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Original Article

EVALUATION OF ANTI-ARTHRITIC POTENTIAL OF PARTITIONED EXTRACTS OF *BOUGAINVILLEA X BUTTIANA (VAR. ROSE)* HOLTTUM AND STANDL

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ABSTRACT

Objective: *Bougainvillea* is a natural source with potential for clinical use, and this plant is routinely employed in traditional Medicine in Mexico. This study planned to evaluate the effect of ethanolic extract partitioned of *Bougainvillea x buttiana* on acute and chronic inflammation.

Methods: The extract from *Bougainvillea x buttiana* partitioned originated two phases the aqueous (*BxbREaq)* and organic (*BxbREop*) phases were employed in anti-inflammatory activity. Acute inflammation was evaluated using the carrageenan model, whereas the chronic inflammation with anti-arthritic potential was explored with complete Freund´s adjuvant (CFA). Arthritis was caused by intradermal inoculation of CFA, and the extract was administered orally at different doses for 21 d. Paw oedema was determined at 7, 14 and 21 d, and serum from the mice were obtained to detect cytokine levels by ELISA and for biological assays.

Results: Phytochemistry studies revealed that these extracts contain alkaloids, carbohydrates, fatty acids, and tannins. The results demonstrated that these extracts significantly inhibited mouse paw oedema for acute and chronic inflammation in a dose-dependent manner. Additionally, *BxbREop* extracts markedly inhibited the production of TNF-α, IL-1β, and IL-6 and remarkably increased IL-10 in serum from mice with control or arthritic groups.

Conclusion: The combined results suggest that *BxbREop* extract shows a potent effect in mice against CFA-induced arthritis for its ability to inhibit paw oedema and arthritic symptoms.

Keywords: Arthritis, Complete Freund´s adjuvant, Anti-arthritis activity.

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INTRODUCTION

Rheumatoid arthritis (RA) is described as a chronic inflammatory systemic autoimmune disease characterized by progressive cartilage degradation with synovial inflammation, proliferation on the synovial linings and irreversible joint damage, and it can alter another body parts [1-4]. RA can cause joint destruction and the aggressive cartilage destruction, progressive bone erosions, swelling, difficulty moving, pain and elevated pro-inflammatory mediator's production [3, 4].

The drug therapies for rheumatism include a) steroids, b) non-steroidal anti-inflammatory drugs (NSAIDs), c) disease-modifying anti-rheumatic drugs (DMARDS) and d) immunosuppressive drugs. Unfortunately, although these drugs have been shown to improve the signs and symptoms, alter the natural history of the disease and improve the quality of life of patients, until now there is still no cure. These available therapies are associated with strong side effects [5-8]. The action mechanisms of these drugs are incompletely understood; they reduce joint swelling and pain, decrease acute phase markers, limit the progressive damage of joint, and improve function. The agents for the RA treatment, include the use of interleukin inhibitors, T cell co-stimulatory blockers, and B cell depletion molecules. Different pro-inflammatory mediators, such as reactive oxygen species, prostaglandins, leukotriene's and cytokines released by macrophages contribute to RA [9, 10]. For the treatment of chronic inflammation, it may be by regulating these mediators secreted by immune cells or by inhibiting enzymes such as cyclooxygenase and lipoxygenase [11, 12]. Currently, the objective of RA treatments is to reduce joint inflammation and pain, maximize joint function, and prevent joint destruction and deformity. The rheumatoid joint includes distinct pro-inflammatory cytokines like interleukin-1 (IL-1), IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, IL-23, tumor necrosis factor-alpha (TNF-α), granulocyte macrophage-colony stimulating factor, and monocyte chemoattractant protein-1. Regarding anti-inflammatory cytokines, they include IL-4, IL-10, IL-11, IL-13, and IL-18 [13]. The imbalance between pro-and anti-inflammatory cytokines, and during RA it causes joint destruction and the angiogenesis in the synovial membrane can contribute to disease progression, as can the production of inflammatory cells that infiltrate and destroy synovial tissue [14-17]. Pro-inflammatory cytokines such as IL-1β and TNF-α are the targets of the strategies used for to the treatment to decrease the signs and symptoms of RA without causing adverse effects [18-22]. There are distinct plants including *Bougainvillea* that has shown ability to reduce chronic inflammation of joints as is the case with RA and osteoarthritis [21-28].

This investigation was designed to check the effect of partitioned *Bougainvillea x buttiana* extract using an animal model of RA and assess the effect of the extract on pro-inflammatory cytokines by examining the TNF-α, IL-1β, and IL-6 levels.

MATERIALS AND METHODS

Chemicals and reagents

RPMI-1640 medium, dimethylformamide, fetal bovine serum (FBS), ethanol, and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Aldrich Chemical Co. (Toluca, Mexico). The monoclonal antibodies used in this assay were anti-mouse capture and biotin-labeled detection and the respective interleukins recombinants were obtained from DB Biosciences Pharmingen (EUA). All other reagents and solvents are of analytical grade.

Collection and identification of plant material

The plant material for *Bougainvillea x buttiana* was procured from Cuernavaca, Morelos, identified with the foil: 33870 in the Herbarium HUMO, CIByC (UAEM).

Preparation of extract

The whole plant material was cleaned, cut into small pieces, and shade dried. The plant material was pulverized in powder and extracted with ethanol by Soxhlet extraction. The solvent was evaporated in a rotary evaporator at 40-45 °C, and the extract obtained was kept on refrigeration at 4 °C. This ethanolic extract (*BxbRE)* was submitted to the bipartition process described in detailed in the patent application (IMPI 10033981514 28/12/2017). In brief, water 200 ml and ethyl acetate 100 ml (ratio of 2: 1). The extract was stirred vigorously for 1 h until dissolved in the immiscible solvent system. After agitation, the system was left to rest until a partitioned portion was obtained in the extract, obtaining two phases, the aqueous and organic phases *BxbREaq* and *BxbREop*, respectively. Each partitioned phase was separated, concentrated and reduced pressure in a rotary evaporator, and kept under refrigeration at 4 °C. Both phases were assayed with respect to the acute inflammation.

Qualitative phytochemical analysis

Phytochemical screening to detect the compounds in each phase purified and dried from an extract of *B. x buttiana* was performed by use of standard methods to identify different constituents including Wagner´s test for detecting alkaloids, Fehling´s test for carbohydrates, colorimetric method for fatty acids, and ferric chloride test for tannins [28].

Animals

BALB/c female mice with (15–20 g) were used for the anti-inflammatory study. The animals were maintained under basic laboratory conditions, feed and water were administered *ad libitum*. All experimental protocols were submitted and authorized by the Committee (CCUAL-FM-UAEM) with registration number 002/2016.

Acute inflammation with use of carrageenan-induced paw oedema

An oedema model was carried out with the carrageenan-induced paw being utilized to assess the acute anti-inflammatory potential [29]. Experimental animals were randomly separated within different groups of six animals. Before treatment, the volume of the right hind paw of each animal was measured by plethysmometer as the initial paw volume (V_0). Control group was injected with 0.9% saline, or standard drug (Indomethacin at 10 mg/kg) or *BxbREaq or BxbREop* extracts (at 40 mg/kg) was given orally to the animals. Thirty minutes after injection of different treatments, each mouse received 1% carrageenan in its right hind paw, and its volume oedema was evaluated at 1st, 2nd, 3rd, 4th, 5th and 6th h after carrageenan injection (Vt). The percent inhibition in oedema volume was obtained by the formula:

$$\% \text{ inhibition of oedema} = \left[1-\left(\frac{v_t\text{-}v_0 \text{ treated}}{v_t\text{-}v_0 \text{ control}}\right) x\ 100\right]$$

Acute toxicity studies

Groups of animals 15–20 g maintained under basic laboratory conditions were used for toxicity testing as described by OECD–Guideline number 425 [30]. Groups of 6 mice were orally treated with aqueous and organic phases purified and dried from *B. x buttiana* extracts at one of four doses (5, 50, 500 and 2000 mg/kg). Animals were individually observed for the control of toxic symptoms such as locomotion, convulsions, and mortality for 72 h. All the observations of the different parameters were systematically recorded and maintained for each mouse individually. In mice injected with 500 and 2000 mg/kg remained under observation for 14 d for additional toxicity study.

Adjuvant-induced chronic arthritis

The intradermal injection of dead mycobacteria (*M. tuberculosis*) suspended in liquid paraffin oil to make 5 mg/ml (of Freund's adjuvant) in mice induced arthritis in the articulations in genetically predisposed animals [31]. Animals randomly selected weighing 15-20 g were separated into seven different groups of 6 animals. Table 1 shows the treatment schedule.

Table 1: Schedule of treatment

Group	Treatment
1	Normal control (saline solution)
2	Complete Freund´s adjuvant (arthritic control)
3	Complete Freund´s adjuvant+Indomethacin (10 mg/kg)
4	Complete Freund´s adjuvant+*BxbREop* 0.04 mg/mice
5	Complete Freund´s adjuvant+*BxbREop* 0.4 mg/mice
6	Complete Freund´s adjuvant+*BxbREop* 4 mg/mice
7	Complete Freund´s adjuvant+*BxbREop* 40 mg/mice

Dosing was started on day 1, 120 min before mouse immunization with heat-killed *M. tuberculosis* (10 mg/2 ml) in complete Freund´s adjuvant (CFA). The immunization dose used of 50 µl in the subplantar region of the right hind paw, and it was continued once a day for 21 d. The volume of both hind paws was assessed with volume differential after 0, 7, 14, and 21 d with the volume of the left paw taken as uninfected paw volume. Percent inhibition was calculated by the difference between right and left paw oedema by the formula:

$$\% \text{ inhibition} = \left[1-\left(\frac{T}{C}\right) x\ 100\right]$$

Where C = mean oedema in control group and T = oedema in the treated group.

Adjuvant-induced chronic arthritis

Animals injected as above described at the end of 0, 7th, 14th, and 21st day were anesthetized and the blood was collected from the retro-orbital plexus. The serum was separated from the rest of the blood using a high-speed centrifuge at 3500 rpm for 20 min and maintained at-20 °C until cytokine determinations.

Measurement of cytokines

The TNF presence was investigated using the standard assay with L929 cells [32]. The production of IL-1β, IL-6, and IL-10 was determined using ELISA in accordance with the manufacturer's instructions from DB Biosciences Pharmingen (EUA) [33].

Statistical analysis

Data were expressed as the mean±SD (n = 6). The repeated measure analysis of variance (ANOVA) followed Bonferroni´s test or Student´s t-test for independent samples. *P* values less than 0.05 ($P<0.05$) were considered significant.

RESULTS AND DISCUSSION

The anti-inflammatory drugs usually prescribed for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, are prescribed in the long run for proper control of the immune system. Therefore, it is a great need to develop secure and valid drugs for long-term use. Many investigations have demonstrated molecules collected from natural sources for the establishment of new treatments for clinical use [34]. In this study for studies of acute toxicity the mice were treated with 2000 mg/kg of *BxbREop* or *BxbREaq* did not show mortality or physical changes in skin, eyes, nasal respiratory rate, circulatory signs or autonomic effects. Since none of the described toxic signs or symptoms or mortality was observed in the animals at the above-mentioned dose, 0.04 up to 40 mg/kg body weight of extract were selected for evaluation of anti-inflammatory activity (data not shown).

The phytochemical qualitative analysis of both partitioned ethanolic extracts contains alkaloids, carbohydrates, fatty acids and tannins (table 2).

Table 2: Phytochemical screening of ethanolic extract

Phytochemical test for	Results	
	BxbREop	***BxbREaq***
Alkaloids	+++	++
Carbohydrates	+++	++
Fatty acids	+++	+
Tannins	++	+

Absent (−), present (+), moderate (++), and abundant (+++)

In different extracts, the major secondary metabolites in plants are the alkaloids and tannins with pronounced pharmacological activities [35, 36]. In *BxbREop* extract shows the different active constituents. Another component group present in plants is the phenolics, which include phenolic acids and tannins that possesses activities, such as anti-mutagenic, antimicrobial, antioxidant and anti-inflammatory [35-37]. Others bioactive agents present in medicinal plants belong to secondary metabolites such as terpenoids or flavonoids in nature are known to possess anti-inflammatory properties [35-37]. The increased activity with increased concentration may be because of the higher concentration of bioactive agents in different extracts.

For the study of acute inflammation mice, carrageenan paw oedema and mycobacterium-induced adjuvant arthritis of chronic inflammation are the model most used to detect the anti-inflammatory effects of many natural products [24]. The effect of the partitioned ethanolic extract on acute inflammatory was determined by the carrageenan-induced hind paw oedema model. We compared acute inflammation in animals orally injected with the aqueous phase and an organic phase (fig. 1). For all animals injected with *BxbREop* extract, the paw oedema volume was significantly smaller when compared to that observed in the control groups and *BxbREaq* extract (P<0.001). In groups injected with indomethacin, the paw oedema volume was similar that obtained for animals injected with *BxbREop* extract at 40 mg/kg. The anti-edematogenic response appeared in a dose-dependent manner (fig. 1).

In this study, the *BxbREop* extract was analyzed for changes in activity with dose variation, it was observed that the increased doses showed increased anti-inflammatory activity. For animal groups injected with *BxbREop* extract the mean of inhibition percentage was 38.33%, 48.33%, 55.26% and 60.50% for 0.04, 0.4, 4 and 40 mg/kg, respectively. In indomethacin groups, the mean of inhibition percent was 69.41% (table 3).

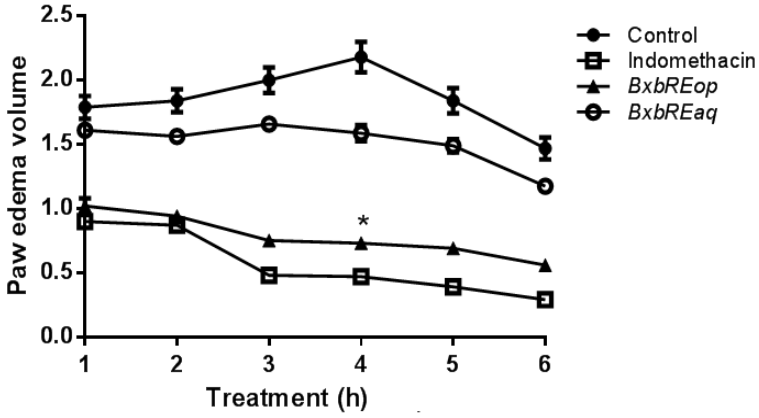

Fig. 1: Effect of *BxbREaq* and/or *BxbReop* extracts on carrageenan-induced hind paw oedema in mice, the animals were treated as above described. In the charts each point represents mean±SD. (n=6) * P<0.001

Table 3: Percentage inhibition of carrageenan-induced mice paw edema by *BxbREop* extract

Treatment	Time (h)						Mean of inhibition %
	1	2	3	4	5	6	
Indomethacin	50	53	76	78.5	79	80	69.41
BxbREop							
0.04 mg/kg	35	32	40	40	48	34	38.33
0.4 mg/kg	40	49	53	63	49	36	48.33
4 mg/kg	40	53	56	59	61	62	55.26
40 mg/kg	43	49	63	80	67	61	60.50

According to the results obtained in the acute inflammation assays, the *BxbREop* extract showed high effectiveness. The effect of *BxbREop* extract on chronic inflammation was determined using the adjuvant-induced arthritis model. The paw volume was measured for twenty-one days. In animal groups treated with *BxbREop* extract, a decrease was observed when compared to arthritic controls. Uninjected paws showed oedema formation at 7 d after mycobacteria immunization. However, the oedema volume in animals injected with *BxbREop* extracts lower than those obtained in arthritic controls. Overall, in treated groups, the inhibition of oedema was dose-dependent manner (fig. 2). The results obtained were found to be significant compared to arthritic controls at P<0.01. A similar reduction of paw volume was obtained after the treatment with indomethacin and/or *BxbREop* extract. For all mouse groups injected with different amounts of extract and/or indomethacin, the paw volume on the 21st day was significantly lower than those obtained in the control group (P<0.01).

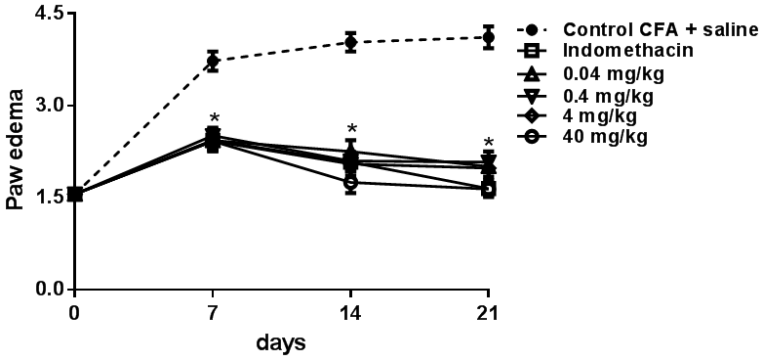

Fig. 2: Paw oedema female BALB/c mice weighing between 15–20 g were grouped and treated as above described. In the charts each point represents the results (mean±SD) from 6 mice. (n=6) * *P*<0.01

The paw oedema inhibition is shown in fig. 3. For the animal groups treated with 40 mg/kg of *BxbREop* extract, the percent inhibition was 17, 48 and 53% at 7, 14 and 21 d, respectively. In the group of animals treated with indomethacin, the mean percent inhibition was 15, 41 and 47% at 7, 14 and 21 d, respectively (fig. 3). The mean percent inhibition obtained in groups of animals treated with *BxbREop* extract and indomethacin was 38.38% and 32.76%, respectively.

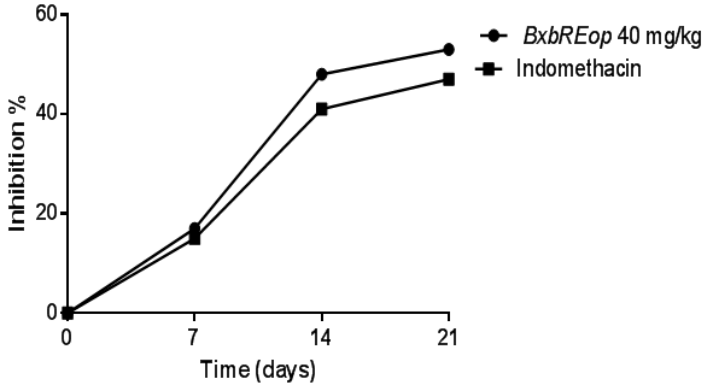

Fig. 3: Paw oedema inhibition percent, female BALB/c mice weighing 15–20 g were grouped and treated as above described. In the charts, each point represents the results (mean±SD) from 6 mice

The *BxbREop* extract has shown a more pronounced effect compared to indomethacin, which indicates the inhibition of chemical mediators of inflammation. In case of adjuvant arthritis, the immune response to mycobacterial antigens has been detected and is involved in the arthritis development [38]. In this study, to verify that the *BxbREop* extract has anti-arthritic activity, the observations in the interleukin production was recorded after the injection of CFA. All doses of *BxbREop* extract, 0.04, 0.4, 4 and 4 mg/kg, reduced the production of TNF-α (fig. 4). The production of TNF-α in the groups treated with *BxbREop* extract was significantly lower than those obtained in control group (*P*<0.01). In groups of mice treated with 40 mg/kg *BxbREop* extract and indomethacin, the TNF-α levels were decreased to 75% on the 21st d.

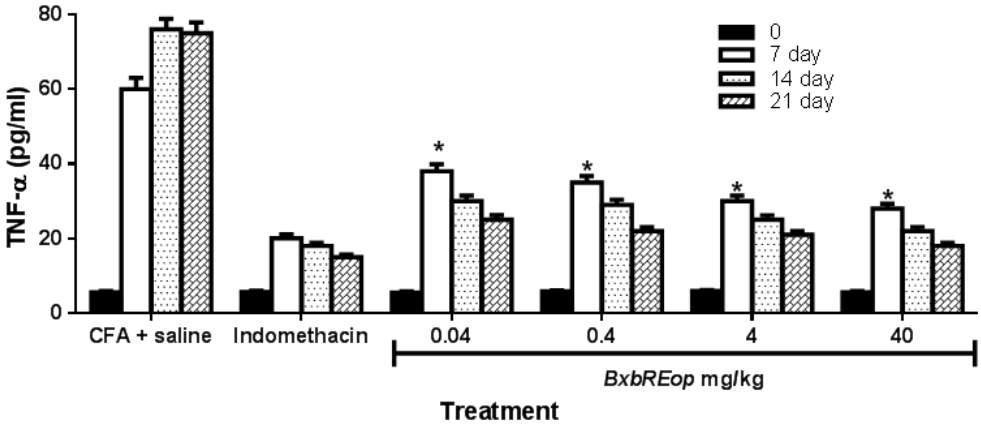

Fig. 4: TNF-α levels in serum from mice, female BALB/c mice weighing 15–20 g were grouped and treated as above described. In the charts, each bar represents the results (mean±SD) from 6 mice. *P<0.01

All doses *of BxbREop*, 0.04, 0.4, 4 and 40 mg/kg also were capable to reduce the IL-6 levels. In groups injected with *BxbREop* extract the levels of IL-6 were significantly lower when compared to the control group (P<0.01). In groups of mice treated with 40 mg/kg *BxbREop* extract the IL-6 levels were significantly decreased to 75% on the 7th, 14th and 21st d (P<0.001). In the same periods in groups treated with indomethacin, the IL-6 production was reduced to 67% (fig. 5).

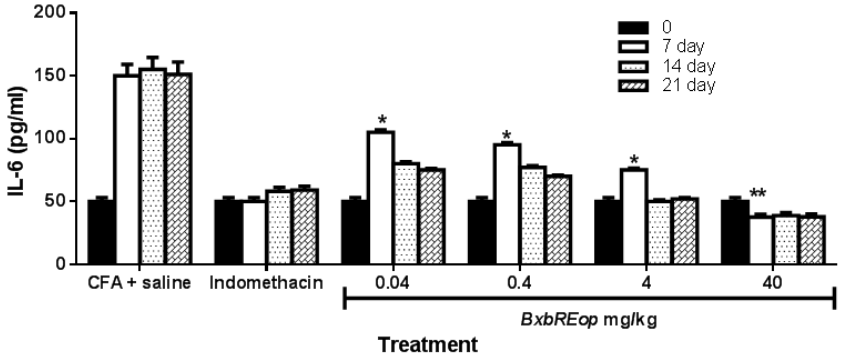

Fig. 5: IL-6 levels in serum from mice, female BALB/c mice weighing 15–20 g were grouped and treated as above described. In the charts each bar represents the results (mean±SD) from 6 mice, *P<0.01 and **P<0.001

In all groups of animals injected with *BxbREop* extract the IL-1β production was reduced (fig. 6). In these groups, the levels of IL-1β were significantly lower when compared to the control group (P<0.01). In groups of mice treated with 40 mg/kg *BxbREop* extract the IL-1β levels were significantly decreased to 75% on the 7th, 14th and 21st day (P<0.001). Similar results were observed in animals injected with indomethacin.

In contrast, the levels of IL-10 in groups of animals treated with 0.04, 0.4, 4 and 4 mg/kg of *BxbREop* extract were increased (fig. 7). Groups of animals injected with *BxbREop* the IL-10 levels were significantly higher than those observed for the control group (P<0.01). The maximum production of IL-10 was obtained in groups of mice injected with 40 mg/kg *BxbREop* extract on the 21st day. Similar results were observed in groups injected with indomethacin (fig. 7).

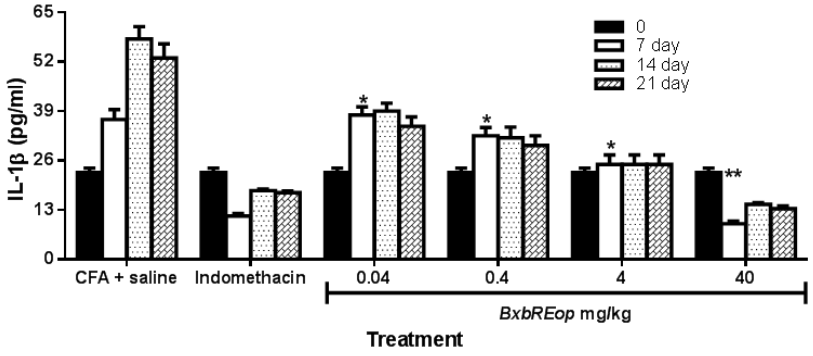

Fig. 6: IL-1β levels in serum from mice, female BALB/c mice weighing 15–20 g were grouped and treated as above described. In the charts each bar represents the results (mean±SD) from 6 mice, *P<0.01 and **P<0.001

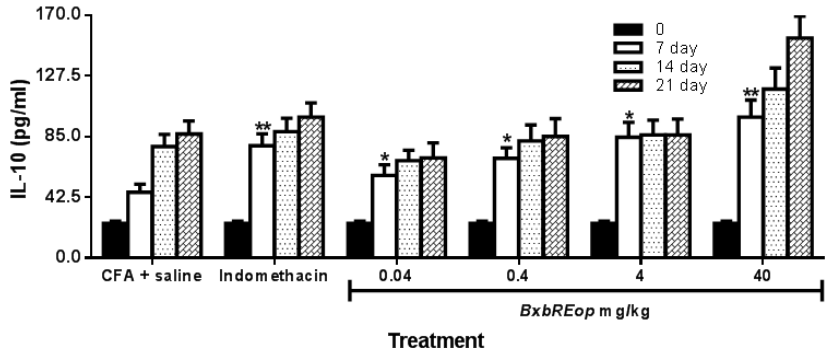

Fig. 7: IL-10 levels in serum from mice, female BALB/c mice weighing 15–20 g were grouped and treated as above described. Each bar represents the results (mean±SD) from 6 mice. *P<0.01 and **P<0.001

The T-cell mediated responses [39, 40] and the cross-reactivity to purified cartilage proteoglycans [41] included in RA [42]. Since RA involves the inflamed joints, the model of adjuvant arthritis model presented similar symptoms. Previously we showed the

immunomodulatory activity of ethanolic extract [43]. The present study results showed that the *BxbREop* extract-treated arthritic animals decreased inflammation of joints. The reduction in paw oedema observed was persistent with the increment of concentration. In this study also found the minor development of arthritis in extract-treated animals. In control group, the highest inflammation was found after 14 d, while in the extract-treated and standard groups the inflammation found was dramatically lower. Our results showed that *BxbREop* extract has anti-inflammatory efficacy in both acute and chronic inflammation. This effect of *BxbREop* extract may be by reason of the decrement of the pro-inflammatory cells [43].

The use of non-steroidal anti-inflammatory drugs cannot block the development and progress of rheumatoid arthritis [44], and the disease-modifying antirheumatic drugs have been impeded by their potential of long-term, side effects, toxicity and immunosuppression [45]. The present study is concerned with the evaluation of the efficacy of *BxbREop* extract as anti-arthritic factors using the adjuvant-induced arthritis model. The present study demonstrated that a single injection of CFA at the plantar surface of mice developed pronounced arthritis in the paws, with 100% incidence. These results agree with the other observation Gonzalez-Gay, *et al.* 2005 [46], who stated that RA is caused by a number of inflammatory mediators released by macrophages. Other investigation reported that characteristics feature of arthritic joints is the persistence of pro-inflammatory cytokines such as TNF-α and IL-1 produced by the inflamed synovium [47]. With respect to TNF-α is considered as an important factor in promoting mechanisms leading to inflammation, whereas IL-1 led to cartilage and bone destruction. In this study, we found that *BxbREop* extract significantly inhibited the levels of three important pro-inflammatory mediators, IL-1, IL-6 and TNF-α, and increased levels of IL-10 in mice. Our results indicated that the *BxbREop* extract exhibits anti-inflammatory properties in arthritic mice. The results of the present study are a coincidence with previous studies that have reported similar observations [48, 49].

CONCLUSION

Our results contribute to the continued research of this extract for the treatment of arthritis. However, no animal model completely depicts the pathophysiology and disease progression in this debilitating disease. Therefore, further research studies will be needed to elucidate the exact mechanism of this extract. From the results obtained, suggest the potential effect of *BxbREop* extract as an anti-arthritic agent towards CFA-induced arthritis in mice.

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AUTHORS CONTRIBUTIONS

Rodolfo Abarca-Vargas and Rigoberto Villanueva Guerrero conducted the experiments, and Vera L. Petricevich analyzed the results, prepared the fig. and wrote the main paper text. All authors reviewed the paper.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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