RESEARCH ARTICLE

ANTI-INFLAMMATORY ACTIVITY OF FOUR FRACTIONS OF ETHANOLIC EXTRACT OF CROTALARIA BURHIA BUCH.-HAM. ROOT IN RATS

Background: Roots of medicinal plants are common ingredients in many folk and herbal medicines system to treat inflammation. The detailed study on *C. burhia* root was lacking to support their anti-inflammatory potential. Henceforth, present investigation was carried out to establish scientific basis for the traditional uses of *Crotalaria buriha* root as anti-inflammatory agent.

Aims & Objective: The present study was aimed to evaluate anti-inflammatory activity of four fractions of ethanolic extract of *Crotalaria burhia* Buch.-Ham. root in W*istar albino* rats.

Materials and Methods: Anti-inflammatory activity of four fractions of ethanolic extract of *Crotalaria burhia* root (EtCB) was evaluated by Carrageenan induced paw edema and cotton pellets induced granuloma in rats. Animals were divided into eight groups (n=6), in which two control groups (25% DMSO and ddH2O), two groups treated with anti-inflammatory drugs (Indomethacin: 10 mg/kg and Diclofenac: 25 mg/kg of body weight) and four fractions treated groups [Hexane (300 mg/kg of bodyweight in 25% DMSO), chloroform (300 mg/kg body weight in 25% DMSO), ethyl acetate (300 mg/kg body weight in 25% DMSO) and aqueous (300 mg/kg of bodyweight in ddH2O)] were treated with oral intubations. Acute anti-inflammatory response was evaluated by measuring paw volume at different time intervals after treatment of test and standard drug. Chronic anti-inflammatory response was evaluated after administration of test and standard treatment for seven consecutive days. On day eighth, four sterile cotton pellets (50 mg) were implanted subcutaneously in the dorsal region of the rats. On the day 16th, the rats were sacrificed and the cotton pellets with granulomatous tissue were taken out, fresh and dry pellets were weighed. Liver tissues was also excised and stored in 0.9% saline at - 20 °C for biochemical analysis.

Results: In acute and chronic anti-inflammatory activity hexane (HF), chloroform (CF), ethyl acetate (EAF) and aqueous (AF) of EtCB were shown significant ($p<0.05$ & $p<0.01$) anti-inflammatory activity when compared to respective control group. However, AF had shown negligible anti-inflammatory activity. Interestingly, EAF was founded more effective than HF and CF in this paradigm. Acute and chronic anti-inflammatory activity of fractions was comparable with positive standard.

Conclusion: The results of present study revealed promising anti-inflammatory and antioxidant activity of EAF. Thus, results indicate that the superiority in anti-inflammatory activity of EAF was largely due to its ability to modulate *In vivo* antioxidant parameters. The results presented here are the first pharmacological studies of EAF of EtCB as anti-inflammatory and antioxidant.

Key Words: Anti-Inflammatory; Antioxidant; *Crotalaria burhia*; Ethanolic Extract; Lipid Peroxidation

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INTRODUCTION

Since antiquity of civilization, people are relying on plants as either prophylactic or therapeutically arsenal to restore and maintain health, and plants are well known as an important source of many biologically active compounds. There has been a growing interest in plants as a significant source of new pharmaceuticals.[1]

Roots of medicinal plants are common ingredients of many folk and herbal medicines as an anti-inflammatory agent. The root extracts of numerous medicinal plants have been reported to have anti-inflammatory activity.[2] The genus *Crotalaria* (Fabaceae) has 300 species world-wide with only eighteen species are found in India. The genus produces mainly pyrrolizidine alkaloids and also some flavonoids and steroids are reported.[3] *C. burhia* or *Khip* is an under shrub, fibrous plant, common in the arid parts of West Pakistan, India and Afghanistan. In ancient Indian medical system of Ayurveda, *khip* has been mentioned as a medicinal plant and various parts of the plant are used. The leaves and branches are used as a cooling medicine, while fresh plant juice is applied on eczema, plant is also very useful in gout, hydrophobia, tumor, pain and swelling.[4] Root extract with sugar is used to cure chronic kidney pain and root decoction is used in typhoid.[5]

Previous reports have shown that pyrrolizidine alkaloids are the main active components of *Crotalaria burhia*. In addition, quercetin and β-sitosterol have been identified from this plant.[6] Various parts of *C. burhia* have shown a

wide array of activities like anticancer, anti-inflammatory and antimicrobial activities.[7] The whole plant has been reported as antibacterial and antifungal agent.[8-9]

Traditionally *C. burhia* is used as remedies for treatment of gout, pain, swelling, tumor and typhoid.[4,7] However, the detailed study on the ethanolic extract of *C. burhia* root was lacking to support their anti-inflammatory activity. Henceforth, we decided and conducted study to evaluate anti-inflammatory activity and establish scientific basis for the traditional uses of *Crotalaria buriha* root as anti-inflammatory agent.

MATERIALS AND METHODS

Materials

Plant Material: The root of *Crotalaria burhia* Buch.-Ham was collected from Rajasthan University Campus, Jaipur, (Rajasthan, India), during month of October. Plant received botanic identification by Mr. P.J. Parmar, Joint Director in Botanical Survey of India (BSI), Jodhpur (Rajasthan, India). A voucher specimen (JNU/JPR/PC/SK-1) was deposited in the BSI, Jodhpur, India.

Chemicals: All chemicals used in the experiments were of analytical grade.

Animal Care and Handling: Healthy, W*istar albino* rats of either sex were procured from departmental animal house for experimentation. The animals were grouped and housed in poly-acrylic cages under standard laboratory conditions (temperature 25 ± 2 °C and dark-light cycle 14-10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The animals were maintained in accordance with CPCSEA guidelines. All the procedures described were reviewed and approved by Institutional Animal Ethical Committee of Suresh Gyan Vihar University, Jaipur, India.

Methods

Extraction and Fractionation: The air-dried and powdered root of *Crotalaria burhia* (1 Kg) was exhaustively extracted with ethanol (95%) by continuous extraction using soxhlet-apparatus. Then filtrate was collected and concentrated by rotary evaporator at 40 °C to yield ethanol extract 78 g (7.8%). The ethanol extract was partitioned between hexane and water (6:1) using separating funnel. This mixture was thoroughly mixed for 15 min and after 6 hrs the hexane fraction was collected. The aqueous layer was further fractionated with chloroform and then with ethyl acetate. All fractions were concentrated by rotator evaporator. The yield of these fractions was 28 g Hexane fraction (HF), 17g Chloroform fraction (CF) and 19g Ethyl acetate fraction (EAF), respectively and constituted about 35.8%, 21.79% and 24.35% of the ethanol extract. The AF (aqueous fraction) was lyophilized and weighed 7.5 g (9.6 % of ethanol extract). Preliminary phytochemical screening of extract was performed using standard procedure.

Acute Toxicity: The up-and-down method for acute toxicity testing was carried out according to method described by Bruce with minor modification and no mortality was observed up to a dose level 2000 mg/kg for all fractions.[10]

Carrageenan-induced Paw Edema in Rats (Acute Inflammation): Rats were divided into eight groups (n=6) and paw edema was produced according to method described by Brich.[11] The test substances such as HF, CF and EAF (300 mg/kg of body weight in 25% DMSO), AF (300 mg/kg of body weight in ddH_2O) and two known anti-inflammatory drugs (As a positive controls): Diclofenac (25 mg/kg of body weight in ddH_2O) and Indomethacin (10 mg/kg of body weight in ddH_2O) were administered by oral intubation. The two control groups received 25% DMSO and ddH_2O. The paw volumes were measured by plethysmometer, before and after injection of 1% carrageenan at different time intervals with reference to the initial volume before giving treatment.

Cotton Pellet-induced Granuloma in Rats (Chronic Inflammation): Rats were divided into eight group (n=6) such as four group of animal were treated with solvent fractions, two group as a positive control treated with anti-inflammatory drugs (Diclofenac and Indomethacin) and two negative control groups treated with 25% DMSO and 25% ddH_2O by oral administration. Treatment was given daily once for seven days to each experimental group. On eighth day, the animals were mildly anesthetized with ether and four sterile cotton pellets with weight of 50 mg were subcutaneously implanted in the dorsal region of rats (two in the axilla and two in the groin regions). On sixteenth day, the rats were sacrificed using anesthetic ether, and the cotton pellets was dissected out without affecting the surrounding granuloma tissues.[12] Liver tissues was also excised and stored in 0.9% saline at – 20 °C for biochemical analysis. The moist pellets was weighed and dried at 60 °C for 48 h and again weighed. The reduced weight of the cotton

pellets observed for the test compounds and anti-inflammatory activity are compared with that the respective negative and positive controls. This method provides a measure to assess the anti-inflammatory effect of the test compounds.

Biochemical Analysis: The liver was excised and ten percent liver tissue homogenate was prepared in Tris-HCl buffer (0.1M, pH 7.4) for estimation of lipid peroxidation[13], antioxidants-glutathione-s-transferase[14], glutathione peroxidase[15], superoxide dismutase[16], catalase[17] and reduced glutathione[18].

Statistical Analysis: All data are expressed as mean ± S.E.M. (n=6). Statistical significance (p) calculated by one-way ANOVA between the treated groups and control followed by Dunnett's test of significance where $p<0.05$ and $p < 0.01$ considered as significant and highly significant, respectively.

RESULTS

Preliminary Phytochemical Screening

The results of preliminary phytochemical screening suggest that the root of *C. burhia* was rich in alkaloids, flavonoids, steroids, terpenes and phenolic compounds.

Acute Anti-Inflammatory Activity

The anti-inflammatory activity of four fractions of *C. burhia* root and standard anti-inflammatory drugs were investigated by measuring paw volume at different time period which is noted in table 1. In both negative control group (DMSO & ddH_2O treated) the paw volume was remain steady up to 4 h and thereafter decline was observed at 8 h, 16 h, and 24 h (table 1). Pretreatment with the anti-inflammatory drugs (DCL and IND) were significantly reduced the paw volume even at 1/2 h and 100% of reduction in paw volume were observed at 24h. Indomethacin even at half the dose of Diclofenac was more effective. At 0.5 h, formers (IND & DCL) were significantly reduced acute inflammation when compared to respective control group. In fractions treated groups, EAF was founded significantly more effective and then followed by HF and CF treated group to reduced inflammation (paw volume) when compared to respective negative control group. Both the fractions HF & CF were showed almost equal anti-inflammatory at 24h after treatment. However AF had negligible anti-inflammatory activity in this paradigm.

Table-1: Effect of solvent fractions of ethanolic extract of *Crotalaria burhia* root and anti-inflammatory drug on carragenan – induced paw edema

Treatment (mg/kg)	Time Intervals (h)						
	0.5	**1**	**2**	**4**	**8**	**16**	**24**
DMSO	0.6 ± 0.06	0.58 ± 0.03	0.56 ± 0.04	0.58 ± 0.03	0.46 ± 0.06	0.41 ± 0.07	0.23 ± 0.02
ddH_2O	0.6 ± 0.05	0.55 ± 0.04	0.55 ± 0.06	0.61 ± 0.03	0.5 ± 0.07	0.43 ± 0.03	0.13 ± 0.02
DCL (25)	0.35 ± 0.03**a	0.33 ± 0.03** a	0.38 ± 0.04* a	0.31 ± 0.06* a	0.2 ± 0.03** a	0.05 ± 0.02** a	0
% Inhibition	-41.66	-40	-30.9	-49.18	-60	-88.37	-100
IND (10)	0.25 ± 0.04* a	0.28 ± 0.03** a	0.35 ± 0.03* a	0.3 ± 0.05* a	0.2 ± 0.04* a	0.03 ± 0.02** a	0
% Inhibition	58.33	-41.09	-39.36	-50.81	-60	-88.37	-100
HF (300)	0.55 ± 0.03 NS, b	0.51 ± 0.03* b	0.5 ± 0.05** b	0.4 ± 0.03** b	0.38 ± 0.06** b	0.25 ± 0.03* b	0.12 ± 0.01* **b**
% Inhibition	-8.3	-12.06	-10.71	-31.03	-17.39	-39.02	-47.82
CF (300)	0.6 ± 0.03 NS, b	0.58 ± 0.04* b	0.45 ± 0.04* b	0.43 ± 0.04* b	0.29 ± 0.04* b	0.24 ± 0.04* b	0.12 ± 0.02* **b**
% Inhibition	0	5.45	-18.18	-29.4	-34.09	-41.46	-47.82
EAF (300)	0.56 ± 0.04NS, b	0.56 ± 0.04* b	0.56 ± 0.06** b	0.5 ± 0.05* b	0.27 ± 0.07** b	0.19 ± 0.04* b	0.08 ± 0.01** **b**
% Inhibition	-6.66	-3.44	0	-13.79	-38.63	-53.65	-65.21
AF (300)	0.61 ± 0.04 NS, a	0.56 ± 0.04* a	0.53 ± 0.09** a	0.58 ± 0.03** a	0.47 ± 0.04* a	0.37 ± 0.04** a	0.12 ± 0.04* **a**
% Inhibition	**1.6**	**1.8**	**-3.63**	**4.91**	**-6**	**-13.95**	-7.6

Data are expressed in centimeters as the Mean ± S.E.M., n=6; a*$p < 0.05$ & a**$p < 0.01$, when compared to vehicle control group (ddH_2O); b*$p < 0.05$ & b**$p < 0.01$, when compared to vehicle control group (DMSO); NS-non significant when compared to respective control group. Percentages refer to reduction in edema size when compared to respective negative control groups. DMSO: dimethyl sulfoxide & ddH2O: double distilled water-negative control.

Table-2: Effect of solvent fractions of ethanol extract of *Crotalaria burhia* root on cotton pellet induced chronic inflammation in rats

Treatment (mg/kg)	Wet weight	Dry weight	Difference	% Inhibition
DMSO + cotton	950.66 ± 2.09	213.33 ± 3.25	737.33	-
ddH_2O + cotton	985.5 ± 2.91	219.5 ± 3.49	766	-
Cotton (50 mg)	992.33 ± 2.3	237.66 ± 4.57	754.67	-
DCL (25) + cotton	334.3 ± 4.03** a	134.83 ± 3.36* a	199.47	-73.56
IND (10) + cotton	321.16 ± 2.12* a	119.5 ± 2.97* a	201.66	-73.67
HF (300) + cotton	692.67 ± 3.72* b	197.5 ± 3.17** b	496.17	-32.7
CF (300) + cotton	709.05 ± 5.87** b	192.66 ± 3.08** b	516.5	-29.96
EAF (300) + cotton	489.33 ± 4.91** b	132.6 ± 3.67* b	356.73	-51.61
AF (300) + cotton	868.83 ± 4.44*a	198.5 ± 4.16* a	670.33	-12.48

Data are expressed in milligram (mg) as the Mean ± S.E.M., n=6; a*$p < 0.05$ & a**$p < 0.01$ when compared to vehicle control group (ddH_2O); b*$p < 0.05$ & b**$p < 0.01$, when compared to vehicle control group (DMSO). A percentage refers reduction in cotton pellet weight when compared to respective negative control groups. The weight of inserted cotton pellet was 50 mg.

Chronic Anti-Inflammatory Activity

Chronic anti-inflammatory activity of the four solvent fractions and two anti-inflammatory drugs were carried out by cotton pellet-induced granuloma in rat. Changes in the cotton pellets weights after treatment were compared with respective negative controls (wet weight-dry weight) and anti-inflammatory response was reported. Both anti-inflammatory drugs were found equally effective and they had reduced approximately 74% mass of the granulomatous tissue formed around implanted cotton pellets. The HF, CF and EAF were shown significant chronic anti-inflammatory activity (Table 2) and their

response was comparable in magnitude with standard anti-inflammatory drug. Only the EAF had shown superior anti-inflammatory activity compared to other fractions. However, in this paradigm also AF was founded less effective (Table 2).

Table-3: Chronic inflammation induced changes in lipid peroxidation and antioxidant in rats liver tissue by solvent fraction of ethanolic extract of Crotalaria burhia root pretreatment

Treatment (mg/kg)	LPO	GST	GPX	SOD	CAT	GSH
Control	2.19 ± 0.57	6.13 ± 0.9	23.84 ± 0.52	12.87 ± 0.12	367.3 ± 10.1	4.1 ± 0.3
DMSO + cotton	3.41 ± 0.82*	3.54 ± 0.76*	15.87 ± 0.67**	8.97 ± 2.1**	271.6 ± 2.7*	2.96 ±0.2*
ddH2O + cotton	4.35 ± 0.23*	3.1 ± 0.81**	15.97 ± 0.3*	8.6 ± 0.4*	257.23 ± 3.8**	3.11 ± 1.2*
HF(300) + cotton	2.97 ± 0.43*[a]	5.17 ± 1.37**[a]	21.98 ± 0.17**[a]	11.61 ± 0.93*[a]	302.1 ± 8.3**[a]	3.19 ± 0.3*[a]
CF (300) + cotton	2.89 ± 0.5*[a]	5.33 ± 0.98**[a]	21.7 ± 0.74**[a]	11.76 ± 0.3**[a]	304.91 ± 3.2*[a]	3.35± 0.2**[a]
EAF (300) + cotton	2.3 ± 0.68**[a]	5.98 ± 0.37*[a]	23.71± 1.4*[a]	12.75 ± 0.53*[a]	362.85 ± 5.7*[a]	4.37 ± 0.7**[a]
AF (300) + cotton	3.1 ± 0.72*[b]	4.71 ± 0.53*[b]	20.89 ± 0.87*[b]	9.27 ± 0.8**[b]	263.9± 11.2*[b]	2.97 ± 1.87*[b]

Data are expressed as the M± S.E.M., n=6. *p<0.05 & **p < 0.01, when compared to control. [a]p<0.5 & [a]p<0.01, when compared to vehicle control group (DMSO). [b]*p<0.05 & [b]**p < 0.01 when compared to control group (ddH_2O). [a]p<0.5 & [a]p<0.01, when compared to vehicle control group(DMSO). LPO: nmoles MDA /mg protein; GST: nmoles CNDB conjugated/min/mg protein; GPx: moles reduced glutathione oxidized/min/mg protein; SOD: units/min/mg protein; CAT: µmoles H_2O_2 consumed/min/mg protein; GSH: µg/mg protein.

Antioxidant Activity under Chronic Inflammation

In chronic inflammation all the major antioxidants in vehicle treated groups were found to be depleted when compared to control groups (Table 2). The lipid peroxidation (LPO) in vehicle treated group was significantly increased by about 56-99 % compared over control group (2.19±0.57 nmoles MDA formed/mg/protein, p<0.05) (Table 3). Solvent fraction, in particular, EAF effectively decreased LPO generated (p<0.01) and also restored other antioxidant as compared to control group. Both the solvent fractions HF and CF showed significant antioxidant activity but it was found less effective as compared to EAF. The results indicated that anti-inflammatory activity of EAF was effectively superior due to its ability to modulate *In vivo* antioxidant.

DISCUSSION

Many experimental protocols are available to evaluate anti-inflammatory potential of medicinal compounds. In the present finding, the anti-inflammatory activity of four fractions of ethanolic extract of *Crotalaria burhia* root and two anti-inflammatory drugs (Diclofenac & Indomethacin) was evaluated in carragenan induced paw edema and cotton pellet induced inflammation in rats to establish scientific basis for the folk use of *C. burhia* root. Treatment with four solvent fractions of ethanolic extract of *Crotalaria burhia* root at the dose of 300 mg/kg body weight was shown significant anti-inflammatory activity in this paradigm when compared to vehicle treated (Table 1, 2 and 3). EAF was found to be more effective to reduced acute and chronic inflammation as compared to other fractions. In acute anti-inflammatory activity study, EAF fraction is effective from 8 h when the inflammation declines by ~39% and moreover continuous reduction was observed at different time and finally ~66% reduction was note at 24 h after treatment. Anti-inflammatory activity of EAF was less and delayed as compared to Indomethacin and Diclofenac.

The acute inflammation has follows two phases reaction: the first phase (within one hour) is characterized by the release of histamine and serotonin; and the second phase (after one hour) is characterized by the bradykinin release via prostaglandins mediator pathways.[19] The delayed inhibitory effect of EAF could be due to its ability to inhibit the bradykinin release or inhibiting prostaglandins mediator pathways.

The chronic inflammation is complex process which happens at the later stage of acute inflammation or generated when body failed to respond against inflammatory agents. This leads to proliferation of fibroblast and granulomatous tissues formation.[20] The EAF treated group was shown potential chronic anti-inflammatory activity (~52% inhibition) that was comparable to the anti-inflammatory drugs treated groups (~74% inhibition). The HF and CF treated group was shown significant chronic anti-inflammatory activity but it was found less effective compared to EAF. These fractions may effectively suppress the granulomatous tissues formation as they have free radical scavenging activity due to presence of large amount of secondary metabolites like alkaloids, flavonoids, steroids, terpens and phenolic compounds. Previously alkaloids,[21] flavonoids[22,23], steroids[24], terpens[25] and phenolic compounds[22,23] reported as anti-inflammatory and antioxidant.

The reactive oxygen species (ROS) participate in process of inflammation in various tissues. ROS produced during chronic inflammation and increase lipid peroxidation and decrease measured antioxidants.[26-27] In EAF treated group was shown significant antioxidant activity by decreased level of lipid peroxidation and increased level of GSH, GPx and other measured antioxidant as compared

to vehicle treated. The results of present study suggest that the fractions of ethanolic extract of *C. burhia* root attenuated the chronic anti-inflammatory activity may be via antioxidant activity.

CONCLUSION

The data reported in these studies confirmed the traditional anti-inflammatory indication of *C. burhia* root and provided biological evidence that *C. burhia* root having anti-inflammatory activity. Thus, present investigation demonstrated, for the first time, *C. burhia* root has relevant anti-inflammatory activity. Further pharmacological and phytochemical investigations are required to elucidate the exact mechanism of action of EAF and isolate the active principles responsible for such effect.

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