

Original Article

COMPARATIVE STUDY OF ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS AND METHANOLIC EXTRACTS OF *HIBISCUS CANNABINUS* LEAF (MALVACEAE)

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Received: 04 Dec 2015 Revised and Accepted: 11 Feb 2016

ABSTRACT

**Objective:** The present study was undertaken to investigate and compare the anti-inflammatory activity of an aqueous and methanolic extract of *Hibiscus cannabinus* (Malvaceae) using carrageenan-induced rat paw edema.

**Methods:** Aqueous and methanolic extracts of *Hibiscus cannabinus* was prepared and tested for anti-inflammatory activity in female spargue dawley rat weighing 150-180 g. The animals were randomly divided into 6 groups of 6 each. First group served as vehicle control, second group served as standard, third and fourth group served as high (400 mg/kg) and low (200 mg/kg) dose of methanolic extract of *Hibiscus cannabinus* leaves (MHCL) respectively and fifth and sixth group as high (400 mg/kg) and low (200 mg/kg) dose of aqueous extract of *Hibiscus cannabinus* leaves (AHCL) respectively. The *In vivo* anti-inflammatory activity was studied using carrageenan induce rat paw edema animal model. The estimation of liver and blood parameters consist of serum glutamic oxalate transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), lipid peroxidation (LPO), reduced glutathione (GSH) and superoxide dismutase (SOD).

**Results:** Both MHCL and AHCL extracts showed significant ( $p < 0.05$ ) inhibition of rat paw edema in dose-dependent manner. The maximum percent inhibition in paw edema was found in MHCL at dose of 400 mg/kg was 52.00% and AHCL at dose of 400 mg/kg was 49.93%. Both MHCL and AHCL at dose of 400 mg/kg reduce LPO level as 31.10 nmol/g and 35.23 nmol/g respectively when compared with standard indomethacin.

**Conclusion:** An anti-inflammatory activity was found in both MHCL and AHCL extracts. But the MHCL showed more significant anti-inflammatory activity.

**Keywords:** *Hibiscus cannabinus*, Anti-inflammatory, Carrageenan, Indomethacin, Edema

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INTRODUCTION

The word inflammation is derived from the Latin "inflammare" (to burn). It is one of the most important processes involved in the defense of an organism against local injury and infections; however it often progresses to a chronic disease requiring pharmacological treatment [1].

According to the world health organization (WHO), about three-quarters of world population relies upon traditional remedies (mainly herbs) for their health care. Herbs/plants are oldest friends of mankind they not only provide food and shelter but also served the humanity to cure different ailments. The herbal medicines also called traditionally, or natural medicine existed in different cultures and civilization. Ayurveda and Chinese medicinal systems are the most acceptable traditional system which has a considerable amount of research on pharmacognosy, chemistry, pharmacology and clinical therapeutics [2]. *Hibiscus cannabinus* belongs to the family Malvaceae is an Indian plant, which has a number of therapeutic value in the traditional system of medicine. *Hibiscus cannabinus* is an erect annual herb, measures up to 2 m tall in the wild and up to 5 m in cultivars. The taproot is well-developed, measures up to 25 cm deep with lateral roots spread horizontally to 1 m and the adventitious roots on lowest stem section. The stem is slender and cylindrical. In cultivation, it is unbranched and smooth, prickly on wild accessions, entirely green, green with red or purple pigmentation or red sometimes lower half green and upper half pigmented [3]. The crude extract of *Hibiscus cannabinus* has shown various activities including haematinic activity [4], fungitoxicity activity [5], antioxidant activity [6], immunomodulatory [7], hepatoprotective [4], antidiabetic activity [8] and anti-hyperlipidemic activity [9]. From the previous studies, *Hibiscus cannabinus* shown good antioxidant and immunomodulatory activity. However, the anti-inflammatory activity on a leaf of *Hibiscus cannabinus* is spare.

Hence, here is an attempt to evaluate and compare the anti-inflammatory activity of an aqueous and methanolic extract of *Hibiscus cannabinus*.

MATERIALS AND METHODS

Plant materials

The plant material was collected from the local vegetable market of panvel, Navi Mumbai in the month of September and authenticated from the Botany Department, khalsa college College Mumbai. (Voucher No ss/130912a). Freshly collected leaves of *Hibiscus cannabinus* were dried under shade. Dried leaves were ground to a coarse powder with an electrical blender.

Extraction and sample preparation

Methanolic extract of *Hibiscus cannabinus* leaves (MHCL)

Plant powder (10 g) was successively extracted with 250 ml of methanol (90%) in a Soxhlet apparatus at 75 °C 10 to 12 h. Extracts were filtered through Whatman no.1 filter paper. The extracts were concentrated by rotary evaporator till all the solvent had completely evaporated from mixtures.

Aqueous extract of *Hibiscus cannabinus* leaves (AHCL)

The powdered plant material was mixed with distilled water (1:5) and magnetically stirred in a separate container for overnight at room temperature. The residue was removed by filtration through Whatman no.1 filter paper, and the aqueous extracts were lyophilized and stored in airtight containers.

Chemicals

Indomethacin (Sigma), Carrageenan (Sigma) and all other chemicals were of analytical grade.

### Animals

Adult female Spargue Dawley rats (150-180 g) were procured from Glenmark Research Centre, Plot no. a-607, TTC industrial area M. I.D. C, Mahape, Navi Mumbai. The animal house was maintained on 12 h light/dark cycle at 22±2 °C, relative humidity 60-70% and the animals were provided with standard laboratory diet and water ad libetum. The study protocol was approved by Institutional Animal Ethics Committee, Mumbai (approval no-IAEC/PR/2012/01) prior to the commencement of experimental work.

### Methods

#### Carrageenan induces rat paw edema model

In this method, rats were divided into 6 groups of 6 animals in each. Paw edema was induced by injecting 0.1 ml of 1% carrageenan in sterile saline subcutaneously into the sub-plantar region of the rat right hind paw of the animals which were pre-treated with normal saline 2 ml/kg (Control), indomethacin 10 mg/kg (Standard), MHCL 200 mg/kg, MHCL 400 mg/kg, AHCL 200 mg/kg and AHCL 400 mg/kg, 30 min before the carrageenan injection. The paw volume was measured plethysmometrically before administering carrageenan, 0.5, 1, 2, 3, 4, 5 and 6 h after. Inhibition of Inflammation was calculated as the increase in volume (ml) of the paw after treatment [10]. The percentage inhibition of edema was calculated by the following equation.

% Inhibition of edema = 100 (1-Vt/Vc) Where Vc is the edema volume in the control group and Vt is the edema volume in tested group.

#### Biochemical parameters

##### Biochemical parameter in serum

After recording the paw edema, the blood samples were collected by retro-orbital route. Serum was separated from each sample and used for the biochemical analysis. The collected serum was used for estimation of SGOT and SGPT enzymes using commercially available standard enzymatic kits [11].

##### Biochemical parameter in liver

After blood collection, the animals were sacrificed. Immediately after sacrificing the animals, liver was separated, wash with pH 7.4 buffer, blotted with dry filter paper and liver weight was recorded. A part of the liver was minced and then homogenized in pH 7.4 buffer to prepare 10% w/v tissue homogenate. The homogenate was centrifuged at 3000 rpm for 15 min at 4 °C and the supernatant was used for estimation of LPO, GSH and SOD [12].

### Lipid peroxidation (LPO)

Reagents-TCA-TBA-HCL Reagent: 15 g of TCA (trichloroacetic acid), 0.375 g (thiobarbituric acid) were dissolved in 100 ml 0.25N HCl. This solution was slightly heated to assist in the dissolution of TBA, Phosphate buffer pH 7.4, Test samples 10 % w/v liver homogenate in phosphate buffer pH 7.4.

Vortex all tubes for few seconds. Heat for 15 min in boiling water bath and then cool to room temperature. Centrifuge the tubes at 1000 g for 10 min then pipette out supernatant in the cuvette and read O. D at 535 nm against blank [12].

Calculation: nmol of MDA/g of liver tissue = O. D × 156m<sup>-1</sup> cm<sup>-1</sup>

### Reduced glutathione (GSH)

Reagents: Precipitating solution: Trichloroacetic acid (10 %w/v), Phosphate solution: 0.1M KHPO<sub>3</sub> solution in H<sub>2</sub>O, DTNB reagent: 0.25 mmol [5, 5'-dithiobis (2-nitrobenzoic acid)]

#### Procedure

Reagent	Blank (ml)	Test (ml)
Tissue homogenate	-	1
Distilled water	1	-
TCA-TBA-HCL	2	2

**Calculation:** GSH content of test samples was calculated by extrapolation method using standard graph. The result was expressed in nmol/g [13].

Another part of the liver was minced and then homogenized in pH 7.0 buffers to prepare 10% w/v tissue homogenate. The homogenate was centrifuged at 3000 rpm for 15 min at 4 °C and the supernatant was used for estimation of superoxide dismutase.

#### Superoxide dismutase (SOD)

Procedure: A 10 %w/v tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.0). The reaction was initiated by addition of 0.5 ml of hydroxylamine hydrochloride to the reaction mixture containing 2 ml of nitroblue tetrazolium (NBT) and 0.1 ml of liver homogenate. Change in absorbance was measured spectrophotometrically at 560 nm [14]. Calculation: The enzyme activity was expressed as a unit of SOD nmol/min/g liver wt.

#### Procedure: Standard: following procedure was followed for preparation of standard curve

S. No.	Tube no/reagent	Volume (ml)				
		1	2	3	4	5
	Conc. Of GSH (µg/ml)	5	10	15	20	25
1.	GSH Dilution	0.2	0.2	0.2	0.2	0.2
2.	Distilled water	1.8	1.8	1.8	1.8	1.8
3.	Precipitating solution	3.0	3.0	3.0	3.0	3.0
Stand for 5 min, filter, 2 ml filtrate added to						
4.	Phosphate solution (in cuvette)	2.0	2.0	2.0	2.0	2.0
5.	DTNB	1.0	1.0	1.0	1.0	1.0

Test: following procedure was followed for the estimation of liver reduced glutathione content of test sample.

S. No.	Reagent	Blank (ml)	Test(ml)
1.	Liver homogenate	-	0.2
2.	Distilled water	-	1.8
3.	Precipitating solution	2	3.0
Stand for 5 min, filter, 2 ml filtrate added to			
4.	Phosphate solution	2.0	2.0
5.	DTNB	1.0	1.0

Read O. D at 412 nm

### Statistical analysis

Results were expressed as mean±Standard Error Mean (SEM). Differences were considered significant at \*\*\*P<0.001, or \*\*P<0.01 or \*P<0.05 when compared test groups v/s control group. Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Dunnett's t-test. \* P<0.05 was considered statistically significant, and all graphs were made by using Graph Pad Prism6 software.

### RESULTS

#### In vivo carrageenan-induced rat paw edema

As seen in table 1, both the extracts of *Hibiscus cannabinus* and indomethacin significantly decreased carrageenan-induced rat paw edema. The anti-inflammatory effects of 400 mg/kg dose of MHCL and AHCL were determined as 52.00 % and 49.93 % respectively at 3 h. For the same hour, a 200 mg/kg dose of MHCL and AHCL produced 46.66 % and 44.00 % anti-inflammatory effects respectively. In comparison, the anti-inflammatory effect of indomethacin was 58.04 % for the same time. These results suggest

that the methanolic and aqueous extract of *Hibiscus cannabinus* leaves exhibits the anti-inflammatory property in the acute phase of inflammation but the anti-inflammatory activity is more significant in methanolic extract than aqueous extract and mechanism of action may be associated with inhibition of the some of the inflammatory mediators like histamine, serotonin, bradykinins and prostaglandins.

*Hibiscus cannabinus* is a very common plant, and it is widely grown as a vegetable or fiber crop. The herb has been used in the treatment of various inflammatory disorders such as rheumatism, asthma, and burning sensation. Hence, there is a need to validate and provide a scientific basis for the claimed medicinal properties of the herb. Inflammation is an integral part of the body's defense mechanism.

Acute inflammation is characterized by vasodilatation, exudation of plasma, release of various inflammatory mediators, cytokines, growth factors and emigration of leukocytes. Carrageenan-induced rat paw edema is a standard experimental model of acute inflammation. Carrageenan is a phlogistic agent of choice for testing anti-inflammatory drugs, as it is not known to be antigenic and is devoid of any apparent systemic effects [10].

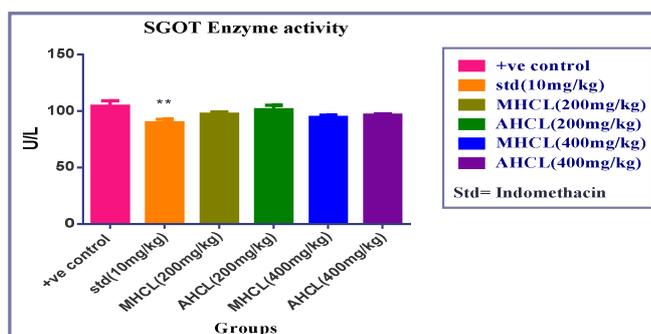
**Table 1: In-vivo anti-inflammatory activity of MHCL and AHCL [mean±SEM, n= 6] significant \* P<0.05 when compared to control**

EDEMA volume (ml)						
Time (h)	Control (vehicle, p. o)	Indomethacin (10 mg/kg)	MHCL (200 mg/kg)	AHCL (200 mg/kg)	MHCL (400 mg/kg)	AHCL (400 mg/kg)
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.27±0.04	0.24±0.03 (11%)	0.26±0.03 (3.70%)	0.26±0.02 (3.70%)	0.25±0.02 (7.40%)	0.25±0.02 (7.40%)
1	0.34±0.02	0.25±0.04* (26.47%)	0.28±0.03* (17.64%)	0.29±0.02* (14.70%)	0.26±0.01* (23.52%)	0.27±0.02* (20.58%)
2	0.55±0.05	0.34±0.01* (38.18%)	0.42±0.03* (23.63%)	0.44±0.02* (20.00%)	0.39±0.05* (29.09%)	0.41±0.02* (25.45%)
3	0.75±0.02	0.31±0.02* (58.04%)	0.40±0.05* (46.66%)	0.42±0.02* (44.00%)	0.36±0.03* (52.00%)	0.38±0.01* (49.93%)
4	0.62±0.07	0.29±0.04* (52.70%)	0.38±0.02 (38.70%)	0.41±0.05 (33.87%)	0.34±0.02* (45.16%)	0.36±0.01 (41.93%)
5	0.51±0.02	0.26±0.05* (48.00%)	0.36±0.04 (29.41%)	0.38±0.05 (25.49%)	0.32±0.02* (37.25%)	0.34±0.01 (33.33%)
6	0.45±0.03	0.29±0.04* (35.55%)	0.39±0.02 (13.33%)	0.41±0.03 (8.88%)	0.35±0.04* (22.22%)	0.37±0.01 (17.77%)

Moreover, the experimental model exhibits a high degree of reproducibility and is least affected by non-specific factors and variations in strain, sex or body weight [12]. Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasations [13], increased tissue water and plasma protein exudation along with neutrophil extravasation, all due to metabolism of arachidonic acid both by the cyclooxygenase and/or lipoxygenase enzyme pathways [14] and also due to activated kinin-forming components and histamine [15]. It has been reported that carrageenan-induced edema is a biphasic response. The 1<sup>st</sup> phase is mediated through the release of histamine and serotonin [16]. The edema maintained between the first phase and the second phase is due to the release of kinin-like substances [17]. Whereas 2<sup>nd</sup>

accelerating phase of swelling is attributed to the cyclo-oxygenase products i.e. prostaglandins [15] and a slow reacting substance with a peak value at 3 h [18]. The prostaglandin phase corresponds with the migration of a large number of leukocytes involved seem to be predominantly the polymorphonuclear type. The mononuclear type may be in lesser numbers than polymorphonuclear cells, at least early in the response [19]. The distinct phases of edema response obtained in our study subsequent to the injection of carrageenan were in confirmation with these reports as shown in table-1. There was a gradual increase in paw edema volume in the control group. The oral pre-treatment with MHCL and AHCL showed an inhibition of edema formation at the 1<sup>st</sup> phase and also in the 2<sup>nd</sup> phase of carrageenan-evoked hind paw edema.

#### Serum glutamate oxaloacetate transaminase (SGOT)



**Fig. 1: Effect of MHCL, AHCL and indomethacin on SGOT level [mean±SEM, n= 6] significant \*\* p<0.01 when compared with control**

Serum glutamate pyruvate transaminase (SGPT)

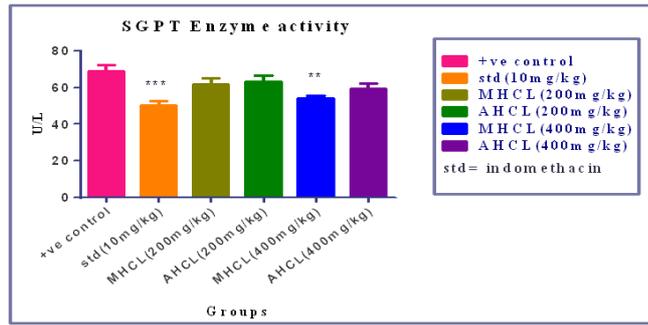


Fig. 2: Effect of MHCL, AHCL and indomethacin on SGPT level [mean±SEM, n= 6] significant \*\* p<0.01, \*\*\* p<0.001 when compared with control

Lipid peroxidation assay

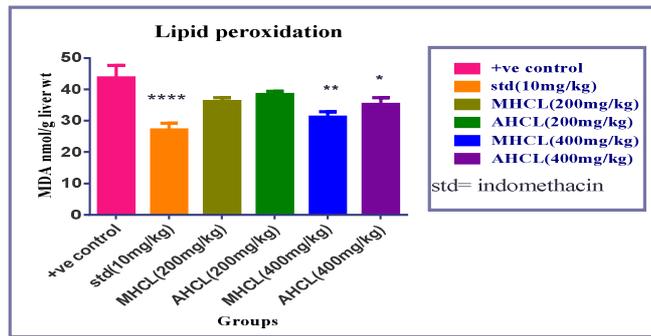


Fig. 3: Lipid peroxidation assay of MHCL, AHCL and Indomethacin [mean±SEM, n= 6] significant \* p<0.05, \*\* p<0.01, \*\*\*\* p<0.0001 when compared with control

Reduced glutathione assay (GSH)

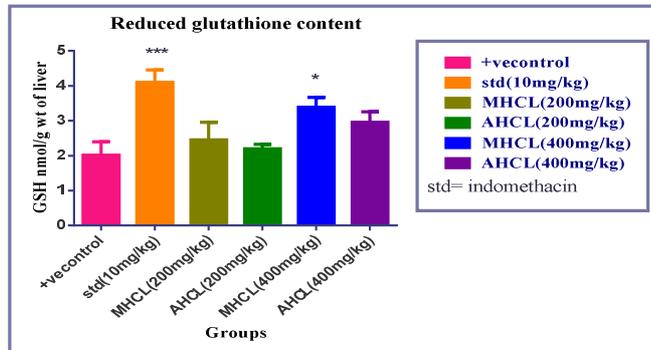


Fig. 4: GSH Assay of MHCL, AHCL and Indomethacin [mean±SEM, n= 6] significant \* p<0.05, \*\*\* p<0.001 when compared with control

Superoxide dismutase assay (SOD)

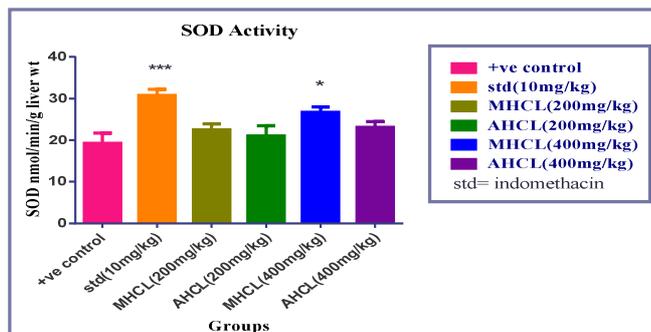


Fig. 5: SOD Assay of MHCL, AHCL and Indomethacin [mean±SEM, n= 6] significant \* p<0.05, \*\*\* p<0.001 when compared with control

Glutamic oxaloacetic transaminases (GOT) is a mitochondrial enzyme present in the highest amount in heart muscle, skeletal muscle, liver, brain and kidney whereas glutamic pyruvic transaminase (GPT) is a cytosolic enzyme primarily present in the liver [20]. Upon damage to the tissues, an appreciable amount of these enzymes escapes into the blood stream, and this is brought out a sharp rise in the serum concentration of these enzymes [21]. The raised activity of these enzymes was reduced in the animals pretreated with indomethacin and the extracts. The results are shown in the fig. 1, 2.

Unsaturated lipids are the normal constituents of the animal tissues, and they undergo oxidation to give rise to toxic peroxide ions (free radicals) [22]. Lipid peroxidation is known to occur in a variety of pathological conditions including rheumatoid arthritis, cancer and degenerative diseases associated with aging [23]. In inflammation, the major source of free radicals is the respiratory burst produced by the inflammatory cells such as macrophages, neutrophils, and eosinophils in response to particular stimuli. Also, the arachidonic cascade releases many free radicals in the process of prostaglandins biosynthesis [24]. Anti-inflammatory drugs are known to inhibit cellular oxidation [25]. Results demonstrate that the inflammation induced by carrageenan is accompanied by an increase in the *in-vitro* output of lipid peroxides by the liver. Carrageenan may elicit a reaction in the paw which is transmitted to the liver which activates lysosomes as defense measure [25]. This activation causes an increase in the output of liver peroxides. Thus, lipid peroxide formation leads to rupture of the lysosomal membrane and allows the release of the contained hydrolytic enzyme [26]. Carrageenan injection to rat paw produced a significant increase in LPO level. However, both the doses of MHCL and AHCL significantly prevented carrageenan-induced increase in LPO level. Indomethacin administration also significantly decreased LPO level when compared to a control group that received carrageenan alone. The 400 mg/kg and 200 mg/kg of MHCL were more effective in decreasing LPO level than 400 mg/kg and 200 mg/kg of AHCL (fig. 3). Carrageenan treatment results in a significant decrease in activity of SOD and GSH level. Which were increased by MHCL, AHCL and indomethacin (fig. 4 and 5).

## CONCLUSION

Anti-inflammatory activity by carrageenan induces rat paw edema model was found in both the extracts i. e. methanolic and aqueous extract of *Hibiscus cannabinus*. But the methanolic extract of *Hibiscus cannabinus* showed more significant anti-inflammatory activity. The study includes the investigation of the effects of MHCL and AHCL on some oxidative parameters such as LPO, SOD and GSH level during the acute phase of inflammation. Thus, a pharmacological basis has been achieved for the use of the plant against rheumatism and other related diseases.

## ACKNOWLEDGMENT

We are thankful to 'Bharati vidyapeeth's college of pharmacy, Belapur, Navi Mumbai for providing necessary facility during this research work.

## ABBREVIATION

MHCL-Methanolic extract of *Hibiscus cannabinus* leaves

AHCL-Aqueous extract of *Hibiscus cannabinus* leaves

## CONFLICT OF INTERESTS

Declared none

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