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ANALGESIC ACTIVITY AND PHYTOCHEMICAL PROFILE OF AQUEOUS, ETHANOL AND DICHLOROMETHANE EXTRACTS OF *PERSEA SCHIEDEANA* LEAVES

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ABSTRACT: Traditionally *Persea schiedeana* is used for headache relief, as anti-hypertensive, nervous relaxant, dermal diseases, among others. In this study, this plant from Salvadorian flora acute toxicity was evaluated *in-vivo* at 500 mg/kg, and the analgesic activity was tested by acetic acid, formalin, and tail flick tests in NIH Swiss mice at doses of 100, 250, and 500 mg/kg body weight, using as a positive control Indomethacin 10 mg/kg and distilled water as a control. Secondary metabolite profile was performed by Thin Layer Chromatography (TLC) and ultra-efficient liquid chromatography-mass spectrometry (UPLC-MS). It was determined by TLC, the presence of terpenoids, flavonoids, tannins, lactonic groups, and coumarins, while UPLC-MS confirmed the presence of scopoletin. The biological assays demonstrated that *P. schiedeana* was deprived from toxic effect at the doses tested and the analgesic activity was confirmed in aqueous, ethanol, and dichloromethane extracts of *Persea schiedeana* leaves. Additionally, it was detected the presence of terpenoids, flavonoids, condensed tannins, lactones, and coumarins, and by UPLC-MS, the identity of coumarin as scopoletin was confirmed. These results are in accordance with the traditional use for pain relief.

INTRODUCTION: Pain is a complex defense process, which indicates damage to a tissue, the sensitive perception of which is subjective, unpleasant, and with a large emotional component. Furthermore, it is a problem that people face every day around the world. The pain can be acute or chronic.

The intensity of pain is subjective due to the differences in the perception and expression of the painful sensation by each patient [1]. The pain-causing stimuli, *noxas*, are detected by specific sensory receptors called nociceptors, which are nerve endings with cell bodies in the dorsal root ganglia ending in the dorsal horn of the spinal cord and that selectively respond to stimuli [2, 3].

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Tissue damage causes the release of numerous chemical agents such as leukotrienes, bradykinins, serotonin, histamine, potassium ions, acids, acetylcholine, thromboxane, substance P, platelet-activating factor, among others. These agents are important factors in the development of continuous pain after acute damage. Prostaglandins are local mediators or cofactors that increase the sensitivity of nerve endings and are closely related to the inflammatory process [2]. The groups of drugs that currently form the basic pillars of analgesic treatment are opioids and steroidal and non-steroidal anti-inflammatory drugs, NSAIDs [4].

NSAIDs are one of the most prescribed groups of drugs and have a wide variety of therapeutic indications [5]. The use of these drugs is limited by the possible appearance of adverse effects that have been known since their introduction [5, 6, 7]. Among the most frequent complications are gastric ulceration and liver damage caused by prolonged use or by overdose.

The use of medicinal plants for the treatment of pain has shown promising results and, in general, has low cost and a minimum of adverse effects [8, 9, 10]. Previous studies indicate that the genus *Persea* shows analgesic activity in *in-vivo* tests in animal models, like conventional therapeutic treatments such as NSAIDs [11]. Various secondary metabolites of this genus have been reported, such as alkaloids, terpenoids, flavonoids, and coumarins, which possess biological activities such as anti-inflammatory, antiparasitic, antifungal, and insecticidal, among others [12, 13].

Persea schiedeana is a tree belonging to the Lauraceae family, which is widely distributed in the Central American region and has ethno-botanical uses for hypertension [14], intestinal parasites, diarrhea, vomiting, headache, nervous relaxant [15], skin diseases, among others. The present study focused on determining the safety of use of *P. schiedeana* leaves, the phytochemical analysis of the extract, and the analgesic activity in mice to provide data that contributes to the scientific validation of popular use in pain medication.

MATERIALS AND METHODS:

Plant Material and Extraction: The leaves of *Persea schiedeana* Nees (Lauraceae) were collected in the Canton El Jocotón, Municipality of Coatepeque, Department of Santa Ana, El Salvador, identified by Jenny Elizabeth Menjívar Cruz, and a specimen of the herbarium was deposited in the herbarium from the Museum of Natural History of El Salvador (J Menjivar-Marvin J Núñez 4205). The leaves were dried at 40 °C for 56 h in a circulating air oven, and then they were ground. Powdered leaves were extracted separately with water (80 g, 2 × 800 mL), ethyl alcohol 95º (100 g, 2 × 1000 mL), and dichloromethane (125 g, 2 × 1200 mL) in a bath VWR ultrasound (Model 97043-988) at room temperature (25 °C) for 120 minutes. A part of each extract (75 mL) was reserved for performing the phytochemical analysis, and the other was concentrated using a rotary evaporator (KNF RC-600) at 40 °C, then it was transferred to a desiccator until the dry extracts were obtained.

Phytochemical Analysis: Silica gel G UV254 was used as the stationary phase for the phytochemical analysis of the extracts by thin-layer chromatography. 20 µL of the sample was placed on the baseline and eluted up to 1 cm before the top edge.

Alkaloid Identification: it was eluted in acetone-ethyl acetate-methanol (9:0.6:0.4), hyoscyamine sulfate was used as a control, and it was visualized with Dragendorff's reagent. The appearance of orange spots indicated the presence of alkaloids[16].

Anthraquinone Identification: the plate was eluted in n-hexane-ethyl acetate (1:1), detected with a 10% solution of KOH in methanol. The appearance of red spots indicated the presence of anthraquinones [17].

Identification of Flavonoids: eluted in ethyl acetate-formic acid-water (8: 1: 1), acetone-quercetin was used as a control. The appearance of fluorescent yellow spots in UV-365 nm light after the addition of 1% aluminum trichloride in ethanol was considered as positive evidence of the presence of flavonoids [16].

Identification of Cardiotonic Glycosides: ethyl acetate-methanol-water (8: 1.5: 0.5) was used as the mobile phase, and control k-strophantidine was used. The plate was developed with a solution of Kedde's reagent (same volumes of 2% 3,5-

dinitrobenzoic acid in methanol and 5.7% KOH in water). The appearance of violet spots indicated the presence of cardiotonic glycosides [17].

Terpenoid Identification: n-hexane-ethyl acetate (1: 1) was used as the mobile phase, it was developed with Komarowski's reagent (50 mL of 2% 4-hydroxybenzaldehyde in methanol and 5 mL of 50% sulfuric acid) and heated (110 ºC) for 5 min. Epifriedelanol and β-sitosterol were used as controls for the triterpene and steroidal nuclei, respectively. Purple pink and blue-green spots, respectively, evidenced the presence of triterpenes and steroids [17].

Identification of Sesquiterpene Lactones: n-hexane-ethyl acetate (7: 3) was used as mobile phase, with 2,3-epoxyjuanislamine as control and Baljet's developer reagent (equal volumes of 1% picric acid in ethanol 95° and 10% sodium hydroxide). Orange spots were taken as positive evidence [17].

Coumarin Identification: n-hexane-ethyl acetate (7: 3) was used as mobile phase, with 6,7-dihydroxy-4-methylcoumarin as control and it was developed with 5% KOH in methanol. The appearance of fluorescent blue spots in UV-365 nm light indicated the presence of coumarins [16].

Identification of Saponosides: the foam test was used. 1 mL of the extract was placed in a test tube, and 9 mL of distilled water was added; the test tube was shaken vigorously (vertically) for 30 seconds, allowed to stand for 15 min. The presence of saponins was evidenced when the foam reaches more than 0.5 cm in height [17].

Tannin Identification: 2 ml of the ethanolic extract were placed in 3 test tubes, 5-10 drops of 10% gelatin were added to the first tube, 5-10 drops of 10% quinine hydrochloride, and one drop were added to the second tube. of 5% $FeCl_3$ in methanol to the third. The appearance of a precipitate in the first two tubes indicated the presence of tannins, and in the third tube, a blue coloration indicated hydrolyzable tannins; and a green color indicated condensed tannins [17].

UPLC-MS Characterization: The characterization of the aqueous and organic extract of *P. schiedeana* was performed using ultra-efficient liquid chromatography-mass spectrometry (UPLC-MS). 1 mg of extract was dissolved in 1 mL of the respective solvent (water, ethanol, and dichloromethane). Then, 0.1 mL was added to 0.9 mL of methanol to be analyzed by UPLC with an ACQUITY QDa Waters mass detector (Milford, MA, USA), under the following conditions, column: ACQUITY UPLC® CORTECS® C18, 1.6 µm, 3.0 × 100 mm; mobile phase A (0.1% formic acid in water), mobile phase B (methanol) and C (5mM ammonium acetate) with a flow gradient (0.5 min, 5.0% A: 85% B: 15%; 3.5 min, 15% A: 75% B: 10% C; 6.5 min, 5% A: 85% B: 10% C; 10.5 min, 90% A: 10% B; 15.0 min, 50% A: 50% B; 18 min, 10% A: 90% B); total running time of 8 min; flow of 0.3 mL / min; injection volume of 1 µL and column temperature of 40 °C.

Experimental Animals and Ethical Considerations of *in-vivo* Testing: Male Swiss albino mice from the Animal Experimentation Laboratory (LEA) at the Centro de Investigación y Desarrollo en Salud (CENSALUD) of the Universidad de El Salvador (UES), and the Facultad de Ciencias Químicas of the Universidad Nacional de Asunción (UNA) in Paraguay. They were kept in a controlled environment (22 ± 2 ° C), in 12/12 h light and dark cycles, fed with a commercial diet and water *ad libitum*. Animals were randomly placed in different groups and received intragastric treatments. The experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care for the care and use of experimental animals [18] and the Standard Working Procedure of the Animal Experimentation Laboratory (LEA) of the Universidad de El Salvador based on protocol 423 of the OECD [19]. Animals were considered biological reagents and were managed by standardized procedures. Each animal was used once and then was euthanized. The minimum number necessary and the shortest observation time required to obtain consistent data were used for each trial.

Acute Toxicity Test of Extracts: Four groups (n = 5) was formed, a control group, which was administered distilled water, and three groups that received the ethanolic, aqueous or dichloromethane extract from the leaves of *P. schiedeana*. Animals were observed after administration, at least for the

first 30 min, periodically for the first 4 h, then daily until day 14. Body weights were recorded at baseline and at 7 and 14 days. Finally, dissection was performed for macroscopic analysis and weight of internal organs [19].

Anti-nociceptive Activity: For each test, three groups of animals treated by extract (aqueous, ethanolic, and dichloromethane from *P. schiedeana*; 100, 250 and 500 mg/kg, po) were used (n = 10), in addition to a control group (water) and a positive control group (indomethacin, 10mg/kg). After each test, the percentage of pain inhibition was calculated by measuring the response variables: number of contortions, accumulated time in seconds of paw licking, and the centimeters traveled per gram of weight; in the acetic acid, formalin, and glue removal tests respectively [11, 20].

Acetic Acid Test: The animals received the corresponding treatment one hour before the administration of acetic acid (0.1 mL, 0.8%, ip), and five minutes later the number of abdominal contortions in all the animals of the groups was counted for 30 min [21].

Formalin Test: One hour before the formalin injection, the groups received the treatments, the painful stimulus was induced by the injection of 20 µL of 1% formalin in the left hind leg of the animal. The accumulated time (seconds), during which the animal licked its paw in two moments, was measured. The first moment was from 0 to 5 min and the second moment from 15 to 30 min [22, 23].

Caudal Pressure Test: Half of the total length of the animal's tail was marked. After administration of the treatments, the animals were placed in consecutive order, each in an immobilizer, and the marked part of the tail was placed at the pressure point of the Randall-Selitto analgesiometer [24]. The reaction to pressure was measured at 30, 60, 90 and 120 min. Thereafter, the measurement of cursor displacement, up to the time of the reaction to pain, was conveniently tabulated according to time.

Statistical Analysis: A normality test was performed on all the results; in the toxicity study, the results of percentage increase in body weight and organ weight were subjected to a comparative analysis of means for independent samples. The results of the evaluation of analgesic activity were statistically analyzed using a one-way analysis of variance (ANOVA); followed by a Tukey test, using the Graphpad Prima® 7 software. The difference between the treated groups and the control group (distilled water) was considered significant when the value of $p < 0.05$.

RESULTS AND DISCUSSION: After phyto-chemical analyzes were carried out on *P. schiedeana*, the presence of condensed tannins, lactones, and coumarins were identified in the three extracts, aqueous, ethanolic, and dichloromethane. The presence of terpenoids in the ethanolic and dichloromethane extracts and the presence of flavonoids in the ethanolic extract were confirmed. By UPLC-MS, the identity of coumarin as scopoletin was confirmed (retention time 1.1 min; molecular weight 192.18 g/mol) in the three extracts. This metabolite was previously reported in the leaves of *Persea americana*, *P. obovatifolia*, and *P. caerulea* [25, 26, 27].

The search for signs and symptoms of toxicity showed that the extracts evaluated did not cause visible alterations, nor did they cause mortality in the animals. Similarly, the weight of the organs: liver, heart, lungs, kidneys (right and left), stomach, spleen, and small and large intestines, were not significantly different, so it is presumed that *P. schiedeana* extracts can be used safely for studies of anti-nociceptive activity. Previous studies for the genus *Persea* are in line with these results since no evidence of organic damage indicating toxicity has been reported either [28, 29, 30].

Anti-nociceptive Activity: Treatment of mice with *P. schiedeana* extracts significantly reduced the number of abdominal contortions in all treated groups; with inhibition values between 36 and 68%, compared to 62% inhibition observed in the group treated with the reference drug, indomethacin **Table 1**. According to these results, the analgesic activity of the extracts of the leaves of *P. schiedeana* was evident, considering that this test allows the detection of analgesic agents with central and peripheral action [31]. Furthermore, these results indicated a possible anti-inflammatory activity of the extracts, since acetic acid causes injury to the peritoneal cell membranes, generating the release of pro-inflammatory prostanoids [32],

therefore the reduction of abdominal contortions is an indication of the reduction in the release of these mediators, and agrees with the presence of scopoletin, a coumarin widely described for its anti-inflammatory activity [33, 34, 35].

As for the formalin test, the biphasic nociceptive response responds to many classes of analgesic drugs. The formalin response mimics some characteristics of post-injury pain and consists of an early phase of short duration response and a second phase of prolonged and continuous response [22]. The initial response is attributed to a direct effect of formalin on nociceptors and it is sensitive to opioid pain relievers. Phase 2 is associated with the release of local endogenous mediators responsible for the sensitization of primary and spinal sensory neurons and the subsequent activation of nociceptors, characteristic of NSAID analgesics [36]. It should be noted that in phase 1 of formalin test **Table 1** the extracts presented varied paw licking times; although they were statistically not significant, they presented inhibition values up to 67% compared to the 22% observed for the group treated with indomethacin. These results clearly demonstrated the therapeutic potential of *P. schiedeana* leaves against neurogenic pain, characteristic of an opioid analgesic [20, 37].

TABLE 1: ANTI-NOCICEPTIVE ACTIVITY OF *PERSEA SCHIEDEANA* AQUEOUS, ETHANOLIC AND DICHLOROMETHANE LEAVES EXTRACT, IN ACETIC ACID, FORMALIN, AND CAUDAL PRESSURES TESTS

Groups and doses in mg/kg		Writhing test (N° contortions)		Formalin test (Licking time)				Caudal pressure (g/cm)	
				0 - 5 min		15 - 30 min			
		Media ± SEM	%	Media ± SEM	%	Media± SEM	%	Media ± SEM	%
Water		27.33 ± 1.51	-	64.95 ± 11.80	-	156.29 ± 13.44	-	78.33 ± 6.00	-
Indomethacin 10 mg/kg		10.33 ± 2.61**	62	50.65 ± 11.21	22	45.64 ± 14.82**	71	286.67 ± 27.88**	266
Aqueous	100	11.50 ± 1.29**	58	39.09 ± 8.15	40	47.18 ± 7.71**	70	235.5 ± 28.43**	201
	250	14.78 ± 1.56**	46	59.36 ± 8.28	9	83.65 ± 14.93**	46	220.75 ± 23.49*	182
	500	9.33 ± 1.65**	66	21.86 ± 3.63*	66	18.38 ± 2.45*	88	169.33 ± 28.43	116
Ethanolic	100	10.78 ± 2.47**	61	59.78 ± 7.10	8	161 ± 26.13	-3	162 ± 23.32	107
	250	08.45 ± 2.41**	69	56.78 ± 8.39	13	12.91 ± 2.53*	92	220.0 ± 62.31*	181
	500	17.44 ± 2.88	36	36.14 ± 5.00	44	78.64 ± 10.68**	50	204.0 ± 19.39*	160
Dichloro-methane	100	9.11 ± 2.64**	67	37.13 ± 11.46	43	23.37 ± 6.10**	85	234.57 ± 15.30*	199
	250	8.67 ± 1.71**	68	25.63 ± 6.14	61	9.23 ± 3.81**	94	385 ± 39.09**	391
	500	13.50 ± 4.24*	51	21.62 ± 5.48	67	2.39 ± 1.55**	98	323.5 ± 48.92**	313

* $p < 0.05$; ** $p < 0.001$; SEM: standard error of the mean; %: Pain inhibition

On the other hand, the extracts were more effective in the second phase of the trial, reaching percentage inhibition values as high as 98%, even exceeding the 71% inhibition of the reference drug **Table 1**, suggesting a possible mechanism in the periphery, which again could be related to the reduction of the synthesis of prostaglandins, related to the nociceptive stimulus through local peritoneal receptors [38]; or it may be due to interference in transduction mechanisms of the primary afferent nociceptors involved in this model. We can affirm that the evaluated extracts act in both phases during the formalin test, although with greater effectiveness in the phase of inflammatory pain. It demonstrates that extracts from *P. schiedeana* leaves have activity similar to a non-opioid pain reliever or NSAID [23], which are active in both phases of the formalin test [39]; but more effective in the second. All this agrees with data reported by Adeyemi *et al*., (2002), who demonstrated that the genus *Persea* analgesic activity in animal models is like NSAIDs [11].

The test of the reaction to the caudal pressure was performed mechanically by means of a Randall-Selitto analgesimeter, whose function is to assess pain at the level of the central nervous system by means of skin mechanoreceptors [24], and is sensitive to the activity of opioid substances with central analgesic activity, so it is used to determine the effectiveness and function of the different opioid receptors located peripherally and centrally [40, 41]. In this sense, there was a general increase in resistance to the pressure force applied to the tail of the groups of animals treated with the extracts evaluated, with percentage inhibition values between 107 and 391%, statistically similar to what was observed in the group. Treated with the reference drug (266%, **Table 1**). We are again faced with results that clearly indicate central

analgesic activity, at this time without the interference of inflammation, since this test does not cause damage to tissues exposed to stimuli, thus avoiding associating it with inflammatory pain [42].

CONCLUSION: The aqueous, ethanolic and dichloromethane extracts of *P. schiedeana* leaves showed that they are safe since no evidence indicating toxicity was found. The pharmacological tests carried out showed that the extracts of the leaves of *P. schiedeana* have an activity homologous both to the effect of a non-opioid analgesic related to inflammatory pain, and to an opioid activity with a central analgesic effect, considering that the results seem indicate anti-inflammatory activity related to prostaglandin inhibition, and this is probably mainly related to the presence of scopoletin. This validates the ethnobotanical importance of this plant species as a method for treating pain.

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