Evaluation of anti-inflammatory activity of methanol extract of Barleria Cristata leaves by in vivo and in vitro methods

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Citation

M Gambhire, A Juvekar, S Wankhede. *Evaluation of anti-inflammatory activity of methanol extract of Barleria Cristata leaves by in vivo and in vitro methods*. The Internet Journal of Pharmacology. 2008 Volume 7 Number 1.

Abstract

The methanol extract of Barleria Cristata leaves (BCM) was evaluated for anti-inflammatory activity using in vivo and in vitro methods. In the in vivo inflammation tests, BCM significantly inhibited edema produced by histamine and serotonin in rats, also reduces significantly acetic acid-induced vascular permeability in mice dose dependently. In the in vitro tests, the probable supporting mode by which BCM mediates its effects on inflammatory conditions was studied on red blood cells (RBC's) exposed to hypotonic solution and thermally induced protein denaturation. BCM exhibited significant membrane-stabilizing property. Thermal induced protein denaturation was significantly inhibited by the extract. The effect was compared with the activity of indomethacin and cyproheptadine as reference standard against different types of inflammation. Results of the study revealed that BCM possesses significant anti-inflammatory activity.

SOURCE OF FINANCIAL SUPPORT RECEIVED

This work was fully supported by grants from University Grants Commission, India.

INTRODUCTION

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. The research into plants with alleged folkloric use as anti-inflammatory agents should therefore be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs[1]. Barleria Cristata Linn (family Acanthaceae) is a shrub found widely in subtropical Himalaya, Sikkim, Khasi Hills, central and southern India at height of 1,350 m.

The chemical constituents of the plant have been identified as flavonoid type phenolic compounds especially apigenin, quercetin, quercetin-3-O-β-D-glucoside, naringenin and apigenin glucuronide[2]. Different parts of Barleria Cristata have been used traditionally for the treatment of variety of diseases including anaemia, toothache, cough and as a hypoglycemic agent. Roots and leaves were used to reduce swellings in inflammation [2]. However, there is no systematic scientific report published indicating utility of this plant material in the treatment of inflammation. Thus the presence of therapeutically active flavonoids as major constituents was the basis of selection and evaluation methanol extract of Barleria Cristata leaves for their anti-inflammatory activity.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh leaves of the plant Barleria Cristata Linn were collected from Mumbai region, India. The plant material was taxonomically identified by Dr. Ganesh Iyer, Prof. in Botany, Ramnarain Ruia college, Mumbai, India. A voucher specimen (No. 9-1/08) has been preserved in our laboratory for future reference. The leaves were dried under shade and then powdered with a mechanical grinder and stored in airtight container. The dried powder material of the leaves was defatted with petroleum ether (60-80) and subsequently extracted with methanol in a soxhlet apparatus. The solvent was completely removed under reduced pressure and methanol extract of Barleria Cristata leaves (BCM) was obtained (yield 12.4%). Solution of BCM was prepared freshly in distilled water and used for the studies.

PHYTOCHEMICAL SCREENING

The BCM extract was screened for the presence of various phytochemical constituents i.e. steroids, alkaloids, tannins, flavonoids, glycosides, etc by employing standard screening tests [3].

CHEMICALS AND DRUGS

Histamine, Serotonin, Egg albumin and Evan's blue were obtained from Himedia Lab [(Mumbai) India], Indomethacin [Recon, (Bangalore) India], Cyproheptadine [Fleming Laboratories Limited, (Hydrabad), India] and all other chemicals used were of analytical grade.

ANIMALS

Wistar albino rats of either sex weighing 180–200 g and Swiss albino mice of either sex weighing 18–22 g were used for animal studies. The animals were grouped in polyacrylic cages and maintained under standard laboratory conditions (temperature 25 ± 2 °C) and relative humidity (50±5%) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet and water ad libitum. The rats and mice were acclimatized to laboratory condition for 10 days before commencement of experiment. The Institutional Animal Ethics Committee had approved the experimental protocols and care of animals was taken according to CPCSEA guidelines.

ACUTE TOXICITY TEST

The animals were divided into six groups containing six animals each. BCM was dissolved in distilled water and administered orally as a single dose to mice at different dose levels viz. 500, 750, 1000, 1250, 1500 and 2000 mg/kg of body weight (b.w.). The Mice were observed periodically for symptoms of toxicity and death within 24 h and then daily for next 14 days [4].

HISTAMINE AND SEROTONIN-INDUCED RAT PAW EDEMA

The paw edema was induced by subplantar administration of 0.1 ml of a 0.1% freshly prepared solution of histamine or serotonin into the right hind paw of rats. The paw volume was measured immediately i.e. at 0 h and after 1 h of histamine or serotonin injection [5]. Different groups of animals were pretreated with BCM (125, 250 and 500 mg/kg) or with 5 ml/kg of distilled water (vehicle control) or 10 mg/kg cyproheptadine (standard drug). The drugs were administered orally 1 h before eliciting paw edema. Percent inhibition of paw edema was calculated by following equation,

ANTI-INFLAMMATORY ACTIVITY (%) INHIBITION = (1-D/C) × 100,

where D represents the percentage difference in increased paw volume after the administration of test drugs to the rats and C represents the percentage difference of increased volume in the control groups.

ACETIC ACID-INDUCED VASCULAR PERMEABILITY IN MICE

This test was followed by the method described by Whittle (1964) [6] with some modifications. Five groups of six mice per group were used for the study. Group I served as vehicle control, groups II, III and IV were treated orally with 125, 250 and 500 mg BCM extract/kg respectively while group V received indomethacin 10 mg/kg orally. One hour after the treatment, 0.2% Evan's blue in normal saline was injected intravenously through tail vein at a dose of 0.1 ml/10 g b.w. Thirty minutes later, each mouse was injected intraperitoneally with 0.2 ml of 0.6% acetic acid in normal saline solution. After 1 h, the mice were sacrificed and the abdominal wall was cut to expose the entrails. The abdominal cavity was washed with 5ml of normal saline to collect pigments in a test tube. After centrifuging the contents of the tube to eliminate contaminants, the solution was subjected to colorimetric analysis using a spectrophotometer at a wavelength of 590 nm. The vascular permeability effects were expressed as the absorbance (A), which represented the total amount of dye leaked into the intraperitoneal cavity.

MEMBRANE STABILIZING ACTIVITY

This test was followed by the method described by Shinde et al (1999) [7] with some modifications. Whole human blood was obtained from a healthy human volunteer and transferred to heparinized centrifuge tube. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) for 10 minutes at 3000g. The test sample consisted of stock erythrocyte (RBC) suspension (0.5 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the BCM extract (0.2- 1.0 mg/ml) or indomethacin (0.1 mg/ml). The control sample consisted of 0.5 ml of RBC suspension mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000g and the absorbance of the supernatant was measured at 540 nm. Each experiment was carried out in triplicate and the average was taken. The percentage inhibition of haemolysis or membrane stabilization was calculated by following equation.

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

Where:

A_1 = Absorption of hypotonic buffered saline solution alone

A_2 = Absorption of test sample in hypotonic solution

EFFECT ON PROTEIN DENATURATION

Test solution (1ml) containing different concentrations (50 - 250 g/ml) of plant extract or indomethacin (100 g/ml) was mixed with 1ml of egg albumin solution (1mM) and incubated at 27 ±1 °C for 15 min. Denaturation was induced by keeping the reaction mixture at 70 °C in a water bath for 10 min. After cooling the turbidity was measured spectrophotometrically at 660 nm [$_{89}$]. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was carried out in triplicate and the average was taken.

STATISTICAL ANALYSIS

The experimental data was expressed as mean ± SEM, the significance of difference among the various treated groups and control group were analyzed by means of one-way ANNOVA followed by Dunnett's t-test using Graphat Instat Software (San Diego, CA, USA).

RESULTS

In this study anti-inflammatory activity of methanol extract of Barleria Cristata leaves was evaluated by different in vivo and in vitro screening methods.

Preliminary phytochemical screening of the methanol extract of Barleria Cristata leaves revealed the presence of steroids, flavonoids, alkaloids and glycosides. Further separation of the specific phytochemicals is in progress. In the acute toxicity assay no deaths were observed during the 72 h period at the doses tested. At these doses, the animals showed no stereotypical symptoms associated with toxicity, such as convulsion, ataxy, diarrhoea or increased diuresis thus the median lethal dose (LD50) was determined to be higher than the dose tested i.e. 2.0 g/ kg b.w.

INHIBITION OF HISTAMINE AND SEROTONIN-INDUCED RAT PAW EDEMA

The BCM extract (125, 250 and 500 mg/kg) significantly ($p<0.01$) and dose-dependently inhibited histamine-induced rat paw edema (19.82, 30.23 and 46.33 %, respectively) and cyproheptadine (55.08 %) produced significant ($p<0.01$) inhibition of histamine-induced rat paw edema when compared with control group after 1 h of histamine injection (Table 1). Also the BCM extract (125, 250 and 500 mg/kg) dose-dependently and significantly ($p<0.01$) inhibited serotonin-induced rat paw oedema. BCM at dose of 500 mg/kg exhibited maximum inhibition (41.64 %) and cyproheptadine produced inhibition of 51.48 % in serotonin-induced rat paw edema after 1 h of the serotonin injection (Table 1).

Figure 1

Table 1: Effect of methanol extract of leaves on histamine and serotonin induced rat paw edema

Treatment (s)	Dose (mg/kg)	Percentage increase in swelling	Percentage of inhibition
Histamine control	Vehicle	51.16 ± 1.72	------
Cyproheptadine (standard)	10	22.97 ± 1.67**	55.08
Methanol extract of *Barleria Cristata* leaves	125	40.81 ± 1.21*	19.82
	250	35.69 ± 1.85**	30.23
	500	27.45 ± 1.07**	46.33
Serotonin control	Vehicle	46.15 ± 2.02	-----
Cyproheptadine (standard)	10	22.39 ± 1.95**	51.48
Methanol extract of *Barleria Cristata* leaves	125	38.36 ± 1.74*	16.88
	250	31.27 ± 1.48**	32.24
	500	26.39 ± 1.15**	41.64

Each value represents the mean ± S.E.M., $n=6$.
* $p<0.05$
** $p<0.01$ compared with control, Dunnett's t-test after analysis of variance.

INHIBITION OF ACETIC ACID-INDUCED VASCULAR PERMEABILITY IN MICE

Effect of BCM extract (125, 250 and 500 mg/kg), indomethacin (10 mg/kg) and control vehicle on acetic acid-induced increased vascular permeability in mice was studied. Results of the activity showed that BCM at dose (125 and 250mg/kg) moderately inhibited the vascular permeability (23.73% and 42.19% respectively), whereas BCM at a dose of 500 mg/kg and indomethacin 10 mg/kg significantly ($p<0.01$) inhibited vascular permeability (63.39% and 68.45% respectively) when compared with vehicle control group (Table 2).

Figure 2

Table 2: Effect of methanol extract of leaves on acetic acid-induced vascular permeability in mice

Treatment (s)	Dose (mg/kg)	Absorption at 590 nm	% Inhibition
Control	Vehicle	0.158	-------
Methanol extract of *Barleria Cristata* leaves	125	0.120 ± 0.31*	23.73
	250	0.091 ± 0.78**	42.19
	500	0.057 ± 0.24**	63.39
Indomethacin	10	0.049 ± 0.75**	68.45

represents the mean ± S.E.M., n=6.
* p<0.05
** p<0.01 compared with control, Dunnett's t-test after analysis of variance.

MEMBRANE STABILIZING ACTIVITY

In the study of membrane stabilization activity the BCM extract at concentration range of 0.60-1.0 mg/ml protect significantly (p<0.01) the erythrocyte membrane against lysis induced by hypotonic solution. Also indomethacin (0.10 mg/ml) offered a significant (p<0.01) protection of the RBC's against the damaging effect of hypotonic solution. At a concentration of 1 mg/ml, the BCM extract showed 91.36 % where as indomethacin at 0.1 mg/ml showed 54.79 % inhibition of RBC haemolysis when compared with blank (Table 3).

Figure 3

Table 3: Effect of methanol extract of leaves on erythrocyte haemolysis

Sample (s)	Concentration	Absorption at 540 nm	% Inhibition of haemolysis
Hypotonic medium	50 mM	0.730 ± 0.06	----------
Methanol extract of *Barleria Cristata* leaves	0.2 mg/ ml	0.480 ± 0.5	34.24
	0.4 mg/ ml	0.384 ± 0.37*	47.39
	0.6 mg/ ml	0.265 ± 0.16**	63.69
	0.8 mg/ ml	0.224 ± 0.29**	69.31
	1 mg/ ml	0.063 ± 0.41**	91.36
Indomethacin	0.1 mg/ ml	0.330 ± 0.24**	54.79

Each value represents the mean ± S.E.M, n=3
* p<0.05.
** p<0.01 compared with blank, Dunnett's t-test after analysis of variance.

INHIBITION OF PROTEIN DENATURATION

The inhibitory effect of different concentrations of BCM on protein denaturation is shown in Table 4. BCM extract (50-250 µg/ml) showed significant (p<0.05) inhibition of denaturation of egg albumin in concentration dependent manner. BCM extract at concentration of 250 µg/ml and indomethacin at concentration of 100 µg/ml showed significant (p<0.01) inhibition (61.24% and 82.67% respectively) of protein denaturation when compared with control.

Figure 4

Table 4: Effect of methanol extract of leaves on protein denaturation

Sample (s)	Concentration	% Inhibition of protein denaturation
Vehicle control	Vehicle	-------
Methanol extract of *Barleria Cristata* leaves	50 µg/ ml	24.65
	100 µg/ ml	31.25
	150 µg/ ml	45.65
	200 µg/ ml	56.42
	250 µg/ ml	61.24
Indomethacin	100 µg/ ml	82.67

Each value represents the mean ± S.E.M., n=3.

DISCUSSION

Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair [10].

The early phase of inflammation mainly mediated by histamine and serotonin, its significant inhibition by BCM extract revealed that the anti-inflammatory activity is possibly backed by its anti-histamine and anti-serotonin activity.

Increased vascular permeability occurs as a result of contraction and separation of endothelial cells at their boundaries to expose the basement membrane, which is freely permeable to plasma proteins and fluid [11]. Histamine and other mediators of inflammation increases vascular permeability at various times after injury. Chemical induced vascular permeability (acetic acid) causes an immediate sustained reaction that is prolonged over 24 h [12] and its inhibition suggests that the BCM extract may effectively suppress the exudative phase of acute inflammation.

The vitality of cells depends on the integrity of their membrane, exposure of RBC's to injurious substances such

as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin [13 14]. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical induced lipid peroxidation [13 14]. It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances [15 16 17 18]. Compounds with membrane-stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators [19]. BCM has shown significant ($p<0.01$) membrane stabilizing property, which suggests that its anti-inflammatory activity observed in this study, may be related to the inhibition of the release of phospholipases that trigger the formation of inflammatory mediators.

Denaturation of proteins is a well documented cause of inflammation and rheumatoid arthritis [8]. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation [20]. Ability of BCM extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity.

The anti-inflammatory activity of BCM extract found may be due to the presence of therapeutically active flavonoids i.e. apigenin, quercetin and quercetin-3-O-β-D-glucoside, naringenin and apigenin glucuronide and which was predicted. The therapeutic applications of flavonoids on inflammation have previously been reported [21 22].

The data obtained from the present study indicated that several factors may contribute to the anti-inflammatory action of BCM. Firstly, BCM significantly inhibited histamine and serotonin induced rat paw edema showing it's anti-histaminic and ant-serotonin ability. Secondly, BCM reduced the increased peritoneal vascular permeability in mice, indicating the suppression of the vascular response in the process of acute inflammation. Finally, BCM exhibited significant membrane-stabilizing property and inhibition of protein denaturation

The data of our studies suggests that BCM showed significant anti-inflammatory activity in both the in vivo and in vitro methods tested. Further studies involving the purification of the chemical constituents of the plant and the investigations in the biochemical pathways may result in the development of a potent anti-inflammatory agent with a low toxicity and better therapeutic index.

ACKNOWLEDGEMENT

This work was supported by grants from University Grants Commission, India. The authors gratefully acknowledge for financial assistance and fellowship from University Grants Commission, India.

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