Analgesic and anti-inflammatory effects in animal models of an ethanolic extract of Taheebo, the inner bark of *Tabebuia avellanedae*

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Abstract. Taheebo, the purple inner bark of the Bignoniaceae tree *Tabebuia avellanedae* Lorentz ex Griseb, which is found in tropical rain forests in northeastern Brazil, has been used as a traditional medicine for various diseases for more than 1,500 years. In the current study, various animal models were used to demonstrate the analgesic and anti-inflammatory properties of its ethanolic extract, thereby investigating its potential as a therapeutic treatment for diseases with pain and inflammation. In the hot plate and writhing tests for the *in vivo* analgesic effect test of Taheebo, a 200 mg/kg dose of the extract induced a significant anti-nociceptive effect and increased the pain threshold by approximately 30% compared with the control. In vascular permeability and tetradecanoylphorbol acetate (TPA)-, arachidonic acid- and carrageenan-induced paw edema tests for anti-inflammatory effects, treatment with 200 mg/kg Taheebo led to significant anti-inflammatory effects and inhibited inflammation by 30-50% compared with the control. At 100 mg/kg, the extract decreased the levels of pain and inflammation in all tested models, but the degree of inhibition was not statistically significant. The results suggest that the ethanolic extract of the inner bark of *Tabebuia avellanedae* has the potential to be developed as a therapeutic or supportive drug against diseases with accompanying pain and inflammation, including osteoarthritis.

Introduction

Among the elderly, osteoarthritis is the most common joint disease and a significant cause of physical illness (1). Symptoms include joint pain, stiffness, limited movement, joint deformity and varying degrees of joint inflammation (2,3). Current therapeutic strategies for osteoarthritis focus on alleviating symptoms, particularly pain and inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are the mainstream treatments for osteoarthritis (4). Although NSAIDs are recommended as an initial drug therapy to reduce joint inflammation and pain, their chronic use is limited by gastrointestinal-related toxicities, including nausea, dyspepsia, upper gastrointestinal bleeding and ulcer perforation (5). To minimize these toxicities, a new generation of NSAIDs, the cyclooxygenase (COX)-2 selective inhibitors (celecoxib, rofecoxib and valdecoxib), have been developed in an attempt to improve gastrointestinal tolerance. However, reported cardiovascular risks, including myocardial infarction and stroke, have led to the removal of rofecoxib from the market (6-8). Although other COX-2 inhibitors provide effective symptomatic relief, their substantial toxicities limit long-term use. Additionally, this therapeutic approach is not curative, but relieves clinical signs and symptoms of the disease; thus, a more effective and safe drug is necessary for the curative treatment of osteoarthritis.

Joins™, a herbal drug combining the extracts of *Clematis mandshurica*, *Trichosanthes kirilowii* and *Prunella vulgaris*, is commonly used for the curative treatment of osteoarthritis in Korea. Clinical studies have demonstrated that Joins relieves joint pain and improves functionality in osteoarthritis patients. Its efficacy may be attributed to cartilage protection and anti-inflammation (9); however, its lack of an immediate analgesic effect is a major drawback. The screening of herbs and natural products for a more efficient compound may lead to the development of a superior therapeutic drug, particularly one with more immediate analgesic effects.

Tabebuia avellanedae Lorentz ex Griseb, a Bignoniaceae, is a tree found in tropical rain forests in northeastern Brazil. Taheebo, a product obtained from the purple bark of the tree, has been traditionally used for over 1,500 years in South America to treat a variety of diseases (10). Its various fractions have been previously reported to exhibit anti-inflammatory, anti-bacterial, anti-fungal, diuretic, anti-coagulant and laxative properties in addition to an anticancer effect (11-14). In

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particular, Taheebo demonstrated anti-nociceptive and anti-edematogenic effects in formalin- or acetic acid-induced nociceptive experimental models in mice and in the rat paw edema model (15). Compared with the anti-inflammatory effect of *Tabebuia avellanedae*, the analgesic effects of Taheebo have not been extensively studied to date (16). For the development as a therapeutic drug against osteoarthritis, the anti-inflammatory and analgesic effects of Taheebo ethanolic extract require further evaluation with various nociceptive and inflammatory experimental animal models. The current study investigated the anti-inflammatory and anti-nociceptive effects of the ethanolic extract of *Tabebuia avellanedae* on various animal models.

Materials and methods

Chemicals. Arachidonic acid, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), acetic acid, carboxymethyl cellulose (CMC), Evans blue, diclofenac, indomethacin and carrageenan were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals. Male 6- to 8-week-old imprinting control region (ICR) mice and Sprague Dawley (SD) rats were obtained from OrientBio Co., Ltd. (Sungnam, Korea). Male ICR mice were used in the following tests: TPA- or arachidonic acid-induced ear edema, hot plate, acetic acid-induced vascular permeability and acetic acid-induced writhing response. SD rats were used in carrageenan-induced paw edema tests. The animals were maintained in plastic cages at 21-24°C under a 12-h light/dark cycle and were provided with free access to pellet food and water. The animals were adapted for at least 1 week prior to the start of the experiment. The subjects were habituated to the behavioral test chambers and handled with special care to minimize stress. All methods were approved by the Animal Care and Use Committee of Kyung Hee University and all procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the Korean National Institute of Health.

Preparation of ethanolic extract of Taheebo. An ethanolic extract from Taheebo, the inner bark of *Tabebuia avellanedae* Lorentz ex Griseb, was prepared as previously reported (17). Briefly, the inner bark of the plant was purchased from GIPI Korea Co. Ltd. (Seoul, Korea), who authenticated the product as Taheebo. Dried inner bark (1 kg) was extracted three times with 70% ethanol (12 l) at room temperature for 3 days. The combined extract was filtered and then concentrated under reduced pressure at 40°C with an end result of ~13% yield (based on the weight of the dried inner bark). The extract was suspended in 0.5% sodium CMC (CMC-Na) solution immediately prior to the start of the experiments. The extract was administered orally to the animals at doses of 100, 200 or 400 mg/kg. Control experiments were performed with 0.5% CMC-Na solution. A combination of diclofenac (25 mg/kg) and indomethacin (1 mg/kg) was used as the positive control.

Hot plate test. The hot plate test was conducted at a fixed temperature of 55±1°C according to a previously reported method (18). Animals were placed into a 50 cm-diameter glass beaker on the heated surface and the time between animal placement and shaking or licking of the paws or jumping was recorded as the index of response latency. Mice exhibiting latency time of 10-30 sec were selected to receive vehicle (0.5% CMC in distilled water), Taheebo extract (100, 200 or 400 mg/kg) or diclofenac (25 mg/kg); 1 h later, the latency time was re-determined.

Acetic acid-induced writhing response. The anti-nociceptive activities of the Taheebo extract were assessed by measuring the response to an intraperitoneal injection of acetic acid solution, which causes contraction of the abdominal muscles and stretching of the hind limbs (19). Each experimental group was administered orally with the vehicle (0.5% CMC in distilled water), Taheebo extract (100, 200 or 400 mg/kg), or diclofenac (25 mg/kg). After 1 h, 1% acetic acid was administered at a dose of 10 ml/kg body weight; measurement of the number of writhes began 10 min later and continued for 20 min.

Acetic acid-induced vascular permeability. The acetic acid-induced vascular permeability test was carried out with a modification of a previously described method (20). Evans blue (10 ml/kg of 2% solution) was injected intravenously into each mouse tail 30 min following oral administration of vehicle (0.5% CMC in distilled water), Taheebo extract (100, 200 or 400 mg/kg) or diclofenac (25 mg/kg). After 20 min, 10 ml/kg of 1% acetic acid in saline was injected intraperitoneally and the mice were sacrificed by cervical dislocation 20 min later. Saline (5 ml) was injected into the abdominal cavity, the washings were collected into test tubes and subsequently centrifuged at 2,000 rpm for 10 min. The absorbance of the supernatant was read at 650 nm with a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and the amount of Evans blue leakage in the abdominal cavity (the vascular permeability) was determined from the absorbance measurement of the supernatant.

TPA-induced ear edema formation in mice. Skin inflammation was induced in the right ear of each mouse by topical application of TPA as previously described (21). Each experimental group orally received the vehicle (0.5% CMC in distilled water), Taheebo extract (100, 200 or 400 mg/kg) or indomethacin (1 mg/kg). After 1 h, TPA solution (1.0 μg dissolved in 20 μl acetone) was applied to the inner and outer surfaces of the ears and 4 h later the mice were sacrificed by cervical dislocation. A mouse ear punch was obtained with a 5-mm dermal biopsy punch and then weighed. The thickness of the punch was measured with calipers (Mitutoyo Corporation, Kawasaki, Japan). The degree of ear swelling was expressed as the increase in ear thickness (mm).

Arachidonic acid-induced mouse ear edema assay. The vehicle (0.5% CMC in distilled water), Taheebo extract (100, 200 or 400 mg/kg) or indomethacin (1 mg/kg) were orally administered 1 h prior to the topical application of 2% arachidonic acid dissolved in acetone (20 μl/ear) to the right ear of the mice (22). After 4 h, the mice were sacrificed by cervical dislocation. A mouse ear punch was obtained with a 5-mm dermal biopsy punch and then weighed. The thickness of the punch was measured with calipers and the degree of ear swelling was expressed as the increase in ear thickness (mm).

Carrageenan-induced paw edema. The anti-inflammatory activity of Taheebo was determined by the carrageenan-induced edema test. Taheebo extract (100, 200 or 400 mg/kg), diclofenac (25 mg/kg) or the vehicle (0.5% CMC in distilled water) was administered orally 1 h prior to the injection of 100 µl of 1% carrageenan in saline into the plantar side of the left hind paws of the rats. Paw volume was measured prior to the carrageenan injection and 1, 2, 3 and 4 h following the administration of the edematogenic agent using a plethysmometer (Ugo Basile, Comerio, Italy). The degree of swelling was determined by the ratio a/b, where a and b are the volumes of the left hind paws following and prior to the carrageenan treatment, respectively. The increase in paw volume (%) was calculated as follows: [(a-b)/b] x 100.

Statistical analysis. Data were presented as the mean ± standard error of the mean (SEM). Comparisons between the experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant result. The program used for the statistical analysis was GraphPad Prism software 5 (San Diego, CA, USA).

Results

Analgesic effect of the ethanolic Taheebo extract in animal models. Oral administration of the Taheebo extract significantly increased the pain threshold of the mice compared with control treatment, as assessed by the hot plate test (Fig. 1). Notably, treatment with Taheebo led to a significant analgesic effect even at 200 mg/kg (~30% greater than control), an effect that was similar to treatment with diclofenac (25 mg/kg). The acetic acid-induced writhing response also revealed the analgesic effect of Taheebo. The cumulative amount of abdominal stretching was associated with the level of acetic acid-induced pain. Thus, Taheebo treatment (100-400 mg/kg) significantly inhibited the number of writhes in comparison to control treatment (Fig. 2). The inhibition of writhes was ~30 and 40% higher than the control at Taheebo doses of 100 and 400 mg/kg, respectively, although this inhibition did not achieve statistical significance at 100 mg/kg.

Anti-inflammatory effect of ethanolic Taheebo extract in animal models. The anti-inflammatory effects of Taheebo extract were assessed through the use of various animal models, including acetic acid-induced vascular permeability, TPA-induced ear edema, arachidonic acid-induced mouse ear edema and carrageenan-induced paw edema. First, in the acetic acid-induced vascular permeability test, the oral administration of Taheebo extract at 100 and 400 mg/kg resulted in the significant inhibition of dye leakage by 30 and 35%, respectively (Fig. 3). The inhibition degree was similar to 100-400 mg/kg, although only the inhibition at 400 mg/kg achieved statistical significance. In the TPA-induced ear edema test, the Taheebo extract led to an inhibition of ear weight and thickness in a dose-dependent manner (Fig. 4). In the arachidonic acid-induced mouse ear edema model, Taheebo extract also exhibited a dose-dependent anti-inflammatory effect (Fig. 5). In the two models, indomethacin (1 mg/kg) inhibited ear inflammation by 50% compared with the control, while the Taheebo extract at 100 mg/kg
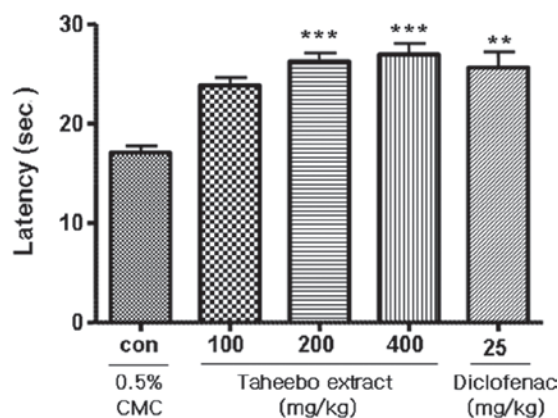

Figure 1. Analgesic effects of Taheebo extract on thermally-induced pain in mice. The hot plate response latency was measured 1 h after oral administration of Taheebo extract (100, 200 or 400 mg/kg), the positive control diclofenac (25 mg/kg) or the negative control (0.5% CMC in distilled water). Values are expressed as the means ± SEM (n=9). **P<0.01 and ***P<0.001 indicate statistically significant differences compared to the vehicle control group. CMC, carboxymethyl cellulose.
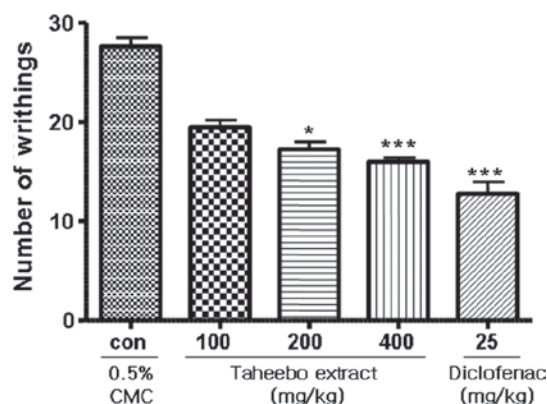

Figure 2. Analgesic effects of the Taheebo extract on acetic acid-induced pain in mice. Acetic acid-induced writhing number was measured 1 h after oral administration of the extract or controls, as described in Fig. 1. Acetic acid (1%) at a dose of 10 ml/kg was injected and 10 min later, the number of writhes was recorded during the subsequent 20-min period. The values are expressed as the means ± SEM (n=9). *P<0.05, **P<0.01 and ***P<0.001 indicate statistically significant differences compared to the vehicle control group. CMC, carboxymethyl cellulose.
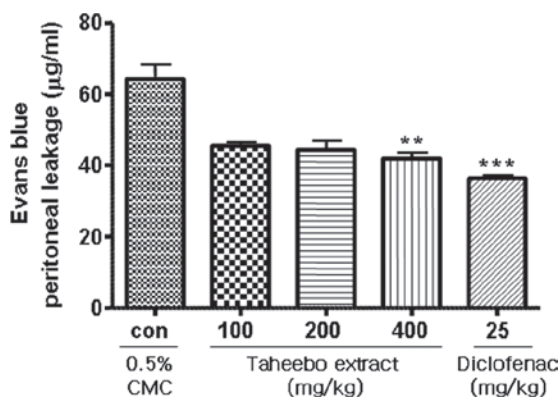

Figure 3. Anti-inflammatory effect of Taheebo extract on acetic acid-induced vascular permeability. The amount of Evans blue leakage in the abdominal cavity was measured as an indicator of inflammation degree. The values are expressed as the means ± SEM (n=9). **P<0.01 and ***P<0.001 indicate statistically significant differences compared to the vehicle control group. CMC, carboxymethyl cellulose.

Table I. Anti-inflammatory effect of Taheebo extract on carrageenan-induced paw edema in rats.

Dose (mg/kg)	Percentage increase of paw edema (mean ± SEM)			
	1 h	2 h	3 h	4 h
CON	29.36±2.66	59.57±3.10	76.28±2.24	76.14±2.52
Taheebo (100)	29.47±1.89	56.24±2.31	62.23±3.95	64.04±2.77
Taheebo (200)	30.73±2.12	50.58±2.60[a]	57.86±2.43[b]	60.78±2.22[b]
Taheebo (400)	30.60±2.11	47.10±2.27[b]	51.32±2.72[b]	52.19±2.30[b]
Diclofenac (25)	27.83±2.82	37.98±2.37[b]	41.18±2.93[b]	35.23±2.69[b]

Values are expressed as the means ± SEM (n=9). [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ indicate statistically significant differences from the vehicle control group. CON, 0.5% CMC-Na solution.
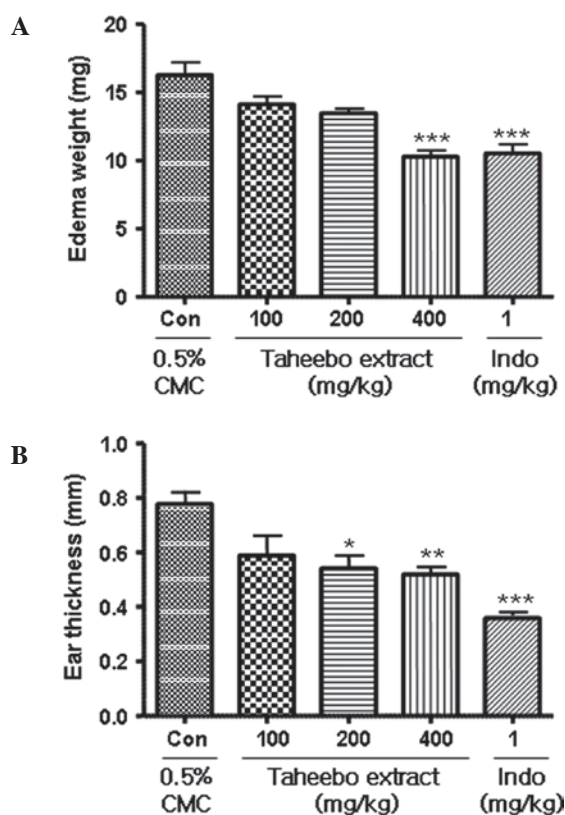

Figure 4. Anti-inflammatory effect of Taheebo extract on TPA-induced ear skin inflammation in mice. The (A) weight and (B) thickness of ear punches were measured as an indicator of the inflammation degree. The values are expressed as the means ± SEM (n=9). **$P<0.01$ and ***$P<0.001$ indicate statistically significant differences compared to the vehicle control group. CMC, carboxymethyl cellulose; indo, indomethacine.
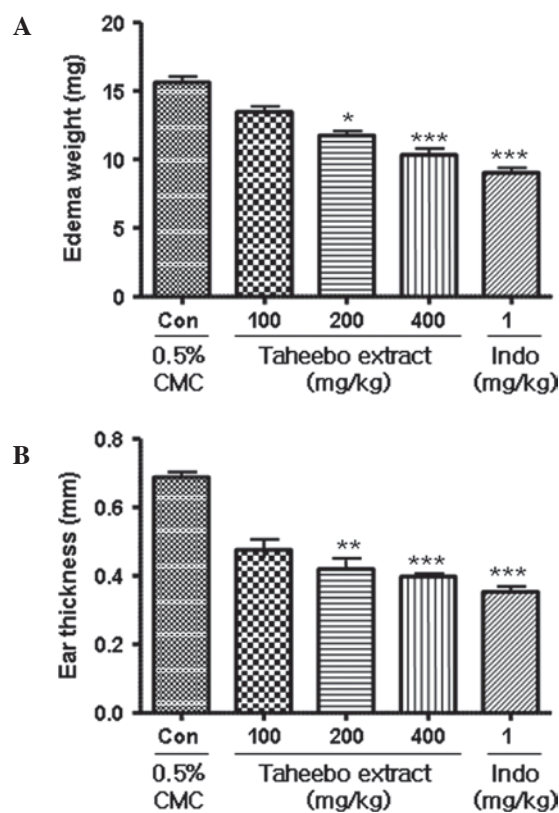

Figure 5. Anti-inflammatory effect of Taheebo extract on arachidonic acid-induced ear skin inflammation in mice. The (A) weight and (B) thickness of ear punches were measured to assess the degree of inflammation following experimental or control treatment. The values are expressed as the means ± SEM (n=9). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate statistically significant differences compared to the vehicle control group. CMC, carboxymethyl cellulose; indo, indomethacine.

inhibited ear inflammation by >25% compared with the control. In the carrageenan-induced hind paw edema test, 200 mg/kg Taheebo extract significantly inhibited ~30% of paw edema 3 h following carrageenan injection, although 100 mg/kg Taheebo extract was unable to significantly decrease edema volume compared with the control at all time points (Table I).

Discussion

Numerous studies have elucidated the pharmacological activities of the Taheebo extract, the inner bark of *Tabebuia*

avellanedae (11-14). However, the few demonstrations of the *in vivo* anti-nociceptive effects were performed in limited animal models (15). Those studies focused on the aqueous and methanolic extracts of *Tabebuia avellanedae*; to the best of our knowledge, the ethanolic extracts have not undergone much study. This investigation has demonstrated that the ethanolic extract of Taheebo significantly attenuated acetic acid-induced writhing (Fig. 2) and the nociception produced by hot-plate thermal stimulation (Fig. 1). Treatment with the extract also decreased the inflammation induced by acetic

acid (Fig. 3), TPA (Fig. 4), arachidonic acid (Fig. 5) and carrageenan (Table I) in animal models.

For nociception induction in animals, acetic acid causes inflammatory pain by inducing capillary permeability (23), while hot-plate-induced pain indicates narcotic involvement (24). Although formalin is known to induce neurogenic and inflammatory pain (25), the formalin test was not performed in this study. However, the demonstration that Taheboo aqueous extract also exerts an analgesic effect on formalin-induced pain (15) suggests that the Taheebo extract has an inhibitory effect on three types of pain induction. These analgesic effects may be partly related to Taheebo's anti-inflammatory neurogenic and narcotic properties. Oral administration of 200-400 mg/kg of the ethanolic Taheebo extract inhibited pain with statistical significance in the two animal models and a dose of 100 mg/kg was extremely effective in the majority of tested animal models. This inhibitory concentration is similar to that of the aqueous extract of *Tabebuia avellanedae* (15). Notably, the inhibitory effect of this aqueous extract at 200 mg/kg was not detected following the 400 mg/kg dose, as the aqueous extract may contain compounds that undercut its own inhibitory action. By contrast, in this study the ethanolic extract caused significant anti-nociceptive effects at doses of 200-400 mg/kg in various animal models, suggesting that the ethanolic extracts contain constituents to relieve pain that differ from the aqueous extracts.

The molecular mechanism by which the extract attenuated the pain level in the animal models has not been defined. Collier *et al* postulated that acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate nociceptive neurons sensitive to NSAIDs and narcotics (26). It has also been proposed that acetic acid induces pain by increasing the amount of PGE_2 and $PGF_{2\alpha}$ at the peritoneal receptors (27,28). On the basis of these studies, the Taheebo extract is thought to regulate the production of PGE_2 and $PGF_{2\alpha}$ in acetic acid-induced animal models of pain. By contrast, as the hot plate test is commonly used for assays of narcotic analgesics, our observations suggest that the extract exerts a central anti-nociceptive effect that may be associated with a reduction in Ca^{2+} influx at the axon termini of the afferent nerves. This reduction may induce a decrease in adenylate cyclase activity, thereby decreasing the levels of cyclic AMP and K^+ efflux and leading to nerve hyperpolarization and an apparent anti-nociceptive effect (29).

Carrageenan, arachidonic acid, TPA and acetic acid were used to induce inflammation in animal models, since edema induced by phlogistic agents is a widely accepted model for inflammation. For example, carrageenan-induced paw edema is a classical model of acute inflammation involving various chemical mediators, including histamine, serotonin, bradykinin and prostgladins (30), in which the involvement of the COX products of arachidonic acid metabolism and the production of reactive oxygen species are well-established (31). Oral administration of the Taheebo extract inhibited the inflammation in these animal models and thus the extract may be presumed to be involved in the downregulation of the production of various chemical mediators of inflammation.

The ethanolic extract of Taheebo exhibited anti-nociceptive and anti-inflammatory effects in animal models, emphasizing its potential for development as a novel therapeutic drug against osteoarthritis. However, studies using other pain and inflammation animal models are required, particularly since the animal models used in this investigation were short-term. The effects of lower doses of Taheebo should be demonstrated in long-term animal models to support its development as a therapeutic drug.

The following major active compounds have been identified in *Tabebuia avellandae*: flavonoids, cyclopentene dialdehydes, benzoic acid and benzaldehyde derivatives, quinones, furanonaphthoquinones, naphthoquinones and anthraquinones (32). Of the 18 most relevant quinines, lapachol and β-lapachone appear to have clinical importance, since they have been associated with anticancer pharmacological activity. Thus, HPLC analysis was conducted to determine the content of lapachol in the ethanol extract of Taheebo (data not shown). Lapachol was detected at 6.642 min retention time. The extract (1 g) contained approximately 0.03 mg of lapachol, although we did not confirm the content of all the compounds. Toxicity was observed with no therapeutic response following oral administration of lapachol and phase I clinical trials of lapachol as an anticancer drug were closed in 1970. The anticancer chemotherapeutic β-lapachone succeeded as the research molecule of note from *Tabebuia avellandae* and the molecular mechanisms associated with its anticancer activity, including topoisomerase inhibition, have been well defined (32). Other compounds, including the furonaphthoquinones and anthraquinones, have been the focus of investigations for various therapeutic roles in several experimental systems. Other molecules considered to be significant in disease treatment should be studied to further understand the molecular mechanisms by which Taheebo extracts relieve pain and inflammation in animal models.

In conclusion, the Taheebo ethanolic extracts demonstrated anti-nociceptive and anti-inflammatory effects in various animal models. These anti-nociceptive effects and the extract constituents related to pain relief require clarification for the development of a safe drug that produces immediate analgesia without adverse effects.

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