

EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF METHANOLIC AND ETHANOLIC EXTRACT OF LEAVES OF *AEGLE MARMELOS* IN RATS

Ankur Choubey^{1*}, Aadarsh Choubey², Ashish Mishra¹, Shilpi Mishra¹, U. K. Patil¹

¹VNS Institute of Pharmacy, Neelbud, Bhopal (M.P), India

²Bundelkhand University, Jhansi

ABSTRACT

Aegle marmelos, belongs to family Rutaceae is generally known as bael fruit. They were evaluated for potential immunomodulatory activity using the *in vitro* Polymorphonuclear leukocyte function test. Both Methanolic and Ethanolic extract of *Aegle marmelos* leaves were evaluated for their immunomodulatory activity. In *in vivo* studies, using rats as the animal model. The extract was tested for hypersensitivity and hemagglutination reactions, using shed red blood cells (SRBC) antigen. The methanol extract exhibited a significant increase in the percentage phagocytosis versus the control. It was noted to exhibit a dose related increase in the hypersensitivity reaction, to the SRBC antigen, at concentration of 100 & 200 mg/kg. It also resulted in a significant increase in the antibody titer value, to SRBC, at doses of 100 and 200 ml/kg in animal studies.

The Methanolic extract was found to stimulate cell mediated and antibody mediated immune responses in rats.

Keywords: *Aegle Marmelos*, Hypersensitivity, Hemagglutination, Immunostimulant

INTRODUCTION

Today, herbal drugs constitute only those traditional medicines which primarily use medicinal plant preparations for therapy. Herbal drugs can be used for enhancing the natural resistance of the body against infection [1]. Several herbal preparations that can enhance the natural resistance of the body are extensively being used in the indigenous system of medicines. There is an upsurge in the clinical usage of indigenous drugs as they are free from serious side effects. Dua *et al*, [2] reported a large number of plants having known immunomodulatory activity.

Stress is a retort to emotional, biological, physical and chemical changes. It consisting of a pattern of behavioral reactions and metabolic that helps in strengthening the organism. If the stress is extreme, the homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened. Under these conditions, stress triggers a wide range of the body changes called General

Adaptation Syndrome (GAS). The stimuli, which produce GAS, are called the stressors. It range from physical to psychological factors including infection, toxins, cold, heat, major personal disappointment etc [3]. In the indigenous system of medicine, there are many herbal drugs and formulations recommended to enable one to withstand stress without altering the physiological functions of the body. This, drug induced state of resistance against aversive stimuli is termed as Adaptogenic activity, and the drugs named Adaptogens [4].

Stress alters the physiological homeostasis of the organism resulting in various endocrinal and visceral responses. Stress and depression have been shown to affect immune system functioning, with both immunosuppression and immune activation. Stress alters the equilibrium of various hormones which have a significant impact on the immune response in general. [5]. Stress involves immunological mechanism, neurological complex

and biochemical which plays a crucial role in the genesis/progression of a variety of disease states such as psychiatric disorder like depression, anxiety, and immunosuppression, endocrine disorder including diabetes mellitus, impotency and cognitive dysfunction.

The tree *Aegle marmelos* (Rutaceae) commonly known as Bael is indigenous to India and found wild all over the Sub-Himalayan forests, in Central, and South India. It is a rich source of coumarins, vitamin C, and riboflavin. The bark as well as fruit is reputed to be a valuable Ayurvedic medicine for dysentery and various intestinal complaints [6, 7]. It possesses potent microfilarial [8], radio protective [9], analgesic [10], antihyperglycemic, antidyslipidemic [11], anticancer, [12, 13] and antidiabetic activity [14, 15]. Literature survey has revealed that leaves of *Aegle marmelos* are prescribed in the treatment of immunological disorders but no scientific study is reported regarding its immunomodulatory activity.

The current study aimed at exploring the immunomodulatory potential of the leaves of *Aegle marmelos*.

Materials and Methods

Preparation of Extracts

Methanolic extraction

Plants materials were dried in oven at 360⁰ c and powdered. Leaves of *Aegle marmelos* were extracted in methanol (99.99%) by cold maceration at room temperature for 24 hrs yields 3.7 %.

Ethanol extraction

A sample of 100 g of leaf powder was extracted with 50% ethanol in a Soxhlet apparatus. The extract was freeze dried and stored at -80°C until further use. An approximate yield of 26% was obtained.

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 Dragendorff'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.

Animals

Random bred albino rats of both sexes were used for acute toxicity and pharmacological studies. The animals were maintained at room temperature and fed with standard pellet diet (Lipton India Ltd.) and tap water, ad libitum. The studies were approved by the Institutional Animals Ethical Committee [CUSEP/ IAEC/ 11/ 2003-2004 and CUSEP/ IAEC/ 12/ 2003-2004].

Polymorphonuclear leucocytes (PMN cells)

PMN cells, collected from normal healthy rats weighting 150-250g with no evidence of bacterial, fungal or viral infection, were used in the study.

Reagents

Candida albicans ATCC-10231, maintained on Sabourads agar HiMedia, was used as the test microorganism in the bioassay. All the solvents, reagents and chemicals used were of analytical grade.

In vitro Phagocytosis Test

By using the PMN function test, all the extracts were evaluated for immunomodulatory activity. Peripheral venous blood, 10 ml, was collected from volunteers in a sterile heparinised tube. Neutrophils were isolated by Ficoll Hypaque density gradient sedimentation [15]. The RBC-PMN pellet was then

subjected to dextran sedimentation. The supernatants, containing more than 90% of PMN cells, were collected and the cell density adjusted to 1×10^6 cells/ml using MEM. *Candida albicans* (cell density adjusted to 1×10^6 cells/ml using MEM) was used as the test microorganism. The PMN cells (cell density adjusted to 1×10^6 cells/ml using MEM) were mixed with 1×10^6 cells/ml of *Candida albicans* and incubated at 37°C for one hour in 5% CO_2 atmosphere, in the presence of the test extracts. The control was the identical solution minus the test extracts. Cytosmears were prepared after incubation. The smear was fixed with methanol, stained with Giemsa and studied under 100 X 'oil immersion objective' to determine the phagocytic activity of PMN cells. Neutrophils (100 nos.) were scanned and the cells with ingested microorganisms were counted [16]. The parameters evaluated were percentage phagocytosis (percentage of PMN cells involved in phagocytosis) and phagocytic index (ratio of number of *Candida albicans* engulfed to the total number of Neutrophils).

In vivo study

Acute toxicity study: The acute toxicity study for the SME was conducted in rats as per the prescribed guidelines [17]. Three animals of either sex were used. Their weights were recorded before beginning the study. They were administered a single bolus dose of the SME (2000 mg/kg) per orally and observed over 14 days for mortality and physical/behavioral changes.

Hypersensitivity reaction

Hypersensitivity reaction which measure the cellular immunity. In vivo study was done for hemagglutination reaction. Hypersensitivity reaction to SRBC was induced in rats, following the prescribed method [18]. The SME (in doses of 50,

100 and 200 mg/kg, body weight) was administered to the test group orally for five days and the vehicle was administered to the control group. Each group consisted of six rats - three male and three female. The SME was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunization (i.e., Days -2, -1, 0, +1, +2).

The rats were immunised by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad on day 0. The animals were challenged seven days later by injecting the same amount of SRBC into the left hind footpad. The thickness of the left hind footpad was measured with a micrometer at 4 h and 24 h after the challenge.

Hemagglutination reaction

Hemagglutination reaction which measure the humoral immunity. In vivo study was done for hemagglutination reaction. The SME (in doses of 50, 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats - three male and three female. The SME was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunisation (i.e., Days -2, -1, 0, +1, +2).

By injecting 0.5 ml of SRBCs intraperitoneally (i.p.) on the day of the immunization, the rats were immunised. Blood samples were collected on the tenth day after the immunisation by puncture retro-orbital of the rat. Antibody levels were determined by the hemagglutination technique [19]. The antibody titer was determined by a two-fold serial dilution of one volume (100 μl) of serum and one volume (100 μl) of 0.1% bovine serum albumin

(BSA) in saline. One volume (100 µl) of 0.1 % SRBCs in BSA in saline was added and the tubes were mixed thoroughly and then settle at room temperature for about 60-90 min until the control tube showed a negative pattern (a small button formation). The value of the highest serum dilution showing visible hemagglutination was taken as the antibody titer.

Result & Discussion

Phytochemical screening of the *Aegle Marmelos*

Phytochemical screening of the Methanolic extract revealed the presence of alkaloids, cardiac glycosides, terpenoids, saponins, tannin, flavonoids, and sterioids, but reducing sugars, carbonyl (aldehyde) and Phlobatanin show negative results and Ethanolic show the same result except the absence of saponin .

Pharmacological investigations

Acute toxicity study

The results of the acute toxicity study indicated that the LD₅₀ of the SME of *Aegle Marmelos* was more than 2000 mg/Kg body weight.

In vitro Phagocytosis test

All the extracts were evaluated at concentrations of 0.5, 1.0 and 2.0 mg/ml. Methanolic and Ethanolic extracts exhibited a significant increase in percentage phagocytosis. The SME showed significant activity at concentrations of 0.5 mg/ml (52%), 1.0 mg/ml (48%) and 2.0 mg/ml (45%) as

compared to 30% in the control. The result was recorded in [Table .2].

Hypersensitivity reaction

Per oral administration of the SME (50, 100 and 200 mg/kg) for five days produced a dose related increase in early (4 h) and delayed (24 h) hypersensitivity reaction in rats. The 4 hour-reaction was found to be of higher magnitude than the 24 hour-reaction. These results indicate that the extract has a greater effect on the early hypersensitivity reaction and a less pronounced effect on the delayed hypersensitivity reaction. The result was recorded in [Table.3].

Hemagglutination reaction

The antigen antibody reaction results in agglutination. The relative strength of an antibody titer is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The antibody titer is useful to measure the changes in the amount of the antibody in the course of an immune response.

Administration of the SME (50, 100 and 200 mg/kg) p.o for five days produced a dose related increase in the antibody titer in rats. The result was recorded in [Table.3].

Conclusion

The leaves of *Aegle Marmelos* showed significant immunostimulant activity on both the specific and non specific immune mechanism.

Table 1 Acute toxicity study of Methanolic and Ethanolic extract of Leaves of *Aegle Marmelos*

Dose(mg/kg body weight)	No of animal died or survived	
	Ethanolic extract	Methanolic extract
50	0/10	0/10
100	0/10	0/10
250	0/10	0/10
500	1/9	1/9
1000	2/8	3/7
1500	4/6	4/6
2000	*5/5	*5/5

Figure – Leaves of *Aegle Marmelos*Table 2: In vitro Phagocytosis test of Methanolic and Ethanolic extract of *Aegle Marmelos*

Test extract	Concentration(mg/ml)	Percentage phagocytosis (Mean± S D)	Phagocytic index (Mean± S D)
Ethanolic	Control	33±0.48	1.72±0.01
	0.5	51±2.35	1.88±0.08
	1.0	47±1.25	1.84±0.04
	2.0	44±3.77	1.82±0.06
Methanolic	Control	35±1.75	1.98±0.02
	0.5	37±2.30*	1.96±0.01*
	1.0	38±0.85*	1.94±0.14*
	2.0	36±2.45*	1.97±0.06*
One way ANOVA		130.08	30.62
		3.10	3.10
		<0.05	<0.05

Value are mean ± S D; N=6 in each group. P<0.05 when compared with respective control group (Dunnett's test)

Table 3. Hypersensitivity and Hemagglutination reaction of Methanolic extract of Leaves of *Aegle Marmelos*

Methanolic extract (50mg/kg. P.O)	Hypersensitivity reaction		Hemagglutination antibody titer	
	4 th (Mean±SD)	24 th (Mean±SD)	Range	Mean±SD
Control	0.43±13	-0.06±0.33		212±67.2
(50 mg/kg. P.O)	0.65±0.22	0.36±0.35	126-258	446±323.3
(100 mg/kg. P.O)	0.76±0.18	0.45±0.25	126-1026	1065±571.56*
(200 mg/kg. P.O)	0.90±0.27*	0.77±0.29*	258-2050	1124±629.1*
One way ANOVA	5.92	5.86	510-2050*	7.35
	<.05	<0.05		<0.05

Reference:

- [1.] CK Atul; ML Sharma; A Kaul; A Khajuria, Agents of plant origin, I: Preliminary screening, *J Ethnopharmacol* 1986,18,133-41.
- [2.] Dua PR, Shanker G, Srimal RC, Saxena KC, Saxena RP, Puri A, et al . Activity of Indian *Panax pseudoginseng*. *Indian J Exp Biol* 1989;27:631-4. † [PUBMED]
- [3.] Selye. H. stress of life. (Mc Graw Hill Books, New York, 1950) 1-11
- [4.] Meera S. and Mustafa S.S. Antistress, Adaptogenic and Immunopotentiating Activity roots of *Boerhaavia diffusa* in mice. *Int. J. Pharmacol.* 3 (5): 416-20 (2007).
- [5.] Synle S. Guide to stress research. (Von Nostrand, New York, 1976). 22-7.
- [6.] A. Shoeb, S. Randhir and S.P. Popli (1973). Coumarins and alkaloids of *Aegle marmelos*. *Phytochemistry*. 12, 2071-2072.
- [7] R. N. Chopra, S.L. Nayar and I.C. Chopra (1956). Glossary of Indian Medicinal Plants. CSIR, New Delhi, pp. 8.
- [8] K. N. Sahare, V. Anandhraman, V.G. Meshram, S.U. Meshram, M.V. Reddy, P.M. Tumane and K. Goswami(2008). Anti-microfilarial activity of methanolic extract of *Vitex negundo* and *Aegle marmelos* and their phytochemical analysis. *Indian J Exp. Biol.* 46, 128-131
- [9] G.C. Jagetia, and P. Venkatesh (2007). Inhibition of radiation-induced clastogenicity by *Aegle marmelos* (L.) correa in mice bone marrow exposed to different doses of gamma-radiation. *Hum. Exp. Toxicol.* 26, 111-124
- [10] V. Shankarananth, N. Balakrishnan, D. Suresh, G. Sureshpandian, E. Edwin and E. Sheeja (2007). Analgesic activity of methanol extract of *Aegle marmelos* leaves *Fitoterapia*. 78, 258-259..
- [11] T. Narender, S. Shweta, P. Tiwari, R.K. Papi, T. Khaliq, P. Prathipati, A. Puri, A.K. Srivastava, R. Chander and S.C. Agarwal, K. Raj (2007). Antihyperglycemic and antidyslipidemic agent from *Aegle marmelos*. *Bioorg. Med. Chem. Lett.* 17, 1808-1811.
- [12] L.V. Costa-Lotufo, M.T. Khan, A. Ather, D.V. Wilke, P.C. Jimenez, C. Pessoa, M.E. De Moraes, M.O. De Moraes. (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *J Ethnopharmacol.* 99, 21-30
- [13] D. Subramaniam, P. Giridharan, N. Murmu, N.P. Shankaranarayanan, R. May, C.W. Houchen, R.P. Ramanujam, A. Balakrishnan, R.A. Vishwakarma and S. Anant (2008). Activation of apoptosis by 1- hydroxy-5, 7-dimethoxy-2-naphthalene-carboxaldehyde, a novel compound from *Aegle marmelos*. *Cancer Res.* 68, 8573-8581.
- [14] M.C. Sabu and R. Kuttan (2004). Antidiabetic activity of *Aegle marmelos* and its relationship with its antioxidant properties. *Indian J Physiol Pharmacol.* 48, 81-88.
- [15] R.T. Narendhirakannan, S. Subramanian, M. Kandaswamy (2006). Biochemical evaluation of antidiabetogenic properties of some commonly used Indian plants on streptozotocin-induced diabetes in experimental rats. *Clin Exp Pharmacol Physiol.* 33, 1150-1157
- [16.] Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968;21:77-89
- [17.] Lehrer RI, Cline MJ. Interaction of *Candida albicans* with human leucocytes and serum. *J Bacteriol* 1969;98:996-1004.
- [18.] Organisation for economic co-operation and development.org [homepage on the Internet]. Paris; OECD environment, health and safety publications, series on testing and assessment No. 24, guidance document on acute oral toxicity testing. Available from: <http://www.cancer-pain.org>.
- [19.] Doherty NS. Selective effect of immunosuppressive agents against the delayed hypersensitive response and humoral response to sheep red blood cells in mice. *Agents Actions* 1981;11:237-42. s
- [20.] Nelson DS, Mildenhall P. Studies on cytophilic antibodies. The production by mice of macropage cytophilic antibodies to sheep erythrocytes: relationship to the production of other antibodies and the development of delayed type hypersensitivity. *Aust J Exp Biol and Med Sci* 1967;45:113-30.

Article History:-----

Date of Submission: 10-03-10

Date of Acceptance: 12-05-10

Conflict of Interest: None

Source of Support: Nil