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Anti-Inflammatory Activities of Aqueous Extract of Fruits and Their Different Fractions of *Melia dubia*.

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ABSTRACT

The anti-inflammatory activity of Aqueous extracts and its fractions of *Melia dubia* (Meliaceae) fruits were screened in-vivo using caragennan induced rat paw edema method and in-vitro by albumin denaturation and membrane stabilization assay at different concentrations. In the present study fruits were extracted successively to obtain various extracts. The aqueous extract was found most active; it was then fractionated into four major fractions (FR) and was evaluated for Anti-inflammatory activity. In-vivo result showed that, FR-III 200 mg/kg & 400mg/kg showed most significant ($P<0.01$) inhibition of edema with 49.11% & 56.24% respectively, which was compared with reference drug indomethacine, that have shown 60.15% most significant ($P<0.01$) inhibition of edema. In-vitro study of FR-III have shown 61.45% inhibition of thermally induced protein denaturation and 61.43% inhibition by membrane stabilization method at concentration of 200µg/ml. From the present study, it is accomplished that FR-III have shown significant dose dependant anti-inflammatory activity.

Keywords: Melia dubia, anti-inflammatory, indomethacin, protein denaturation, membrane stabilization.

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INTRODUCTION

The common complaint of most of patient suffering from diseases is pain and inflammation. Inflammation is a normal protective response shown by living tissue against the injury caused by noxious chemicals, physical shock or microbiological agents [1,2]. Bacterial infection leads to increase numbers of neutrophills, which cause to the production of oxidative burst at the site of microbial invasion [3]. However inflammation remains unchecked, it leads to the cause of disease such as vasomotor rhinitis and atherosclerosis [4]. Most of the users of non steroidal anti inflammatory agents usually sustain some gastrointestinal problems due to the inhibition of the protective cyclooxygenase enzyme in gastric mucosa [5]. Melia dubia belongs to the family meliaceae; the pulp of the fruit has a bitter, nauseous taste and is mainly used in colic[6]. Fruit also used in skin diseases and as anthelmintic [7], hypoglycaemic and antidiabetic activity [8] Leaves and seeds were reported to possess two tetranotriterpenoids, compositin and compositolide [9], fruits showed the presence of bitter principle, Salannin [10] The plant have been used as folk remedies for pain and inflammation condition. Therefore effort has been made to determine anti-inflammatory activity of *M. dubia* fruits.

MATERIAL AND METHODS

Plant material:

Fruits of *M.dubia* were procured from local market and authenticated by Mr. P.G. Diwakar at Botanical Survey of India, Pune. A voucher specimen (CDKMED2) was deposited in the herbarium of BSI Pune for further reference.

Chemicals and drugs

Caragennan (Sigma Aldrich US), indomethacin (Loba chem. Mumbai, India), Petroleum ether, Chloroform, Ethanol and Methanol all solvents were used of Analytical grades, Saline water (Claris life sci., India)

Animals

Wistar albino rats of either sex weighing between 150-200g were used for Anti-inflammatory studies. Animals were grouped in clean polyacrelic cages and maintained at standard laboratory condition (temp 25±2^{o}C) and relative humidity (50±5%) with dark and light cycles (12/12 hrs). animals were allowed to free access to standard dry pellets diets and water ad libitum for two days. The institutional animal ethics committee has approved the experimental protocols and was performed in accordance with the guidelines for the care and use of laboratory animals as adopted and promulgated by institutional animal committee. (CPCSEA, India Reg. No.1211/ac/08/CPCSEA)

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Extraction and fractionation

Dried fruits were crushed to coarse powder and extracted by successive solvent extraction in soxhlet extractor with petroleum ether, chloroform, ethanol and methanol and lastly marc was reflux with water. All the extracts were vacuum dried, which were labeled as PETE (13.48%), CHME (3.14%), EOHE (9.72%), MEOH (5.38%) and AQSE (4.13%) respectively.

Column chromatography

On preliminary pharmacological evaluation of anti-inflammatory activity, AQSE was found most active extract and it was then subject to column chromatography. Aqueous extract was dissolved in small volume of water and applied to column (3x60 cm) which was eluted by Methanol yielding (FR-I, 7.42%), Methanol: water (9:1) yielding (FR-II, 8.35%), Methanol: water (1:1) yielding (FR-III, 6.57%), Water (FR-IV,4.23%)

Phytochemical evaluation

Various extracts obtained were screened by qualitative phytochemical test[11] and it was found to contain steroid, alkaloids, tannins, flavonoids and carbohydrates.

Acute toxicity

Acute toxicity of aqueous extracts was carried out using acute toxic class method as described in OECD [organization of economic co-operation and development]. It was found to be safe up to dose of 2000mg/kg body weight; hence 200 mg/kg and 400mg/kg dose was used for evaluation.

Evaluation of anti-inflammatory activity

Caragennan induced paw edema in rats

Anti-inflammatory activity [12] was studies for different fraction of aqueous extract of *M. dubia* by caragennan induced paw edema in rat model. Test Solution in saline water administered orally. The wistar albino rats were divided into ten groups (n=5). Group-I serve as control and orally administered vehicle only. Group II-V administered orally (200mg/kg) of FR I-IV respectively and Group VI-IX administered orally (400mg/kg) of FR I-IV respectively. Group X administered orally with Indomethacine (10mg/kg) as reference drug. One hour after the respective treatment caragennan (0.1ml of 1% w/v suspension) was injected into sub planner side of the right hind paw of rats. The paw volume was measured at 1, 2, 3 and 4 hours using plethysmometer. Anti-inflammatory effects of fractions were calculated by using the following equation.

$$\% \text{ inhibition of edema} = (Vc-Vt/Vc) \times 100$$

Where Vt is paw volume in test group and Vc is paw volume in control group.

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Statistical analysis

All the experimental data was expressed as mean± SEM, significance of difference among the various groups and control group were carried out using one way ANOVA followed by dunnett's t test using grapat Instat software . Where *P <0.05 was considered as significant, while **P < 0.01 was considered as more significant of test group compared with control group.

Inhibition of albumin denaturation

In this method[13], extract (1ml) of different concentration (50-200µg/ml) or indomethacine (100µg/ml) was mixed with 1ml (1%) aqueous solution of egg albumin and incubated at 37±1^{o}C for 20 min. Denaturation was induced by keeping the reaction mixture at 57±2^{o}C in water bath for 20 min. after cooling the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated with control where no drug was added. Each experiment was carried out in triplicate and its average was taken to calculate Percentage inhibition of protein denaturation by the following equation:

$$\% \text{ inhibition} = Ac- At/Ac \times 100$$

Where Ac= absorption of control , At = absorption of test samples

Membrane Stabilization Test

Preparation of Red Blood Cells suspension

In this method[14], Fresh human blood (10ml) was collected and transferred to heparized centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline. The assay mixture contains the test drug at various concentration, 5ml phosphate buffer (7.4pH),2.0 ml normal saline and 0.5 ml of RBC suspension. Indomethacine (100µg/ml) was used as reference drug. Control sample consist of 0.5ml of RBC suspension mixed with normal saline solution. All the assay mixture were incubated at 37^{o}c for 30 min. and centrifuged. Absorbance of supernant solution was estimated using spectrophotometer at 560nm. The % Inhibition of haemolysis was calculated by using following equation.

$$\% \text{ Inhibition of haemolysis} = 100 \times (A_1- A_2/A_1)$$

Where: A_1= absorbance of control, A_2 = absorbance of test sample.

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RESULTS

In-Vivo Anti-inflammatory activity

Caragennan induced rat paw edema Test

In this study aqueous extract of *M.dubia* was carried out for acute toxicity assay, no death was observed during 72 h period at the dose (2000mg/kg) tested also does not shown any symptoms of diarrhea, seizure or increased dieresis, thus the dose of 200 and 400mg/kg was used in the study. In the caragennan induced rat paw edema test, aqueous extract fractions FR-I, II, III & IV were given orally at a dose of 200mg/kg & 400mg/kg. The effect of various fractions in all experimental groups were studied at 1, 2, 3 & 4 h for inhibition of rat paw edema compared with control group are shown in Table-I. At the end of 4 h, FR-III 200 mg/kg & 400mg/kg showed significant (P<0.01) inhibition of edema with 49.11% & 56.24% respectively, which was compared with reference drug indomethacine, that have shown 60.15% significant (P<0.01) inhibition of edema.

Table 1: Anti-inflammatory activity of *M. dubia*. by carrageen induced rat paw edema method.

Group		Increase in rat paw volume in ml ± SEM (% inhibition)			
		1 hr	2 hr	3 hr	4 hr
Control	Dose mg/kg	0.73±0.05	0.93±0.02	1.1±0.09	1.61±0.01
FR-I	200	0.66±0.11 (10.72%)	0.80±0.15 (14.12%)	0.89±0.07* (20.13%)	1.09±0.03* (31.27%)
	400	0.64±0.03 (12.76%)	0.78±0.05 (16.73%)	0.81±0.02* (26.80%)	0.98±0.06* (38.15)
FR-II	200	0.58±0.04 (21.03%)	0.69±0.08 (26.82%)	0.74±0.02* (32.91%)	0.88±0.06** (46.30%)
	400	0.59±0.01 (22.78%)	0.63±0.02 (33.08%)	0.70±0.05** (37.28%)	0.84±0.07** (49.89%)
FR-III	200	0.57±0.06 (22.42%)	0.62±0.07* (33.46%)	0.67±0.04** (39.38%)	0.81±0.02** (49.11%)
	400	0.52±0.07 (28.97%)	0.59±0.02* (37.03%)	0.63±0.04** (43.38%)	0.71±0.05** (56.24%)
FR-IV	200	0.59±0.03 (19.12%)	0.71±0.08* (24.14%)	0.73±0.02** (34.19%)	0.99±0.08** (37.82%)
	400	0.61±0.01 (16.89%)	0.66±0.05* (28.64%)	0.71±0.04** (34.62%)	0.79±0.02** (44.29%)
Indomethacin	10	0.50±0.02 (35.13%)	0.55±0.07** (41.28%)	0.59±0.07** (46.80%)	0.66±0.03** (60.15%)

Values are expressed as mean± SEM (n=5) animals in each group
*P<0.05 & **P<0.01 significant compared to control group by one way ANOVA followed by Dunnet multiple comparision test

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Table 2: In-vitro anti-inflammatory activity of *M. dubia*

Treatment	Concentration (µg/ml)	Protein denaturation		Membrane Stabilization	
		Absorbance (nm)	%inhibition	Absorbance (nm)	%inhibition
Control	-	0.49	- -	0.57	--
FR-I	50	0.46±0.007	06.52	0.50±0.004	12.26
	100	0.44±0.002	10.78	0.46±0.002	19.31
	150	0.41±0.003	16.47	0.44±0.018	22.81
	200	0.39±0.009	20.81	0.41±0.006	28.12
FR –II	50	0.30±0.004	38.87	0.37±0.003	35.12
	100	0.28±0.009	42.98	0.35±0.001	38.61
	150	0.24±0.001	51.74	0.31±0.007	45.59
	200	0.22±0.004	55.82	0.28±0.004	50.92
FR –III	50	0.27±0.005	45.21	0.34±0.008	40.38
	100	0.24±0.011	51.02	0.31±0.003	45.42
	150	0.20±0.009	59.57	0.27±0.002	52.71
	200	0.19±0.002	61.45	0.22±0.005	61.43
FR –IV	50	0.37±0.006	24.57	0.39±0.004	31.62
	100	0.35±0.001	28.92	0.37±0.002	35.06
	150	0.31±0.009	36.95	0.34±0.005	40.33
	200	0.28±0.002	43.19	0.30±0.002	47.37
Indomethacine	100	0.18±0.003	63.87	0.21±0.007	63.18

Values are expressed as SEM of three readings

In-Vitro Anti-inflammatory activity

Inhibition of albumin denaturation

The different concentrations of test fractions as shown in Table-II, were studied for their inhibiting heat induced albumin denaturation. Maximum inhibition 61.45% (FR-III) was observed at 200µg/ml. IC_{50} value was found to be 87.80 and correlation coefficient value (r) 0.97. Indomethacine at a concentration of 100 µg/ml showed inhibition of 63.67 %.

Membrane Stabilization Test

The different concentrations of test fractions (50- 200µg/ml) were studied for their stabilization of RBCs membrane. The inhibition of heat induced haemolysis of RBCs to varying degree are shown in Table-II, FR-III showed the maximum inhibition 61.43% at concentration of 200µg/ml and IC_{50} was observed at 125.92 and correlation coefficient value (r) at 0.99 and indomethacine at a conc. of 100 µg/ml showed inhibition of 63.18%.

DISCUSSION

In the present study *M. dubia* extracted by soxhlet apparatus, vacuumed dried extracts were preliminary screened for anti-inflammatory activity, the most active aqueous extract was further carried out for acute toxicity assay, no death was observed during 72 h period at the

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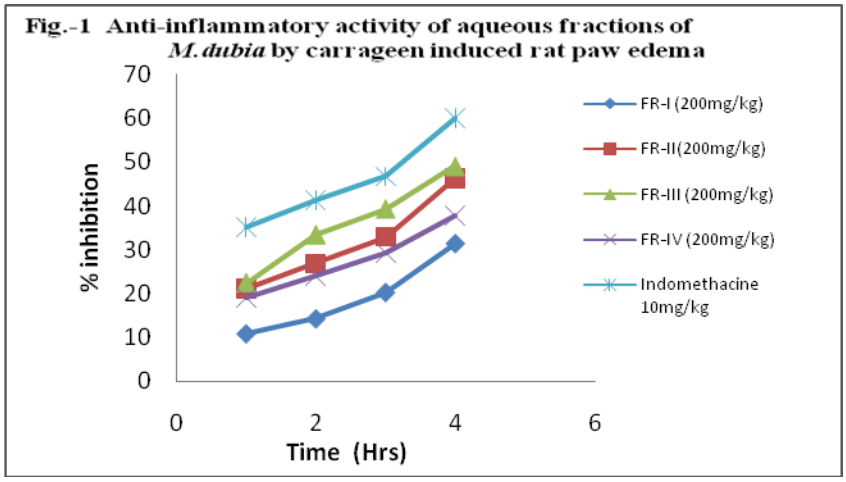
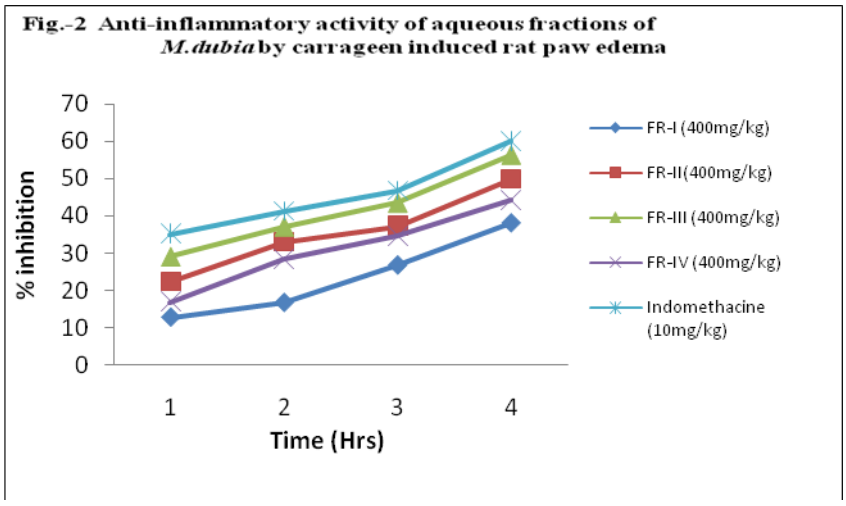
dose tested also does not shown any symptoms of convulsion, diarrhea or increased dieresis, thus the moderate dose of 200 and 400mg/kg was used in the In-vivo anti-inflammatory study. Aqueous extract (AQSE) was further fractionated in four major fractions by using column chromatography. Inflammation is a biphasic process [15]. Early phase (1-2h) of caragennan model is mainly mediated by histamine and serotonin in the mast cells. The later phase is mediated by prostaglandin, bradykinin, protease and lysosome [16], the products of cycloxygenase and lipoxygenase enzymes. Formations of arachidonic acid via cycloxygenase and lipoxygenase pathway represent two important classes of inflammatory mediator. The product of cycloxygenase pathway mainly prostaglandin E2 is known to cause cardinal sign of inflammation and the product of lipoxygenase pathway mainly leukotrine B4 is mediator of leukocyte activation in the inflammation. From the result FR-II & III at a dose of 200 mg/kg (Fig-1) & 400mg/kg (Fig-2) showed significant ($P<0.01$) inhibition against caragennan induced rat paw edema which is comparable with reference drug indomethacine. FR-III was found more potent than FR-II. Reference drug is cycloxygenase inhibitor but anti-inflammatory activity against caragennan induced rat paw edema also shown by lipoxygenase inhibitor; hence inhibition of caragennan induced paw edema by test extract may be due to inhibitory activity of lipoxygenase enzymes.





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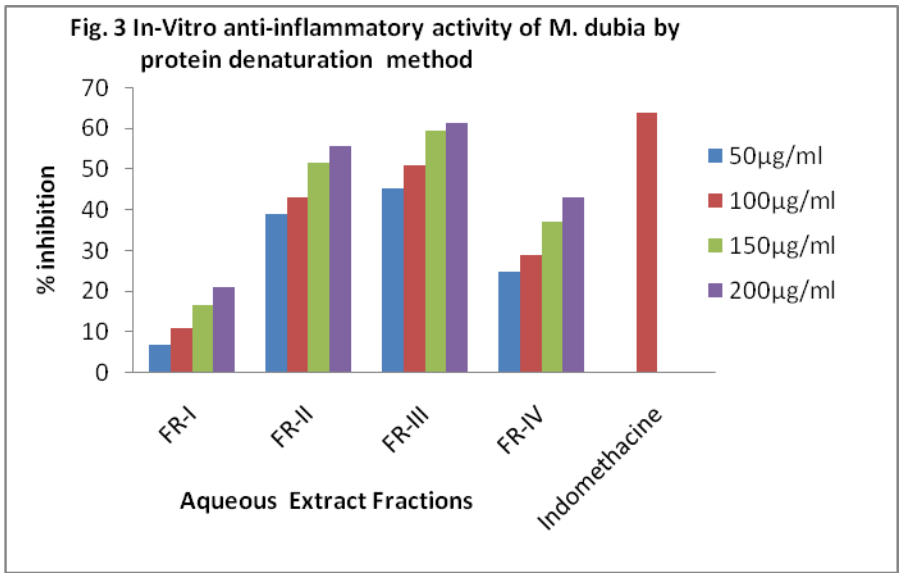

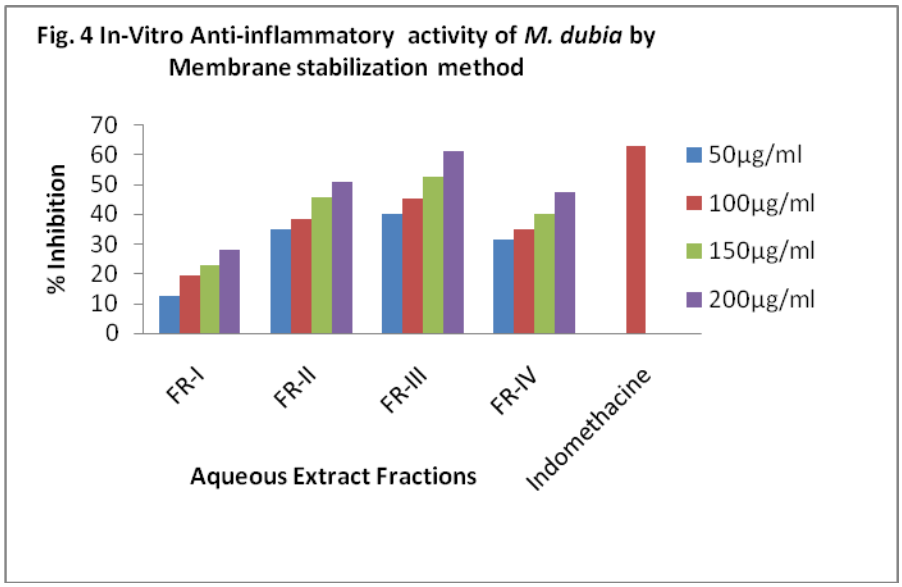

In-vitro study was conceded out by inhibition of thermally induced protein denaturation and membrane stabilization method, in which FR-III (Fig-3) have given away more percentage of inhibition of thermally induced protein denaturation and also given away significant HRBCs membrane stabilization (Fig-4) at a conc. of 200µg/ml, which was analogous with reference indomethacine drug. The extracts exhibited membrane stabilization effect by inhibiting hypotonicity induced lyses of erythrocyte membrane. The erythrocyte membrane is comparable to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in preventive the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and smash up extra cellular release.

From the present study, it is accomplished that AQSE fractions of *M.dubia* fruits have shown significant dose dependant anti-inflammatory activity. These results support the traditional claim of this plant in the treatment of inflammation condition. Potent inhibition of caragennan induced rat paw edema, showed inhibition of prostaglandins synthesis is major mechanism by which the plant extract may showed anti-inflammatory activity.

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