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Phoradendron bathyoryctum Eichler relieves acute nociceptive pain stimulus and carrageenan-induced inflammation in mice

Phoradendron bathyoryctum Eichler alivia el estímulo de dolor nociceptivo y la inflamación aguda inducida por carragenina en ratones

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ABSTRACT

Background: The genus *Phoradendron* belongs to the Santalaceae family and possesses several species with antitumor, cytotoxic, and immunomodulatory activity, where pain and inflammation are common symptoms. It is used in venereal and liver diseases in Paraguayan folk medicine. In addition, it claims to have tonic properties for the heart and central nervous system. Previous studies have shown that crude extract of *Phoradendron bathyoryctum* Eichler (*Pb*) has anxiolytic and antidepressant activity. **Objective:** This work aimed to determine the anti-nociceptive and anti-inflammatory activity of *Pb* using acute models in mice. **Methods:** the anti-nociceptive activity of *Pb* was evaluated using mechanical pressure (Randall-Selitto test), acetic acid (writhing test), and heat (hot plate test) noxious stimulus in mice. The anti-inflammatory activity was assessed through carrageenan-induced plantar edema in mice previously treated with *Pb*. **Results:** Presences of high polarity alkaloids, steroids/ free triterpenoids, leucoanthocyanidins, and tannins were detected in phytochemical studies. Oral doses of 30 ($p<0.01$) and 300 ($p<0.001$) mg/kg of *Pb* denoted a significant dose-dependent increase in pain threshold, using the Randall-Selitto and Writhing test (30; $p<0.05$; and 300; $p<0.01$. In addition, consistent with the above anti-nociceptive effect, an increase in the reaction latency time after oral administration of *Pb* at a dose of 300 mg/kg ($p<0.05$) in the hot plate test was denoted. Finally, a significant reduction of edema (30 mg/kg; $p<0.01$) induced by 1% carrageenan was evidenced, demonstrating a potential anti-inflammatory activity of *Pb* compared to the positive edema control. Interestingly, the anti-edematous activity of *Pb* showed a similar intensity response compared to the group treated with 10 mg/kg indomethacin ($p<0.01$). **Conclusion:** This work revealed that the crude extract of *Pb* can increase pain threshold, be compatible with an analgesic effect, and reduce edema (anti-inflammatory) induced by Carrageenan in mice. Further pharmacological and chemical studies are being conducted to elucidate molecular mechanisms and components involved in the observed effects.

Keywords: *Phoradendron bathyoryctum*, anti-nociceptive, anti-inflammatory, Randall-Selitto, writhing test, hot plate, plethysmometer.

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RESUMEN

Antecedentes: El género *Phoradendron* pertenece a la familia Santalaceae y posee varias especies con actividad antitumoral, citotóxica e inmunomoduladora, donde el dolor y la inflamación son síntomas comunes. Se utiliza en enfermedades venéreas y hepáticas en la medicina popular paraguaya. Además, se le atribuyen propiedades tónicas para el corazón y el sistema nervioso central. Estudios anteriores han demostrado que el extracto crudo de *Phoradendron bathyoryctum* Eichler (*Pb*) tiene actividad ansiolítica y antidepresiva. **Objetivo:** Este trabajo tuvo como objetivo determinar la actividad anti nociceptiva y antiinflamatoria del *Pb* utilizando modelos agudos en ratones. **Métodos:** la actividad anti nociceptiva del *Pb* se evaluó mediante estímulos nocivos de presión mecánica (prueba de Randall-Selitto), ácido acético (prueba de contorsión) y calor (prueba de placa caliente) en ratones. Mientras que la actividad antiinflamatoria se evaluó mediante el edema plantar inducido por carragenina en ratones previamente tratados con Pb. **Resultados:** En los estudios fitoquímicos se detectaron presencia de alcaloides de alta polaridad, esteroides/triterpenoides libres, leucoantocianidinas y taninos. Dosis orales de 30 ($p<0,01$) y 300 ($p<0,001$) mg/kg de *Pb* denotaron un aumento significativo dosis-dependiente en el umbral del dolor, utilizando la prueba de Randall-Selitto y de contorsiones (30; $p<0,05$; y 300; $p<0,01$. Además, concordante con el efecto anti nociceptivo anterior, se denotó un aumento en el tiempo de latencia de reacción después de la administración oral de *Pb* a una dosis de 300 mg/kg ($p<0,05$) en la prueba de la placa caliente. Finalmente, se evidenció una reducción significativa del edema de pata (30 mg/kg; $p<0,01$) inducido por carragenina al 1%, demostrando una potencial actividad antiinflamatoria del *Pb* en comparación con el control positivo del edema. Curiosamente, la actividad antiedematosa del *Pb* mostró una respuesta de intensidad similar en comparación con el grupo tratado con 10 mg/kg de indometacina ($p<0,01$). **Conclusión:** Este trabajo reveló que el extracto crudo de *Pb* posee la capacidad de aumentar el umbral del dolor, compatible con un efecto analgésico, y capacidad de reducir el edema (antiinflamatorio) inducido por carragenina en ratones. Se están realizando más estudios farmacológicos y químicos para dilucidar los mecanismos moleculares y los componentes implicados en los efectos observados.

Palabras clave: *Phoradendron bathyoryctum*, anti nociceptivo, antiinflamatorio, Randall-Selitto, prueba de contorsión, placa caliente, pletismómetro.

1-.INTRODUCTION

Pain is a serious problem for many patients in Intensive Care Units, either because of their pathology or because of the number of therapies or diagnostic techniques to which they are subjected. Most acute or chronic inflammatory pathologies in humans involve pain, which, depending on its intensity, can interfere with the patient's well-being or even incapacitate it (1). Pain has been undertreated for years, among other reasons, due to a lack of knowledge of the link between pain and physical and/or emotional damage or fear of possible side effects of drugs, such as respiratory depression or cardiovascular instability, that opioids can induce. At the same time, during the painful illness and the medical procedures it entails, a level of stress can be generated in the patient that leads to relevant organic and/or psychiatric disorders such as depressive conditions. On the other hand, depression generates neuroendocrine, metabolic, and emotional responses and induces a significant reduction of pain threshold that is detrimental to recovery, especially in those patients who are resistant to treatment and worsens their general condition

Antidepressant medications are effective in treating the pain associated with nerve damage (neuropathic pain). At least one-third of patients with neuropathic pain who took traditional antidepressants (such as amitriptyline), among others (venlafaxine), obtained moderate or better pain relief (2). However, about one-fifth of those taking these pain medications discontinue treatment due to adverse effects. Neuropathic pain can be treated with antidepressants, the effect of which is independent of any effect on depression (3).

P. bathyoryctum Eichler is used in venereal and liver diseases in Paraguayan folk medicine. In addition, it claims to have tonic properties for the heart and central nervous system. Against this background, and taking into account previous studies demonstrating the anxiolytic and antidepressant effect of *P. bathyoryctum* Eichler (4), the idea of exploring its potential analgesic activity in rodents is supported by the fact that antidepressant drugs are used in chronic pain conditions. Therefore, there are academic (generate new knowledge), medical (potential alternative or complementary therapy), socioeconomic (development of new drugs), and innovation support to implement the use of this plant or its derivatives in the generation of well-tolerated and effective phytopharmaceuticals for pain and/or inflammation treatments.

Finally, given the evidence in the scientific literature showing anti-inflammatory activity in several species of the *Phoradendron* genus, the availability of potent antitumor phytopharmaceuticals, and considering the intricate association of depression, pain, and inflammation, it is rational to investigate the potential anti-nociceptive and antiedema activity of crude extract of *P. bathyoryctum* (Pb). Therefore, the present work proposes to evaluate the influence of Pb in acute models of pain and inflammation induced experimentally in mice subjected to acute

oral treatment in order to determine its potential analgesic and anti-inflammatory activity.

2-. MATERIALS AND METHODS

2.1. Plant Material and extract preparation

Whole plants of *P. bathyoryctum* Eichler growing on branches of "Guajayvi" trees (*Cordia americana* (L.) Gottschling & J.S.Mill.) were collected in the Acclimatization Garden of Native and Medicinal Plants of the Faculty of Chemical Sciences, at the National University of Asunción, San Lorenzo, Paraguay. All collected plants were authenticated in the Department of Botany at the Faculty of Chemical Sciences, and an identified voucher was deposited in the Herbarium (FCQ), with code number 4.661, R. Degen and M. Ortiz.

The collected fresh material was dried in an oven at 40°C, then ground (leaves and stems together) to obtain a fine powder. Usually, drying a plant in a shade is always the best. However, in our experience, the fleshy leaves of *P. bathyoryctum* dry very slowly and lead to the growth of fungi (high humidity in Paraguay summer time); therefore, drying by oven at low temperature avoids this inconvenience. A 400 g of this powder was extracted with 2000 mL of a cold mixture of ethanol-water (70:30), sonicated for 15 minutes at 30°C, then allowed to stand for 30 minutes, repeating the process three times, and vacuum filtered. The same procedure was repeated twice more, and the filtrates were pooled, homogenized, and evaporated under reduced pressure by rotary evaporation in a bath at 50°C. The concentrated crude extract of *P. bathyoryctum* (Pb) was frozen and finally lyophilized for use in all biological studies. A total of 41.1 grams of freeze-dried extract was obtained from the 400 grams of powder with a yield of 10.3 %. In addition, crude extract was subjected to qualitative phytochemical profiling and analytical thin-layer chromatography (TLC) on a silica gel-precoated plastic plate, eluted with a mixture of ethyl acetate: formic acid: acetic acid: water (100:11:11:26) and revealed by spraying with appropriate reagents accordingly, and heating at 100°C and then exposed to UV light at 254 - 366 nm wavelength to visualize the spots better (5)

2.2. Animals

One hundred twenty female Swiss albino mice, weighing between 25 and 35 g, from the biotherium of the Department of Pharmacology of the Faculty of Chemical Sciences were used. All animals were maintained in a room with an air-conditioned controlled environment (22-25°C temperature and 55 ± 5% relative humidity) and 12/12 hours fluorescent light/dark cycle. The animals were separated randomly into groups of 6 female mice each, and, the night before the experiments, food was withdrawn to keep 8 hours of fasting condition, with free access to water. The experimental procedures, handling, and treatment of animals were conducted following international Animal Welfare Standards established by the European Community Ethics Committee (6). The experimental protocol was submitted to the institutional Ethics Research Committee of the Faculty of Chemical Sciences and approved on April 14, 2021 (code CEI-703/2021).

2.3. Drugs

Drugs of analytical quality were used. Carrageenan, Indomethacin, and acetic acid were obtained from Sigma-Aldrich (USA), and sodium chloride was acquired from Wako (Japan). Morphine, ethanol, and propylene glycol for pharmaceutical use were obtained from Lasca Pharmaceutical Company (Paraguay).

2.4. Pharmacological assays

P. bathyoryctum extract (Pb) was dissolved in 0.9% saline for oral administration to mice. Indomethacin (10 mg/kg, b.w.) was dissolved in a solution containing 10% ethanol, 40% propylene glycol, 50% distilled water (V/V) and with the addition of 0.05 mL of 1% of sodium bicarbonate buffer. This vehicle is used in pharmaceutical formulations to dissolve low-solubility drugs intended for injectable parenteral therapy in humans (Diazepam, Indomethacin, etc.) and was administered as negative control in all experiments. Morphine, dissolved in saline, was used at a dose of 6 mg/kg. The doses of Pb used were selected from previous work performed in our laboratory (4). The volume of all samples administered was made at a dose of 0.1 mL per 10 g body weight (b.w.).

2.4.1. Assessment of the analgesic activity of Pb.

2.4.1.1. Determination of the influence of oral administration of Pb on caudal mechanical pressure-induced painful stimulus in mice (Randall-Selitto Test).

Female Swiss albino mice (25-35 g b.w.) were randomly selected into 5 groups of 6 mice each. Group 1 (positive control) received 10 mg/kg of Indomethacin (p.o.); groups 2, 3, and 4 were

treated orally with 3, 30, and 300 mg/kg of Pb, respectively. Group 5 (negative control) received the vehicle (0.1 mL/10g of b.w.; p.o.). After one hour of the treatments, the caudal sensitivity to noxious pain stimuli induced by mechanical pressure was measured (7) using an analgesy meter (Randall-Selitto Test). The midpoint of the tail, previously marked with ink, was submitted to increasing mechanical pressure with an automatized device. The maximal applied mechanical pressure was limited to 250 g to avoid caudal injury.

2.4.1.2. Determination of the influence of oral administration of Pb on chemically induced painful stimulus in mice (Writhing test).

A trial was conducted using female Swiss albino mice (25-35 g b.w.) divided randomly into 5 groups of 6 mice each. Group 1 (positive control) received 10 mg/kg of Indomethacin (p.o.); groups 2, 3, and 4 were treated orally with 3, 30, and 300 mg/kg of Pb, respectively. Group 5 (negative control) received the vehicle (0.1 mL/10g of b.w.; p.o.). All animals were sequentially injected intraperitoneally one hour after the treatments with 0.8% acetic acid diluted in saline. Contortions were counted every 5 minutes for 30 minutes (8,9).

2.4.1.3. Determination of the influence of oral administration of Pb on the reaction latency to thermally-induced painful stimulus in mice (hot plate).

The experiment was conducted using female Swiss albino mice (25-35 g b.w.) divided aleatorily into five groups of 6 mice each. Group 1 (positive control) received 6 mg/kg of morphine (i.p.); groups 2, 3, and 4 were treated orally with 3, 30, and 300 mg/kg of Pb, respectively. Group 5 (negative control) received the vehicle (0.1 mL/10g of b.w.) by oral route. After 60 minutes, the animals were individually placed on a hot plate (56°C) device in their corresponding sequence (10). The reaction time of the animal to the thermal stimulus is characterized by the licking behavior or raising of the paws, considered a signal either of nociception or time to remove the animal from equipment. The maximum time the animal remained on the hot plate was 30 seconds to avoid injury (8,11).

2.4.2. Evaluation of the anti-inflammatory activity of the Pb

Determination of the influence of oral administration of Pb on carrageenan-induced paw edema in mice.

The experiment was conducted using female Swiss albino mice (25-35 g b.w.) divided arbitrarily into five groups of 6 mice each. Group 1 (positive anti-inflammatory control) received 10 mg/kg of Indomethacin (p.o.); groups 2, 3, and 4 were treated orally with 3, 30, and 300 mg/kg of Pb, respectively. Group 5 (negative control) received the vehicle (0.1 mL/10g of b.w.) by oral route. After 1 h of treatment, the animals were injected in the sub plantar region (s.p.) of the right hind legs with 40 µL of Carrageenan (1 %) except for the saline-treated animals, which constitute the blank group and were therefore not injected with Carrageenan, to validate the method for edema formation (12). Paw volume measurements were recorded immediately before, at 30 minutes, and up to 3 h after carrageenan injection. Briefly, the procedure consisted of immersing the rear legs up to the lateral malleolus inside the digital plethysmograph vessel (LE 7500 Panlab, Harvard Apparatus, Spain). The displaced volume is automatically recorded and tabulated as an individual value and associated with edema (8,11,13). The volume differences between legs were considered the edema value for each animal.

2.5. Statistical Analysis

The results obtained in the different experimental groups under study were expressed as mean ± standard deviation. The statistical analysis of the data was performed by applying parametric ANOVA followed by Tukey multiple comparison tests using the software GraphPad Prism 7.0; $p < 0.05$ was considered statistically significant.

3-. RESULTS

3.1.- Qualitative phytochemical analysis of crude extract.

The qualitative phytochemical profile of the hydro-alcoholic extract of *P. bathyoryctum* showed the presence of high polarity alkaloids, steroids/ free triterpenoids, leucoanthocyanidins, and tannins, as seen in Table 1. Data on TLC are not shown.

Table 1. Preliminary phytochemical analysis of crude extract of *P. Bathyoryctum* (*Pb*)

Chemical groups	Assay	HCl 5%	Soluble in $CHCl_3$	Soluble in $CHCl_3$-Et-OH	Phenolic alkaloids	Quaternary ammonium/ amine oxide
Alkaloids	Dragendorff	++	-	+	+	-
	Mayer	+	-	+	-	-
	Valser	++	-	+	-	-
	Reineckate	-	-	-	+	-
Flavonoids	Cyanidin	-				
	HCl 10%	+				
Nafto and/or Anthraquinone	Borntrager-Kraus	-				
Tannins	Gelatine-salt	+				
	$FeCl_3$	+				
Saponins	Foam	-				
Steroids y/o triterpenoids	TLC + Liebermann-Burchard.	+				
			Plate W Vanillin	Plate X Hydroxamate	Plate Y Raymond	Plate Z Vanillin
Coumarins						
Cardiotonic			-	-	-	-
Terpene's lactones						

3.2. Effect of *Pb* on mechanical pressure-induced pain response in mice (Randall-Selitto test).

In the Randall-Selitto test, a statistically significant increase in pain threshold to noxious mechanical pressure was observed with doses of 30 (558.0 ± 136.2 g; $p<0.01$; 60%) and 300 (616.5 ± 169.2 g; $p<0.001$; 77%) mg/kg of *Pb*, when compared to the vehicle-treated group (349.1 ±124.2 g) respectively, compatible with a potential analgesic effect and shown in figure 1. The lower sensitivity to a mechanical pain stimulus is dose-dependent, and the higher dose of *Pb* represents 92% of the maximal intensity of the positive control indomethacin. However, the dose of 3 mg/kg of *Pb* has no significant effect (343.6 ± 113.1 g) on the mice's pain threshold when compared to the vehicle-treated group. The statistically significant increase in pain threshold caused by 10 mg/kg indomethacin (666.6 ± 122.9 g; $p<0.0001$), positive control, compared to the blank group, effectively validates the method used.

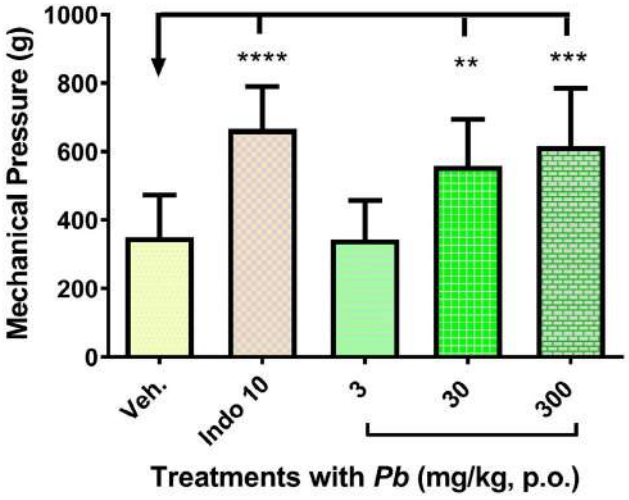

Figure 1. Effect of Pb on mice submitted to the caudal mechanical pressure test (Randall-Selitto). 1h before the application of mechanical noxious stimuli, the animals were subjected to administration of vehicle (Veh.), Indomethacin (Indo), and doses of crude extract of *P. bathyoryctum (Pb).* The bars represent the means ± standard deviation. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. N=6, ****$p<0.0001$; ***$p<0.001$; **$p<0.01$ significantly different from the vehicle-treated group.

3.3. Effect of *Pb* on chemically-induced painful stimulus with acetic acid in mice (Writhing test).

Figure 2 shows that the groups of animals orally treated with doses of 30 (32.29 ± 19.73; 44%; $p<0.05$) and 300 mg/kg (26.25 ± 7.87; 55%; $p<0.01$) of *Pb* showed a significant reduction in the number of abdominal contortions in comparison to the vehicle-treated group (57.67 ± 11.17), compatible with analgesic activity. Likewise, a statistically significant reduction in the number of abdominal contortions of 76% was induced in the group of animals orally treated with 10 mg/kg of Indomethacin (14.0 ± 4.0; $p<0.001$) when compared to the vehicle group, which validates the experiment for the detection of substances with a potentially analgesic activity. In contrast, the 3mg/kg dose of *Pb* did not modify the number of abdominal contortions (33.6 ± 16.9) induced by acetic acid as a noxious stimulus.

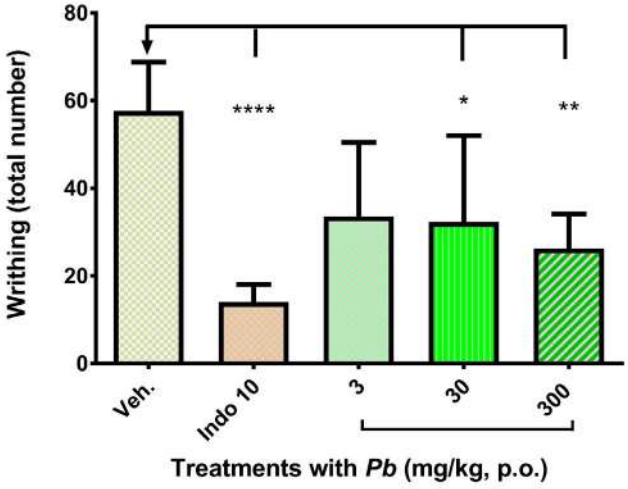

Figure 2. Effect of Pb on mice submitted to 0.8% acetic acid-induced abdominal contortions (writhing test). 1h before the application of chemical noxious stimuli, the animals were subjected to administration of vehicle (Veh.), Indomethacin (Indo), and doses of crude extract of *P. bathyoryctum* (Pb). The bars represent the means ± standard deviation. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. N=6, ****$p<0.0001$; **$p<0.01$; *$p<0.05$ significantly different from the vehicle-treated group.

3.4. Effect of Pb on thermally-induced noxious pain stimulus in mice (Hot Plate Test).

The oral dose of 300 mg/kg Pb showed a significant increase of 155% of the latency time in the reaction to the thermal painful stimulus (16.25 ± 3.67 sec; $p<0.05$) compared to the vehicle-treated group (6.37 ± 1.44 sec) and is visualized in figure 3. In the same sense, a statistically significant difference was observed between the latency time of the group of animals treated with 6 mg/kg morphine (26.85 ± 4.45 sec; $p<0.001$; positive analgesic control) increased by 322% compared to the group of animals treated with the vehicle (6.37 ± 1.44 sec; 0.9% saline), validating the method used. However, groups of animals treated orally with 3 (10.96 ± 6.73 sec) and 30 (11.64 ± 5.20 sec) mg/kg of Pb did not induce significant modifications in the latency time of reaction to the thermal painful stimulus when compared to the animals treated with the vehicle (6.373 ± 1.44 sec).

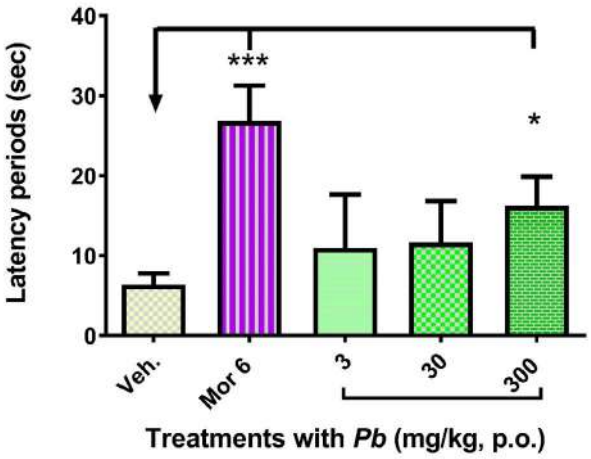

Figure 3. Latency periods variation of mice subjected to the painful thermal stimulus reaction test (hot plate). 1h before the application of thermal noxious stimuli, the animals were subjected to administration of vehicle (Veh.), Morphine (Mor), and doses of crude extract of *P. bathyoryctum (Pb).* The bars represent the means ± standard deviation. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. N=6, ***$p<0.001$; *$p<0.05$ significantly different from the vehicle-treated group.

3.5. Effect of oral *Pb* administration on carrageenan-induced plantar edema in mice.

After 3 hours of the sub-plantar injection of Carrageenan, the group of animals orally treated with 30 mg/kg Pb (5.50 ± 3.42 µL; **$p<0.01$) showed a significant anti-edematous (anti-inflammatory) capacity of 51 % (anti-inflammatory) concerning the positive edema control group (Carrageenan; 11.14 ± 2.73 µL); as can be seen in figure 4. The doses of 3 (8.29 ± 2.69 µL) and 300 (10.00 ± 2.27 µL) mg/kg of Pb did not significantly modify the edematous capacity of Carrageenan. On the other hand, a statistically significant difference between the volume of plantar edema induced by Carrageenan (11.14 ± 2.73 µL; ***$p<0.001$) when compared to the vehicle-treated group (2.75 ± 2.50 µL) was observed. The last fact thus validates the edema induction method used. Furthermore, a significant anti-

edema difference of 50% between those treated with 10 mg/kg of Indomethacin (5.56 ± 2.88 µL; **p<0.01) concerning the edema positive control group (Carrageenan-induced) was observed. Thus, validating the anti-edema capacity of Indomethacin and certifying the sensitivity and efficiency of the method for the determination of substances with anti-inflammatory properties.

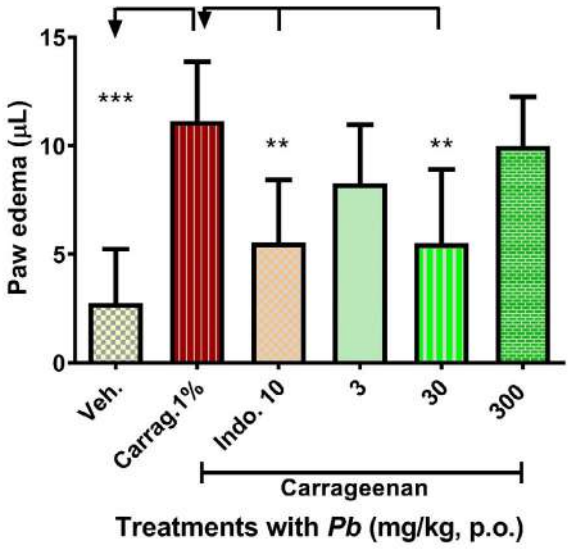

Figure 4. Variation of the volume, in µL, of plantar edema induced by Carrageenan and determined 3 h after phlogistic stimulation, in groups of animals previously treated (1h before) with vehicle (Veh.), Carrageenan (Carrag. 1%), Indomethacin (Indo), and doses of crude extract of *P. bathyoryctum* (Pb). The bars represent the means ± standard deviation 3 hours after the administration of the Carrageenan. The statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison test. N=6, ***p<0.001, significantly different from the vehicle-treated group, and **p<0.01, significantly different from the carrageenan-treated group

4-. DISCUSSION

This study evaluates the anti-nociceptive and anti-inflammatory activity of orally administered hydro-alcoholic extract of *P. bathyoryctum* (*Pb*), which we have recently reported as safe, well-tolerated, and having antidepressant activity in mice (4).

In spite of some controversy, the International Association for the Study of Pain (IASP) does define pain as "an unpleasant sensory or emotional experience associated with actual or potential tissue damage" (14–16). Pain usually can be considered pragmatically as nociceptive, inflammatory, and pathological pain (17). Nociceptive pain is usually caused by the normal activation of peripheral nociceptors ("alarm") to protect the body against noxious stimuli, characterized by a high threshold to pain, and often accompanied by a withdrawal reflex. The inflammatory pain is caused by tissue damage and is activated by the infiltration of inflammatory mediator cells (macrophages, neutrophilic mast cells, and granulocytes) it is characterized by a low pain threshold. Finally, the pathological pain (neuropathic and dysfunctional) occurs as a consequence of nervous system injury (central or peripheral) or abnormal central processing respectively (1,17). Pain is considered an unpleasant subjective experience that cannot be easily measured. A complex set of pathways transmits pain messages from the periphery to the central nervous system, where control occurs from higher centers. Nociception is the transmission of potentially noxious stimulus to the brain via neural pathways, and signaling systems. Primary afferent pain fibers synapse with second-order neurons in the dorsal horn of the spinal cord. Ascending spinothalamic and spinoreticular tracts convey pain signals to the brain, where they are processed by the thalamus and sent to the cortex. Descending pathways (via the midbrain periaqueductal grey and nucleus raphe magnus) modulate the pain from higher centers, which can both increase or decrease the severity. These mechanisms involve both peripheral and central sensitization and as a whole pain experience is influenced by psychological, biological, and social factors (18). Cells with ON- and OFF properties were recently identified in rostral ventromedial spinal cord circuits for brainstem pain modulation. Functional pain disorders excessively affect women, but most of the preclinical pain research in animals has been conducted in males and very little in females. Furthermore, both ON and OFF cells showed a sensitized response to somatic stimuli in females subjected to persistent inflammation, and ON and OFF cells responded to systemically administered morphine in a dose sufficient to produce behavioral antinociception, and is the rationale for the use of females animals in studies the descending modulation of pain (19).

According to the scientific information accessed, the genus *Phoradendron* reports the presence, among others, of pentacyclic triterpenic acids, polyphenols, and small proteins known as thoinins (20). In addition, triterpenoids (oleananes, lupanes, ursanes, and sterols) and flavonoids are the typical families of compounds in European and American mistletoe and related to its biological activities (21). More than 50 different compounds were detected in samples of *P. microphyllum* and *P. mucronatum* from Brazil. Among others, the presence of

alkaloids, diterpenes, triterpenes, sterols, alcohols, aldehydes, fatty acids and hydrocarbons were detected (21). Likewise, *P. piperoides showed the presence of alkaloids, flavonoids*, saponins, tannins, and triterpenes (22). The *Phoradendron* genus is also in the sights of scientists as a source of new materials such as mucilage viscine (highly branched non-cellulosic polysaccharide) with relevant pharmaceutical potential as a thickener among other properties (23,24). For all the above, it is clear that the complexity of the analysis of biological activities found in the *Phoradendron* genus is not only due to the different species but also to the diversity of components and their variability according to the seasonal time of collection (25).

A postpartum antihemorrhagic activity is ascribed to *Pb* by The Caboclo river-dwellers. (26). *Pb* showed anxiolytic and antidepressant capacity and given the duality between depression/pain or pain/depression, we have considered its pharmacological evaluation appropriate using preclinical models of experimental pain and inflammation in mice. In the Randall and Selitto trial, the influence of oral administration of *Pb* showed an efficient capacity to increase the pain threshold, concordant with analgesic effect at doses of 30 and 300 mg/kg. The pain threshold was increased by 77% with the highest dose of *Pb* compared to the vehicle-treated group, and moderate in intensity compared to the effect obtained with Indomethacin (analgesic reference drug). The lower dose of *Pb* (3 mg/kg) did not modify the pain threshold in mice submitted to mechanical pressure-induced nociceptive pain.

Independently, the significant reduction in the number of abdominal contortions provoked by *Pb* (30 mg/kg, 44%; and 300 mg/kg, 55%; respectively) to the painful chemical stimulus (acetic acid) concerning the control group (100 %), reinforces that *P. bathyoryctum* possess the significant anti-nociceptive capacity but of lower intensity compared to reference drug Indomethacin-treated group, which showed a potency of 75% compared to the vehicle. Curiously, methanolic extract of *P. piperoides* had manifested the same dose-dependent tendency either in mechanical pressure (Randall-Sellito) or chemically-induced pain behavior (Writhing tests) when evaluating the anti-nociceptive capacity in mice (22). Likewise, in the thermally-induced pain behavior (hot plate test), it could be seen that the highest dose of *Pb* used (300 mg/kg) showed a potent capacity to increase the pain threshold by 155% in comparison to the vehicle-treated group. This was evidenced by an efficient increase of latency time before the jump of the hot plate or plantar licking compatible with the analgesic effect of the maximum dose of *Pb* (300 mg/kg). However, the effect intensity was lower (48%) when compared to the reference drug used, Morphine (100%). Indeed, Morphine caused an increase in the pain threshold by 322% compared to the vehicle-treated group. It should be noted that Morphine and congeners are agents with the ability to efficiently modulate pain pathways triggered by thermal stimuli and regulate them at the level of the central nervous system, unlike Indomethacin, which only regulates peripheral pain.

In contrast, none of the doses of *P. piperoides* extract used showed a significant anti-nociceptive effect in mice submitted to the hot plate test (22). Other studies with *P. piperoides,* disagree with the above results since tests in acute models (chemical and thermal stimuli) and persistent pain model (formalin test) showed a decrease in the pain threshold, pointing to a lack of anti-nociceptive activity (27). Logically, the lack of coincidence may be due to many factors. Among others, we can mention the animal's sensitivity, the extraction procedure, the region and season time of collection, and the type of tree or substrate on which the species under study is growing. The bioavailability of crude extract has never studied and is another factor to consider in future studies. However, it can be accepted that extracts have adequate absorption by the oral route because a relevant effect has been noted in previous and present work. Almost all of the above-mentioned factors may generate variations in the components of the final extracts.

Remarkably, *Pb* (30 mg/kg) was able to reduce the acute edema caused by sub-plantar injection of Carrageenan 3 h after administration by 51%, being slightly more potent than the reference drug (10mg/kg Indomethacin; 50%). Carrageenan-induced paw edema in the mouse is associated with nociceptive changes in the paw and migration of inflammatory cells to the injection site (17,28) and is sensitive to non-steroidal anti-inflammatory drugs such as Indomethacin. (8,29). Further, this finding is concordant with anti-inflammatory activity of *Pb*.

The molecular mechanism by which *Pb* increases the pain threshold and reduces edema is unknown. Considering new and broad scientific advances in pain research, it can be hypothesized that the effects induced by *Pb* may result from peripheral or central modulation. Certainly, it could be potentially due, among others, to modulation of transient receptor

potential channels, inhibiting cyclooxygenase activities (COX-1 and COX-2), regulating oxygenated free radicals' level, or reducing oxidative stress or cellular infiltration. The molecular mechanism of mechano-sensation is not completely understood. Some members of transient receptor potential (TRPA) are probably directly activated by mechanical forces, altering the distribution of ions involved with the mechano-transduction of pain. Interestingly, the release of reactive compounds or intracellular calcium, induced by mechanical damage, could indirectly activate transient receptor potential toward modulation of mechanosensory signaling instead of a mechano-transduction system (30,31). It is known that the nociceptive response begins when the primary sensory fibers are activated by a noxious stimulus (chemical, thermal, or mechanical). Therefore, some of the TRP channels (TRPV1 and TRPA1), are transducers of both thermal and mechanical stimuli, acting as molecular integrators for a range of diverse noxious stimuli, where *Pb* or compound (s) present(s) in the extract may interact, and mediate the observed analgesic or anti-inflammatory effect (32). Besides, the presence of high polarity alkaloids, steroids/ free triterpenoids, leucoanthocyanidins, and tannins are related to antioxidant and free radical scavenger activities (33).

Likewise, the activation of cyclooxygenase 2 (COX-2), elevated prostaglandin production (by the action of cyclooxygenases 1 and 2, COX-1 and COX-2), elevated oxygenated free radicals (NO derived from eNOS and iNOS) and neutrophil infiltration, recently has been recognized as associated with the second phase of the inflammatory response after carrageenan injection into the paw of mice (28,33–35). Also, oxidative stress has been associated with a huge of disturbs, including among others, the metabolic imbalance (carbohydrate and lipid metabolism), and inflammation, which are conditions associated with noncommunicable diseases (NCDs) pathophysiology, including diabetes mellitus, cancer, and cardiovascular diseases that are leading global causes of death (33). Therefore, as mentioned above, Pb shows anti-inflammatory activity by reducing the second phase edema and it remains to be determined which of the above-mentioned pathways are affected and which component(s) are involved in and inflammation. In the same sense, work with other species of the genus *Phoradendron* using the same nociceptive model obtained similar results, but with a lesser anti-inflammatory effect (22,36).

Due to the aforementioned, and given the complexity and molecular diversity of the response to noxious stimuli, the study of pain in animals involves limitations in accurately measuring physical damage and the intensity of emotional involvement. Accordingly, it should be noted that no test can therefore measure pain in animals directly. The presumably unpleasant emotional experience of pain is inferred from pain-like behaviors which can include the withdrawal of a body part from a stimulus, reduced ambulation, agitation, an increase in grooming of the affected area, and vocalizations upon sensory stimulation. The distinction between nociception and pain thus underlines a key difference in terminology when referring to communicating and non-communicating subjects. Similarly, as it cannot be said that the animal feels pain, analgesia and analgesic intervention cannot take place, only anti-nociception and anti-nociceptive interventions can be made experimentally. Consequently, as pain cannot be directly measured in rodents, it has been necessary to develop indirect methods to quantify and evaluate pain-like behaviors in non-anesthetized animals which are reliable, reproducible, sensitive and specific (37). Finally, our report on the efficient anti-nociceptive and anti-edematous properties of *P. bathyoryctum* increases the knowledge and literature available on this natural resource potentially useful in human health for pain and inflammation treatments but requires additional specific chemical and biological studies.

5-. CONCLUSION

Presences of high polarity alkaloids, steroids/ free triterpenoids, leucoanthocyanidins, and tannins were detected in phytochemical studies. The pharmacological study showed that oral administration of crude extract of *P. bathyoryctum (Pb)* in mice revealed an efficient anti-nociceptive and anti-edematous capacity in the pre-clinical models used. In acute noxious stimulus applied to peripheral nociceptor models (mechanical pressure, chemical, and thermal-induced pain assays), an effective dose-dependent pain threshold increase was observed in mice orally treated with *Pb*, concordant with significant analgesic activity. Correspondingly, *Pb* showed the capacity to reduce Carrageenan-induced edema with a comparable potency of Indomethacin that is compatible with the anti-inflammatory effect. Finally, additional studies are needed to elucidate in detail the specific mechanism of action of the extract and the structure(s) of the bioactive molecule(s) involved in the above-observed effects.

Conflict of interest: All authors have none to declare

AUTHORS' CONTRIBUTIONS

This research was initiated and developed by MOR and MCH-I. OYH, and WA, were involved in the design of the study, the experimental implementation, evaluation of the data, and making a review of the preliminary written manuscript. MOR elaborate extract, and RDA performed botanical studies. MCH-I and DAI were involved in coordinating the study, supervising the work, and involved in writing the final form of the manuscript. All authors read and approved the final manuscript.

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