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Research Article

Investigation of centrally and peripherally acting analgesic and anti-inflammatory activity of biological immune response modulator (an Amazonian plant extract) in animal models of pain and inflammation

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ABSTRACT

Background: Biological immune response modulator (BIRM) - An aqueous extract of dried roots of the species *dulcamara* (family Solanaceae) grown in Ecuador, considered as a natural remedy for various disease is promoted as a natural herbal medicine. Our aim of the study was to assess the central and peripheral analgesic and anti-inflammatory property of BIRM and to study its mechanism of action.

Methods: Peripheral analgesic and anti-inflammatory activity was evaluated using acetic acid induced writhing test and carrageenan paw edema test in male Swiss Albino mice (n=8 per group). Formalin test was taken up to evaluate BIRM's centrally, as well as peripheral antinociceptive action.

Results: We observed through our studies that BIRM when administered repeatedly for 7 days (4 ml/kg, p.o.) was able to exert its anti-nociceptive and anti-inflammatory activity through central and peripheral mechanism. BIRM was able to significantly inhibit both acetic acid induced writhes and carrageenan-induced paw edema indicating it's possible peripheral analgesic and anti-inflammatory action. BIRM was also able to inhibit both neurogenic and inflammatory pain in the formalin test indicating its action through central and peripheral nervous system.

Conclusion: Our study results show that BIRM has the potential anti-inflammatory property and is able to exert its anti-nociceptive effect through both central and peripheral mechanisms.

Keywords: Anti-inflammatory, Anti-nociceptive, Central analgesic, Peripheral analgesic, Biological immune response modulator

INTRODUCTION

In the era of new analgesics and non-steroidal antiinflammatory drugs (NSAIDs), plants still remain to be major possible source of new drugs and chemicals. They continue to be the source of lead structures for synthetic modifications and optimization of bioactivity. Due to severe side effects associated with available analgesics and NSAIDs, medicinal products derived from plants are preferred, and are becoming part of the integrative health care systems in industrialized nations. 1 A dramatic increase is seen in the number of patients opting for complementary and alternative medicine and consuming plant extracts from folklore medicine.2 Along with mechanism of action being broader than that of NSAIDs and analgesics, herbal medicinal products has lesser side effects. Even when exact mechanism of action of herbal medicinal products remains elusive, it is for sure that most of the herbal medicinal products exert their efficacy/potency through several pathways, which include inhibition of cyclooxygenase (COX) and/or lipoxygenase (LOX), inhibition of cytokine release, inhibition of elastase or hyaluronidase and may induce anti-oxidative activity.³ In line with the above hypothesis, herbal medicinal product of our choice, biological immune response modulator (BIRM) is thought to exert its potential efficacy through inhibition of COX in therapeutic area of pain and inflammation. Jäggi et al.⁴ have studied mother tincture of *Solanum dulcamara* - source of BIRM through *in-vitro* studies and found that it inhibits production of COX-1 and COX-2, but do not inhibit the production of leukotriene LTB₄ by 5-LOX.

Pain, as defined by The International Association for the Study of Pain Taxonomy, is an unpleasant sensory and emotional experience associated with actual or potential tissue damage.⁵ Pain in a way protects us from potential

injury. However, when the painful sensation persists after removal of the stimulus, it becomes mandatory to take steps towards the pain management.

BIRM is an oral solution extracted from Amazonian plant formulated by a physician (Edwin Cevallos). Based on the local folklore of the Ecuadorian native population, it is promoted as a natural herbal medicine in South America. BIRM is considered to be as a natural remedy for various diseases such as cancer, HIV-1-infection and so on.^{6,7} Dandekar et al.⁸ have shown through their *in-vitro* and *in-vivo* studies that BIRM have anti-proliferative property against prostate cancer cells. However, even though the COX inhibitory property of BIRM is known from sometime the efficacy of this drug in ameliorating pain is yet to be assessed hence, we decided to study BIRM in a systematic way in *in-vivo* models of pain and inflammation to evaluate its anti-nociceptive and anti-inflammatory properties.

METHODS

Animals and housing condition

Healthy male Swiss Albino mice (6-8 weeks old) weighing 25-35 g and Sprague-Dawley (SD) male rats (8-10 weeks old) weighing 200-230 g were procured from AAALAC approved vivarium facility of GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of 1-week prior to experiment initiation. Animals were group housed for their respective experiments in polypropylene cages under ambient conditions. Room temperature and humidity were maintained at 22-25°C and 65-70%, respectively. 12 hrs light/dark cycle was maintained. Standard laboratory rodent diet and potable drinking water were provided ad libitum. Experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) according to Committee for the purpose of Control and Supervision of Experiments of Animals (CPCSEA), India. All animal procedures were performed in accordance with guidelines of CPCSEA.

Test compound

BIRM was a gift from BIRM Inc. (Quito, Ecuador). It is an aqueous extract of dried roots of a plant of the species dulcamara (family Solanaceae) grown in Ecuador. It is marketed as a greenish-brown suspension with a mild bittersweet smell. The inactive ingredients in BIRM comprise 16% solid particles, likely root fibers and the remainder, a lipid-free liquid. For all the studies reported here, BIRM was clarified by centrifugation at 10,000 g prior to use. BIRM was administered orally for 7 days as a pretreatment in all the tests performed.

Diclofenac and gabapentin used as reference drugs were obtained commercially from Sigma-Aldrich Chemie GmbH.

Determination of peripheral analgesic activity

Acetic-acid induced writhing test

Test groups and dosing regimen

It was performed using male Swiss Albino mice. Total of 32 animals were used and divided into four groups (n=8 per group): Group I - Vehicle control (4 ml/kg, p.o., distilled water), Group II - BIRM (4 ml/kg, p.o., 7 days pretreatment), Group III - Diclofenac (20 mg/kg, p.o., single dose at 30 mins pre-treatment), and Group IV - BIRM + diclofenac (BIRM: 4 ml/kg, p.o., 7 days pre-treatment + diclofenac: 20 mg/kg, p.o., single dose at 30 mins pretreatment on day 7).

Test procedure

The test was carried out according to the method described by Koster et al. BIRM was administered orally through oral gavage needle for 7 days prior to acetic acid treatment. Diclofenac was administered orally at a dose level of 20 mg/kg as a single dose on the day of assessment (day 7). 30 mins later, acetic acid (0.6% v/v in distilled water, 10 ml/kg, intraperitoneal [i.p]) was administered to mice to induce the characteristic writhing. Animals were placed in a plexiglass box immediately post acetic acid administration and writhing response (abdominal constriction, trunk twisting, and extension of hind limbs) was counted for 20 mins and expressed as the pain response.

Carrageenan-induced paw edema test

Test groups and dosing regimen

This test was performed using male Swiss Albino mice. Total of 24 animals were divided into three groups (n=8 per group): Group I - Vehicle control (4 ml/kg, p.o., distilled water), Group II - BIRM (4 ml/kg, p.o., 7 days pre-treatment), and Group III - Diclofenac (20 mg/kg, p.o.; single dose at 30 mins pre-treatment).

Test procedure

Paw edema was induced in male Swiss Albino mice by injection of 100 µl of 1% carrageenan diluted in saline in the plantar surface of left hind footpad. 10 In a similar manner, $100 \,\mu l$ of 0.9% saline solution was administered in the plantar surface of right hind footpad to serve as a control reference for the tested paw. The paw volume was measured through water displacement method using water plethysmometer (LE 7500, Panlab SI) immediately before intraplatar injection of carrageenan and at 2, 3, 4, and 5 hrs thereafter. Each paw was marked at the lateral malleolus in order to emerge it always at the same extent in the measurement chamber. The assessment of paw volume was performed in a blind fashion. The change in paw volume was calculated by subtracting the initial paw volume of left hind paw (basal) from the paw volume of left hind foot measured at each time point. The percentage inhibition of paw edema was calculated by using the following formula:11

Percentage of edema inhibition = $(Vc-Vt/Vc) \times 100$

Vc = Volume of paw edema in the control group,

Vt = Volume of paw edema in the treated group.

Dissociation between central nervous system (CNS) and peripheral analysesic activity

Formalin-induced paw licking test

Test groups and dosing regimen

This test was performed using male SD rats. Total of 15 male SD rats were selected for the study and were divided into three groups (n=5): Group I - Vehicle control, Group II - BIRM (4 ml/kg, 7 days, p.o.), and Group III - Gabapentin (50 mg/kg, single dose, i.p. on day 7). 12

Test procedure

On day 7, animals were administered with formalin (50 μ l of 2.5% concentration) ¹³ subcutaneously into the plantar surface of the rat left hind paw using a 27-gauge needle. Prior to formalin administration, animals were acclimatized in an open plexiglass chamber for 30 mins.

Post formalin administration, animals were returned back to the observation chamber (open plexiglass chamber) with a mirror angled at 45° positioned behind to allow an unobstructed view of the paws. The frequency of formalininduced behavior in terms of frequency of pain response (it includes paw lifting, flinching, biting, and licking) was recorded continuously for 60 mins (Phase 1: 0-10 mins, Phase 2: 11-60 mins).

Statistical analysis

Results were expressed as mean ± standard error of the mean of the pain response measured. Data were analyzed using Graphpad Prism (version 4.1). One-way ANOVA followed by Tukey's multiple comparison test was used to analyze data generated from acetic acid induced writhing assay and formalin test. For carrageenan induced paw edema, two-way repeated measures ANOVA was used followed by Bonferroni's post-test. p<0.05 was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the Figures 1-3.

RESULTS

Acetic acid induced writhing test

Intraperitoneal injection of 0.6% acetic acid to animals caused an average of 57 writhes in a 20 mins interval. The treatment with BIRM alone, repeatedly for 7 days could reduce (41%) the writhing response significantly (p<0.001) as compared to vehicle control. However, BIRM when administered as combination therapy with standard analgesic diclofenac,

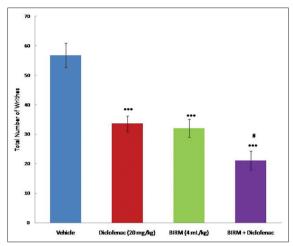


Figure 1: Effect of repeated administration of biological immune response modulator (4 ml/kg, 7 days p.o.) on nociception induced by acetic acid in writhing test as a standalone and in combination with standard drug, diclofenac (20 mg/kg, single dose p.o.).

***p<0.001 as compared to vehicle control group, #p<0.05 as compared to diclofenac group. Data was analyzed using ANOVA followed by Tukey's multiple comparison test.

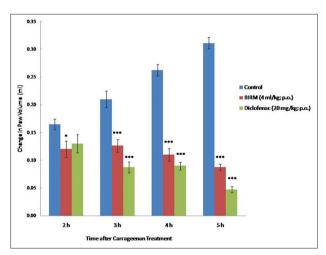


Figure 2: Effect of repeated administration of biological immune response modulator (4 ml/kg, p.o. for 7 days) on inflammation induced by intraplantar injection of carrageenan in hind foot pad of Swiss Albino mice. *p<0.05 and ***p<0.001 as compared to vehicle control. Data were analyzed using two-way repeated measure ANOVA followed by Bonferroni post-test.

could significantly reduce the occurrence of writhes (63%) as compared to vehicle control (p<0.001) (Table 1 and Figure 1).

Carrageenan induced paw edema test

The intra-plantar administration of carrageenan induced gradual increase in paw edema. Repeated oral treatment of BIRM (4 ml/kg) for 7 days significantly reduced the

carrageenan-induced paw edema at 2 hrs (p<0.05), 3 hrs, 4 hrs, and 5 hrs (p<0.001) post carrageenan treatment as compared to vehicle control. BIRM exhibited highest reduction of 71.89% in paw edema at 5 hrs post carrageenan treatment. As reported, diclofenac too showed a significant reduction in paw edema at 3, 4, and 5 hrs (p<0.001) post carrageenan treatment as compared to vehicle control (Table 2 and Figure 2).^{14,15}

Formalin-induced paw licking test

The repeated administration of BIRM orally for 7 days prior to formalin administration resulted in a significant (p<0.001)

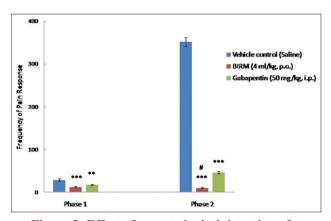


Figure 3: Effect of repeated administration of biological immune response modulator (4 ml/kg, p.o. for 7 days) on pain behavior induced by formalin in the formalin test. **p<0.01 and ***p<0.001 as compared to vehicle control.*p<0.01 as compared to gabapentin. ANOVA followed by Tukey's multiple comparison test.

Table 1: Effect of repeated administration of BIRM on nociception induced by acetic acid in writhing test.

| | • | |
|-----------------|-------------------------|-------------------|
| Group | Total number of writhes | Inhibition (%) |
| Vehicle control | 56.75±3.56 | - |
| Diclofenac | 33.63±2.25*** | 44 |
| BIRM | 32±3.06*** | 41 |
| BIRM+diclofenac | 21.13±2.78**** | 63 |

Data represented as mean±SEM. ***p<0.001 as compared to vehicle control; *p<0.05 as compared to diclofenac (ANOVA followed by Tukey's multiple comparison test), BIRM: Biological immune response modulator. SEM: Standard error of the mean

reduction in overall pain response, which includes frequency of flinching, biting, licking, and paw lifting in Phase 1 and Phase 2 as compared to vehicle control. Gabapentin, when administered as a single i.p. dose produced significant reduction in pain response in Phase 1 (p<0.01) and Phase 2 (p<0.001) as compared to vehicle control but to a lesser extent as compared to BIRM in Phase 2 (Table 3 and Figure 3). This observation with respect to gabapentin is in line with the reported data where Phase 2 is concerned. 12,16

DISCUSSION

In the current study, in order to ascertain BIRM's peripheral analysesic activity and its anti-inflammatory property we employed the acetic acid-induced writhing test and carrageenan-induced paw edema test, respectively. Moreover, formalin-induced paw licking test was used to confirm whether BIRM's anti-nociceptive property is mediated through central or peripheral nervous system.

Acetic acid-induced writhing test was mainly performed to assess the peripheral analgesic activity of the compound in question. In general, acetic acid causes spontaneous pain by secretion of endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins, and substance P Deraedt et al.¹⁷ have shown increased presence of PGE, in the peritoneal fluid post acetic acid administration. PGs along with local peritoneal receptors are thought to be responsible for abdominal constriction and activation and sensitization of the peripheral chemo-sensitive nociceptors 18-20 and causing inflammatory pain.²¹ BIRM significantly reduced the frequency of the writhing in mice subjected to i.p acetic acid administration, similar to the conventional NSAID diclofenac sodium. BIRM as standalone treatment was found to be equally efficacious as compared to standard diclofenac, but BIRM when administered as combination therapy along with diclofenac was found to be more effective in terms of inhibition of writhes as compared to BIRM or diclofenac alone.

Carrageenan induced inflammation model is used extensively in the development of NSAIDs and selective COX-2 inhibitors and in assessing the contribution of mediators involved in vascular changes associated with acute inflammation.²² Carrageenan-induced paw edema test, commonly used as an experimental model for acute inflammation, is observed to be biphasic. Acute inflammation observed in both phases leads to leakage of plasma elements from blood vessels to

Table 2: Anti-inflammatory effect of repeated BIRM administration in Carrageenan-induced paw edema test.

| Group | Change in paw volume of left hind paw post-Carrageenan administration at | | | |
|-----------------------------|--|---------------|---------------|---------------|
| | 2 hrs | 3 hrs | 4 hrs | 5 hrs |
| Control | 0.17±0.009 | 0.21±0.015 | 0.26±0.010 | 0.31±0.010 |
| BIRM (4 ml/kg; p.o.) | 0.12±0.015* | 0.13±0.011*** | 0.11±0.011*** | 0.09±0.006*** |
| Diclofenac (20 mg/kg; p.o.) | 0.13±0.016 | 0.09±0.010*** | 0.09±0.007*** | 0.05±0.005*** |

Data represented as mean±SEM. *p<0.05, ***p<0.001 as compared to vehicle control; (two-way repeated measures ANOVA followed by Bonferroni's post-test), BIRM: Biological immune response modulator, SEM: Standard error of the mean

Table 3: Effect of BIRM on pain behavior in formalin-induced nociception assay.

| Group | Cumulative pain response observed post formalin administration | | |
|-----------------------------|--|----------------------|--|
| | Phase 1 (0-10 mins) | Phase 2 (11-60 mins) | |
| Vehicle control | 28.60±2.86 | 351.80±10.41 | |
| BIRM (4 ml/kg, p.o.) | 11.80±1.66*** | 10.00±1.38*** | |
| Gabapentin (50 mg/kg, i.p.) | 17.00±1.30** | 46.20±3.18*** | |

Data represented as mean±SEM. **p<0.01 and ***p<0.001 as compared to vehicle control. *p<0.01 as compared to gabapentin. ANOVA followed by Tukey's multiple comparison test, BIRM: Biological immune response modulator, SEM: Standard error of mean

the inflamed tissue, and the infiltration of neutrophils.^{23,24} Histamine, serotonin, bradykinin, PGs, hydrogen sulfide, and nitric oxide are some of the inflammatory mediators which play a role in this model.²² It was noticed in the current study that BIRM administration significantly reduced the carrageenan-induced paw edema at all-time points of the study. The observed reduction was comparable to the reference drug, diclofenac used in this study.

Edema observed in the first phase (mainly 1-6 hrs) of the carrageenan model is believed to be of little intensity as compared to the second phase (24-72 hrs) with more pronounced edema. ^{10,25} However, Posadas et al. ²⁵ have reported age and weight of mice as the critical issue while studying this model. They have clearly shown through their studies that biphasic edema and consistent inflammatory pattern in response to carrageenan was observed in first phase in animals of 7-8 weeks with weight range of 32-35 g as compared to their younger counterpart (4-5 weeks age with 18-20 g weight range). Hence, in the present study, our observation was limited up to the first phase only.

As we know, formalin test is capable of discriminating between neurogenic pain (early phase which is considered to be CNS modulated and non-inflammatory) and inflammatory pain (chronic and peripheral pain). The neurogenic pain caused due to direct chemical stimulation of nociceptive afferent fibers (predominantly C fibers) could be attenuated by opiates like morphine, ²⁶ whereas inflammatory pain caused by the sensitization of spinal cord mediated through activation of N-methyl-D-aspartate receptors and release of inflammatory mediators like histamine, PGs, bradykinin, serotonin in the peripheral tissue could be attenuated by opiates, NSAIDs, etc., ²⁷ Repeated treatment of BIRM was able to inhibit both neurogenic, as well as inflammatory pain significantly indicating it's centrally as wells as peripherally acting analgesic activity.

In the present study, it is observed that BIRM when administered orally to rats for 7 days prior to formalin administration, inhibits overall pain response like biting, paw licking, lifting, and flinching behavior. Contrary to many reported results, reference compound gabapentin used too showed unusual inhibition of pain response in Phase 1 as compared to vehicle control. 12,16 The reason for this change is not clear but as opined by few earlier authors, who too observed similar result, could be attributed to difference in methodologies such as species, strain, development stage, environmental stress, ambient temperature, and formalin injection site. 28,29

PGs, which are thought to play an important role in nociceptive transmission at peripheral sites and in the spinal cord³⁰⁻³² are synthesized in tissues by COX, an enzyme involved in the metabolism of arachidonic acid into PGs. COX-2 an isoform of COX is highly inducible in response to cytokines, growth factors or other inflammatory stimuli. 30 There are reports indicating that COX-2 inhibitors are effective in producing an anti-nociceptive effect in rat inflammatory pain models thus proving that COX-2 has a major role in nociceptive transmission in both the spinal cord and at the peripheral sites. 31,33 There are also several reports indicating COX-2 mediated increase in PGE, production in the CNS as the major player in inducing inflammatory pattern and pain response in carrageenan-induced paw edema model.^{34,35} It has been reported that administration of carrageenan in the paw leads to increased mRNA levels of COX-2 in the spinal cord and other regions of CNS, thus indicating its major role in induction of inflammation.³⁶ Several other studies by Seibert et al.; Ibuki et al.; Guay et al. also have shown elevated levels of COX-2 very early on (1-6 hrs) in paw tissues and in the CNS following carrageenan-induced inflammation.35,37,38 Diclofenac and other NSAIDs drugs such as indomethacin and celecoxib are found efficacious through their inhibitory action on COX-2. As seen in the case of all the three models, PG has a critical role in mediating pain and inflammation. As reported by Jäggi et al.,4 mother tincture of S. dulcamara - source of BIRM is able to inhibit the production of PGs through COX-1 and COX-2. This finding indicates that BIRM has potentials to inhibit COX-2 resulting in a reduction of PGE. the major mediator of inflammation and nociception, which subsequently leads to suppression of pain and inflammation.

CONCLUSION

We observed repeated administration of BIRM to be effective in attenuating pain and inflammation occurring due to CNS activation and peripheral inflammatory mediators, thus showcasing its anti-nociceptive and anti-inflammatory role as peripherally and centrally acting compound. Second, we observed that BIRM when administered in combination with conventional NSAIDs is found to be more efficacious in attenuating pain thus rendering BIRM to be used as standalone or in combination to conventional therapy. The anti-inflammatory and anti-nociceptive property exhibited by BIRM in above animal models could be attributed to

its inhibitory action on COX-2 and thereby inhibiting the production of PGE₂ - The major mediator of inflammation or BIRM having the possible ability of hindering the endogenous synthesis or release of inflammatory mediators such as PGs, histamine, serotonin, bradykinin, and leukotrienes. The latter mechanism of amelioration of pain by BRIM however, remains to be evaluated.

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Conflict of interest: None declared

Ethical approval: All experimental protocols were approved by the IAEC (Institutional Animal Ethics Committee) according to CPCSEA (Committee for the purpose of Control and Supervision of Experiments of Animals), India. All animal procedures were performed in accordance with guidelines of CPCSEA.

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