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Acute Toxicity and Evaluation of the Antinociceptive Activity of *Amanoa almerindae* Leaf Aqueous Extract in Experimental Animals

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ABSTRACT
The aim of the present study was to evaluate the antinociceptive activity and toxicity of an aqueous extract obtained from leaves of *Amanoa almerindae*. Abdominal constriction, formalin and tail flick test were use to determine the antinociceptive properties of the extract. The extract was administered both orally and using intraperitoneal injections. The results indicate that this aqueous extract exhibit dose dependent antinociceptive activity when tested by the different methods. Moderate toxicity was more evident following intraperitoneal administration. This confirms that oral administration is safer and should be performed when evaluating natural products as proposed by OMS/TMR (2002). In spite of toxicity signs shown by intraperitoneal/oral administration, there was no evident direct tissue damage (toxicity). The analgesic activity of the drug seems to affect both the peripheral and central nervous system. Therefore, it is important to ascertain the metabolites responsible for these actions in the phytochemical screening.

Keywords: *Amanoa almerindae,* acute toxicity, antinociceptive activity.

INTRODUCTION

The genus Amanoa belongs to subfamily Phyllanthoideae of the Euphorbiaceae, and comprises about 15 species, which are spread in tropical regions of America and Africa. In Venezuela six are present in the Guayana region. Amanoa almerindae Leal, known by the vernacular name "reventillo", is a medium-sized tree that is widespread in the Venezuelan Amazonas. [1] Recently, our research group reported chemical constituents of the species from samples collected on the borders of the Sipapo River, Amazonas State, Venezuela. Triterpenes, steroids, lignans and biflavones were the main compounds isolated. [2] The literature shows few studies of the genus Amanoa. The most published known are of *Amanoa oblongifolia* Muell., which report the isolation of terpenes and lignans [3-5], and its antiviral activity. [6] In the continuation of our investigation, of phytochemical and pharmacological evaluation of *Amanoa almerindae*, the aim of the present study was to evaluate its oral and intraperitoneal acute toxicity and its antinociceptive activity, and elucidate its possible mechanism of action.

MATERIALS AND METHODS

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Plant material

The aerial parts of *Amanoa almerindae* Leaf, were collected on the borders of the Sipapo River, Amazonas State, Venezuela, in May 2000. The plant material was authenticated by Dr. Anibal Castillo, botanist at the Jardín Botánico de Caracas. A voucher specimen (AC 8989) has been deposited in the Herbarium "Dr. Víctor Manuel Ovalles (MYF22909) of the Facultad de Farmacia, Universidad Central de Venezuela.

Preparation of the aqueous extract

Amanoa almerindae leaves were dried at room temperature, and later triturated. An aqueous extract (AE) was obtained by decoction of plant material (100 g) boiled in distilled water for 30 min. The resulting decoction was filtered, frozen at -20^{o}C and lyophilized. The yield of the extract was about 25% (w/w). At the time of use, the extract was reconstituted in distilled water at the required concentrations.

Animals

Experiments were conducted using male albino mice, NIH strain, weighing 18-20 g. Animals were housed in cages under standard environmental conditions, with free access to food and water. The animals were acclimatized to the laboratory for at least 24 hours before testing. Experiments reported in this study were performed in accordance with the current guidelines for the care of laboratory animals (NHI guide, 1996) and the ethical guidelines approved by the Committee of the School of Pharmacy, Universidad Central de Venezuela, Caracas, Venezuela.

Drugs

The following drugs were used for all the assays. An aqueous solution of AAAE in a concentration of 4 g/mL, was used as stock solution. All dilutions were obtained from the stock solution using distilled water to obtain the required volume. Other reagents used were: acetic acid and formaldehyde purchased from Merck, (USA), acetylsalicylic acid (Bayer), tramadol® (morphine clorhidrate, Biosano Lab., Santiago, Chile), ketamine® (Hueber, Biotec), naloxone and arabic gum (Sigma).

Experimental procedure

Acute toxicity

This assay was carried out to evaluate any possible toxic effects or changes in normal behavior of the experimental animals. The TD_{50} of the AAAE was determined by oral and intraperitoneal administration, following the method described by Litchfield and Wilcoxon [7], with small modifications. [8] Mice selected for this study were weighed, and randomly divided into six groups each containing five animals. By intraperitonial administration (ip), the control group was treated with physiological solution, and the remaining groups with increasing doses (12.5, 25.0, 50.0, 125.0, 250.0, and 500.0 mg/kg) of AAAE. By oral administration (po), the control group was treated with distilled water, and the animals under treatment with increasing doses of AAAE (12.5, 62.5, 125.0, 250.0, 500.0, 1000.0, 2500.0 mg/kg). In both administrations a volume 0.1 mL for 10 g/kg of body weight was used. The mice were observed at 10, 30, 60, 90 min, 24 hours, and 15 days after treatment, to detect changes in behavioral responses, signs of toxicity, and mortality. At the 15th day the animals were anesthetized and sacrificed by decapitation. The internal organs and tissues such as, kidney, heart and liver were removed for histological examination, and preserved in buffer with 10% formaldehyde.

Table 1: DT_{50} of Amanoa almerindae for two methods of administration

DT_{50} (mg/kg)	
ip	po
22.87	376.49

Antinociceptive tests

Acetic acid-induced abdominal writhing test

The acetic acid-induced abdominal writhing test was carried out by the procedure described by Lima *et al.* [9] The antinociceptive activity was determined *in vivo* using the abdominal writhing test induced by 0.6% acetic acid (0.1 mL/10 g, ip). The AAAE was administered orally (47, 94 and 188 mg/kg) 30 min priori to injection of acetic acid. The number of writhings was recorded for 30 min, starting 5 min after the injection of acetic acid. Control animals received an equal volume of vehicle and acetylsalicylic acid (ASA, 200 mg/kg, po); tramadol (40 mg/kg, ip) was used as a positive control.

Formalin test

The test was performed according to Hunskaar and Hole. [10] Briefly, 30 min after administration of the AAAE (47, 94 and 188 mg/kg, p.o.), acetylsalicylic acid (ASA, 200 mg/kg, p.o.), tramadol (40 mg/kg, ip) or distilled water, 20µL of formaldehyde 2.5% (v/v in distilled water) was injected subcutaneously into the plantar surface of the left hind paw of the mice. The behavioural responses to nociception, including biting, licking and scratching of the injected paw, were noted and the time spent was recorded up to 30 min. The first 5 min was considered as early phase (neurogenic phase) and the period of 15-30 min as the late phase (inflammatory phase) of the nociceptive response.

Thermal nociception

The antinociceptive effect, expressed as the time required for mouse tail flick after exposure to a source of radiant heat (Letica®), was evaluated according to Davies. [11] Animals were placed in a Plexiglas box that allowed the animal's tail to be free for exposure to a radiant heat source at a distance of 5 mm. Animals were divided in groups of five individuals. The intensity of the heat stimulus was adjusted so that the intact animal flicked its tail within 2-4 s after stimulation, with a cut-off time of 15 seconds to avoid injury to the tail. The time of withdrawal of the tail was measured 30 minutes after administration of aqueous AAAE (188 mg/kg, ½ DT50, po), acetylsalicylic acid (200 mg/kg, po), and tramadol (40 mg/kg, po) at 15 min. The latency time for withdrawal of the tail was an average of three determinations.

In order to verify the involvement of the opioidergic system of the AAAE induced antinociception, separate groups of mice were pre-treated with the non-selective opioid receptor antagonist, naloxone (10 mg/kg) which was given subcutaneously (sc) on the animal's back in a volume of 0.1 mL/10 g, 10 min before the extract (188 mg/kg, po) or tramadol (40 mg/kg, ip). To identify whether the endogenous opiod system was involved in the antinociceptive effects of the extract, tail-flick latency was assessed at 30 and 60 min after administration the different doses of AAAE.

Statistical analyses

TD_{50} was determined according to the method of Litchifield and Wilcoxon. [7] Values were graphically obtained from the dose-effect curves. Results are reported as values of mean ± S.E.M. The differences were estimated by ANOVA analysis of two factors, followed by the Student- Newman-Keuls' test (GraphPad®). Significance level was set at $p<0.05$.

RESULTS

Acute toxicity

The effects of AAAE given to mice as single doses by oral and intraperitoneal routes indicated that at the dose of 1000 mg/kg, by ip route, 75% of mortality had occurred, while by the oral route at a dose of 3000 mg/ kg, 50% of deaths were found. For both routes of administration, the predominant toxic effects noticed were piloerection and intestinal contraction, which were dose dependent but were observed at different dose ranges, with 12.5 to 2500 mg/Kg for po and 12.5 to 500 mg/Kg for ip (Fig. 1a,b). The main behavioral sign of toxicity was intestinal contraction and it was chosen to determine the DT_{50} by the Probit method (Table 1). By oral and intraperitoneal routes the values obtained were 376.49 mg/kg and 22.87 mg/kg, respectively.

Histological analyses were performed on different tissues fifteen days after the administration of higher doses of AAAE for both routes; there were no changes in the structure in any of the tissues evaluated (Results not shown).

Acetic acid-induced abdominal writhing test

The effect of administration of AAAE using the abdominal constriction test in mice is shown in Figure 2. It was found that AAAE was able to inhibit significantly ($p<0.001$) the nociceptive effects induced by administration of acetic acid, the effect being dose-dependent. Interestingly, at doses of 94 and 188 mg/kg of aqueous extract, levels of antinociceptive

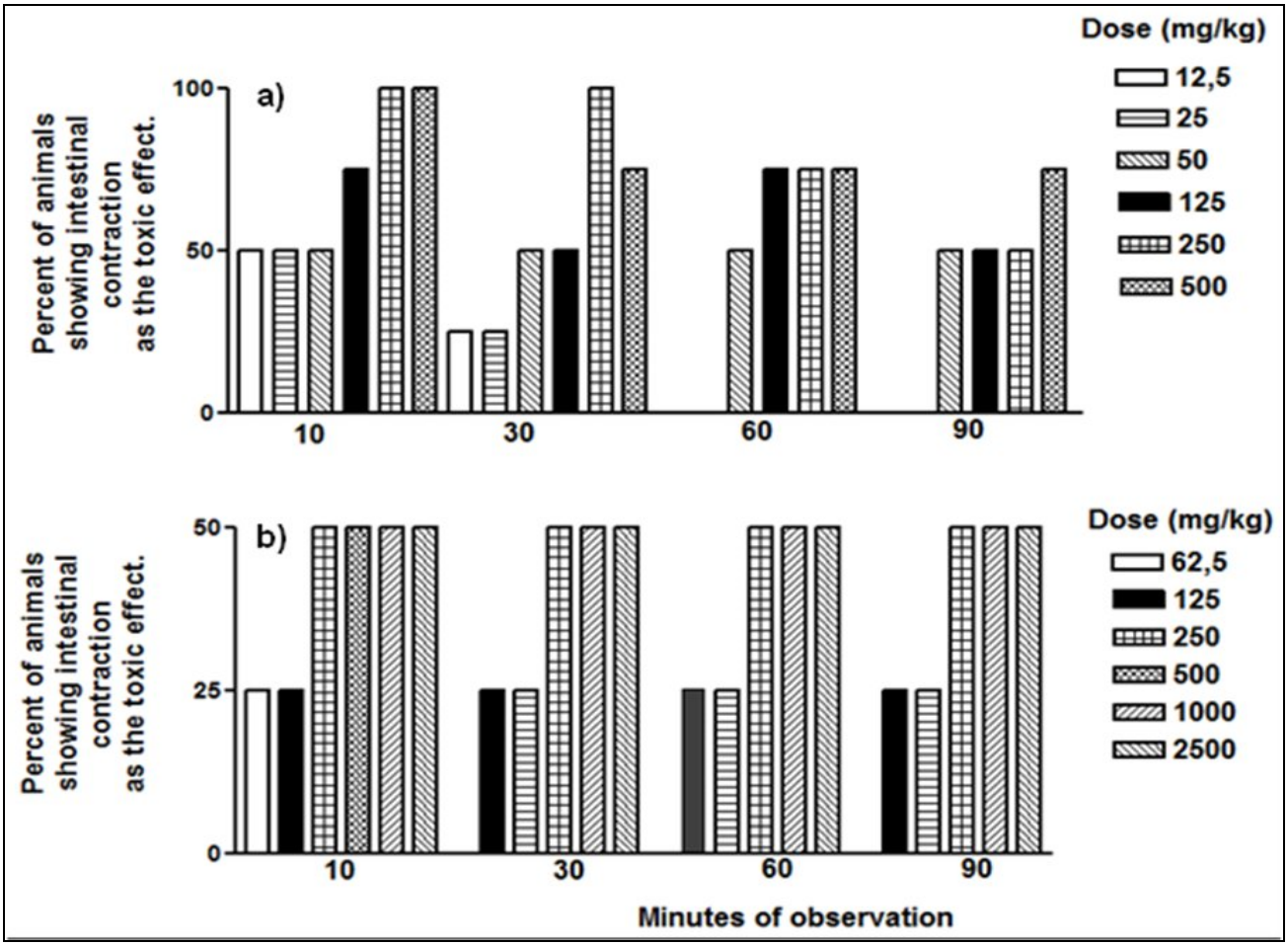

Fig. 1: Percent of animals showing intestinal contraction as the toxic effect of the aqueous extract of *Amanoa almerindae*, in function of time and dosage administered by intraperitoneal (a) and oral (b) routes

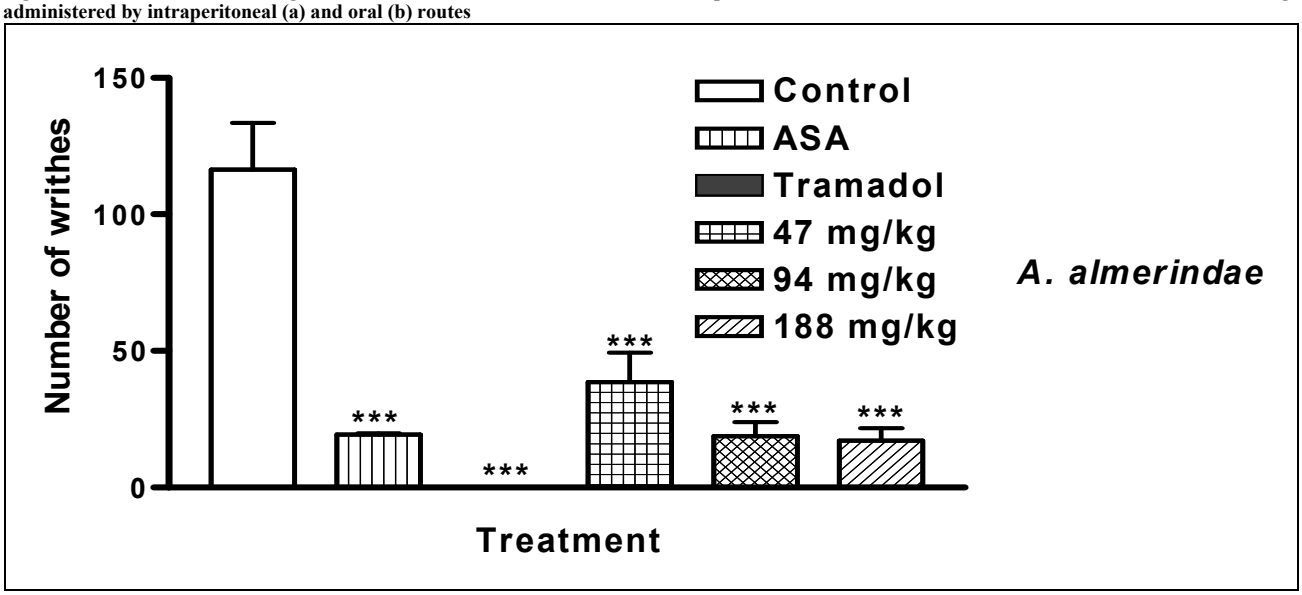

Fig. 2: The peripheral antinociceptive profile of the aqueous extract of *Amanoa almerindae* leaves, assessed by the method of abdominal constrictions induced in mice by acetic acid. Data are expressed as means ± S.E.M. *** p<0.001, as compared with the vehicule group. Student-Newman-Kells test (GraphPad®)

activity comparable to that produced by acetylsalicilic acid were found.

Formalin-induced nociception

The results of the antinociceptive effect of AAAE evaluated by the formalin test in mice is depicted in Figure 3, and show that AAAE produced significant ($p<0.001$) antinociceptive activity in the early phase of the formalin-induced nociception. Interestingly, this effect was not dose dependent, as there was no statistical difference between the different doses tested. However, in the late phase the antinociceptive

effect was minor and only at higher doses (94 and 188 mg/kg) was it statistically significant relative to control. Moreover, the antinociception produced by AAAE was not like the effect produced by the drugs used as controls, tramadol and aspirin. The antinociceptive effect produced by AAAE was independent of dose in neurogenic pain while the extract produced an antinociceptive effect dose-dependent in inflammatory pain. ASA and tramadol were significantly active in both phases ($p<0.001$).

Thermal nociception

Fig. 4 shows the antinociceptive effect produced by AAAE in the thermal method. At a dose of 188 mg/kg (1/2 TD_{50}) the antinociceptive effect was statistically significant ($p<0.001$) and similar to tramadol. It was noticeable that the effect of AAAE was still maintained at 60 min. Naloxone was able to reverse the antinociceptive effect of both AAAE and tramadol.

DISCUSSION

During the phytochemical screening of *A. almerindae,* a series of interesting molecules were isolated including, triterpenes, steroids, the known lignan podophylotoxin, and three biflavones. [2] It was also interesting that in the preliminary pharmacological screening a considerable antinociceptive effect was observed in the experimental animals. No medicinal uses of *A. almerindae* had been reported until now, but in view of the interesting compounds isolated, it was considered a good candidate to investigate the toxic effects that its use should produce.

The fact that the dosage for TD_{50} obtained via oral administration was sixteen times greater than that by intraperitoneal injection, shows that some compounds present in the AAAE are not absorbed by the oral route; they may excrete, and this could be a reason for obtaining a minor toxicity compared with the intraperitoneal administration. Another reason to explain this difference is that some metabolites in the AAAE will be metabolized by the entero-hepatic route, producing inactive substances and toxic agents, but the results herein reported strongly support that for toxicities in the study of plants the level of toxicity obtained is more confident when administered by oral ingestion. [8]

The results of the present study also demonstrated its antinociceptive activity on the periferic and central nervous systems. The abdominal constriction test has been widely used to evaluate the peripheral analgesic action exerted by extracts, and is considered a sensitive method to detect compounds with antinociceptive action at very small dose. [12] It has been postulated that acetic acid induces the production of mediators such as prostaglandins E (PGE_2) and $PGF_{2\alpha}$, and lipooxigenases, products of the peritoneal fluids which stimulate the nociceptive neurones sensitive to non-steroidal anti-inflammatory drugs (NSAIDS). [13-14] AAAE produced an effect similar to aspirin; therefore, the result of the acetic acid induced writhing strongly suggests that the antinociceptive mechanism AAAE may be linked partly to inhibition of lipooxigenase and/or cyclooxigenase in peripheral tissues, thereby reducing PGE_2 synthesis and interfering with the mechanism of transduction in the primary afferent nociceptor.

The formalin test is used to evaluate the mechanism of action and efficacy of antinociceptive effects of analgesic drugs. Phase I is thought to be produced by the acute activation of primary afferent fibers by formalin injection with needle insertion, whereas phase 2 might be due to the local release of neuroactive substances, including serotonin (5-HT), histamine, bradykinin, and prostaglandins, that are responsible for the sensibilization of primary afferent fibers and subsequent activation of the dorsal horn neurons in the spinal cord. [15]

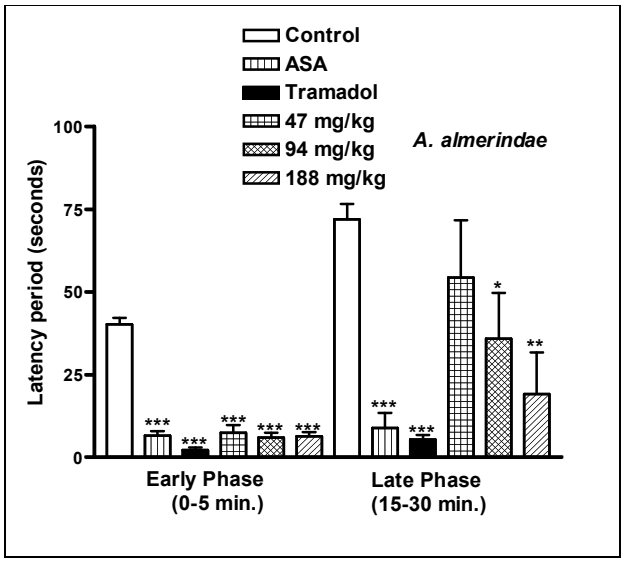

Fig. 3: The antinociceptive profile of the aqueous extract of *Amanoa almerindae* leaves assessed by the formalin test in mice. Each column indicates the mean + S.E.M. ***p<0.001, **p<0.01, *p<0.05 as compared with the control group. Student-Newman-Kells test (GraphPad®)

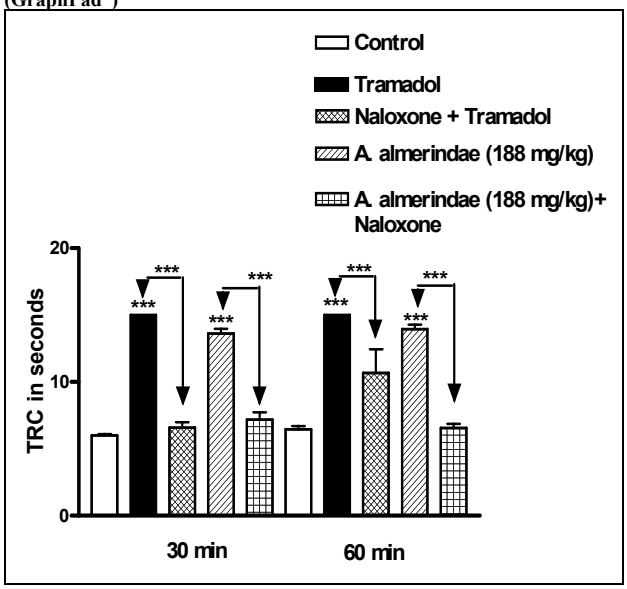

Fig. 4: Effect of naloxone (10 mg/kg,s.c.) on the antinociceptive effect caused by aqueous extract of *Amanoa almerindae* (188 mg/kg, po); Tramadol (40 mg/kg), using the thermal method. Each column indicates the mean ± S.E.M. *** p<0.001 as compared with the control group. Student-Newman-Kells test (GraphPad®)

The centrally acting drugs, such as narcotics, inhibit both phases equally, while peripherally acting drugs inhibit only the second phase. [16] In this case AAAE produces inhibition of both phases, but exhibits a non-dosage dependent antinociceptive activity only in the first phase. It is possible to speculate that only a small concentration of AAAE is necessary to block the nociceptive path, as was reported by Tripathi [17], who established that high concentrations of

drugs may cause reduced activity. Also considered is that, at high concentrations of drugs, desactivation of the antinociception receptors occur. Further studies are required to elucidate the mechanism of antinociceptive activity of AAAE.

The effect on the second phase was dose dependent and of minor intensity, it is possible that the metabolites present in AAAE may cause antinociceptive activity on peripheral and central levels.

The results of the formalin test suggest that AAAE inhibits nociceptive activity via direct action on the nociceptor (early phase), or indirectly through the inhibition of release of inflammatory mediators (late phase). The fact that it shows inhibition on both phases suggests that some of the metabolites present in AAAE are endowed with direct antinociceptive action and anti-inflammatory activity. The central analgesic effect of the aqueous extract may be supported by the results recorded in the thermal test, which is a selective method able to screen centrally acting opiate analgesic drugs [18]; this is supported by the fact that naloxone, an opioid antagonist, was able to reverse the effect of the aqueous extract.

In the phytochemical studies of *Amanoa almerindae*, compounds as daucosterol and betulinic acid between others were isolated. [2] Betulinic acid isolated from *Ipomoea pes-caprae* showed pronounced antinociceptive properties in the writhing test and formalin test in mice [19], results that support the analgesic activity as evidenced to the aqueous extract of the leaves of *A. almerindae*.

The leaves of *Amanoa Almerindae* also showed the presence of biflavones, such as the amentoflavone, sequoiaflavone and putraflavone. [2] Studies carried out by Kim *et al.* [20] showed that the amentoflavone produced a potent anti-inflammatory activity in the rat carrageenan paw edema model, and potent analgesic activity in the acetic acid writhing test. Some pharmacological activities of biflavonoids had been described, such as inhibition of histamine release from mast cells and inhibition of lymphocyte proliferation, suggesting the anti-inflammatory/antiallergic potential of the biflavonoids. These molecules also exhibit phospholipase A_2 and cyclooxygenase-2 inhibitory activity. [21] This evidence supports the existence in the leaves of *Amanoa almerindae* of metabolites with analgesic and anti-inflammatory activity. The study demonstrates that AAAE has antinociceptive activity, which is evident in all the nociceptive models, indicating the presence of both central and peripherically mediated activities. In an aqueous extract there exists a diversity of compounds, where several of them may be responsible for the toxicity, and others of the analgesic activity.

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Animal Rights

The institutional and international guide for the care and use of laboratory animals was followed. See the 'materials and methods' part for details.

REFERENCES

1. Steyermark JA, Berry PE, Yatskievych K, Host BK. Flora of the Venezuelan Guayana. Vol 5. Missouri Botanical Garden Press. St Louis, USA 1999, pp. 95- 99.
2. Leong KI, Alviarez PF, Compagnone RS, Suárez AI.Isolation and structural elucidation of chemical constituents of *Amanoa almerindae*. Pharmaceutical Biol. 2009; 47: 496-499.
3. Fang XD, Dhammika NP, Phoebe CH Jr, Pezzuto JM, Kinghorn AD, Farnsworth NR. Plant anticancer agents XXXVII. Constituents of *Amanoa oblongifolia*. Planta Med. 1985; 4: 346-7.
4. Nanayakkara DNP, Kinghorn AD, Farnsworth NR. New hydroxylated Eudesmane sesquiterpenoids from *Amanoa oblongifolia.* J Chem Res. 1986; 12: 454-455.
5. MacRae WD, Hudson JD, Towers GH. Alpha-(-)-peltatin, an antiviral constituent of *Amanoa aff. Oblongifolia.* J Ethnopharmacol. 1988; 22: 223-236.
6. Jassim SAA, Naji MA. Novel antiviral agents: a medicinal plant perspective. J Applied Microbiol. 2003; 95: 412-427.
7. Litchfield J T, Wilcoxon FA. A simplified method of evaluating dose-effect experiments.J Pharmacol Exp Ther. 1949*;* 96: 99-113.
8. OMS. Pautas generales para las metodologías de investigación y evaluación de la medicina tradicional. WHO/EDM/TRM/2000.1. 2002. Ginebra: Organización Mundial de la Salud, pp.81
9. Lima JA, Oliveira AS, de Miranda AL, Rezende CM, Pinto AC. Anti-inflammatory and antinociceptive activities of an acid fraction of the seeds of Carpotroche brasiliensis (Raddi) (Flacourtiaceae). Braz J Med Biol. 2005; 38: 1095-103.
10. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain.Pain. 1987; 301: 103-14.
11. Davies OL, Raventos J, Walpole AL. A method for evaluation of analgesic activity using rats. Br J Pharmacol. 1946; 1: 255-64
12. Bentley GA, Newton SH, Starr J. Evidence for an action of morphine and the enkephalins on sensory nerve endings in the mouse peritoneum**.** British J Pharmacol. 1981; 73: 325-32.
13. Derardt R, Jougney S, Delevalcee F, Falhourt M. Release of prostaglandins E and F in an algogenic reaction and its inhibition. Eur J Pharmacol. 1980; 51: 17-24.
14. Collier, HDJ, Dinneen LC, Johnson CA, Schneider C. The abdominal response and its suppression by analgesic drugs in the mouse. British J Pharmacol. 1968; 32: 295-310.
15. Dickenson AH, Sullivan AF. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurons. Neurosci Lett. 1987; 83: 207-11.
16. Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic biphasic pain response. Pain. 1989; 38: 347-352.
17. Tripathi KD, editor. In: Essentials of Medical Pharmacology, 4 Edn, Jaypee Brothers, Medical Publishers, New Delhi, India, 2001, pp. 52-53.
18. Abbott FV, Melzack R. Brainstem lesions dissociate neural mechanism of morphine analgesia in different kinds of pain. Brain Res. 1982*;* 251: 149-155.
19. Krogh R, Kroth R, Berti C, Madeira AO, Souza MM, Cechinel-Filho V, Delle-Monache F, Yunes RA. Isolation and identification of compounds with antinociceptive action from Ipomoea pes-caprae R. Br. Pharmazie. 1999; 54: 464-6.
20. Kim HK, Son KH, Chang HW, Kang SS, Kim HP. Amentoflavone, a plant biflavone: A new potential anti-inflammatory agent. Arch Pharm Res. 1998; 21: 406-410.
21. Kim HP, Park H, Son KH, Chang HW, Kang SS. Biochemical pharmacology of biflavonoids: implications for anti-inflammatory action. Arch Pharm Res. 2008; 31: 265-73.