ORIGINAL RESEARCH

Anti-inflammatory Activity of the Hexane Extract of *Byrsonima crassifolia* Seeds in Experimental Animal Models

Alethia Muñiz Ramirez, MCs; Luis B. Flores Cotera, PhD; Rosa Martha Perez Gutierrez, PhD

ABSTRACT

Context • *Byrsonima crassifolia* is a tropical tree, commonly known as *nance* and distributed widely in Mexico and Central and South America. Since pre-Hispanic times, the seeds of the fruits have been used in folklore medicine as an anti-inflammatory; however, currently no researchers have examined its potential pharmacological properties in scientific studies.

Objective • This study investigated the anti-inflammatory activity of extracts obtained with the solvents n-hexane, chloroform, and methanol from seeds of *B crassifolia*.

Design • The research team induced edemas in Wistar rats with 12-O-tetradecanoylphorbol (TPA), formaldehyde, carrageenan, and histamine to study the anti-inflammatory activity of the three organic extracts of seeds from *B crassifolia*. The team also used the cotton-pellet granuloma method to induce edemas in Wistar rats and study the inhibitory effect of the three extracts from *B crassifolia*. Finally, the team examined the participation of the nitric oxide (NO) system in the anti-inflammatory activity of the hexane extract of nance seeds (NS), diclorofenac, and L-NAME as well as the effects of L-arginine and D-arginine on the anti-inflammatory actions of the compounds.

Setting • This research was conducted in the Laboratory of Research of Natural Products, School of Chemical Engineering, National Polytechnic Institute (IPN-ESIQIE) and Department of Biotechnology and Bioengineering, Cinvestav-IPN, Av. IPN 2508, Col. San Pedro Zacatenco, Mexico D.F., CP 07360, Mexico.

Outcome Measures • The research team measured the edema that the solvents caused, either in the ears of rats for tetradecanoylphorbol or in the paws for formaldehyde, carrageenan, and histamine. To study the antiproliferative effects of the extracts after implantation of the cotton-pellet granuloma, the team determined the wet and dry weights of the pellets, after drying at 70°C for 1 hour in the second case. To study the participation of the NO system in the anti-inflammatory activity of the hexane extract of NS, diclofenac, and L-NAME, the research team measured paw edema.

Results • Among the extracts tested, NS showed the most significant anti-inflammatory activity. That extract decreased the paw edema that carrageenan, formaldehyde, histamine, and cotton pellet-induced, either by oral or topical administration at doses of 200 mg/kg, with 31%, 66%, 83%, and 58.2% inhibition respectively. In addition, NS inhibited the ear edema that TPA induced by 62%. Methanol and chloroform extracts produced a small effect, so the team does not present the results in this article. L-arginine, a precursor of NO, significantly inhibited the anti-inflammatory effects of NS and L-NAME, an anti-inflammatory drug, on mouse paw edema, but D-arginine did not. In contrast, neither D-arginine nor L-arginine inhibited the anti-inflammatory effects that diclofenac produced. These results indicate that the anti-inflammatory effect of NS on mouse paw edema occurs via the inhibition of NO production, as does the anti-inflammatory effect of L-NAME but not the anti-inflammatory effect of diclofenac. The anti-inflammatory activity of NS was comparable to standard anti-inflammatory drugs such as indomethacin, dexamethasone, and sodium diclofenac.

Conclusions • The hexane extract from seeds of *B crassifolia* exhibited significant anti-inflammatory activity in both acute and chronic inflammatory models with a partial contribution of inhibitory actions on some cellular inflammatory responses. The anti-inflammatory mechanism of NS may be related to the other isoform (iNOS). (*Altern Ther Health Med.* 2013;19(1):26-36.)

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Byrsonima crassifolia (B crassifolia) is a tropical tree, commonly known as *nance* and distributed widely in Mexico and Central and South America. Nance fruit is edible and bright yellow when ripened; it has a sweet taste and a slightly bitter aftertaste. In Mexico, nance is consumed as juice, liquor, jelly, and candy. Since pre-Hispanic times, it has been used as an anti-inflammatory.

Reports on ethnobotanical uses include (1) using the bark to promote bleeding in females, to facilitate childbirth, and to treat snakebites; (2) using the leaves as a diuretic, as an antipyretic, as a way to expel the placenta, and as a treatment for diarrhea; (3) using the fruit to treat fever and to induce a pleasant dizziness; (4) using the bark and branches to assist in tightening loose teeth; and (5) using the seeds to treat dysentery, heal wounds, and treat inflammation.[1] Phytochemical studies indicate that the nance plant contains esters,[2] epicatechins,[3] and glycolipids.[4] A pharmacological study of its leaf and bark extracts demonstrated spasmogenic effects.[5] In another study, a chloroformic extract from the bark showed anti-inflammatory activity when the researchers evaluated it using the Croton oil model.[6] Furthermore, Silva et al have determined the antioxidant activity of extracts from the leaves, fruits, and bark.[7] The ethyl-acetate extract of its roots exhibit antibacterial activity.[8] Also, the aqueous extract of its leaves inhibits some dermatophytes.[9] The ethanol extract of the leaves shows trypanocidal activity against *Leishmania mexicana* promastigotes (trypanosome parasites).[10]

Based on the traditional uses of seeds from *B crassifolia*, and given the lack of scientific studies on their potential pharmacological properties, the objective of this work was to study the anti-inflammatory activity of nance seed (NS) extracts obtained with hexane, chloroform, and methanol on acute and chronic phases of inflammation, and to also compare their anti-inflammatory effect potencies with indomethacin, dexamethasone, and diclofenac sodium, which are well known as anti-inflammatory drugs.

MATERIALS AND METHODS

Plant Material

B crassifolia belong to the Malpighiaceae family. The research team collected the tree's fruit in Morelos, Mexico, in October 2010. The plant material was identified by biologist Martha Arreguin of the Department of Botany, Escuela Nacional de Ciencias Biologicas, Instituto Politécnico Nacional (ENCB-IPN). A voucher specimen (No. 8976) was deposited at the Herbario as reference.

Experimental Animals

The research team conducted the study in Wistar rats weighing between 150 g and 200 g and in CD1 mice weighing between 20 g and 25 g. The team procured all animals from the bioterio of ENCB-IPN and housed them in Microlon boxes in a controlled environment (temperature 25°C ± 2°C), with a standard laboratory diet and water ad libitum. Prior to the experiments, the team acclimatized the animals to the new environment for 3 days. The research team observed the ethical clearance in animal handling as described in the US National Institutes of Health's publication No. 85-23 (revised 1985).

Preparation of Extracts

The seeds of *B crassifolia* were air-dried at 38°C and powdered. The dried powders (400 g), were extracted with 1.5 L of hexane, chloroform, and methanol of form consecutively under reflux condition for 3 hours using a Soxhlet apparatus followed by filtration through a Whatman No. 42 filter. The solvent was removed under reduced pressure at 30°C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) to obtain a residue used for the determination of its bioactivity. The averages for extract yields (weight/weight [w/w]) were as follows: 10.1% for hexane, 0.9% for chloroform, and 12.3% for methanol.

Anti-inflammatory Activity

Formaldehyde-, Carrageenan-, and Histamine-induced Paw Edema in Rats. Wistar albino rats of either sex (150-200 g) in 24 groups (each n = 6) were fasted for 12 hours prior to the induction of edema, but water was available ad libitum. Rats were deprived of water only during the experiment to ensure uniform hydration and to minimize variability in edematous response. Inflammation of the hind paw was induced by injecting 0.2 mL of formaldehyde (1% weight/volume; w/v),[11] or 0.1 mL of 1% carrageenan,[12] or 1 mg/mL of histamine[13] in a normal isotonic saline solution into the subplantar region of the right-hind paw. The control group orally received a saline solution (0.1 mL), and the positive standard groups received indomethacin (10 mg/kg),[14] or diclofenac sodium orally. The groups treated with NS extracts (hexanic, chloroformic, and methanolic) received doses at 50 mg/kg, 100 mg/kg, and 200 mg/kg, PO. All drug treatments were given 1 hour before the inflammatory injection. Edema was measured with a digital plethysmometer

Figure 1. General Procedure Used to Prepare Extracts of *B crassifolia* Seeds Using Hexane

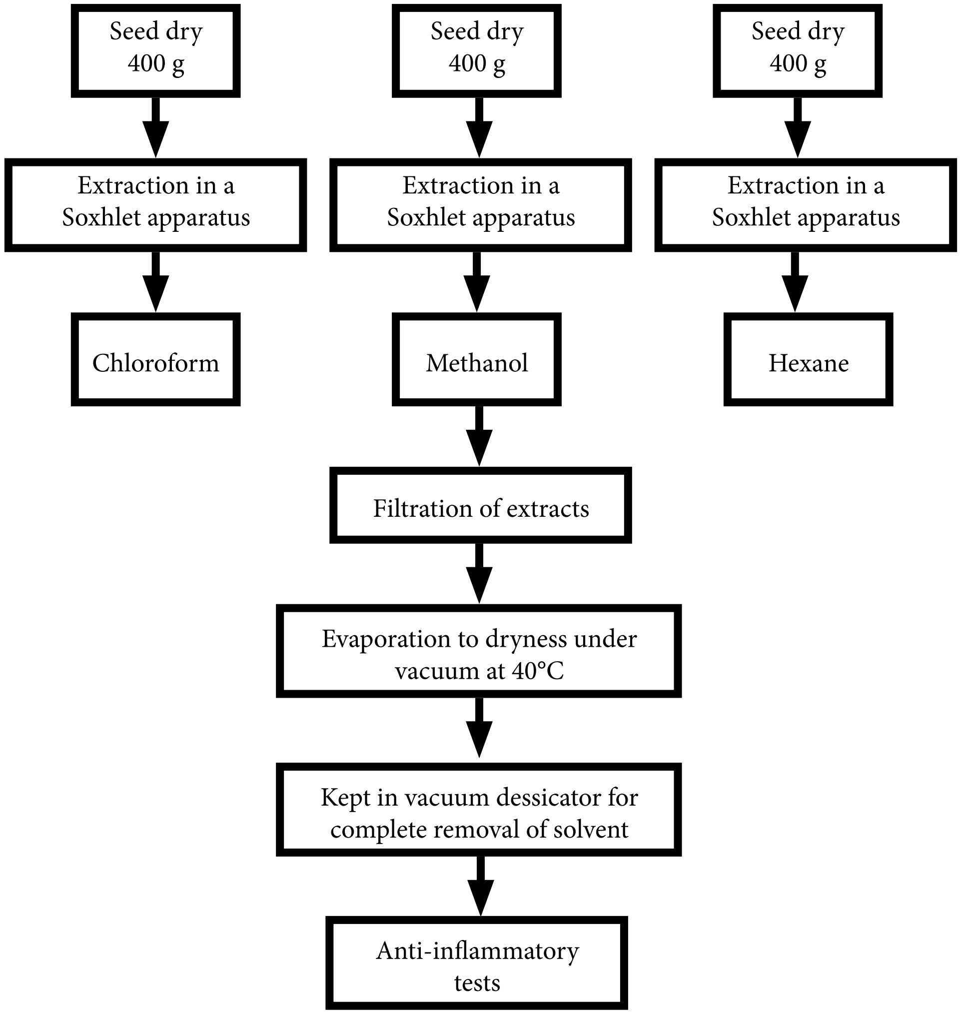


Table 1. Screening of the Anti-inflammatory Activity of the Hexane Extract From *B crassifolia* Seeds (NS) on Different Inflammation Models

Treatment	Inflammatory drug	Standards
Tetradecanoylphorbol-induced ear edema in mice	Tetradecanoylphorbol	Dexamethasone
Formaldehyde-induced paw edema in rats	Formaldehyde	Indomethacin
Carrageenan-induced paw edema in rats	Carrageenan	Indomethacin
Cotton-pellet granuloma test	Cotton pellet	Indomethacin
Histamine-induced paw edema in rats	Histamine	Diclofenac
Treatment of carrageenan edema with L-arginine and D-arginine	Carrageenan	Diclofenac and L-NAME

(Ugo Basile Sri, Comerio, Italy) before and after the inflammatory injection (ie, at 0.5, 2, 4, and 6 hours).[12]

Edema was expressed as the relative increase in paw volume that the inflammatory injection induced (ie, the edema was proportional to the volume difference between 0 hours and the other times [0.5, 2, 4, and 6 hours]).

The percentage of rise in paw volume was as follows[13]:

% rise = Vt – Vc/Vc × 100
Where: Vt = paw volume at time t
Vc = paw volume at time 0

Tetradecanoylphorbol-induced Ear Edema in Mice. TPA (l μg) dissolved in acetone (20 μL) was applied to the right ear of mice by means of a micropipette, delivering a volume of 10 μL to the inner and outer surface of the ears. The samples of each extract (0.12 mg/ear, 0.25 mg/ear, 0.5 mg/ear), and the control (water) and dexamethasone (0.05 mg/ear) as the drug reference were applied topically about 30 minutes before TPA treatment. For ear thickness determinations, a pocket thickness gauge (Mitutoyo, Kawasaki, Japan) was applied to the tip of the ear. The gauge had a range of 0 mm to 9 mm, graduated at 0.01-mm intervals and modified to increase the contact surface area to reduce tension. The ear thickness measured before the first treatment and 6 hours after TPA treatment: (TPA + water and TPA + extract). The following values were then calculated: edema A as induced by TPA alone (B – A) and edema B as induced by TPA + extract (B – A).

Inhibitory ratio (%) = ([edema A – edema B]/edema A) × 100

Each value was the mean of individual determinations from five mice. The 50% inhibitory dose (ID_{50}) values were determined by probit-graphic interpolation for four dose levels.[15]

Cotton-pellet Granuloma Test. The effects of the hexane extract (NS) and indomethacin on the proliferative phase of induced-inflammation in rats were studied by a cotton-pellet granuloma test. A 200-mg/kg dose of NS and a 10-mg/kg dose of indomethacin were administered to two rats groups separately. The same volume of distilled water was applied to the control group. After 30 minutes, the animals were anesthetized with 25 mg/kg thiopental sodium (IP). Under sterile conditions, cotton pellets, weighing 7 mg each, were implanted at an interscapular distance under the skin. The same doses of NS and indomethacin were supplied once a day for a period of 7 days. The rats were sacrificed by a high anesthesia dose on the eighth day; the cotton pellets, surrounded by granuloma tissues, were dissected, and then the wet and dry weights of the pellets were determined (in the second case after drying them at 70°C for 1 hour).[16] The antiproliferative effect of the NS was compared with the control and indomethacin groups.

Effect of L-arginine and D-arginine on the Anti-inflammatory Activity of *B crassifolia*, Diclofenac, and L-NAME in Mice. An intraplantar injection of 30 μL of 2% carrageenan stimulated edema development at 30 minutes in the control mice treated orally with 0.45% ethanol solution 10 minutes before the injection. The paw edema increased 6 hours after the injection. The anti-inflammatory effect of the hexane extract was evaluated as the area under the curve (AUC) during the period between 30 minutes and 6 hours after carrageenan injection[17] at doses of 1:1000 and 1:100 equivalent to 18.5 mg extract per 100 μL.[18] L-arginine or D-arginine was intraperitoneally administered 2 hours before the peak time of the anti-inflammatory effects; peak times were determined using the data obtained earlier in the test with carrageenan. The anti-inflammatory effects of diclofenac (3.1-50 mg/kg PO), a nonsteroidal anti-inflammatory drug (NSAID), and L-NAME (1-100 mg/kg subcutaneously SC), a NOS inhibitor, in a carrageenan-induced mouse paw edema were examined to compare the effects with the anti-inflammatory effects of the NS.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM) for six or eight rats per group. Parametric data were assessed using one-way analysis of variance (ANOVA), followed by Dunnet's *t* test; *P* values <.05 were considered to be significant.

RESULTS

Figure 1 shows the general procedure used to prepare extracts of *B crassifolia* seeds using hexane, chloroform, and methanol because these solvents have different abilities to extract substances from plants. The extract prepared with hexane showed the highest percentage of reduction of the induced edema in comparison with those of the other two solvents. Methanol and chloroform extracts produced a small effect, so those results are not presented here. In the present study, the main focal point is the efficacy of the NS on anti-inflammatory activity. Such activity may be associated with the presence of terpenoids. A few of them have been used for therapeutic purposes for decades as an anti-inflammatory agent. Table 1 shows a screening of the anti-flammatory activity of the hexane extract from *Byrsonima crassifolia* seeds (NS) on different inflammation models induced by formaldehyde, carrageenan, histamine, TPA, and cotton pellets.

Formaldehyde-induced Edema in Rats

The hexane extract supplied orally for 6 days at 50 mg/kg, 100 mg/kg, and 200 mg/kg doses decreased the edema induced by formaldehyde by 35% ($P < .005$), 46% ($P < .005$), and 66% ($P < .001$), respectively. The edema decreased 73% ($P < .001$) with a dose of 10 mg/kg indomethacin. Table 2 shows the mean paw volume of different rat groups.

Table 2. Effects of NS and Indomethacin on Formaldehyde-induced Paw Edema in Rats

NS doses mg/kg	Mean values for paw volumes Before inf	After 6 d inf	Anti-inflammatory effects (%)
Control	0.89 ± 0.65	1.64 ± 2.34	–
50	0.86 ± 0.38	1.42 ± 1.28[a]	35
100	0.88 ± 0.57	1.34 ± 1.45[a]	46
200	0.89 ± 0.73	1.19 ± 1.32[b]	66
Indomethacin, 10 mg/kg	0.90 ± 0.19	1.14 ± 1.63[b]	73

Note: The data are expressed as mean ± for six rats.
[a]$P < .005$.
[b]$P < .005$ compared with vehicle inflammation (inf).

Table 3. Effects of the Hexane Extract of *B Crassifolia* Seeds on Acute Inflammation Induced by Carrageenan[a]

Doses, mg/kg	Percentage rise in paw edema at different time intervals				
	1 h Mean ± SEM	2 h Mean ± SEM	3 h Mean ± SEM	4 h Mean ± SEM	6 h Mean ± SEM
Carrageenan control	52 ± 3.81	65 ± 3.76	69 ± 2.19	86 ± 4.12	82 ± 3.68
50	28 ± 3.48[b]	38 ± 6.02[b]	41 ± 5.31[b]	58 ± 3.96[b]	42 ± 2.86[b]
100	23 ± 4.23[b]	35 ± 6.13[b]	38 ± 4.36[b]	48 ± 4.54[b]	39 ± 4.93[b]
200	16 ± 3.78[b]	22 ± 4.15[b]	27 ± 3.78[b]	44 ± 4.07[b]	36 ± 4.80[b]
Indomethacin, 10 mg/kg	2 ± 3.47[b]	19 ± 3.89[b]	24 ± 6.01[b]	36 ± 5.64	33 ± 5.13[b]

[a]The results of the increase in edema are expressed as the percentage of the postdrug value of the paw volume to the predrug value. The results are expressed during the period from 30 minutes to 6 hours after carrageenan injection. The means and standard errors of the means represent data for groups of six mice.
[b]$P < .01$ when compared to vehicle-treated controls.

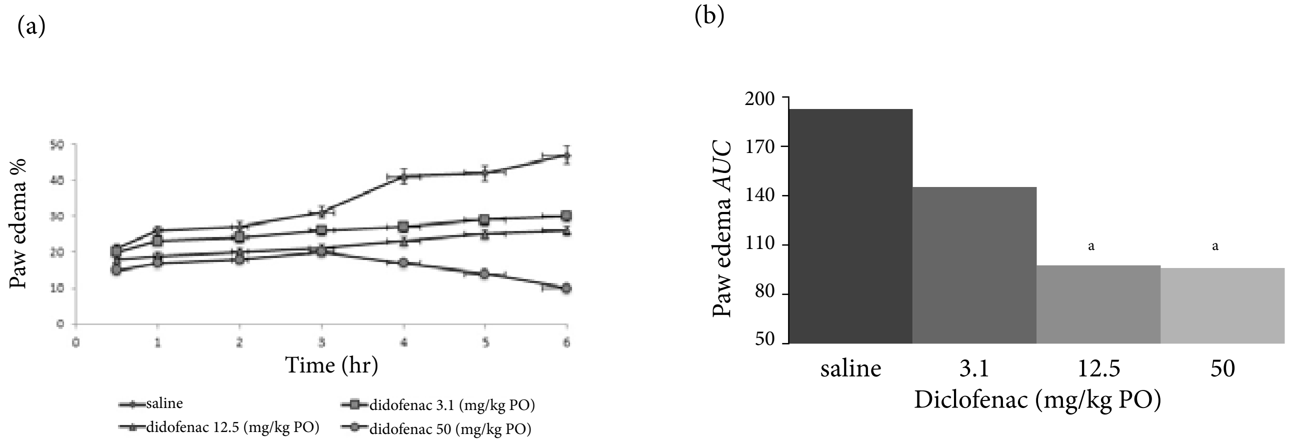
Acute Carrageenan-induced Edema in Rats

The results of the effect of NS on carrageenan-induced paw edema are shown in Table 3. The hexane extract exhibited significant reduction in the percent rise of carrageenan-induced rat paw edema. The maximal inhibition in the percent rise of edema volume was observed at the doses of 200 mg/kg when compared to the control, decreasing after 6 hours. Figure 2 shows anti-inflammatory activity of diclofenac on rat paw edema caused by carrageenan. Results show the dose-dependence of the anti-inflammatory effect of diclofenac and NS.

Histamine-induced Edema in Rats

In this study, the team evaluated the anti-inflammatory activity of the extract on the phlogistic agent histamine, which is a known mediator of inflammation. Table 4 shows that the hind paw edema of rats peaked at 2 hours; then, it rapidly decreased from 3 hours after the injection. The left-hind paw used as the control showed no increase in paw volume during the entire experiment (data not shown), whereas the injection of histamine successfully induced the edema for the right-hind paw. Administration of the NS by intraperitoneal injection at 100 mg/kg and 200 mg/kg dosages showed significant and increasing inhibition of the edema at 1, 2, and 3 hours after injection. These data indicate that the anti-inflammatory effect of NS is time-dependent. The NS showed strong and dose-dependent inhibition on the paw edema in the early stage of inflammation (1 hour after histamine injection) at 50 mg/kg to 200 mg/kg dosages. However, in the late phase (2 hours after histamine injection), the NS affected the paw edema to a lesser extent with dose-dependence. Diclofenac sodium also showed a significant inhibition of paw edema from 1 hour after histamine injection.

Figure 2. Anti-inflamatory Activity of Diclofenac on Rat Paw Edema Caused by Carrageenan

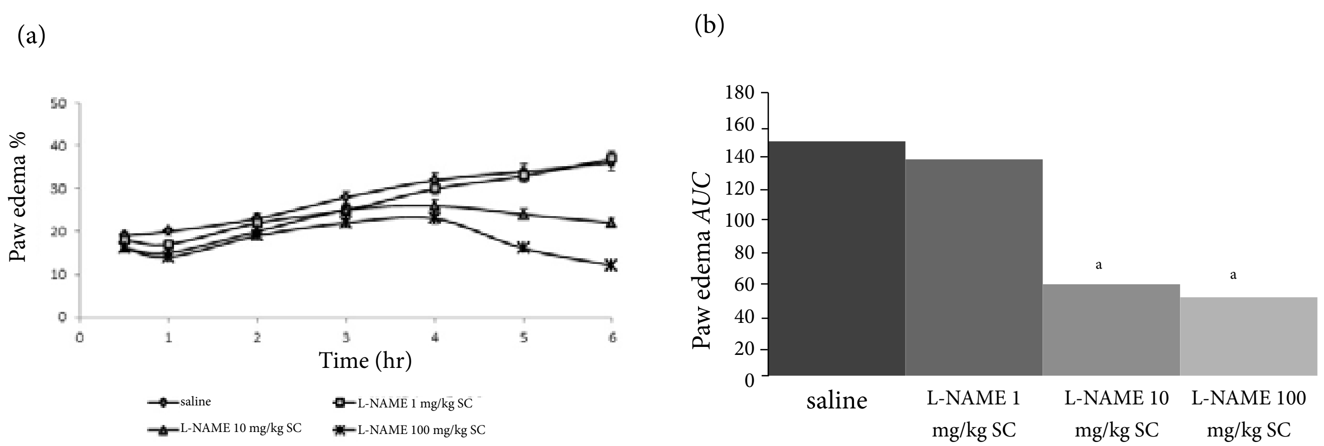


[a]$P < .01$ when compared to water-treated controls.

(a) Results of edema increase are expressed as the percentage of the postdrug value of paw volume to the predrug value. $P < .05$.

(b) Dose-dependence of the anti-inflammatory effect of diclofenac. The results are expressed as AUC during the period between 30 min and 6 h after carrageenan injection. These data are given as the means and SEM for groups of six rats. $P < .01$ when compared to water-treated controls.

Figure 3. Anti-inflamatory Effect of L-NAME on Rat Paw Edema Caused by Carrageenan



[a]$P < .01$ when compared water-treated controls.

(a) Time course of L-NAME or saline. The results of edema increase are expressed as the percentage of the postdrug value of paw volume to the predrug value.

(b) Dose-dependence of the anti-inflammatory effect of L-NAME. The results are expressed as AUC during the period from 30 min to 6 h after carrageenan injection. These data are given as the means and SEM for groups of six rats, $P < .05$. $P < .01$ when compared to water-treated controls.

Table 4. Anti-inflammatory Effects of the Hexane Extract of *B Crassifolia* Seeds on Histamine-induced Paw Edema in Rats [a]

	% Increase in Paw Edema Size		
Doses, mg/kg	1 h Mean ± SEM	2 h Mean ± SEM	3 h Mean ± SEM
Control	46 ± 2.78	70 ± 6.43	66 ± 5.72
50	33 ± 5.15[b]	27 ± 3.69[b]	23 ± 1.27[b]
100	28 ± 3.46	22 ± 3.54[b]	19 ± 2.89[b]
200	24 ± 3.38[b]	19 ± 2.70[b]	11 ± 2.23[b]
Diclofenac sodium, 10 mg/kg	17 ± 4.12	14 ± 2.89[b]	9 ± 3.62[b]

[a]The results of edema increase are expressed as the percentage of the postdrug value of paw volume to the predrug value. The results are expressed during period from 1 hour to 3 hours after histamine injection. The means and standard errors of the means represent data for groups of six mice.
[b]$P < .01$ when compared to vehicle-treated controls.

Tetradecanoylphorbol-induced Edema in Mice

Evaluation of the topical anti-inflammatory activity of the NS was performed in the TPA-induced mouse ear edema. The phorbol ester (TPA) provides a skin inflammation model suitable for evaluation of both topical and systemic anti-inflammatory agents and it has been extensively applied in studies of anti-inflammatory products. As shown in Table 5, topical application of the NS significantly suppressed the extent of swelling by 38%, 51%, and 62% at the doses of 0.125, 0.25 and 0.50 mg/ear, respectively. The anti-inflammatory activity of NS is less than that obtained with dexamethasone (0.05 mg/ear).

Cotton-pellet Granuloma Test

In this assay, the team calculated the anti-inflammatory effect of indomethacin and NS from the weight of cotton pellets procured from the rats. The mean weight of wet pellets removed from rats in the control group was 205 mg ± 30.3 mg. In contrast, the mean weights in rats given 200 mg/kg NS and indomethacin were 85 mg ± 15.4 mg ($P<.005$) and 75 mg ± 26 mg ($P<.001$), respectively (Table 5). According to these data, the antiproliferative effect of the NS and indomethacin was 57.7% and 62.7%, respectively. The mean dry weight of pellets was 26.5 mg ± 1.76 mg ($P < .005$) and 24.3 mg ± 1.45 mg ($P<.005$) in the rat groups treated with the NS and indomethacin, respectively. Based on the mean dry weight, the NS and indomethacin administration inhibited inflammation by 58.2% and 56%, respectively.

Effects of L-arginine and D-arginine on the Anti-inflammatory Actions of *B crassifolia*, Diclofenac, and L-NAME

To determine the participation of the NO system in the anti-inflammatory mechanism of NS, diclofenac, and L-NAME, the research team studied the effect on the anti-inflammatory activity of the extract and the two compounds of L-arginine and D-arginine administered intraperitoneally.

Table 5. Effects of NS on Tetradecanoylphorbol-induced Ear Edema

Doses mg/ear	Δ Ear Thickness mm (% Reduction)
Control	0.091 ± 0.12
NS 0.125	0.056 ± 0.30[b] (38)
NS 0.25	0.044 ± 0.47[a] (51)
NS 0.50	0.034 ± 0.42[b] (62)
Dexamethasone, 0.05 mg/ear	0.072 ± 0.98[a] (80)
Indomethacin, 10 mg/kg	–

Note: The data are expressed as mean ± SEM for six rats.
[a]$P < .005$.
[b]$P < .001$ compared with vehicle.

The administration of L-arginine or D-arginine was realized 2 hours before the peak time of the anti-inflammatory activity in each case; peak times were determined using the data obtained earlier in the test with carrageenan. Figures 2 and 3 respectively show the growth of paw edema induced by carrageenan injection as well as the effect of diclofenac (3.1-50 mg/kg PO) and L-NAME (1-100 mg/kg SC). In both cases, the research team administered the drug 10 minutes prior to the carrageenan injection. The evaluation of AUC during the period between 30 minutes and 6 hours after the carrageenan injection shows the significant anti-inflammatory effects of diclofenac (12.5, 50 mg/kg) and L-NAME (10, 100 mg/kg). Figure 3 shows the anti-inflammatory effects of L-NAME on

Figure 4. Effect of L- and D-arginine on the anti-inflamatory effect induced by NS. Each arginine was given IP 1h after carrageenan injection. The results are expressed as AUC during the period from 2 to 6 h after carrageenan injection. These data are given as the means and SEM for groups of six rats.

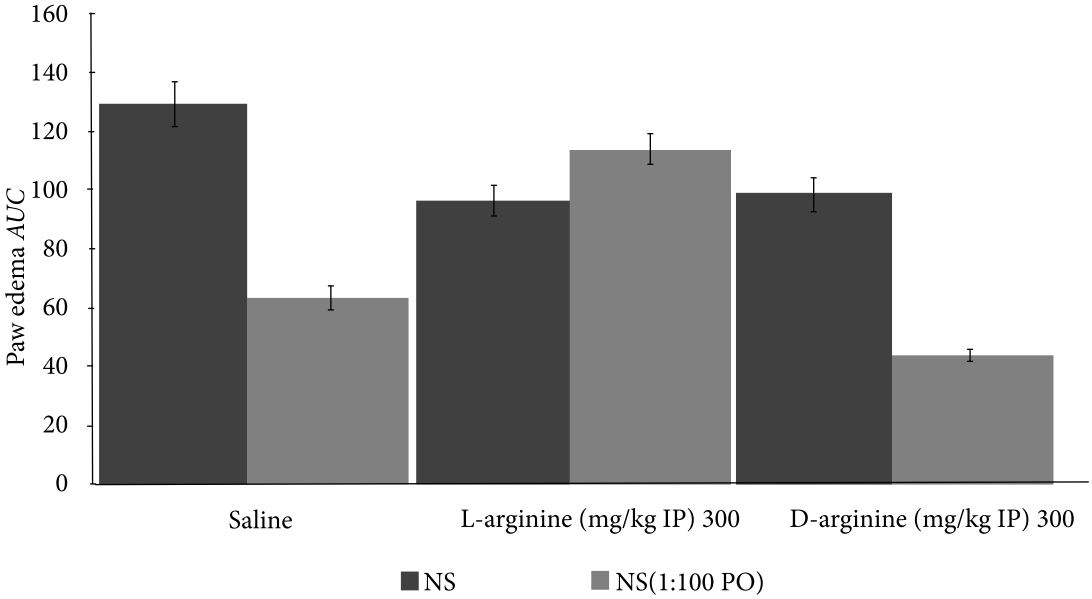
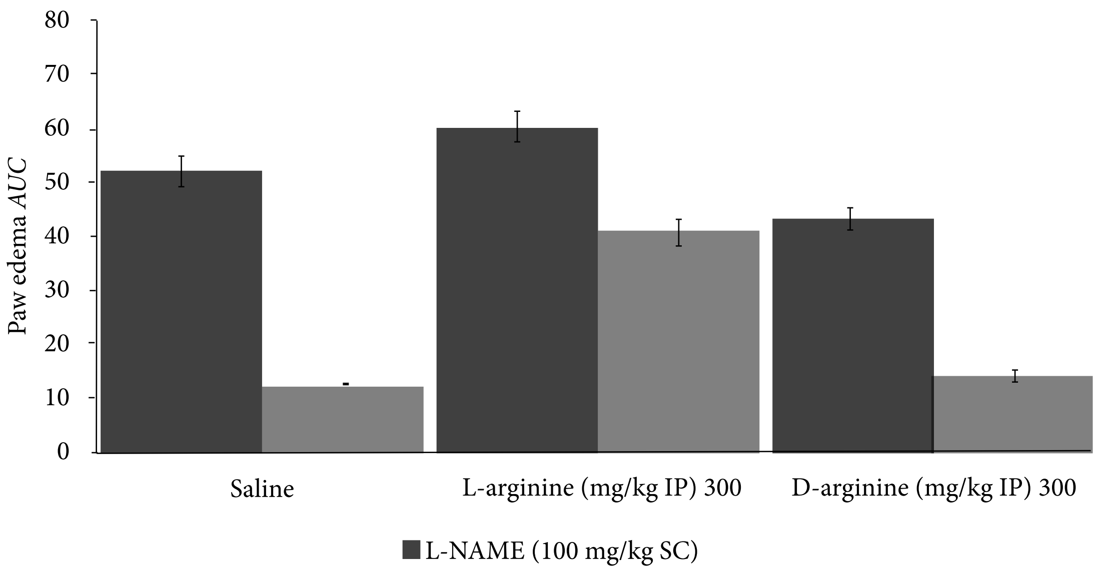


Figure 5. Effect of L- and D-arginine on the anti-inflamatory effect induced by L-NAME. Each arginine was given IP 1h after carrageenan injection. The results are expressed as AUC during the period from 2 to 6 h after carrageenan injection. These data are given as the means and SEM for groups of six rats.

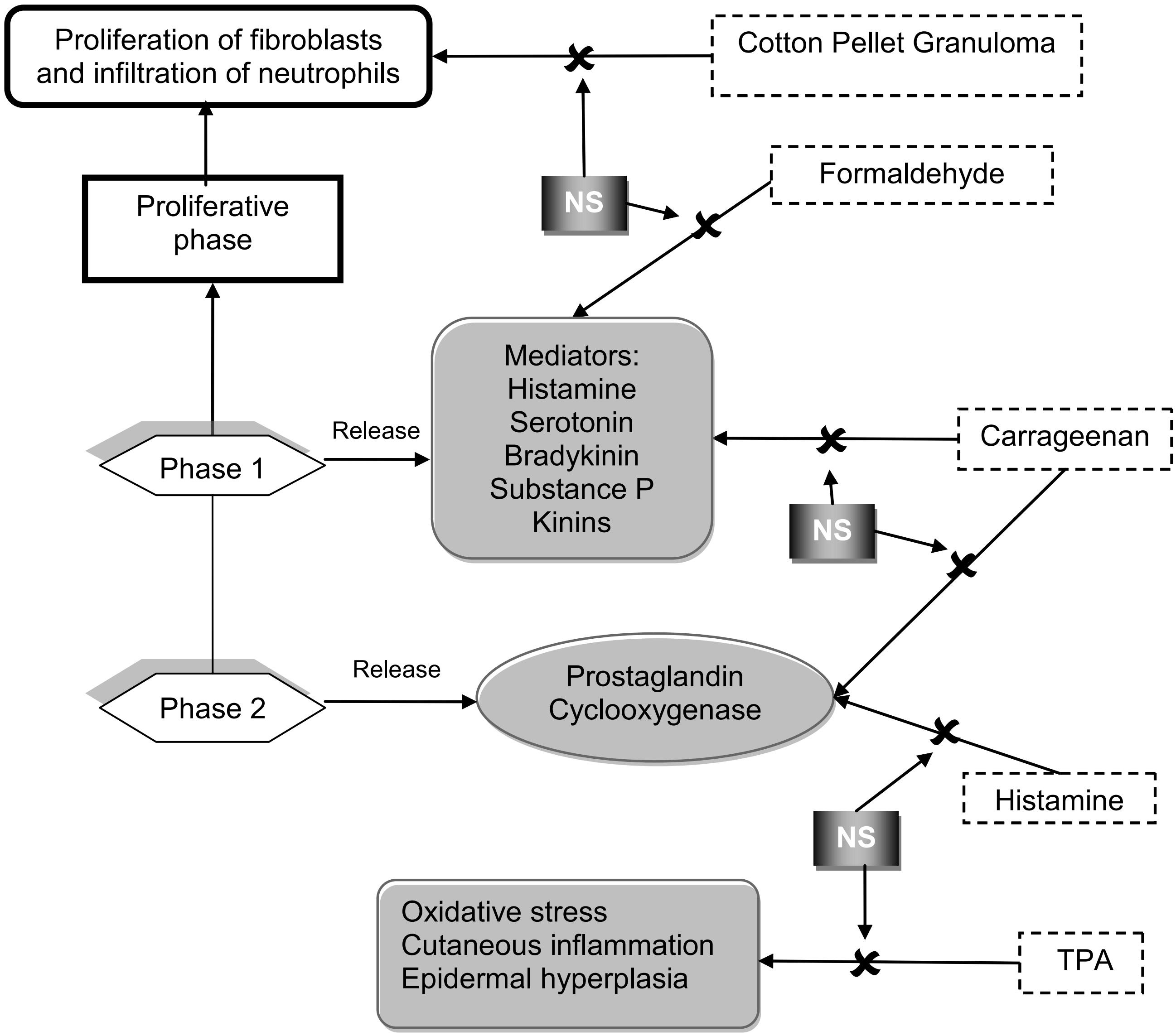


rat paw edema that carrageenan induced. Figure 4 shows the effect of the administration of arginine D or L on the rat paw edema carrageenan induced. Each arginine was given IP 1 hour after carragenin injection. Treatment with L-arginine (300 mg/kg) significantly inhibited the anti-inflammatory effect of the NS. In contrast, neither D-arginine nor L-arginine inhibited the anti-inflammatory effect of diclofenac (50 mg/kg). The single administration of the arginines (300 mg/kg) did not significantly affect the paw edema.

DISCUSSION

Traditional medicine for the treatment of various diseases is becoming more popular. Therefore, the present study aimed to evaluate the scientific basis for the traditional use of seeds from *B crassifolia*, employing six different types in vivo inflammatory models. The study's data indicate that NS exerts anti-inflammatory effects in rats and mice, and specifically that these effects could be associated with the presence of terpenoids.[19]

Figure 6. Schematic Representation of the Events After Treatment of NS on Inflammatory Process



The paw edema induced by formaldehyde is another of the most suitable test methods to evaluate anti-inflammatory agents. The method is commonly considered as a close model to human arthritis.[20] Histamine, serotonin, bradykinin, prostaglandin, and P-substance have roles in formaldehyde-induced edema in rat hind paws (Figure 6). The formaldehyde-induced inflammation usually involves two distinct phases. Researchers suggest that the first phase involves the direct stimulation of nociceptors, and the second may be associated with activity of inflammation mediators.[21] Some studies have shown that the P-substance receptor antagonists slow down the progression to the second phase of formaldehyde-induced edema, and P-substance has a role in this response. Formaldehyde-induced paw edema becomes visible after a short period of time following formaldehyde injection, and acute inflammation symptoms (tumor, rubor, and color) reach the peak at 3 to 6 hours.

Researchers have widely used carrageenan-induced edema in rats' hind paws for the discovery and evaluation of anti-inflammatory drugs, since for most drugs tested with this model, the relative potency estimates tend to reflect clinical experience.[22] The intraplantar injection of carrageeman in rats leads to paw edema in two phases (Figure 6). The first phase occurs within an hour of injection and is the result of the concurrent release of histamine, serotonin, and kinins; the second is associated with elevated production of prostaglandins, oxygen-free radicals, and inducible cyclooxygenase (COX-2) and the local infiltration and activation of neutrophils.[23,24] Prostaglandins play a major role in the development of the second phase, which usually occurs after 3 hours.

The increase in paw size usually quantifies the inflammatory response, but inhibitors, such as NSAIDs, can modulate this response.[20] The extract of seeds from *B crassifolia* significantly ($P < .05$) decreased the edema size during the initial 4 hours after treatment as compared to control rats. The extract most likely decreased the paw edema by acting at both phases of the carrageenan-induced inflammation. The effect is similar to that of indomethacin. Thus, the NS extract may inhibit the synthesis or release of mediators leading to the acute phase, like histamine, serotonin, or other proinflammatory mediators, which usually appear in the early phase of inflammation (Figure 2). Moreover, the effect in the second phase of inflammation may be through the inhibition of COX-2, which leads to the inhibition of prostaglandin synthesis.

Histamine is likely one of the most important mediators of inflammation. Histamine may increase vascular permeability and act with prostaglandins to induce edema (Figure 6). The secretory granules store these two mediators, but mast cells release them during their activation.[25] Silva et al propose that prostaglandins act through specific receptors on the nearby vasculature to induce plasma extravasation.[26] The hexane extract and the diclofenac, a reference drug, significantly decreased inflammation 1 hour after histamine injection.

Topical application of TPA, a protein kinase well characterized as an activator and tumor promoter, is a suitable model to screen compounds for potential topical anti-inflammatory therapy. A single application of TPA induces oxidative stress, cutaneous inflammation, and epidermal hyperplasia due to enhanced keratinocyte proliferation (Figure 6).[22] Researchers have used the mouse ear edema test to identify the potential allergens on the basis of increases in ear thickness in sensitized animals. The inhibition of this dermal reaction can be expressed as the decrease in ear edema or ear thickness as compared to a control group. Topical application of NS markedly suppressed the ear thickening and epidermal hyperplasia. Dexamethasone, a glucocorticoid agonist, has an anti-inflammatory action that is thought to involve phospholipase A_2 inhibitory proteins and lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes. Unbound dexamethasone crosses cell membranes and binds with high affinity to specific cytoplasmic receptors.[27]

Inflammatory granuloma is a typical feature of an established chronic inflammatory process (Figure 6). Researchers have employed the cotton-pellet granuloma method widely to evaluate the transudative, exudative, and proliferative components of chronic inflammation. Generally, the dry weight of the cotton pellets correlates well with the amount of granulomatous tissue.[28] The use of NS decreased the dry weight of implanted cotton pellets, indicating that it inhibits the proliferative phase of inflammation. Chronic inflammation is a reaction arising when the acute response is insufficient to eliminate proinflammatory agents and includes proliferation of fibroblasts and the infiltration of neutrophils and exudation. Chronic inflammation occurs by means of the development of proliferative cells. These cells can be either of spread or granuloma form.[29] The NS was more effective on chronic inflammation compared to acute inflammation. Separate administration of NS and indomethacin prevented the growth of granuloma tissue that the cotton pellets induced, at comparable levels.

Indomethacin decreased the granuloma tissue arising from the cellular response, which inhibits the granulocyte infiltration to the foreign cotton body implanted.[30] The NS was more effective on chronic inflammation than on acute inflammation, and both NS and indomethacin prevented weight increases of granuloma tissue induced by cotton pellets at almost the same level.

Three isoforms of NOS synthase produce NO from L-arginine.[31] Two of them, namely endothelial NOS and neuronal NOS, are calcium-dependent and constitutively expressed enzymes. The other isoform (iNOS) is calcium independent; so consequently its inhibition causes an anti-inflammatory effect on rat paw edema. The separate administration of L-NAME (100 mg/kg) or NS showed significant but similar anti-inflammatory activity. Moreover, L-arginine, a substrate of NOS, significantly inhibited the anti-inflammatory effects of NS and L-NAME, but D-arginine did not. Also, L-arginine did not inhibit the diclofenac-induced anti-inflammation mediated through COX-2 inhibition.[32] The results suggest that NS and L-NAME inhibit only the catalytic activity of iNOS and not iNOS expression. Regardless of the mechanism implied, it may be that *B crassifolia* produces an anti-inflammatory activity through the inhibition of NO production. The anti-inflammatory mechanism of NS may be related to iNOS and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). NS may be used as a pharmacological agent for controlling acute and chronic inflammation in experimental models of diseases in which free radical formation is a pathogenic factor.

CONCLUSION

In conclusion, the hexane extract from seeds of *Byrsonima crassifolia* exhibited significant anti-inflammatory activity in both acute and chronic inflammatory models with a partial contribution of inhibitory actions on some cellular inflammatory responses. The anti-inflammatory mechanism of NS may be related to iNOS. Further studies are needed to elucidate the precise mechanism of action and effective constituents of *B crassifolia.* Isolation of the active constituents and evaluation of their anti-inflammatory activity are in progress. This study also confirms the folklore medicinal uses of the plant to treat various ailments related to inflammatory processes.

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