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ORIGINAL ARTICLE

Immunomodulatory activity of methanolic fruit extract of *Aegle marmelos* in experimental animals

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KEYWORDS

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Abstract *Aim:* The aim of the present study was to investigate the immunomodulatory action of methanolic extract of *Aegle marmelos* fruit (FEAM) in experimental model of immunity.
Methods: Cellular immunity was carried out by neutrophil adhesion test and carbon clearance assay, whereas, humoral immunity was analyzed by mice lethality test and indirect haemagglutination assay. FEAM dose was selected by Stair case method (up and down) and administered at 100 and 500 mg/kg orally. The *Ocimum sanctum* (OSE, 100 mg/kg, *p.o*) was used as standard.
Results: FEAM at 100 and 500 mg/kg produced significant increases in adhesion of neutrophils and an increase in phagocytic index in carbon clearance assay. Both high and low doses of FEAM significantly prevented the mortality induced by bovine *Pasteurella multocida* in mice. Treatment of animals with FEAM and OSE significantly increased the circulating antibody titre in indirect haemagglunation test. Among the different doses, low one was more effective in cellular immunity models than the high. However, all the doses exhibited similar protection in humoral immunity procedures.
Conclusion: From the above findings, it is concluded that FEAM possesses potential for augmenting immune activity by cellular and humoral mediated mechanisms more at low dose (100 mg/kg) than high dose (500 mg/kg).

1. Introduction

A global reliance on alternative system of medicine for chronic and acute ailments resulted in an intense area of research and discovery of a number of herbs with potential to curb diseases. Among them, ample number of herbs has been exploited for modulation of immune system from Ayurvedic formulation either alone or in combinations.

Traditionally, various parts of the plant, *Aegle marmelos* Corr. (Rutaceae), used for the treatment of a variety of disorders (Nadkarni, 1986). The plant is reported to have multiple

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therapeutic properties such as anti-inflammatory, antipyretic and analgesic (Arul et al., 2005; Shankarananth et al., 2007), anti diabetic (Kamalakkannan et al., 2003; Arumugam et al., 2008; Kesari et al., 2006), anti diarrheal (Shoba and Thomas, 2001), anti hyperlipidemic (Vijaya et al., 2009), antifungal (Rana et al., 1997), antimicrobial, antibacterial and anti parasitic (Ulahannan et al., 2008), anti cancer (Gangadevi and Muthumary, 2008), anti malaria (Elango et al., 2009), hepatoproctective (Singh and Rao, 2008) and cardioprotective (Vimal and Devaki, 2004) potentials.

Environmental pollutants and dietary habits cause disturbances in immune activities and diet containing micronutrients and antioxidants are known to prevent these alterations (Bafna and Mishra, 2010). The use of herbs as immunomodulators in the indigenous system of medicines, indeed, can modulate the body's defence mechanism. The following active constituents of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the immune system in different experimental *models* (Shivaprasad et al., 2006). The fruit of *Aegle marmelos* is reported to contain many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants. In addition, it also has many vitamins and minerals including vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus (Das and Das, 1995). Therefore, the chemical profile indicates *Aegle marmelos* as good sources of immunomodulatory agents. Further, the fruit of the plant has been used for many disorders such as chronic diarrhoea & dysentery and act as a tonic for the heart and brain. It is widely used as indigenous traditional medicine for variety of stress disorders including immunodeficiencies (Das and Das, 1995). However, till date no scientific evaluations are conducted for confirming its role as immunostimulant. Thus, this study was designed to study the immunomodulatory activity of extract of *Aegle marmelos* fruit extract in different experimental models of cellular and humoral immunity in animals.

2. Materials and methods

2.1. Experimental animals

Laboratory bred Wistar albino rats (180–200 g) and albino mice (20–25 g) of either sex were housed at 25° ± 5 °C in a well-ventilated animal house under 12/12 h light/dark cycle. The mice were procured from Drug Testing Laboratory, Bangalore. The animals had free access to standard food pellets (Amrut Laboratory Animal feed, Maharashtra, India) containing (% w/w) protein 22.10, oil 4.13, fibre 3.15, ash 5.15, sand (silica) 1.12, and water *ad libitum*. Bedding material was removed and replaced with fresh paddy husk as often as necessary to keep the animals clean and dry. The animals were maintained under standard conditions in an animal house approved by Committee for the purpose of control and supervision on experiments on animals (CPCSEA). The experimental protocol was approved by Institutional ethical committee (KCP/IAEC-24/2008-09). The animals were subjected for quarantine (10 days) prior to experimentation

2.2. Procurement of plant material and extraction

Aegle marmelos fruits were purchased from S.K.R. Market Bangalore (India). The plant was identified and authenticated by Regional Research Institute (Bangalore, India) (RRCBI-Mus/02) The fruits were given to Phytotech Extracts Pvt. Ltd. (Bangalore, India) to get methanol fruit extract of *Aegle marmelos* (FEAM). The ethanolic extract of *Ocimum sanctum* leaves (Natural remedies, Bangalore, India) was used as standard immunomodulatory agent (Mediratta et al., 2002).

2.3. Chemicals and their sources

Leishmann's stain and gluteraldehyde were purchased from Merck (Mumbai, India). Indian ink from HIMEDIA (Mumbai, India). WBC diluting fluid and EDTA from Nice Chemicals (Cochin, India). *Pasteurella multocida* of bovine origin and its vaccine were (Institute of Animal Health and Veterinary Biologicals, Bangalore, India). Nylon fibers (Local market, Bangalore, India).

2.4. Antigen preparation

Fresh sheep blood was collected from the local slaughterhouse. Sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenge (Thomas et al., 2007).

2.5. Preliminary phytochemical screening of extract

Preliminary phytochemical analysis was carried out to check and identify the active constituents of the methanolic extract of *Aegle marmelos* fruit such as alkaloids, carbohydrates, flavonoids, terpenes and steroids, saponins and tannins by using test methods of Draggendorff's and Mayer's test, Molisch's and Fehling's test, lead acetate and magnesium ribbon test, Liebermann–Burchard test, foam formation test, ferric chloride test and gelatin test, respectively (Trease and Evans, 1983).

2.6. Acute toxicity studies (Ghosh, 1984)

The acute toxicity study was carried out to select the dose, by using up and down or stair case method. Two mice were selected with a dose of 50 mg/kg orally and examined for a period of 24 h for mortality. The subsequent doses are then increased by 1.5 factors to attain maximum non-lethal and minimum lethal dose. The extract was found to be safe at the dose of 5 g/kg *p.o.* According to office of pollution prevention and toxics (OPPT) guidelines (http://www.epa.gov/oppts/home/guideline.htm) (Kubavat and Asdaq, 2009), 1/10th and 1/50th of the maximum safe dose (5 g/kg) corresponding to 500 mg/kg and 100 mg/kg were selected as high and low doses, respectively.

2.6.1. Experimental protocol

The drug solutions were prepared in distilled water for oral administration. Immunomodulatory activity was checked both at cellular and humoral levels. Cellular immunity was evaluated by neutrophil adhesion test and carbon clearance assay, whereas, humoral immunity was analyzed by mice lethality test and indirect haemagglutination assay. All the experimental models had four common groups consisting of six animals each. Group I, was served as control and received (vehicle 1 ml/100 g, *p.o*), group II, received the ethanolic extract of

Ocimum sanctum (OSE) at a dose of (100 mg/kg, *p.o*) Sharma et al., 2001, whereas groups III and IV were administered low (100 mg/kg, oral) and high dose (500 mg/kg, oral) of FEAM, respectively. However, in mice lethality test, an additional negative control group was also present.

2.6.2. Neutrophil adhesion test (Fulzele et al., 2003; Shinde et al., 1999)

The rats were pre-treated orally with vehicle or extracts for 14 days. At the end of treatment day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg nylon fibres/ml for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC, respectively to give neutrophil index of blood samples. The percent neutrophil adhesion was calculated as follows:

$$\text{Neutrophil adhesion } (\%) = \frac{\text{NIu} - \text{NIt} \times 100}{\text{NIu}} \quad (1)$$

where NIu is the neutrophil index of untreated blood samples and NIt is the neutrophil index of treated blood samples.

2.6.3. Carbon clearance test (Jayathirtha and Mishra, 2004; Gokhale et al., 2003)

Swiss albino mice were administered FEAM, vehicle and OSE treatment orally for 10 days in their respective groups. Forty-eight hours after the last dose of the drug, animals of all the groups received intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) via the tail vein. Blood samples were withdrawn from each animal by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. 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 formula:

$$K = \frac{(\text{Log}_e\text{OD1} - \text{Log}_e\text{OD2})}{15} \quad (2)$$

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

2.6.4. Mice lethality test (Ramanatha et al., 1995)

Swiss albino mice were pretreated with FEAM and OSE orally for 21 days in their respective groups. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine) through subcutaneous route. On the 21st day, the animals were challenged subcutaneously with 0.2 ml of lethal dose ($25 \times LD_{50}$) of *Pasteurella multocida* (bovine origin) containing 10^7 cells per ml. The animals were observed for a period of 72 h and the mortality percentage was determined.

2.6.5. Indirect haemagglutination test (Fulzele et al., 2003)

Rats of various groups were pretreated with the drugs for 14 days and all rats of entire groups were immunized with 0.5×10^9 sheep red blood cells (SRBCs) intraperitoneally. The day of immunization was referred to as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions (50–100 μl) with SRBC (0.025×10^9 cells) in microtitre plates. The plates were incubated at room temperature for 2 h and examined visually for agglutination. The minimum volume of serum showing heamagglutination was expressed as heamagglutination (HA) titre.

2.6.6. Statistical analysis

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

3. Results

3.1. Neutrophil adhesion test

Incubation of blood with nylon fibres (NF) produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. Both doses of FEAM and OSE showed significant increase in the neutrophil adhesion when compared to control. The low dose of FEAM was found to be more effective than high dose of FEAM. There was also rise in neutrophil count in untreated blood of all treatment groups (Table 1).

3.2. Carbon clearance test

Both doses of *Aegle marmelos* extract and OSE showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best with low dose of FEAM and OSE (Table 2).

Table 1 Effect of FEAM and OSE on neutrophil adhesion test.

Treatment	TLC (10^3/mm^3) (A)		Neutrophil% (B)		Neutrophil index (A × B)		Neutrophil adhesion (%)
	UB	NFTB	UB	NFTB	UB	NFTB	
Control	6.6 ± 0.16	6.5 ± 0.16	24.3 ± 0.80	23.5 ± 0.8	160.58 ± 5.4	153.5 ± 4.5	4.4 ± 0.6
OSE 100 mg/kg	7.6 ± 0.18	6.8 ± 0.15	27.6 ± 1.08	19.6 ± 0.4	209.23 ± 6.4	135.0 ± 4.2	35.4 ± 0.6***
FEAM 100 mg/kg	7.7 ± 0.12	6.8 ± 0.13	28.0 ± 1.33	17.6 ± 1.0	220.13 ± 4.7	121.3 ± 8.5	45.1 ± 1.2***
FEAM 500 mg/kg	7.3 ± 0.86	6.9 ± 0.49	25.5 ± 1.23	18.6 ± 1.2	186.88 ± 8.8	129.9 ± 9.1	30.7 ± 2.0***

All values are expressed as mean ± SEM of six observations.
UB, untreated blood; NFTB, nylon fiber treated blood; OSE, *Ocimum sanctum* extract; FEAM, fruit extract of *Aegle marmelos*. Group I-control; Group II-OSE treated; Group III-FEAM 100 mg/kg and Group IV-FEAM 500 mg/kg.
*** $P < 0.001$ when compared to control.

Table 2 Effect FEAM and OSE on phagocytic index and HA titre.

Treatment	Phagocytic index in carbon clearance assay	Haemagglutination (HA) titre (μl)
CONTROL	0.0175 ± 0.0018	0.0875 ± 0.2562
OSE (100 mg/kg, *p.o*)	0.0483 ± 0.002***	0.0019 ± 0.0003***
FEAM (100 mg/kg, *p.o*)	0.0423 ± 0.0027***	0.0019 ± 0.0003***
FEAM (500 mg/kg, *p.o*)	0.0416 ± 0.0016***	0.0044 ± 0.0008***

All values are expressed as mean ± SEM of six observations.
OSE, *Ocimum sanctum* extract; FEAM, fruit extract of *Aegle marmelos*. Group I-control; Group II-OSE treated; Group III-FEAM 100 mg/kg and Group IV-FEAM 500 mg/kg.
*** $P < 0.001$ when compared to control.

3.3. Mice lethality test

Mortality was found to be 100% within 72 h in control group upon administration of *Pasteurella multocida*. There was 83.33% mortality in vaccinated group without any prior treatment of drug. The low and high doses of FEAM as well as OSE reduced the mortality percentage to 66.66% (Table 3).

3.4. Indirect haemagglutination test

The haemmagglutinating antibody (HA) titre value was significantly increased in animals that received vaccination along with low or high dose of FEAM or OSE compared to animals that received vaccination alone (Table 2).

4. Discussion

In this study we found that methanolic extract of *Aegle marmelos* (FEAM) posseses immunomodulatory activity in experimental models of cellular and humoral immunity. The extract was found to be most effective at low dose (100 mg/kg, *p.o*), whereas, high dose (500 mg/kg, *p.o*) of FEAM was moderately effective in modulating immune system. The study was carried out using four different methods, each of which provides information about effect on different components of the immune system. The variety of plant products can modulate immune reaction either by stimulation or suppression and may assist as a supportive therapy along with conventional drugs in immune compromised patients (Wagner, 1984).

The neutrophil adhesion to nylon fibres describes the margination of polymorphonuclear lymphocyte in the blood vessels and the number of macrophages reaching the site of inflammation (Shinde et al., 1999). Both low and high doses of FEAM (100 and 500 mg/kg, *p.o*) showed a substantial rise in the neutrophil adhesion to nylon fibres. This might be due to the upregulation of the $\beta 2$ integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres (Srikumar et al., 2005). Hence, it was inferred that FEAM causes stimulation of neutrophils towards the site of inflammation.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Gokhale et al., 2003). Since both doses of FEAM as well as OSE showed remarkable augmentation in the phagocytic index, it is speculated that it might be due to increase in the activity of the reticuloendothelial system by prior treatment of animals with FEAM and OSE.

The mouse lethality test is one of the commonly employed tests to assess serological responses in animals immunized with vaccines. *Pasteurella multocida* is pathogenic to mice (Sabri et al., 2001). The mouse lethality test involves injecting mice with the vaccine before administration of the bacterial culture and determining the mortality percentage (Finco Kent et al., 2001). The survival of animals is dependent on the ability of drug to produce adequate antibodies that can counter the pathogen. Both low and high doses of FEAM as well as OSE prevented the death of 33.33% of animals at the end of 72 h.

The indirect haem agglutination test was performed to confirm the effect of *Aegle marmelos* extract on the humoral immune system. It is composed of interacting B cell with antigens and subsequently proliferating and differentiating into antibody producing cells. Antibody works by binding with

Table 3 Effect FEAM and OSE on mice lethality test.

Treatment dose	Mortality first day	Second day	Third day	Mortality percentage
No drug, no vaccination	2	4	–	100
No drug, vaccination	1	3	1	83.33
OSE (100 mg/kg, *p.o*) + vaccination	–	1	3	66.66
FEAM (100 mg/kg, *p.o*) + vaccination	–	2	2	66.66
FEAM (500 mg/kg, *p.o*) + vaccination	–	3	1	66.66

OSE, *Ocimum sanctum* extract; FEAM, fruit extract of *Aegle marmelos*. Group I-normal control without vaccination; Group II-vaccinated without treatment; Group III-OSE treated with vaccination; Group IV-FEAM 100 mg/kg with vaccination and Group V-FEAM 500 mg/kg with vaccination.

antigens and neutralizing it or facilitating its elimination by cross linking to form latex that is more readily ingested by phagocytic cells (Fulzele et al., 2003). The results showed that levels of circulating antibodies are increased if the test animals are pretreated with *Aegle marmelos* extract or OSE.

In conclusion, both low dose (100 mg/kg, *p.o*) as well as high dose (500 mg/kg, *p.o*) of *Aegle marmelos* stimulates immune system by acting through cellular and humoral immunity in experimental models of immunity in animals. However, low dose was found to be most effective than the high dose. However, further studies should be carried out to elucidate the reason behind low efficacy of high dose than the low dose of *Aegle marmelos*.

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