2 & 15 min after injection. A 25 μl sample was mixed with 0.1% sodium carbonate solution (2 ml) and the absorbance was measured at 660 nm. The carbon clearance was calculated using the following equation (15, 16).

$$\text{Carbon clearance} = (\log \text{OD1} - \log \text{OD2}) / (\text{T2} - \text{T1})$$

OD1 and OD2 are optical densities at T1 and T2 respectively.

T1 = 2 min, T2 = 15 min

Antigen

Fresh blood was collected from sheep sacrificed in local slaughter house in Alsever's solution. Sheep red blood cells (SRBC) were washed three times in normal saline and RBC of this suspension was adjusted to a concentration of 5×10^9 cells per ml for immunization and challenge (17).

SRBC-induced Delayed Type Hypersensitivity Reaction (DTH response)

The rats were divided into 7 groups of 6 rats in each. DTH response was induced in rats using SRBC as an antigen according to Doherty method (18). Group-I act as control group, Groups- II to VI treated with SME at different doses and Group-VII was treated with Levamisole-50 mg/kg orally. SME at different doses (100, 200, 300, 400 and 500 mg/kg b.wt) were administered on day 0 and continued till the day of challenge. The rats were primed with 0.1 ml of SRBC suspension containing 5×10^9 cells i.p. on day 8 and challenged on day 13 with 0.05 ml of 5×10^9 SRBC on the right hind foot pad. The contra lateral paw received equal volumes of saline. The thickness of the foot pad was measured at 24 h after the challenge using vernier caliper (Schnelltester, Germany). The difference in the thickness of the right hind paw and the left hind paw was used as a measure of DTH.

Humoral Antibody Response (HA response)

The rats were divided into 7 groups of 6 in each. Group-I act as control group, Groups- II to VI treated with SME at different doses and Group-VII was treated with Levamisole-50 mg/kg orally. SME was administered at different doses (100, 200, 300, 400 and 500 mg/kg b.wt) on day 0 and continued till the day of the experiment. On day 8, the rats were immunized with 10% suspension of SRBC, i.p. Blood samples were collected from the retro orbital plexus of individual animals on day 13 and their antibody titre from serum was determined according to Puri et al. (19). Briefly, an aliquot (25 μ l) of 2-fold diluted sera in saline was challenged with 25 μ l of 0.1%v/v SRBC suspension in micro titre plates. The plates were incubated at 37°C for 1 h and then examined for haemagglutination and expressed as HA titre.

Haematological Profile

After 8 days of administration of the extract, blood was collected via retro orbital plexus of each rat. Various haematological parameters such as white blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils were estimated. White blood cells were estimated by haemocytometer and remaining parameters by Leishman's stain method (20).

Statistical Analysis

Data were expressed as mean \pm SEM. Data were analyzed by using Analysis of variance and Dunnett's 't' test. The difference was considered to be significant at $p < 0.05$ level.

Results

Preliminary Phytochemical Screening

This investigation showed the presence of alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins and triterpenoids in the methanolic extract of *Syzygium cumini* seeds (SME).

In vivo Carbon Clearance Method

The methanolic extract of *Syzygium cumini* seeds (SME) produced a significant increase in carbon clearance from the blood in a dose-dependent manner (Refer table 1) in mice. The maximum carbon clearance was observed with the 500 mg/kg of SME. The SME effects were compared with the standard drug, Levamisole-50 mg/kg.

Table 1. Effect of methanol extract of *Syzygium cumini* seeds on carbon clearance, DTH response and HA titre

Treatment	Carbon clearance	DTH response (% paw thickness)	HA titre
Control	0.070 \pm 0.014	10.74 \pm 0.91	6.33 \pm 0.73
SME – 100 mg/kg	0.124 \pm 0.018*	10.85 \pm 0.75	6.64 \pm 0.64
SME – 200 mg/kg	0.134 \pm 0.016*	12.20 \pm 1.11*	8.27 \pm 0.66*
SME – 300 mg/kg	0.150 \pm 0.018*	15.13 \pm 1.12*	9.14 \pm 0.77*
SME – 400 mg/kg	0.160 \pm 0.020*	15.92 \pm 1.94*	9.16 \pm 0.78*
SME – 500 mg/kg	0.164 \pm 0.020*	16.13 \pm 1.83*	9.18 \pm 0.80*
Levamisole-50 mg/kg	0.160 \pm 0.018*	16.02 \pm 1.86*	9.12 \pm 0.82*

SME: Methanolic extract of *Syzygium cumini* seeds

* Significance at $p < 0.05$ (Compared to control)

DTH Response

SME produced a significant dose related increase in DTH reaction in rats (Refer table 1). The edema reached a peak at 24 h, the percent edema being 10% for the control group, after which it subsided. The maximum effect was observed with the 500 mg/kg of SME. The effects were compared with standard.

HA Response

SME produced a significant increase in humoral antibody titres in a dose-dependent manner (Refer table 1) in rats. The maximum effect was observed with the 500 mg/kg of SME. The SME effects were compared with the standard drug.

Haematological Profile

SME produced a significant increase in total WBC, neutrophils and lymphocytes count in dose-dependent manner (Refer table 2). Insignificant changes were observed in monocytes, eosinophils and basophils count. The maximum effect was observed with the 500 mg/kg of SME. The SME effects were compared with the standard drug.

Table 2. Effect of methanol extract of *Syzygium cumini* seeds on haematological parameters in rats

Treatment	Total WBC ($10^3/\text{mm}^3$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Control	6.91 ± 0.40	31.18 ± 0.90	68.02 ± 2.10	3.02 ± 0.20	3.02 ± 0.23	0 ± 0
SME – 100 mg/kg	9.98 ± 0.54*	40.95 ± 0.19	70.02 ± 1.90	3.18 ± 0.22	3.34 ± 0.45	0 ± 0
SME – 200 mg/kg	11.12 ± 0.48*	41.42 ± 0.72*	76.02 ± 1.90*	3.26 ± 0.24	3.22 ± 0.54	0 ± 0
SME – 300 mg/kg	11.93 ± 0.29*	42.18 ± 0.72*	77.02 ± 1.60*	3.32 ± 0.34	3.18 ± 0.32	0 ± 0
SME – 400 mg/kg	13.02 ± 0.36*	43.02 ± 0.87*	78.77 ± 2.50*	3.25 ± 0.33	3.15 ± 0.37	0 ± 0
SME – 500 mg/kg	13.81 ± 0.37*	44.35 ± 0.78*	79.02 ± 1.40*	3.18 ± 0.32	3.02 ± 0.27	0 ± 0
Levamisole-50 mg/kg	13.02 ± 0.99*	44.02 ± 0.14*	80.77 ± 2.80*	3.32 ± 0.33	3.16 ± 0.31	0 ± 0

SME: Methanolic extract of *Syzygium cumini* seeds

* Significance at $p < 0.05$ (Compared to control)

Discussion

Modulation of immune response through stimulation or suppression may help in maintaining a disease free state (21). Increased carbon clearance is an indicator of enhanced *in vivo* phagocytic activity and competency of granulopoietic system in removal of foreign particle, thereby an indicator of enhanced immunological response against foreign particles or antigens (22). SEM was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rat of carbon clearance.

DTH is a part of the process of graft rejection, tumor immunity and most importantly, immunity to many intracellular infections of microorganisms, especially those causing chronic diseases (23). In DTH, circulating T cells sensitized to the antigen from prior contact react with the antigen and induce specific immune response, which includes mitosis and the release of soluble mediators. This reaction process involved antigen presentation by macrophages, releases interleukin-I and tumor necrosis factor- α (TNF- α) from activated macrophages, releases interleukin-II and IFN- γ from activated T cells (24) and promotes phagocytic activity and increases the concentration of lytic enzymes for more effective killing (25). This DTH directly correlates with cell-mediated immunity, was found to be highest at the maximum dose of SME (500 mg/kg).

SEM was tested on SRBC hemagglutination antibody titre in rats. SEM was found to be significantly enhanced the circulating antibody titre when compared to untreated immunized rats. This indicates the enhanced responsiveness of T and B lymphocyte subsets involved in antibody synthesis (26).

The majority of all the cells type involved in the immune system are produced from common haemopoetic stem cells of bone marrow. Bone marrow also provides microenvironment for antigen dependent differentiation of B-cells. Since SEM increased circulating antibody titre, it was thought worthwhile to evaluate their effect on peripheral blood counts. In the present study, SEM showed an enhancement in total WBC, neutrophils and lymphocytes counts indicating their effect on haematopoiesis (27).

Our phytochemical investigation revealed the presence of saponins, triterpenoids, flavonoids, tannins, steroids and alkaloids in the methanolic extract. Various research works in the current literature indicate the immunomodulatory activities of these constituents (28-30) and the presence of these constituents may be responsible for the immunomodulatory activity exhibited by this plant.

From the results observed in the current investigation, it may be concluded that the methanolic extract of *Syzygium cumini* seeds possesses immunomodulatory activity by stimulating both cell mediated and humoral immune responses. This study warrants the investigation to isolate and identify the active principles and to elucidate the exact mechanism of action.

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