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Isolation and Antioxidant Activity of *Agle marmelos* Leaf Extract in Animals

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ABSTRACT

The aim of the current research was to isolate and evaluate the antioxidant action of *Aegle marmelos* extract (LEAM) in experimental animal models of cellular and humoral immunity due to its antioxidant nature was carried out by using following experimental models, cyclophosphamide-induced neutropenia, serum immunoglobulin estimation, humoral antibody (HA) and delayed type hypersensitivity (DTH) response. Administration of LEAM (500 and 1000 mg/kg, *p.o*) and *Ocimum sanctum* (100 mg/kg, *p.o*), produced a significant increase in TLC and neutrophils against cyclophosphamide-induced neutropenia. Both high and low doses of LEAM and OSE showed a highly significant rise in the serum immunoglobulin levels. Treatment of animals with LEAM and OSE significantly increase the circulating antibody titer in humoral antibody (HA) test and increase in paw edema in DTH response. All the treatment exhibited similar protection in humoral immunity procedures. Moreover, from the above results, it can be concluded that LEAM stimulates both cellular and humoral immune response is assumed the presence of antioxidant active constituents.

INTRODUCTION

Herbal medicinal history is as old as human civilization. Many of the documents revealed that plants were used for the medicinal importance in China, India, Egypt, and Greece before the beginning of the Christian era. Most of the medicinally active substances identified in the nineteenth and twentieth centuries were used in the form of crude extracts. In these aspects, Indians worked meticulously to examine and classify the herbs, which they come across, into a group, called *Gunas.* A large portion of the Indian population even during modern time depends on the Indian system of medicine called *Ayurveda:* Ancient science of life. The well-known treaties in Ayurveda are the *Charak Samhita* and *Sushrutha samhita*[1].

Aegle marmelos (Rutaceae) is commonly known as Bael, found in the dry deciduous forests of Himalayas. The major chemical constituents of leaves are alkaloids including aegelenine and aegeline. Anthraquinones-7,8-Dimethoxy-1-hydroxy-2-methyl anthraquinone and 6-hydroxy-1-methoxy-3-methyl anthraquinone. Coumarins. Leaves also contain condensed tannins[2]. Traditionally, the various parts of the plant are used to treat ailments viz, abdomen pain, palpitation of the heart, urinary troubles. The plant is reported to possess different pharmacological properties like anti-inflammatory, antipyretic and analgesic[3,4], anti-diabetic activity[5,6], antidiarrheal activity[7], anti hyperlipidemic[8], antifungal[9], antimicrobial, antibacterial and ant parasitic activity[10], anti cancer[11], anti malaria[12], hepatoproctective[13], It has been reported that furanocoumarin marmesin in isolated from *Aegle marmelos* have the protective effect against the damage caused by experimental myocardial injury[14]. The immune system is influenced by the environmental hazards and dietary habits and it is believed that diet rich in micronutrients and free radical scavenging property can boost the immune system[15]. From earlier studies, it is evident that the leaf extract, by its free-radical scavenging activity, possesses the radioprotective effect in rodents[16]. *Aegle marmelos* is reported to contain many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants[17]. Some of the plants with known immunomodulatory activity include; *Tinospora cordifolia, Viscum album, Panax ginseng, Asparagus racemosus,* etc. Components such as polysaccharides, lectins, proteins and peptide present in plants have been shown to stimulate the immune system[18]. Therefore, the chemical profile may suggest that *Aegle marmelos* would be a promising immunomodulatory agent because of its antioxidant nature.

Botanical Description

Aegle marmelos is a slow-growing sharp tree and medium in size, about 12 to 15 meter in height with short trunk, thick, soft, flaking bark, and the lower ones drooping. The tree is armed with straight sharp axillaries thorns, 2.5 cm long, ferete, and leaflets 5- 10 by 2.5-6.3 cm, ovate or ovate-lanceolate, flower greenish white, sweetly scented about 2.5 cm across, two sexual. New foliage is glossy and pinkish-maroon in color. Mature leaf emits a disagreeable odor when bruised. Fragment flowers, in clusters of 4 to 7 along young branchlets, have four recurved, fleshy petals, green outside, yellowish inside and 50 or more greenish-yellow stamens. The fruits are round, pyriform oval, or oblong, 5-20 cm in diameter, may have a thin, hard, woody shell or a more or less soft rind, grey-green until the fruit is fully ripe, when it turns yellowish[19,-23.]

ORIGIN AND DISTRIBUTION:

The bael plant is widely distributed in eastern ghat and central India. It is native to India and bael tree is usually available in the range of Himalaya to West Bengal, in central and south Asia. It grows around foothill of Uttar Pradesh, Bihar, Chhattisgarh, Madhya Pradesh, Uttaranchal, Jharkhand, The Deccan Plateau, the East coast, Myanmar, srilanka[22-25].

Medicinal Uses:

The medicinal part of the plants is leaves, fruits, and bark. It had numerous uses in traditional medicine. The leaves and fruit hull of Aegle marmelos have been used for hundreds of years in Southeast Asia as a medicine for skin infection, wound, dysentery, and diarrhea. Apart from the above stated, it also processes some very useful pharmacological properties like anti-inflammatory, antipyretic and analgesic[3,4], anti-diabetic activity[5,6], antidiarrheal activity7, anti hyperlipidemic[8], antifungal[9], antimicrobial, antibacterial and ant parasitic activity[10], anti cancer[11], anti malaria[12], hepatoproctective[13].

2. MATERIALS AND METHODOLOGY

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. The animals had free access to standard food pellets and water *ad libitum*.

2.2. Procurement of plant material and extraction- Aegle marmelos leaves were collected from the fields of Southern parts of Karnataka (India). The plant was identified and authenticated. The leaves were given to outside to get methanol leaf extract of *Aegle marmelos* (LEAM). The ethanolic extract of *Ocimum sanctum* was used as a standard immunomodulatory agent.

2.3. Chemicals- Leishmann's stain, glutaraldehyde. WBC diluting fluid, EDTA, cyclophosphamide (Endoxan Injection).

2.4. Antigen

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 1×10^8 cells/ml for immunization and challenge.

Acute toxicity studies[26] - The acute toxicity study was carried out according to the up and down or staircase test described in the fundamentals of experimental pharmacology. The animals were administered a test dose of 50 mg/kg orally and observed for a period of 24h for mortality, the subsequent dose was increased by 1.5 factor. The extract was found to be safe at the dose of 10 g/kg; *p.o*. According to OPPTS guidelines, [27,] $1/10^{th}$ and $1/20^{th}$ of the maximum safe dose corresponding to 1000 mg/kg and 500 mg/kg were chosen as high and low doses respectively.

Experimentation- The drug solutions were prepared in distilled water for oral administration. The following models of cellular and humoral immunity carried out evaluation of the antioxidant immunomodulatory effect. The animals were distributed into four groups consisting of six animals in each group. The first group served as control (vehicle 1 ml/100 g, *p.o*), the second group, received the extract of *Ocimum sanctum* (OSE) at a dose of (100 mg/kg, *p.o*) [28,] the third and fourth groups were administered low (100 mg/kg, oral) and high dose (500mg/kg, oral) of LEAM respectively.

Cyclophosphamide-induced neutropenia[29] - Swiss albino mice received the drug or vehicle orally for 10 days. On the 10th day, a neutropenic dose of cyclophosphamide (200 mg/kg, *s.c*) was injected and this day was labeled as day zero. Blood was collected; the total leukocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of the control group.

Serum immunoglobulin[30] – The rats are treated with drug or vehicle orally for 21 days. Six hours after the last dose of the drug, blood was collected and the serum was used for immunoglobulin level estimation following a method described by Mullen (1975). As explained, for every sample of serum to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulfate solution was 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 hr at room temperature. The first tube served as blank and the second tube was taken as a sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulfate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acids. The turbidity obtained with this solution was expressed as 20 zinc sulfate turbidity (ZST) units.

HA titer and DTH response using SRBCs as an antigen[31, 32] -The pre-treatment time of 15 days was based on the method demonstrated by Sharma et al. (1996). Schedule for drug administration was −7 days prior to immunization and 7 days after immunization. Mice of various groups were pre-treated with drugs for 7 days and all mice of entire groups were immunized by injecting 0.1 ml SRBCs suspension containing 1×10^8 cells intraperitoneally on day 7. The day of immunization was referred to as day 0. The drug treatment was continued for another 7 more days. Blood samples were collected from each animal at the end of the drug treatment and the titer value was determined by titrating serum dilutions with 0.025 ml of 1% suspension of SRBC in saline in microtiter plates. The plates were incubated at room temperature for 1 h and examined visually for agglutination. The minimum volume of serum showing haemagglutination was expressed as haemagglutination (HA) titer.

On day +7, the thickness of the right hind footpad was measured using vernier caliper. The mice were then challenged by injecting 1×10^8 SRBCs into right hind footpad. Foot thickness

was measured again +24 h after this challenge. The difference between the pre and post-challenge foot thickness expressed in mm was taken as a measure of DTH.

RESULTS

Effect on cyclophosphamide-induced neutropenia – Administration of cyclophosphamide reduced the TLC in control animals by 57.65%. Pretreatment of animals with LEAM for 10 days before cyclophosphamide administration produced 31.93% and 39.26% reduction in TLC with low and high doses respectively. The pretreatment of animals with OSE showed a 41.09% fall in TLC when compared to initial values. The percentage reduction in the neutrophil count was found to be 56.81 and 46.96 in control and OSE groups respectively. The low and high doses of LEAM demonstrated 43.23% and 45.96% reduction in neutrophil count compared to initial values [refer below Table 1].

Table.1: Effect of LEAM and OSE on cyclophosphamide-induced neutropenia

Treatment	Total leucocytes count (cells/mm^3)		% Reduction	% neutrophils		% Reduction
	Before	After		Before	After	
CONTROL	6350± 232.02	2700± 178.42	57.65± 1.6	14.33± 0.66	6.16± 0.30	56.81
OSE (100 mg/kg, *p.o*)	7545± 157.43	4441± 156.75	41.09± 1.7***	13.83± 0.47	7.0± 0.51	46.96*
LEAM (500mg/kg, *p.o*)	7340± 79.87	4951 ± 159.17	32.56± 1.8***	14.16± 0.47	8.3± 0.42	41.24***
LEAM (1000 mg/kg, *p.o*)	7033± 122.00	4300± 202.90	38.98± 1.9***	15.16± 0.47	8.0± 0.36	47.13*

All **v**alues are expressed as mean ± SEM of six observations, ***P<0.001 when compared to control.

OSE- *Ocimum sanctum extract;* LEAM-leaf extract of *Aegle marmelos*

Effect on serum immunoglobulins- The low dose of LEAM and OSE showed a highly significant rise in the serum immunoglobulin levels when compared to control. The ZST unit was found to be significant with a high dose of LEAM compared to control [refer below Table 2].

Effect on HA titer and DTH response- Both the test group, as well as OSE, showed statistically significant in HA titer value.

The low dose exhibited a significant increase in paw edema 24 h after the challenge with antigen, whereas a high dose of test extract and OSE showed a significant increase in paw edema. [Refer below Table 2]

Table. 2: Effect of LEAM and OSE on serum immunoglobulin level, DTH response, and HA titer.

Treatment	Serum immunoglobulin level(ZST units)	DTH response paw edema (mm)	Haemagglutination (HA) titer
CONTROL	14.14± 0.284	0.35± 0.007	0. 1250±0.025
OSE (100 mg/kg, *p.o*)	24.50 ± 0.396***	0.41 ± 0.011***	0.0015±0.0003***
LEAM (100 mg/kg, *p.o*)	24.31 ± 0.327***	0.45 ± 0.007***	0.0023±0.0003***
LEAM (500 mg/kg, *p.o*)	21.88 ± 0.720***	0.41 ± 0.011***	0.0046±0.0006***

OSE- *Ocimum sanctum extract*, LEAM-leaf extract of *Aegle marmelos*

DISCUSSION

In this paper, we report that methanolic extract of *Aegle marmelos* (LEAM) exhibited an immunomodulatory effect in experimental models of both cellular and humoral immunity in animals. The extract was found to be most effective at a low dose (500mg/kg, *p.o*), whereas, high dose (1000 mg/kg, *p.o*) of LEAM was moderately effective in modulating the immune system. The study was performed by four different methods, each of which provides information about the effect on different components of the immune system.

The cyclophosphamide-induced neutropenia model based on the protective effects against cyclophosphamide-induced myelosuppression in the experimental animals.[33] Both low and high doses of LEAM caused a decrease in the cyclophosphamide-induced neutropenia indicating that it diminishes the effect of cyclophosphamide on the hemopoietic system.

The serum immunoglobulin levels indicate the number of antibodies present in the serum. The zinc sulfate turbidity test is performed to assess the approximate amount of immunoglobulins present in the serum. A measured volume of serum was added to a zinc sulfate solution and allowed to incubate at room temperature for 1 h. Zinc sulfate will cause precipitation of the immunoglobulins, which makes the solution cloudy instead of clear [34].

The turbidity is expressed as ZST units, which in turn indicate the amount of immunoglobulin present in the sample. LEAM at both the doses showed a significant increase in the serum immunoglobulin levels.

Administration of 1×10^8 SRBCs to mice i.p. sensitizes them for elicitation of DTH and induces antibody formation. Hence, this system has the major advantage that it allows us to measure two component of the immune response in the same species under the ideal condition and is relatively simple and ease to perform[35] LEAM low dose produced significant increase in both the parameters, i.e. antibody production and delayed type hypersensitivity when compared to control. LEAM high dose and OSE produced moderately increase in paw edema as well as circulating antibody.

In conclusion, both low dose (500 mg/kg, *p.o*), as well as high dose (1000 mg/kg, *p.o*) of *Aegle marmelos*, stimulates the immune system by acting through cellular and humoral immunity in experimental models of immunity in animals is assumed to be the presence of antioxidant active constituents.

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