

ANTI-INFLAMMATORY STUDIES IN RELATION TO CHEMICAL EVALUATION ON TWO SPECIES OF AN INDIGENOUS DRUG 'BARLERIA'

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Received: 07 Oct 2014 Revised and Accepted: 08 Nov 2014

ABSTRACT

Objective: The present study was aimed to assess the anti-inflammatory activity profile of *Barleria* spp. viz., *B. prionitis* Linn. and *B. cristata* Linn. against different models of inflammation in female rats.

Methods: Seven different extracts viz., mother extract (methanolic, ME, maceration), hexane (HE), chloroform (CE), ethyl acetate (EAE), butanol (BE) and left aqueous (LAE) extract obtained after partitioning of ME and total aqueous extract (TAE, maceration) of each *Barleria* spp. were evaluated chemically and biologically. The LD₅₀ of ME was found to be more than 2000 mg/kg p.o. Hence, the resultant extracts were evaluated at 100, 200 and 400 mg/kg p.o. dose against different inflammogens.

Results: Both *Barleria* species showed anti-inflammatory results with matching chemical profile. *B. prionitis* with more intense spots of major compounds exhibited maximum protection at 400 mg/kg for methanolic extract with per cent protection of 73.83 (3 h) in the carrageenan model and 42.04 (wet basis) in cotton pellet induced inflammation, respectively. A promising activity against histamine and dextran induced inflammation was shown by methanolic extract of *B. prionitis* at 200 mg/kg dose.

Conclusion: Owing to the present findings it can be concluded that the iridoid enriched extracts might collectively be responsible for its anti-inflammatory activity.

Keywords: *Barleria*, Anti-inflammatory, Acute and Sub-acute models.

INTRODUCTION

Inflammation, a defence reaction of the body to eliminate or limit the spread of an injurious agent, is characterized by oedema formation, leukocyte infiltration and granuloma formation [1-3]. The cardinal signs of inflammation are rubor (redness), tumor (swelling), calor (heat), dolor (pain) and loss of function (function laesa) [4]. Inflammation is caused by the release of pro-inflammatory mediators like prostaglandins, leukotrienes, nitric oxide, adhesion molecules etc through induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX), [1, 5-7] which further mediates the initiation, progression and persistence of acute and chronic inflammatory pathological conditions [8]. A large number of plants are mentioned in Ayurveda for the treatment of inflammatory disorders [9]. One of the reputed plants of Ayurveda which enjoy high status for its versatile use in several ailments including inflammation is *Barleria*. The genus *Barleria* of family Acanthaceae comprises of small shrubs or undershrubs, which are distributed in warmer parts of the world. It has about 300 species, of which nearly 32 species are reported to occur in India and the important medicinal species are *B. buxifolia* Linn., *B. courtallica* Nees., *B. cristata* Linn.

B. longifolia Linn., *B. prionitis* Linn., *B. lupulina* Lindl. and *B. strigosa* Willd [10-14]. The well documented traditional uses of *Barleria* employs whole plant for various ailments like catarrhal affections of children which are accompanied by fever and much phlegm, leaves to relieve toothache, roots for boils and glandular swellings, bark for cough and anasara and decoction of the plant in dropsy and as an anti-inflammatory. Some of the important Ayurvedic formulations of *Barleria* are Sahacaradi taila, Nilikadya taila, Astavarga kvathaurna

and Rasnarandadi kvathaurna [15-18]. The genus *Barleria* has gained importance in recent years for the treatment of various diseases such as liver disorders, diabetes, neurological disorders, immunodeficiency, inflammation, ulcers, HSV-2 viral diseases etc. because of its safety, efficacy and cost effectiveness [16-28]. Few reports are available on the acute [29-32] and sub-acute [30] anti-inflammatory study using different plant parts viz., flowers and roots and only one report comprises of preliminary acute anti-inflammatory study of four fractions of the whole plant of *B. prionitis* using various inflammogens at a single dose of 100 mg/kg [33]. In other reports, the methanolic extract of only leaves of *B. cristata* was subjected to *in vivo* and *in vitro* anti-inflammatory studies [34-36]. In Ayurveda, the whole plant of *Barleria* is used for the treatment of various inflammatory disorders. Hence in the present study, the anti-inflammatory evaluation of multiple extracts (seven in number) at safe multiple doses for the two most common species of *Barleria* was done. Further the study employed different inflammogens with an aim to explore the mechanism of action and also to validate the traditional claims of the plant which is being used against inflammation. The study also included generation of a comparative chemical profile to establish correlation (if any) with the activity profile which will help the herbal industry for the selection of a suitable species.

MATERIAL AND METHODS

Plant material

The plant material of *B. prionitis* Linn., and *B. cristata* Linn. was collected during August to September 2008 from the Medicinal Plants Garden of University Institute of Pharmaceutical Sciences, Panjab

University, Chandigarh. The authenticity of the samples was duly confirmed by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (Ref. NISCAIR/RHMD/Consult/-2008-09/1127/158) and voucher specimens of the same have been deposited in the Museum-cum-Herbarium of University Institute of Pharmaceutical Sciences-Centre of Advanced Study, Panjab University, Chandigarh, India, under the voucher numbers 1460 and 1461 for *B. prionitis* Linn. and *B. cristata* Linn. Respectively.

Preparation of extract and fractionation

Shade dried whole plant material (600 g) of *B. prionitis* was coarsely powdered and extracted thrice with methanol (750 ml) each time for 48 h at room temperature. The extract was filtered and the solvent was removed under vacuum to obtain a viscous residue (59 g). The total methanolic extract, (ME-52 g) was suspended separately in 200 ml of water and extracted successively with solvents of increasing polarity to obtain respective partitioned fractions viz., hexane soluble (HE-8 g), chloroform soluble (CE-7 g), ethyl acetate soluble (EAE-8 g), butanol soluble (BE-9 g) and left aqueous extract (LAE-17 g). The total aqueous extracts (TAE) was also prepared by maceration technique and dried by lyophilization using freeze dryer to get 9 g of TAE. The extracts of *B. cristata* were prepared in similar fashion with 650 g as the starting material and the quantities obtained were as ME (49 g), HE (6 g), CE (7 g), EAE (7 g), BE (9 g), LAE (12 g) and TAE (7 g). Further extracts were prepared as and when more quantities were required. The acute toxicity was evaluated for mother extract (methanolic) of *B. prionitis* while all extracts were subjected to acute and sub-acute anti-inflammatory activity using different inflammogens and also for generating comparative TLC chemical profiling.

Phytochemical screening

The genus *Barleria* was screened for alkaloids, tannins, flavonoids, sterols, triterpenoids, anthraquinones, iridoids and carbohydrates by giving treatment with different chemical reagents using standard and well known methods (Table 1).

Chemical profiling

Different extracts of both the species as prepared above were subjected to comparative TLC fingerprint profiling using precoated silica gel G aluminium plates 60F-254 (20 cm × 10 cm, 0.2 mm thickness; Cat.no.1.05554.0007, E. Merck, Darmstadt Germany). Plates were developed in solvent system (ethyl acetate:methanol:formic acid::7.5:2.0:0.5) up to a distance of 8 cm and were visualized under UV light.

Animals

Female Sprague-Dawley rats (150-180 g), bred in the central animal house of Panjab University, were used in the study. Animals were housed under standard conditions (25 ± 2° C, 60-70 % relative humidity and 12 h photoperiod) and were maintained on standard rodent pellet diet (Ashirwad India Ltd, Chandigarh) and water *ad libitum*. The rats were acclimatized to laboratory conditions for 3 days before commencement of the experiment. All experimental procedures were reviewed and approved by Institutional Animal Ethics Committee (IAEC), vide ref. no. CAH/09/50 dated 04.06.2009 of Panjab University, Chandigarh.

Acute toxicity study

Acute toxicity of the methanolic extract. was performed using Sprague-Dawley rats (n=5). A 2000 mg/kg dose was selected as the test dose. One animal was dosed at the test dose and as the animal survived, four additional animals were dosed sequentially so that a total of five animals are tested. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days. Individual weights of animals were

determined shortly before the test substance was administered and weekly thereafter. All animals were subjected to gross necropsy after a period of two weeks. The *p.o.* LD₅₀ was found to be more than 2000 mg/kg, with no signs of abnormalities or any mortality observed for 14 days after single dose administration. Acute oral toxicity of methanol extract was performed using Sprague-Dawley rats (n=5) following OECD 425 guidelines (Up and Down Procedure) [37].

Treatment

Different extracts were evaluated at three dose levels viz., 100, 200 and 400 mg/kg (*p.o.*) using ibuprofen (50 mg/kg, *p.o.*) as a standard drug. All doses were freshly prepared in 2%, v/v tween 80 in normal saline (0.9%, w/v). The control group received only vehicle. The oedema was measured using plethysmographic recordings of paw volume (Volume differential meter, Model 7140, Ugo Basile, Italy) of the experimental rats.

Acute anti-inflammatory study

Carrageenan induced rat paw oedema

Acute oedema was induced by sub plantar administration of 0.1 ml of freshly prepared (1%, w/v) carrageenan (Sigma Aldrich, USA) suspension in normal saline. The study was done for 24 h and the paw volume was measured at pre determined intervals of 0, 1, 3, 6, 9 and 24 h.

Histamine induced rat paw oedema

Oedema in left hind paw was induced by the sub plantar injection of 0.1 ml freshly prepared 0.1 %, w/v histamine (Sigma Aldrich, USA) in normal saline. The oedema was quantified before and 1 h after the histamine injection.

Dextran induced rat paw oedema

Acute oedema in left hind paw was induced by the sub plantar injection of 0.1 ml freshly prepared 6.0 %, w/v dextran (Sigma Aldrich, USA) in normal saline. The paw volume was measured before and 1 h after the phlogistic administration.

Different doses of the test and reference drug were given *per orally* 1 h before the carrageenan, histamine and dextran administration.

Sub-acute anti-inflammatory study

Cotton pellet induced granuloma in rats

Foreign body granulomas can be provoked by subcutaneous implantation of sterilized compressed raw cotton pellets (20 ± 1 mg) in the right side of shaved and disinfected (70 % ethanol) scapular region of rats anaesthetized with ethyl ether. The animals were treated with the test sample, vehicle and standard drug for 7 consecutive days orally. On day 8, the animals were sacrificed and the pellets were removed and wet weight of cotton pellet was determined. Further, wet cotton pellets were dried at 60° C for 18 h in a hot air oven and dry weight of granuloma was also determined.

Statistical analysis

All the results were expressed as mean ± SEM. Data was analysed using one-way Anova followed by Dunnett Test as post hoc analysis. The *p* value of < 0.05 was considered as the criteria of statistically significant values.

RESULTS AND DISCUSSION

The phytochemical screening showed the presence of flavonoids, steroids, terpenoids, anthraquinones, and iridoids in methanolic extract of both the species. Further details are given in Table 1.

Acute oral toxicity

Administration of the methanolic extract of *B. prionitis* did not cause any behavioral, toxic symptoms and mortality at a dose of 2000 mg/kg

p.o. Therefore, 100, 200 and 400 mg/kg were selected as safe doses for investigating the anti-inflammatory activity.

TLC studies

The TLC fingerprint profiles developed for *Barleria* were visualized under 366 nm before derivatization (Plate 1 and 3) and visible light after derivatization with 0.5 % anisaldehyde-sulphuric acid reagent spray followed by heating (Plate 2 and 3). The number, nature and intensity of spots corresponding to different components varied from non-polar to polar extracts.

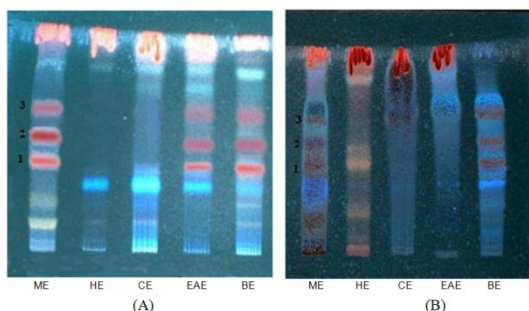


Plate 1: It shows TLC chromatogram of different extracts of *B. prionitis* (A) and *B. cristata* (B) at 366 nm before derivatization where ME: Methanol extract, HE: Hexane extract, CE: Chloroform extract, EAE: Ethyl acetate extract, BE: Butanol extract, LAE: Left aqueous extract and 1, 2 and 3 are the three targetted markers

The chemical profile of the different extracts showed three major red coloured spots at Rf 0.37, 0.47 and 0.60 for *B. prionitis* and 0.55, 0.65 and 0.75 in methanol extract of *B. cristata*. All the three major spots (1-3) were seen in methanol, butanol and ethyl acetate extracts, the spots 1 and 2 in total aqueous extract and none of these were present in hexane and chloroform extract of

B. prionitis. Similar results were observed with TLC fingerprint profiles of methanol and butanol extracts of *B. cristata*. In total aqueous extract, only spot 1 was seen and none of the three components was observed in hexane, chloroform and ethyl acetate extracts. Moreover, the intensity of these spots which is an indication of their quantity in the plant, was much less in *B. cristata* than *B. prionitis*. Further, a correlation was drawn between chemical and activity profiling after carrying out anti-inflammatory studies in different models using different inflammogens. Inflammation is a body's response to disturbed homeostasis caused by infection, trauma penetrating injury, blunt trauma, thermal injury, chemical injury, and immunologically mediated injury (humoral or cellular) and as a result of the loss of blood supply (ischemia). An increase in vascular permeability and cellular infiltration leading to oedema formation, exudation of fluid and

proteins and accumulation of leukocytes at the inflammatory site are characteristic features of acute inflammatory response. Histamine, serotonin, bradykinin, prostaglandins (PG), nitric oxide (NO) are some of the several mediators involved in increased vascular permeability and inflammation. Carrageenan-induced rat paw oedema is a widely used test to determine the anti-inflammatory activity and it has been well established that 1% carrageenan solution induces a marked powerful acute oedema with a biphasic response (early phase and late phase) and displays all the noticeable biochemical and cellular features. Other mediators like histamine (0.1 %), dextran (6.0 %), bradykinin, leukotrienes etc shows immediate transient response of short life of 15-30 min and shows endothelial cell contraction which further leads to intercellular gaps in post capillary venules and is a reversible process [3,38].

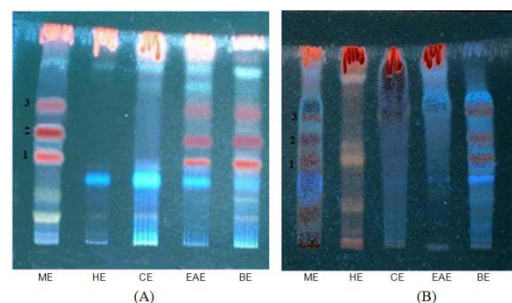


Plate 2: It shows TLC chromatogram of different extracts of *B. prionitis* (C) and *B. cristata* (D) under visible light after derivatization where ME: Methanol extract, HE: Hexane extract, CE: Chloroform extract, EAE: Ethyl acetate extract, BE: Butanol extract, LAE: Left aqueous extract and 1, 2 and 3 are the three targetted markers

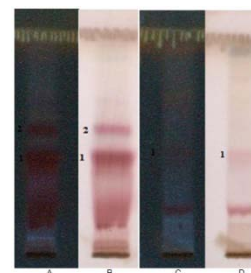


Plate 3: It shows TLC chromatogram of total aqueous extract (TAE) of *B. prionitis* - A at 366 nm before derivatization, B under visible light after derivatization and *B. cristata* - C at 366 nm before derivatization, D under visible light after derivatization and 1 and 2 are the targetted markers.

Table 1: It shows Phytochemical screening of different extracts of *B. prionitis* and *B. cristata*

Phytochemical group	<i>B. prionitis</i>						<i>B. cristata</i>							
	ME	HE	CE	EAE	BE	LAE	TAE	ME	HE	CE	EAE	BE	LAE	TAE
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	+	-	-	+	-	-	+	+	-	-	+	-	-	+
Steroids	+	+	++	-	-	-	-	+	+	++	-	-	-	-
Terpenoids	+	-	+	+	-	-	-	+	-	+	+	-	-	-
Anthraquinones	+	-	+	+	-	-	+	+	-	+	+	-	-	+
Carbohydrates	-	-	-	-	-	+	+	-	-	-	-	-	+	+
Iridoids	+++	-	-	+	+++	+	++	+++	-	-	-	+++	+	++

+ sign indicates the intensity of the colour and - indicates the absence; where ME: Methanol extract, HE: Hexane extract, CE: Chloroform extract, EAE: Ethyl acetate extract, BE: Butanol extract, LAE: Left aqueous extract, TAE: Total aqueous extract.

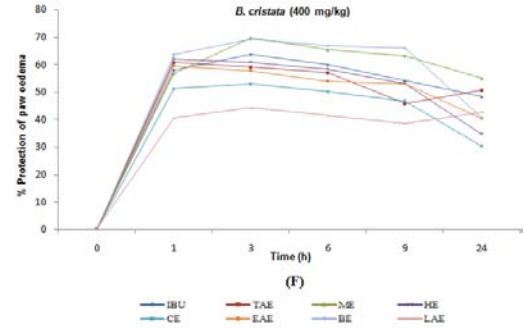
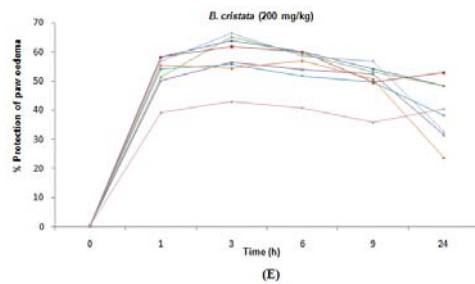
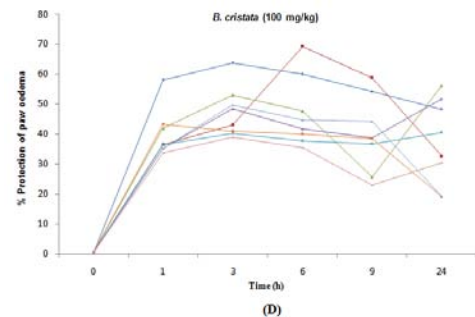
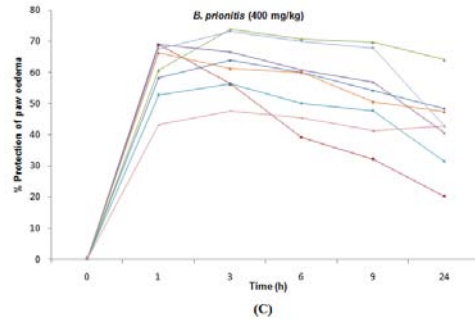
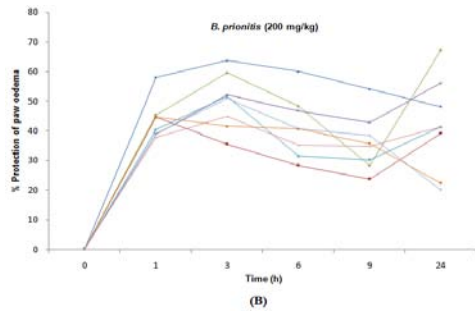
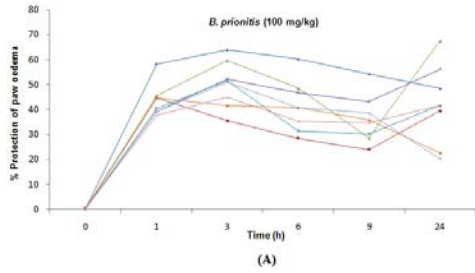


Fig. 1: A to F: It shows effect of various extracts of *B. prionitis* and *B. cristata* at various dose levels on carrageenan induced rat paw oedema.

IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract.

The % protection against paw oedema at all dose levels and time intervals is statistically significant at $p < 0.05$ except for LAE at 3, 6 and 9 h at 200 mg/kg for *B. prionitis* and BE and LAE at 3, 6 and 9 h at 200 mg/kg for *B. cristata*

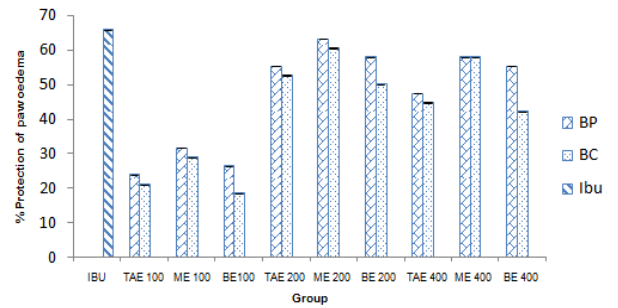


Fig. 2: It shows effect of different extracts of *B. prionitis* and *B. cristata* on histamine induced inflammation.

The % protection against paw oedema at all dose levels is statistically significant at $p < 0.05$

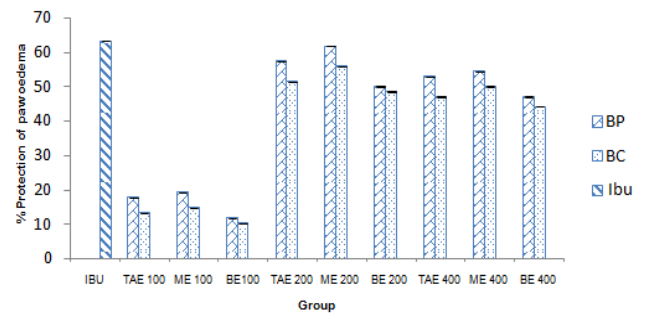


Fig. 3: It shows effect of different extracts of *B. prionitis* and *B. cristata* on dextran induced Inflammation

The % protection against paw oedema at all dose levels is statistically significant at $p < 0.05$

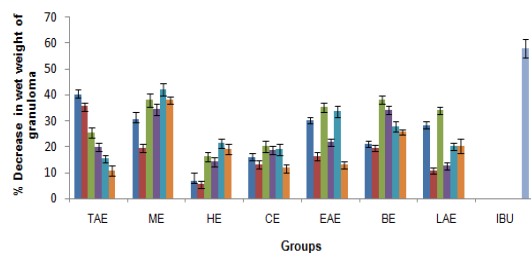


Fig. 4: It shows effect of various extracts of *B. prionitis* and *B. cristata* on cotton pellet induced wet granuloma

All doses in mg/kg. The % protection against wet granuloma formation of all extracts at different dose levels is statistically significant at $p < 0.05$

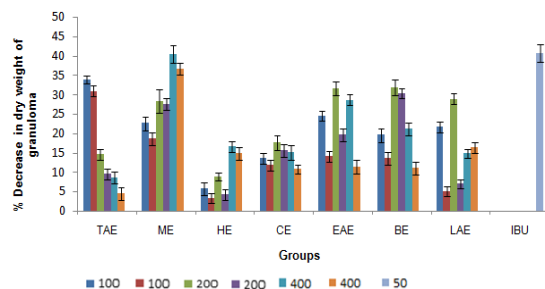


Fig. 5: It shows effect of various plant extracts of *B. prionitis* and *B. cristata* on cotton pellet induced dry granuloma

All doses in mg/kg. The % protection against dry granuloma formation of various extracts at different dose levels is statistically significant at $p < 0.05$ except for HE at 100, 200 mg/kg for BP and BC, TAE at 200, 400 mg/kg for BC, 400 mg/kg for BP and LAE at 100 and 200 mg/kg

In acute model of inflammation using carrageenan as an inflammogen, seven different extracts of each of the two *Barleria* species were screened at three dose levels of 100, 200 and 400 mg/kg. In case of *B. prionitis*, a definite and significant ($p < 0.05$) protection was observed with all extracts except for 200 mg/kg dose of left aqueous extract at 3, 6 and 9 h. The maximum activity during early phase of inflammation at 1h was shown by total aqueous extract of *B. prionitis* at 200 mg/kg dose with 72.97 per cent protection followed by ethyl acetate extract with 66.22 per cent protection at 400 mg/kg dose. Methanol, chloroform, butanol and left aqueous extracts of *B. prionitis* showed prominent response at 3 h. The best per cent protection of 73.83 was shown by 400 mg/kg dose of methanol extract, closely followed by butanol extract with 73.15 per cent reduction in oedema at 400 mg/kg dose. However, the maximum activity shown by chloroform extract was at 200 mg/kg with 64.43 per cent protection against carrageenan challenge and that of left aqueous extract at 400 mg/kg with 47.65 per cent inhibition of oedema. A gradual fall in protection of oedema was observed from 3 h onwards till 9 h. But interestingly, total aqueous, methanol and hexane extracts showed a reversal with augmented activity at 100 mg/kg at 24 h with per cent protection of 39.33, 67.42 and 56.18 respectively (Fig. 1).

A definite and significant ($p < 0.05$) protection was also observed for *B. cristata* where in it was statistically non-significant for butanol and left aqueous extract at 3, 6 and 9 h at 200 mg/kg. The maximum activity during the initial phase of inflammation was depicted by methanol

extract at 400 mg/kg with 69.80 per cent protection. The two extracts *viz.*, total aqueous at 100 mg/kg and butanol at 400 mg/kg exhibited nearly same per cent protection with promising anti-inflammatory activity. Total aqueous extract showed maximum activity at 6 h with 69.23 per cent protection at 100 mg/kg and butanol extract was found to be active at 400 mg/kg with 69.13 per cent inhibition of inflammation at 3 h. Further, both hexane and ethyl acetate extracts also showed promising results at 1 h with 62.16 and 59.45 per cent reduction in swelling respectively at 400 mg/kg dose. The chloroform and left aqueous extracts showed protection at 3 h with 55.70 (200 mg/kg) and 44.30 (400 mg/kg) per cent reduction in oedema at respective dose levels. Although both species of *Barleria* showed significant anti-inflammatory activity but the comparative overall activity profile of *B. cristata* was found to be less than *B. prionitis* (Fig. 1).

Thus, various extracts (especially methanolic, total aqueous and butanol at their respective dose levels) showed significant protective effect thereby indicating their ameliorating effect in carrageenan induced inflammation which may be due to the suppression of the various inflammatory mediators both during early and late phases. The methanol extract of *B. prionitis* and *B. cristata* also showed potent activity against mediators like histamine and dextran induced inflammation at 200 mg/kg. The maximum per cent protection was found to be 63.16 and 61.76 for *B. prionitis* against histamine and dextran and that of *B. cristata* was 60.53 and 55.88 per cent respectively (Fig. 2 and 3).

The cotton pellet-induced granuloma has been widely used to evaluate the transudative and proliferative components of inflammation and the wet and dried weight of the pellets correlates well with the amount of granulomatous tissue formed. In this sub-acute model, inflammation is induced due to implantation of the foreign body. Sub-acute inflammation is a reaction arising when acute response is insufficient to eliminate pro-inflammatory agents. Sub-acute inflammation induces a proliferation of fibroblasts and the infiltration of neutrophils and exudation and occurs by the development of proliferative cells. In sub-acute cotton pellet induced granuloma, total aqueous extract of both the species showed best activity at lower dose of 100 mg/kg and methanol and hexane extracts were active at high dose of 400 mg/kg whereas chloroform, ethyl acetate, butanol and left aqueous were active at 200 mg/kg

(Fig. 4 and 5). It is proposed that the active extracts (methanol, total aqueous and butanol) may have caused a decrease in granuloma tissue formation by inhibiting proliferation of fibroblasts and granulocyte infiltration.

A correlation of chemical profiling was made with the activity profile of the two *Barleria* species. It was observed that a higher protection against oedema formation with *B. prionitis* corresponded well with higher intensity of major components seen in methanol extract of the plant and as the content of these components varied in different extracts, there was a shift in activity. However, none of the three major spots were seen in hexane and chloroform extracts but these still exhibited protection of oedema and this probably could be because of the presence of some steroidal components like β -sitosterol (which is a known anti-inflammatory agent) present in non-polar extracts.

Thus the extracts were found to be active against the acute and sub-acute inflammogen induced oedema. The carrageenan oedema is inhibited by at least two mechanisms: inhibition of the primary mediators involved in the swelling or inhibition of the prostaglandin amplification mechanism [3,38]. The activity profile generated by different extracts showed that majority of the test samples were active at 3 h and afterwards the activity either decreased or was almost the same except in few cases where reversal of fall in activity was observed. According to the general release mechanism of various mediators, it is known that the cellular fluid is enriched in histamine, bradykinin and less of prostaglandins during the early phase (till 6 h).

Hence, it is proposed that the mechanism of inhibition of oedema in carrageenan induced inflammation could be related to inhibition of the primary mediators involved in the swelling or inhibition of the prostaglandin amplification mechanism. In cotton pellet induced oedema, it is proposed that the active extracts may have caused a decrease in granuloma tissue formation by inhibiting proliferation of fibroblasts and granulocyte infiltration possibly by attenuating iNOS activity.

CONCLUSION

The present study provides the anti-inflammatory profile of different extracts of whole plant of *B. prionitis* and *B. cristata* indicating their ameliorating effect in inflammation induced by different types of phlogistic substances. The activity profile generated by the active extracts matches with the TLC profile which clearly indicates that iridoid enriched extracts are highly active against inflammogens, thereby, indicating the active role of iridoids in reducing the inflammation. In acute and sub-acute models, the best activity was exhibited by methanol extract of *B. prionitis*. It was observed that anti-inflammatory response depicted by active extracts in terms of percent protection of paw oedema is in close proximity to the response depicted by the standard drug ibuprofen. Therefore, based on this justification the active extracts of both the species were further evaluated against different inflammogens and in sub-acute model to generate the mechanism of action. Both *B. prionitis* and *B. cristata* showed anti-inflammatory activity at various dose levels. Thus from the generated anti-inflammatory pattern we can conclude that the different extracts of *Barleria* showing their maximum activity during the early or late phase might be due to their inhibition of the release of different mediators involved in different types of acute and sub-acute studies. Thus the traditional use of *Barleria* mentioned in Ayurveda as an anti-inflammatory agent is justified by the present study. The data generated through in this work has provided the base to work further on the pure compounds of the plant by taking into consideration the protection shown by active extracts of *Barleria*.

CONFLICTS OF INTEREST

All authors have none to declare.

ACKNOWLEDGEMENT

One of the authors, Preet Kawal is grateful to University Grants Commission, New Delhi for the award of fellowship under Research Fellowship for Meritorious Students scheme.

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