



Anti-inflammatory Activity and Total Flavonoid Content of *Aegle marmelos* Seeds

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ABSTRACT

The anti-inflammatory effect of aqueous and methanol extracts of *Aegle marmelos* seeds was evaluated using carrageenan induced paw edema and cotton pellets induced granuloma in rats. To prove the dependency of pharmacological activity on certain phytoconstituent, total flavonoid contents were estimated, using a spectrophotometric technique. The Quercetin was used as standard compound and the total flavonoids were expressed as mg / g quercetin equivalents (Standard curve equation: $y = 0.0009X + 0.0011$, $R^2 = 0.9189$). The total flavonoid varied from 3.267 to 12.933 mg/g in extracts. The maximum flavonoid content was found in the methanolic extract (65.20 ± 0.2 mg/g). The methanolic extract administered orally at doses of 200 and 400 mg/kg, b.w. showed highly significant ($P < 0.01$) anti-inflammatory activity. The aqueous extract also produced significant ($P < 0.05$) anti-inflammatory property at the same doses.

Keywords: *Aegle marmelos*, Flavonoid, Quercetin, Inflammation, Carrageenan.

INTRODUCTION

Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses. [1] Although, it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases. [2] Currently used anti-inflammatory drugs are associated with some severe side effects. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is necessary. The demand for plant based medicines, health products; phyto pharmaceuticals, health supplements, cosmetics etc. are increasing in both developing and developed countries, because of the growing recognition, that the natural products are non-toxic, less side effects and available at affordable price. [3] As far as the presence of phytochemicals is responsible for their respective medicinal value, some of them are in toxic nature also through direct ingestion or indirectly may alter in the gene through mutation or chromosomal abrasion. Therefore the safety profile of complete category of phytochemical is to be establishing

through the quantitative estimation of a broader category like total phenolics, total alkaloids, total carbohydrate, total protein etc., through which a researcher can image a tentative idea of about nature of medicinal value of the compound extract of any plant part. [4] Flavonoids are a large class of naturally occurring polyphenolic compounds, with C6-C3-C6 backbone, widely present in fruits, vegetables, and beverages derived from plants. These flavonoids are chemically divided into subgroups of flavones, flavanones, flavonols, dihydroflavonols and chalcones. [5-6] Reports have suggested that these compounds might be useful for the prevention of a number of diseases. [7-10] The medicinal value of any plant or plant part may be assumed by estimating the presence of total flavonoid content. A no. of approaches has been made for this purpose. [11-15]

Bael (*Aegle marmelos* Linn.), family rutaceae, is also known as Bale fruit tree, is a moderate sized, slender, aromatic tree, 6.0 -7.5 m in height, and 90 to 120 cm in girth, with a somewhat fluted bole of 3.0-4.5 meter growing wild throughout the deciduous forests of India. [16] It is indigenous to Indian sub continents and mainly found in tropical and subtropical regions. [17] A no. of phytoconstituents have been isolated and reported from the different plant parts of *Aegle marmelos*. [18]

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MATERIAL AND METHODS

Chemical Used: Methanol and ethanol, (Merck, Germany) Quercetin (Sigma, Germany), Carrageenan (Sigma Chemical

Co., USA), were incorporated in study. All the chemicals and reagents used were of analytical grade.

Collection and authentication of plant material

The fresh, unripe fruits of *Aegle marmelos* were collected from healthy trees were growing at very hygiene and polluted free area in the month of May-June, located at various regions of Jaipur, Rajasthan. The seeds and plants were identified and authenticated from the department of Botany, University of Rajasthan, Jaipur, Rajasthan, and voucher specimen was deposited, viz no. RUBL: 20866.

Preparation of extract: Freshly collected seeds of *Aegle marmelos* were dried at 30°C and at 18.9 % relative humidity condition and milled with sieve to remove excess of mucilaginous hair. The plant extract was prepared using two different laboratory grade solvents (double distilled water & methanol);

Preparation of aqueous extract: The dried powdered plant part (1.0 Kg of *Aegle marmelos* seeds) was extracted with 4.0 liters of double distilled water for 72 hours in a round bottom flask, by placing on water bath, attaching reflux water condenser. After filtering and concentrating under vacuum the crude extract (reddish brown) was obtained.

Preparation of methanolic extract: The powdered plant material (1.0 Kg of *Aegle marmelos* seeds) was extracted with 4.0 liters of analytical grade methanol for 72 hours in a round bottom flask, on water bath attaching reflux water condenser. After filtering and concentrating under vacuum the crude extract (yellow reddish) was obtained. The % yields of both the extracts (i.e. aqueous and methanol) were 19.71% and 10.84 %, respectively

Estimation of total flavonoid contents

The total flavonoid content in aqueous and methanol extract of *Aegle marmelos* seeds was determined as method described by Chang *et al.*, 2002. [14]

Preparation of standard curve of Quercetin

Stock solution of Quercetin of 1000µg/ml was prepared by dissolving 100 mg of quercetin in 100 ml of 80 % ethanol (v/v); further, various dilutions were made to prepare 6.25, 12.5, 25.0, 50.0, 80.0 and 100µg/ml solution of quercetin; absorbance was recorded at 415 nm; standard curve (Fig. 1) was plotted between various concentrations and its absorbance.

Both the extracts were evaporated to dryness under vacuum rotary evaporator and re-dissolved in 80 % ethanol (v/v). 100 mg of each dried extracts were dissolved in 10 ml of 80% ethanol; 1 ml of each samples (separately) was mixed with 3 ml 95% ethanol (v/v), 0.2 ml 10% aluminium chloride, 0.2 ml of 1 mol/liter potassium acetate and 5.6 ml of double distilled water to make final volume up to 10 ml, and incubated for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm.

A volume of test sample and aluminium chloride were substituted by the same volume of distilled water and taken as blank solution.

Anti-inflammatory activity

Animals: The male Wistar rats were (180-200 g) were used for the study. They were housed in polypropylene cages and were left for two days for acclimatization to animal room, was maintained under controlled condition of (12 hours light and dark cycle at 22 ± 3°C), and were kept on standard pellet diet and water *ad libitum*. Before the study the animals were fasted overnight with the free access to water. The study protocol was approved by the Institutional Animal Ethical

Committee (IAEC) of CPCSEA (Committee for the purpose of control and supervision of experiments on experiments on animals).

Carrageenan induced rat paw edema

Rats were divided in six groups of 6 animals each. Group I served as control and received normal saline; group II, IV received 200 mg/kg, b.w and group III, V received 400 mg/kg, b.w of aqueous and methanolic extracts of *Aegle marmelos* seeds, sequentially. Group VI was treated with Indomethacin (10 mg/kg, b.w.); served as standard. Paw oedema was induced by injecting 0.1 ml of 1% carrageenan prepared in normal saline solution into sub plantar tissue of right hind paw. Paw volume was measured with the help of Plethysmometer by mercury displacement method at 0, 1, 2, 3, 4, 6 and 24 h. [19] All the doses were administered orally. The percentage inhibition of paw edema was then calculated by using Formula;

$$\text{Percentage Inhibition} = (1 - V_t / V_c) \times 100$$

Where;

V_t = Paw volume after drug and extract treatment

V_c = Paw volume in the control

Cotton pellets induced Granuloma in Rats

Rats were divided into 6 different groups, each containing 6 animals and shaved on the back of neck. Cotton pellets weighing 50 mg each were sterilized by autoclaving at 120±1°C for 1 hour and implanted subcutaneously, one on each side of the subscapular region under the light ether anaesthesia. Following the implantation of cotton pellets, all the established six groups were treated orally for 7 consecutive days. Group I served as control, group II and IV were treated with 200 and 400 mg/kg b.w of aqueous extract, group III and IV were treated with 200 and 400 mg/kg b.w. of methanolic extract respectively. Group VI was treated with Indomethacin (10 mg / kg, body weight), served as standard. [20]

On 8th day, the animals were sacrificed with excess of ether and pellets were dissected out. The pellets were dried at 60°C and dried weight was determined. The difference between the initial and final weight of cotton pellets was considered to be the weight of granulomatous tissue produced.

Statistical Analysis: Data are expressed as the mean ± standard error of mean (S.E.M.) and statistical analysis was carried out employing one way analysis of variance (ANOVA) followed by Dunnet test.

RESULT

Estimation of total flavonoid content: The absorbance for various dilutions of quercetin was found as described in Table 1. Found standard curve equation was; $y = 0.0009X + 0.0011$, $R^2 = 0.9189$. The absorbance for aqueous and methanol extract of *Aegle marmelos* seeds were found 0.305 and 0.1175. The total flavonoid contents (Quercetin equivalents, mg/g) in methanol extract and aqueous extract were calculated as 12.933 mg/g and 3.267 mg/g, respectively (Table 2).

Anti-inflammatory activity

Carrageenan induced rat paw edema: In carrageenan induced rat paw edema (Table 3 & 4), at the end of 6th hour of the study, methanolic extract at the dose of 400 mg / kg b.w. and at the end of 3rd hour Indomethacin at 10 mg/kg b.w., were found to inhibit carrageenan induced edema up to more than 50 % in compare to the control group of animals.

Likely the similar effects were also observed with same methanolic extract at 200 mg / kg b.w. after the end of 24th hour, at the same time methanolic extract at 400 mg / kg b.w. and Indomethacin at 10 mg / kg b.w. had shown total anti-inflammatory response, which were found to be 52.85 % and 53.28 % inhibition. On the second hand, aqueous extracts had failed to produce standard drug like effects even after end of 24hours at both the 200 mg / kg b.w and 400 mg / kg b.w. doses.

Table 1: Absorbance recorded for standard quercetin curve

S. No.	Concentration of standard quercetin (µg/ml)	Absorbance
1.	6.25	0.0079
2.	12.5	0.0156
3.	25.0	0.0316
4.	50.0	0.0439
5.	80.0	0.0641
6.	100	0.0756
7.	125	0.1375

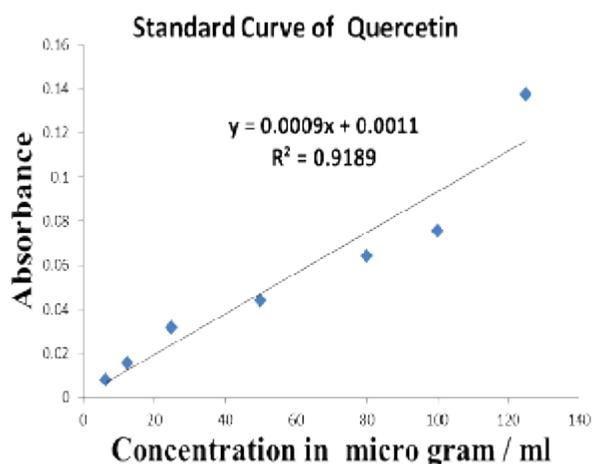
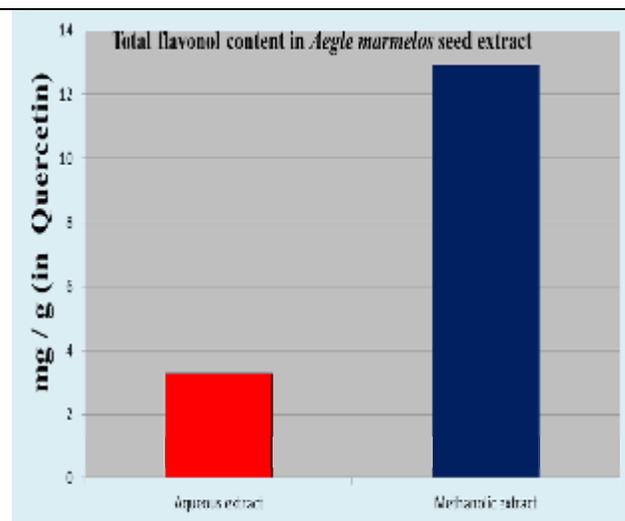


Fig. 1: Standard curve of Quercetin

Table 2: Total flavonol contents in *Aegle marmelos* seed extracts

S. No.	<i>Aegle marmelos</i> seed	Total flavonol content (mg/g) equivalent of Quercetin content
1.	Aqueous extract	3.267 mg/g
2.	Methanolic extract	12.933 mg/g



Cotton pellets induced Granuloma in Rats: Significant reduction of cotton pellet induced granuloma was observed in rats by methanol extract at both the doses i.e. 200 mg / kg b.w and 400 mg / kg b.w., and standard drug Indomethacin at 10 mg / kg b.w., whereas aqueous extract at 200 mg / kg b.w and 400 mg / kg b.w. did also show significant reduction in cotton pellet induced granuloma but the difference is easily recognizable as shown in Table 5.

DISCUSSION

The present study was based to establish the pharmacological effect of seeds of *Aegle marmelos*, where seeds were extracted with aqueous and methanolic solvents. In view of that, we aimed to calculate the comparative total flavonoid estimation in both the extracts, in term of quercetin equivalent and our observation revealed that methanolic extract was 400 % richer in quercetin content than aqueous extract. However, it is must to say that excessive generation of reactive oxygen species, particularly nitric oxide which leads to inflammation, and certain molecules which have flavonoid moiety have been reported to have anti-inflammatory activity.

Table 3: Anti-inflammatory effects of aqueous and methanolic extracts of *Aegle marmelos* seed on carrageenan-induced rat paw oedema

Group	Treatment	Dose (mg/kg b.w., Oral)	Percentage of hind paw edema at time (h) after carrageenan						
			0h	1h	2h	3h	4h	6h	24h
I	Control	2 ml/kg	100.00±2.76	171.00±4.76	209.50±8.91	213.75±8.07	217.39±5.96	220.06±9.01	215.72±4.44
II	Aq. extract	200	100.00±3.71	117.16±4.31*	131.79±6.55*	143.53±3.47*	152.65±1.11*	158.57±3.06*	131.76±7.17*
III	Aq. extract	400	100.00±3.13	117.27±2.31*	129.68±4.14*	139.92±4.65*	148.79±7.76*	156.44±6.76*	130.90±6.16*
IV	Meth. extract	200	100.00±4.14	109.89±2.75**	114.71±2.02**	117.06±3.15**	114.55±4.16**	113.06±2.88**	106.78±3.16**
V	Meth. extract	400	100.00±2.12	108.99±6.66**	113.72±5.05**	115.76±4.14**	111.90±6.06**	107.76±6.16**	101.72±1.71**
VI	Indomethacin	10	100.00±1.09	106.77±7.71**	106.22±2.01**	105.23±1.03**	103.17±1.92**	102.06±2.27**	100.79±6.17**

Values are given as Mean ± SEM for groups of six animals each. *P<0.05 as compared to vehicle control; **P<0.01 as compared to vehicle control.

The development of carrageenan induced edema is believed to be biphasic, of which the first phase is mediated by release of histamine, serotonin and kinins in the first hour after administration of carrageenan, while the second phase is related to release of prostaglandin like substances. Result of present study suggested that methanolic extract of *Aegle marmelos* seed were quite appreciable or quite able to inhibit prostaglandin like substances. The repairing phase of inflammation is inhibited as proliferation of fibroblast and

multiplication of small blood vessels. Proliferating cells penetrate the exudate, producing a highly vascularised reddened mass known as granulation tissue. Both the Tables (4 & 5), when observed simultaneously indicates that methanolic extract at both the doses (200 mg/kg b.w and 400 mg/kg b.w.) in compare to aqueous extract at the same doses were more liably to inhibit the prostaglandin like substances and active in the proliferative phase of inflammatory process. This could be attributed to

Table 4: Inhibitory effect of extracts of *Aegle marmelos* seed at different time interval in respect of control (Group I)

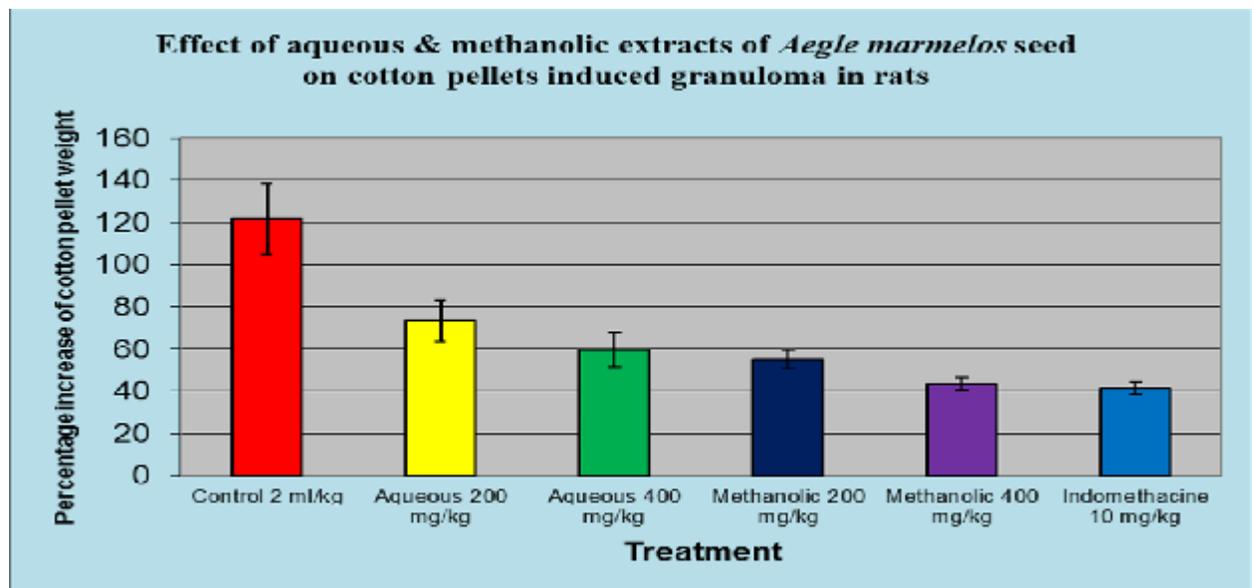
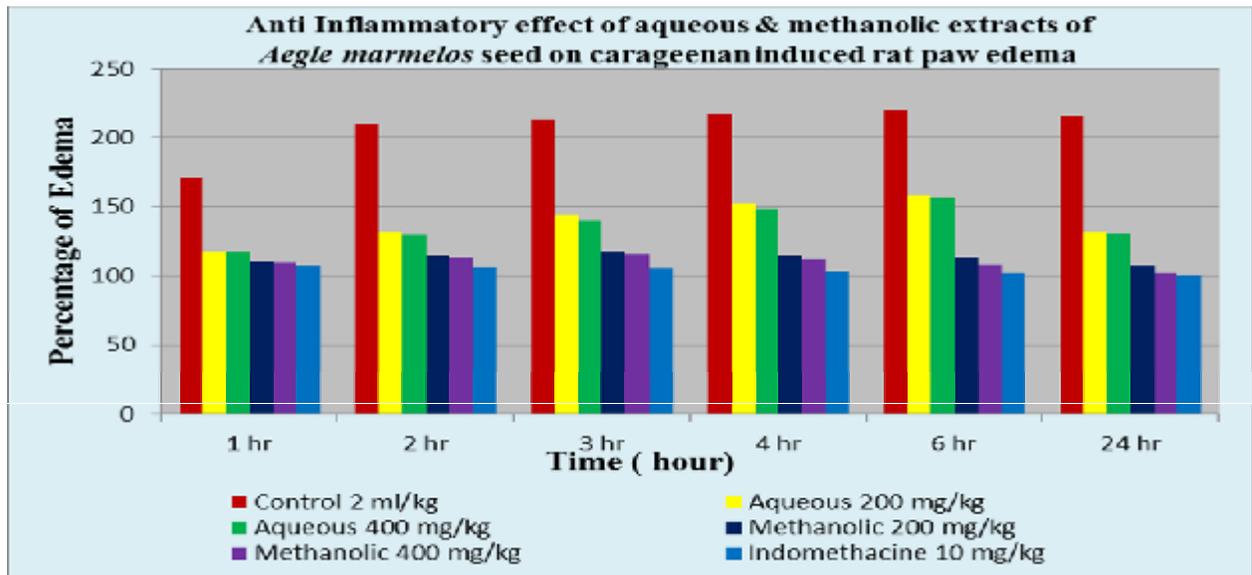
Group	Treatment	Dose (mg/kg, b.w., Oral)	Time (h)					
			1h	2h	3h	4h	6h	24h
II	Aq. Extract	200	31.49	37.09	32.85	29.78	27.94	38.92
III	Aq. Extract	400	31.42	38.10	34.54	31.56	28.91	39.32
IV	Meth. Extract	200	35.74	45.25	45.24	47.31	48.62	50.50
V	Meth. Extract	400	36.26	45.72	45.84	48.53	51.03	52.85
VI	Indomethacin	10	37.56	49.29	50.77	52.54	53.62	53.28

Table 5: Anti-inflammatory effects of aqueous and methanolic extracts of *Aegle marmelos* seed on cotton pellet granuloma in rats

Group	Treatment	Dose (mg / kg, b.w.)	Percentage increase of cotton pellet weight
I	Saline control	Saline (2 ml /kg) orally for 7 days	121.75 ± 16.72
II	Aq. Extract	200	73.67 ± 9.76*
III	Aq. Extract	400	59.62 ± 4.60*
IV	Meth. Extract	200	55.12 ± 8.12**
V	Meth. Extract	400	43.72 ± 3.06**
VI	Indomethacin	10	41.66 ± 2.99**

Values are given as Mean ± SEM for groups of six animals each.

*P<0.05 as compared to vehicle control; **P<0.01 as compared to vehicle control.



high content of total phenolic content, but especially due to 400% difference in presence of quercetin equivalent flavonoid content

Both methanolic and aqueous extract of *Aegle marmelos* seeds showed good anti-inflammatory activity against the acute inflammation (carrageenan induced rat paw edema) and

chronic inflammation (cotton pellet induced granuloma) in animal models, at both the doses (i.e. 200 mg/kg b.w. and 400 mg/kg b.w.), although the better anti-inflammatory activity of methanol extract than the aqueous extract might be due to the presence of higher concentration of flavonoid contents.

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