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RESEARCH ARTICLE

EXTRACTION OF PHENOLIC COMPOUNDS FROM *PINUS PATULA* BARK USING ETHANOL-WATER MIXTURES AND THE ANTI-INFLAMMATORY ACTION OF THE ETHANOLIC EXTRACT

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ABSTRACT

Interaction between factors to take into consideration for extraction of bioactive phenolics compounds from *Pinuspatula* bark was evaluated. Contact time, particle size, temperature, ethanol: water ratio, bark: solvent ratio and revolutions per minutewere evaluated to identify the highest percentage of extraction. The optimal extraction conditions resulting in the highest percentage of phenolics and procyanidins compounds were 6 hours of contact, less than 1.18 mm of particle size, with a temperature of 60°C, and ethanol: water ratio of 30:70, and bark: solvent ratio of 1:10 g:mL, with 250 revolutions per minute. The combination of this factors resulted in 8.56 ± 0.86% of extract of *Pinuspatula* bark, with 1610 µg/g of total phenols and 560.82 µg/g of procyanidins. Concerning their anti-inflammatory activity, *P. patula* bark extract exhibited a broad activity towards eicosanoids concentration produced by the polymorph nuclear leukocytes of rats. Results indicated that the hydroalcoholic extract of *Pinuspatula* bark has anti-inflammatory activity. ANOVA analysis showed significant differences between treatment groups ($p < 0.05$) receiving drug (Ketoprofen®) versus ethanolic extract (*Pinuspatula* bark).

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INTRODUCTION

Pinuspatula Schiede ex Schltdl. and Cham belongs to the Pinaceae family, and is commonly known as pine, Colorado pine, candlestick pine, patula pine (Colombia), and weeping pine (Mexico). It is extensively planted outside of subtropical habitats in the tropics and temperate zones, since the 1940s, including in southern Africa, southern Rhodesia, South America and Australia. In Colombia, *P.patula var. patula* its grown in Valle del Cauca, Cauca, Antioquia, Cundinamarca and Santander departments. Bark (periderm and sections of the secondary phloem) has different characteristics and functions depending on the plant species. It provides structural support for the tree, conducts nourishments from leaves to roots, and serves as protection from wood-eating insects. Tree bark is a particularly rich source of a wide variety of kinds of medicinal compounds, and this has been known to many cultures for many centuries (Greada and Ferguson, 1993). Decoctions or infusions of barks and leaves were commonly used as poultices and therapies throughout Europe and Asia (Hough, 1984). In Colombia, *Pinuspatula* is use to timber, paper pulp mainly (Moncada *et al*., 2016). *Pinuspatula* Schiede ex Schltdl. And Chamalso has traditional medicinal uses (antiseptic, diuretic, tonic and anti-inflammatory), as well as cultural uses (charcoal, pigment, resin and wood). Likewise, *Pinuspatula* bark is use as infusion and oral administration to headache and influenza by in habitantse indigenous of the zone (Horák *et al*., 2014). *Pinus* bark has been demonstrated to have antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory, and cardio-cerebrovascular protective effects, among others (Li *et al*., 2016). Karonen *et al* have found interesting anti-inflammatory properties in the extract from *Pinusmaritima* bark and have identified some compounds, including flavonoids and other polyphenols with anti-inflammatory properties, acting on two substances in particular that are involved in this type of process(nitric oxide and E_2 prostaglandins) (Karonen *et al*., 2004). Bark chemical

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composition is complex, changes between tree species, and it is influence by morphological elements. Bark has the same components as wood, although these are found in different proportions. The fibrous fraction consists of polysaccharides such as cellulose (30%), hemicelluloses (4-15%) and lignin (15-30%). Moreover, bark contains a relatively high percentage of components in addition to suberin, tannins, phlobaphenesand other phenolic compounds. Suberin content of cork range between 20-40% (Sharkov, 1972), while minerals, in form ofashes, compose 5% of the structure. In the case of pine bark, the suberin value is between 1.4-2%.Bark has hydrophilic and lipophilic extractives, although there is not a distinguishable barrier between them. They represent 20-40% of bark's dry biomass. The lipophilic fraction is soluble in non-polar solvents (ethyl ether, dichloromethane, etc.) such as fat, wax, terpenes, terpenoids and superior aliphatic alcohols. The hydrophilic fraction is extractable in water or organic solvents (acetone, ethanol, etc.) and contains primarily phenolic compounds. Phenolic compounds that can be extracted from pine bark includegallic acid, ferulic acid and p-hydrobenzoic acid, monomers such ascatechin, epicatechin and taxifolin, and condensed flavonoids such as procyanidins. It is thought that procyanidins can protect against cardiovascular diseases. The antiviral and antimicrobial activities of procyanidins have also been described (Ikigai, 1993; Chung, 1998) as well as theirantifungal activities (Valcic, 1996; Chung, 1998). Moreover, procyanidins have been shown to have preventive or therapeutic properties against gastrointestinal disorders. It is believed that at the intestinal level, procyanidins are fundamentally involved in acid secretion, intestinal motility, and bactericidal activity against diverse enteropathogenicgerms (Bujanda, 1999).

The importance of procyanidins asanticarcinogenic agents is well appreciated because of their proliferative effects in gastrointestinal (Stavric, 1994; Yang, 2001; Mackenzie *et al*., 2009), lung (Hertog, 1994; Le Marchand, 2000), ovary (Stavric, 1994), mammary (Stavric, 1994; McKay, 2002), colon, esophageal and stomach cancers (Tajima, 1985; Tsubono, 2001;), both in experimental animals and in human beings.At the onset of inflammation, a diverse population of leukocytes migrates through the postcapillaryvenules surrounding the damaged tissue. The composition of the leukocyte population and leukocyte activities at the site of inflammation are regulated both in intensity and duration by the mediators of inflammation. Substances such as leukocytes, prostaglandins, thromboxanes, lipoxins and leukotrienes (Samuelsson, 1983), which are eicosanoids, are produced from arachidonic acid released by mechanical, enzymatic, hormonal and immunological stimuli through the action of 5-cyclooxygenase and 5-lipoxygenase enzymes. The 5-lipoxygenase enzyme catalyzes the hydroperoxidation of arachidonic acid (C20) (CH_3CH_2 $(CH_2)_2$ CH_2 $(CH{=}CHCH_2)_4$ $(CH_2)_2COOH$) via an oxygen-dependent reaction to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE) or an unstable intermediate and LTA_4, which is an epoxy derivative. By the action of an epoxide hydrolase, LTA_4 can also be transformed into LTB_4, which is a powerful chemo attractant of additional white cells to the site of damaged tissue (Higgs, 1981). The majority of NSAIDs (non-steroid anti-inflammatory drugs, derived from enolic or carboxylic organic acids) have no significant effect on the 5-lipoxygenase pathway. As a result, drugs that act on both the 5-lipoxygenase and 5-cyclooxygenase pathways have greater analgesic and anti-inflammatory capacity because the mediators of inflammation generated by the two regulatory enzymes will decrease, resulting in a better therapeutic activity. The vast majority of NSAIDS, by inhibiting 5-cyclooxygenase, interfere with arachidonic acid metabolism, increasing the formation of 5-lipoxygenase products and causing breathing problems. Drugs such as Ketoprofen® and diclofenac® directly inhibit phospholipase, avoiding the production of prostaglandins and leukotrienes; this feature makes them particularly effective and safe in asthmatic people. However, these drugs are not exempt from producing side effects, namely gastritis and renal toxicity. From the above considerations, the goal of this study was to evaluate the efficiency of the hydroalcoholic extract of *Pinuspatula* bark in inhibiting mediators of inflammation (leukotriene B_4) produced by the 5-lipoxygenase pathway.

The beneficial effects of the ethanolic extract of the bark of *Terminaliacatappa L* is associated with antioxidant, immunomodulatory and anti-inflammatory activities, which can be attributed to the presence of phenolic compounds (Abiodum, 2016). Extraction of phenolic compounds from plant matrix is influenced by several factors such as their chemical nature, the extraction method used, the size of the particles, time and storage conditions and the presence of interfering substances. Therefore, optimization of the extraction process is fundamental for an accurate assessment of phenolic compounds from different vegetal matrices (Mokrani and Madani, K, 2016). Although the phenolic compounds have been extracted from different species of pine(Chiang *et al*., 2017; Chupin *et al*., 2015) no data was reported about the effect of extracting parameters on the survey of phenolic from *Pinuspatula* bark. In this work, the best conditions for the extraction of phenolic compounds present in *Pinuspatula* bark and their anti-inflammatory capacities were studied using a Sprague-Dawley rat animal model under the supervision of the ethics committee from the Research Vice-Rectory at the Universidad del Cauca-Colombia.

MATERIALS AND METHODS

Samples

Pinuspatula bark samples were collected from proximately sixteen-year-old trees in the Smurfit Carton de Colombia's forester subnucleus (*Cabuyerita*) at Popayán, northwest in the Cauca department (Colombia), latitude (1° 56´ north), longitude(77° 10´ west), 1700m.Bark samples were dried by 24 hours and mashed in an electric mill and separated into size fractions using sieves with a size between 0.05 a 1.00 mm.Samples were mixed with 99.8% ethanol and deionized water and were submitted to agitation and warming. After the extraction, the mixture was vacuum filtered, and thehydroalcoholic extract was rot evaporated at 40°C. The obtained extract percentage was calculated using Durling *et al*., (2007) methodology. The extraction variables temperature, extraction time, particle size, solvent-to-bark ratio, solvent composition and agitation, were systematically varied as shown in Table 1.

Proximal analyses

The protein (Kjeldalh method), fat (Sohxlet with hexane), ashes (calcination at 550°C), fiber (acid-basic digestion) and non-nitrogenous extract NNE(difference content)(AOAC, 1980) contents of bark samples were determined.

Cellulose and lignin

Thelignin (Klason method) (Schwanninger, 2002) and cellulose (Kurschener and Hoffer methods) (Melcer, 1976) contents of the bark samples were determined.

Total phenolic content

Total phenolic content was determined via a colorimetric method using the Folin–Ciocalteureagent (BDH). Ethanolic extract was treated with the Folin-Ciocalteureagent and a sodium carbonate solution. The absorbance was measured at 765 nm using a Spectronic 21. Calibration curves were performed using solutions of gallic acid.

Identification of procyanidins

The vanillin assay was applied (Sun *et al*., 1998). The extract was mixed with vanillin in methanol and sulfuric acid. The absorbance was measured at 500 nm. The calibration was performed with solutions of (+)-catechin.

Biological tests of the hydroalcoholic extract of Pinuspatula bark (lethal dose 50)

Due to the phenolic composition of the extract of *Pinuspatula* bark, its anti-inflammatory capacity was studied in processes of leukotriene inhibition using an animal model. For this, it was first necessary to establish the dose of the extract that does not cause mortality or toxic effects. Thus, a lethal dose 50(LD50) test was performed. The method developed by Lagarto and collaborators (Lagarto, 1997) was used, with some modifications. From the hydroalcoholic extract of *Pinuspatula* bark, dispersions were prepared using tween solution as an emulsifier, with concentrations of 1000, 500, 250 and 125 mg/kg, which were applied at the rate of 0.1 mL/10 g mouse weight. To test the dosages, 24 albino Swiss malemice were chosen with an average weight between 30 and 40 grams and were distributed into four groups of six animals each. Each group was given a different extract dose. During the 10 days following the administration of the extract, the mice were monitored for symptoms and any changes.

Levels of leukotriene B_4 produced by polymorphonuclear leukocytes as indicators of anti-inflammatory activity

Once the adequate dose of extract was determined, its efficiency in inhibiting processes of leukotriene production was studied in a rat model of LTB_4reduction in polymorph nuclear leukocytes. These cells are isolated from a peritoneal washing with a buffer by means of ionophore and calcium chloride solutions. 5-lipoxygenase was activated for the production of leukotrienes, which were measured. For this purpose, 12-month-old Sprague-Dawleyrats were used, weighing between 200 and 300 g and fed with commercial Conejina® concentrate (Salari *et al*., 1985).

Cell suspension

Glycogen was injected (1 g/kg weight of the animal) into the peritoneum of the rodents (rats) to obtain enough leukocytes, which are the biological entity affected by possible anti-inflammatory activity. After 4 hours, rats were sacrificed to remove leukocytes from the peritoneal cavity using phosphate-buffered saline (PBS), pH 7.2, at 0 °C (two washes of 5 mL each). The peritoneal lavage (rich in polymorph nuclear leukocytes) of each rat was divided into three parts, each receiving a different treatment: the first group received 0.2 µL of buffer, serving as the negative control; the second group served as the positive control and received 0.2 µL of Ketoprofen®, the drug of reference, at 0.5 g/mL. Ketoprofen is an NSAID drug with recognized activity against 5-oxygenase and arachidonate 5-lipoxygenase pathways, leading to a decrease in the production of LTB_4. The third group received 0.2 µL of 1 g/mL vegetable extract. A phosphate-buffered solution (137 mMNaCl, 2.7 mMKCl, 2 mM KH_2PO_4, 5mM $NaHPO_4$, 5 mM glucose) was then added to each group up to a final volume of 25 mL at pH 7.2, and the samples were incubated in a water-bath at 35 °C for 10 min.

Incubation of cells

The incubated solution was preheated at 37 °C for 10 min with 200 µL of a calcium ionophore solution, A23187, at 1 µg/mL. The role of the ionophoreis to activate the enzyme responsible for the production of LTB_4 (Kale *et al*., 2007). Subsequently, 500 µL of 87.5 mM calcium chloride was added for a final concentration of 1.75 mM in a total volume of 25 mL. The decrease in the production of the leukotriene B_4 was considered an indicator of efficient inhibition by the plant extract, using Ketoprofen® as a reference (positive control).

Extraction of LTB_4 in the peritoneal wash samples

The activity of 5-lipoxygenase in the production of LTB_4 was interrupted by incubating the samples at 0 °C for 10 min. Samples were subsequently centrifuged, and 8 mL of absolute ethanol was added to the resulting supernatant. This solution was then filtered, dried in a speed-vacuum at 35 °C and was then kept at 0 °C until the levels of leukotriene B_4 were measured.

Identification of LTB_4 levels

To quantify the levels of leukotriene B_4, the chromatographic method reported by Salariand collaborators was used (Salari *et al*., 1985). A Nova-Pak C_{18} chromatographic column was employed at room temperature with the following specifications: 5.9 X 150 mm, a volume of injection of 10 µL, a flow of 1.0 mL/min, an isocratic elution, a mobile phase composed of methanol/water /acetic acid(72 /27.8/0.2), pH 4.9 and UV detection at 280 nm. The sample obtained from the peritoneal wash was dissolved in 300 µL of methanol and was then filtered by 0.45 µm. To identify the leukotriene LTB_4, three types of samples subjected to different treatments were separately injected to compare the production of LTB_4 leukotriene in the presence and absence of the vegetable extract and the reference drug (Ketoprofen®). The presence of leukotriene was confirmed using LC-MS.

RESULTS AND DISCUSSION

Proximal analyses

The humidity content of all samples was approximately 16.52% with a 1.94%RSD, and based on this, the proximal analyses and the lignin and cellulose content quantification in dry base were realized. Proximal analyses of *P.patula* bark samples indicated 0.76% of brute protein content, similar to

Table 1. Extraction variables used for each experiment

Experiment	(mm)	(°C)	(h)	B:S	EtOH	(rpm)
(mm)	1.18-2.36	40	6	1:6	70	380
(°C)	1.70	28-60	6	1:6	70	380
(h)	1.70	40	3-9	1:6	70	380
B:S	1.70	40	6	1:6-1:10	50	380
EtOH	1.70	40	6	1:6	50-100	380
(rpm)	1.70	40	6	1:6	70	155-550

Temperature (°C), extraction time (h), particle size (mm), solvent-to-bark ratio (B:S), solvent composition (Ethanol: EtOH) and agitation (rpm)

Table 2. Optimal phenolics extraction conditions from P. patula bark

Parameter	Condition
Time	6 hours
Particle size	Smaller than 1.18 mm
Temperature	60°C
Solvent Ratio (water: ethanol)	30:70%
Bark: Solvent Ratio	1:10 (g:mL)
Agitation	250 rpm

Table 3. Average areas for the signal with a retention time of 5.690 min in HPLC

Control sample			Sample with drug				Sample with extract			
Areas	Area average	Standard deviation	Areas	Area average	Standard deviation	Decrease (%)	Areas	Area average	Standard deviation	Decrease (%)
24190			3188				19140			
23870	24093.33	0.805	2910	3082.66	4.890	87.20	18960	19103.00	0.673	20.71
24220			3150				19209			
34210			3060				27400			
35238	34669.33	1.508	3156	3109.00	1.545	91.03	26940	27146.67	0.86	21.70
34560			3111				27100			
42137			2800				32700			
41780	42455.67	2.071	2785	2759.67	2.079	93.50	31900	32483.33	1.572	23.49
43450			2694				32850			
				Average decrease		90.58		Average decrease		21.97

other pine species including *P. ponderosa*(1.10%) cultivated in Chile and *P. oocarpa* (0.76%) from Colombia. Fat content was 2.54%, which is very similar to *P. ponderosa* (2.50%) and *P. oocarpa* (2.41%). This is different for the ashes content of 0.83%, which is lower than *P. ponderosa* (1.10%), *P. oocarpa* (6.79%) and *P. pinaster* (1%) (Fradinho *et al*., 2002). The low level of ashes content of *P. patula* indicates a low mineral content (Sjöstrom, 1981).

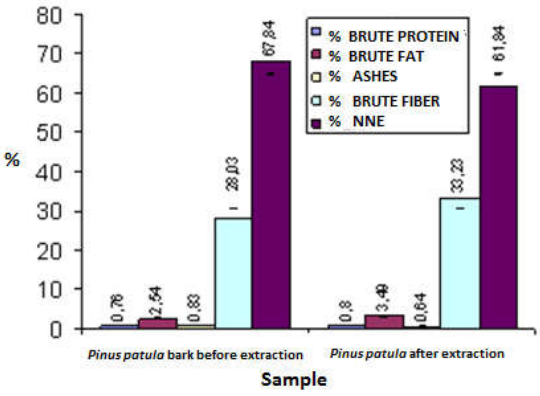

Fig. 1. *P. patula* bark proximal analyses data before and after the phenolic extraction

Barks with a high mineral content are suggested to be used in compost and/or as a substrate additive for plant growth. The carbohydrates percentage of *P. patula*bark, expressed as 67.84% NNE,is superior to those of *P. ponderosa* (46.30%) and *P. oocarpa* (56.87%). The high NNE content in *P. patula*bark may be related to the cellulose, hemicellulose and lignin content; furthermore, pine bark contains hydrolysable polysaccharides in different proportions(Ernst, 1982).

The proximal analyses results, together with the carbon, oxygen and nitrogen content (C: 52.40, N: 2.70, O: 38.30) of other pine species(Ernst, 1982), indicates that this species is an important source of nourishment for animals because saccharides and combined foods are obtained from it.Based on field surveys, estimated that the *Pinuspatula* processing generated approximately 65% of residues and only the 35% was used as end wood product. One of these residues are pinus barks with no commercial uses in Colombia (Moncada *et al*., 2016).X: Sample (*P. patula* bark before extraction – *P. patula* bark after extraction, Y: Percentage, brute protein%, brute fat%, ashes%, brute fiber%, NNE%.

Lignin and cellulose content

P. patula bark lignin content is similar to those presented by *P. resinosa*, *P. elliotti*, *P. oocarpa* and *P. ponderosa*. In addition to lignin, pine bark presents other types of compounds such as suberin, phlobaphenesand other phenolics (Sharkov, 1972). Cellulose content is lower than thatpresented by *P. resinosa*, *P. elliotti*, *P. oocarpa* and *P. ponderosa*. X: Sample (*P. patula* bark before extraction – *P. patula* bark after extraction, Y: Percentage, lignin%, cellulose%. Higher values are related to bark polymerstructural characteristics and to the presence of hemicelluloses in cellulose samples (Carballo *et al*., 2004). In Figure 2, the results of *P. patula* bark cellulose and lignin content are presented. The lignincontentto *Pinuspinasters* barkdetermined by the Klasonmethod, aftertheextraction of polyphenolics (tannin-richfraction) fromthebark byalkalisolution, is 33.2%. Cellulose content is about 24%. Hemicelluloses (about 15% of barkdryweight) (Fradinho *et al*., 2002).

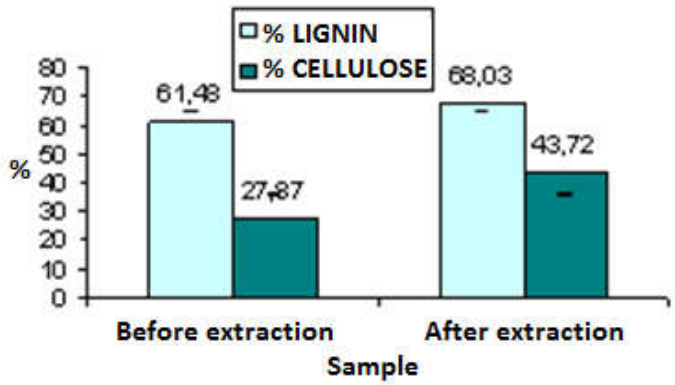

Fig. 2. Data of *P. patula* bark lignin and cellulose content before and after the phenolicextraction

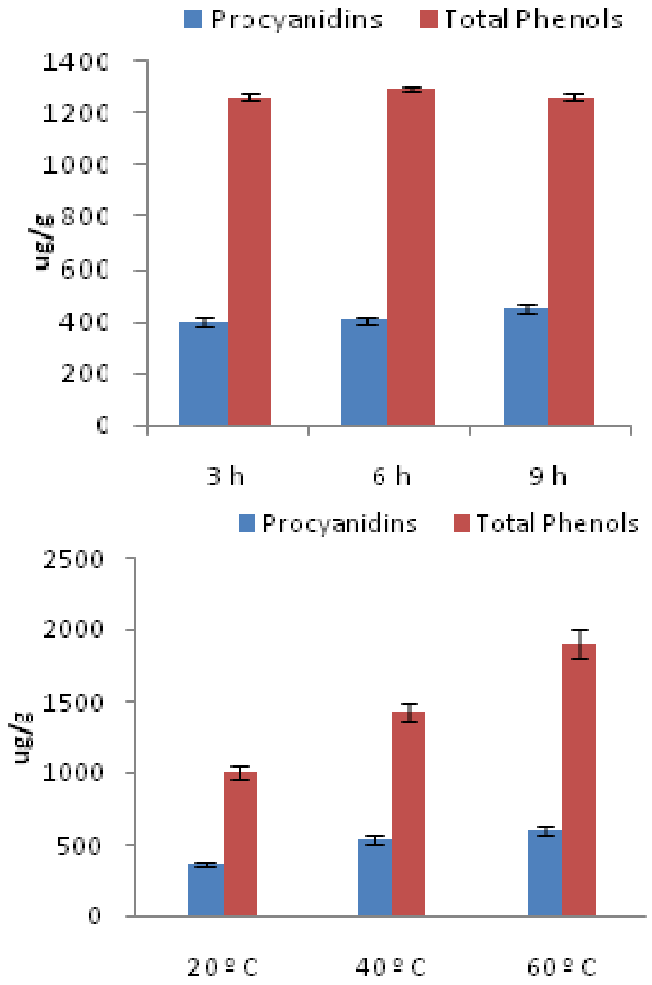

Fig. 3. Procyanidins and total phenols as function of A extraction time and B temperature

Evaluation of phenol extraction conditions

For this study, the best phenol extraction conditions for *P. patula* bark were determined using spectrophotometric methods to quantify total phenol and procyanidins content (Valls *et al*., 2000). Using ethanol (100%), the biggest percentage of total extract was obtained, along with the total phenolic and procyanidin contents. However, very similar percentages were obtained using a 70:30 ethanol: water mixture, which, industrially, is better considering that water is less volatile than ethanol, thus avoiding losses during the extraction process. With water: ethanol (50:50) and water (100%), the percentages of total extract were lower at 8.96% and 6.02%, respectively, and this was also the case for total phenolic and procyanidin contents. The use of methanol and methanol-water mixtures for *P. radiate* bark phenol extraction (water 100%, water: methanol 7:3, water: methanol 1:1, water: methanol 3:7, methanol 100%) has allowed one to obtain average extraction percentages of 8.3, 11.98, 16.04 and 21.93% from this species, respectively; these extractions were made from 27-year-old-bark samples, with a particle size obtained from passing the bark through 40-60 mesh, an agitation of 100 rpm, and 70°C of warming. It was noted that increasing the methanol percentage increases the extraction percentage. The appropriated time for obtaining *P. patula* bark extracts was 9 hours with a 6.02% total extract. With 3 and 6 hours, 4.7 and 5.30% of total extract was obtained, respectively. Increasing the extraction time to greater than 6 hours increased extraction costs. In *P. pinaster* bark extracts, extended times of extraction are not necessary for phenolic compounds liberation from the bark structure (Jerez *et al*., 2006). The temperature for the biggest *P. patula* bark phenol extraction was 60°C with 9.95% total extract, which is superior to what was obtaine d at ambient temperature (6.09%) or at 40°C (9.37%). Figure 3 shows the levels of procyanidins and total phenols as a function of extraction time and temperature.

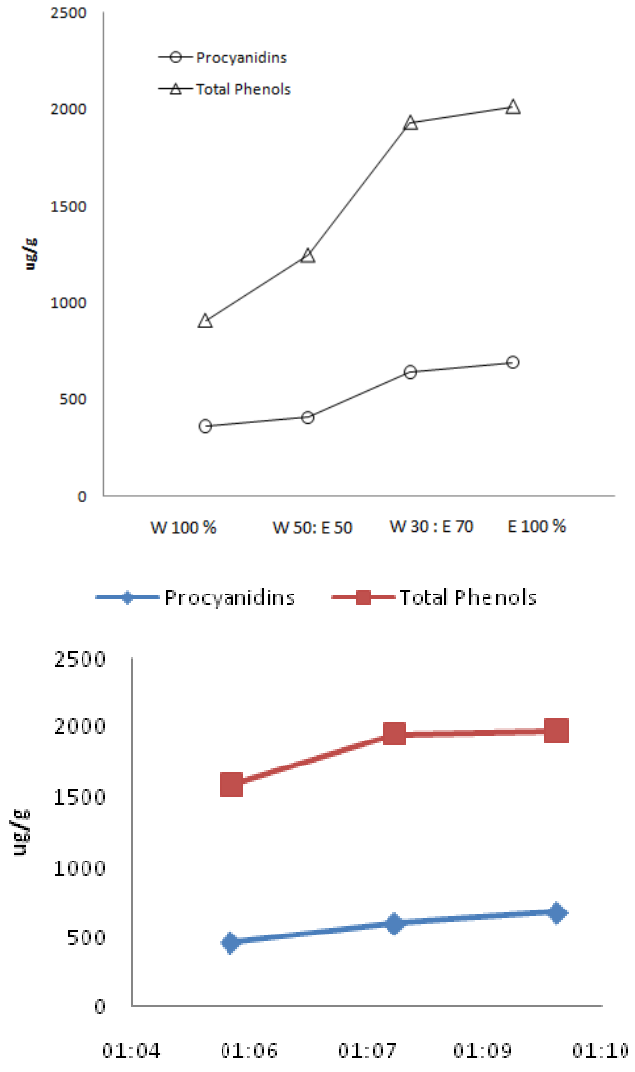

Fig. 4. Procyanidins and total phenols as function of A solvent water: ethanol ratio, B solvent: bark ratio

Revolutions above 250 rpm (250, 380 and 550 rpm) showed constant total extract percentages (9.56, 10.19 and 9.98%), indicating the need for a vigorous and constant agitation to obtain the best extraction.

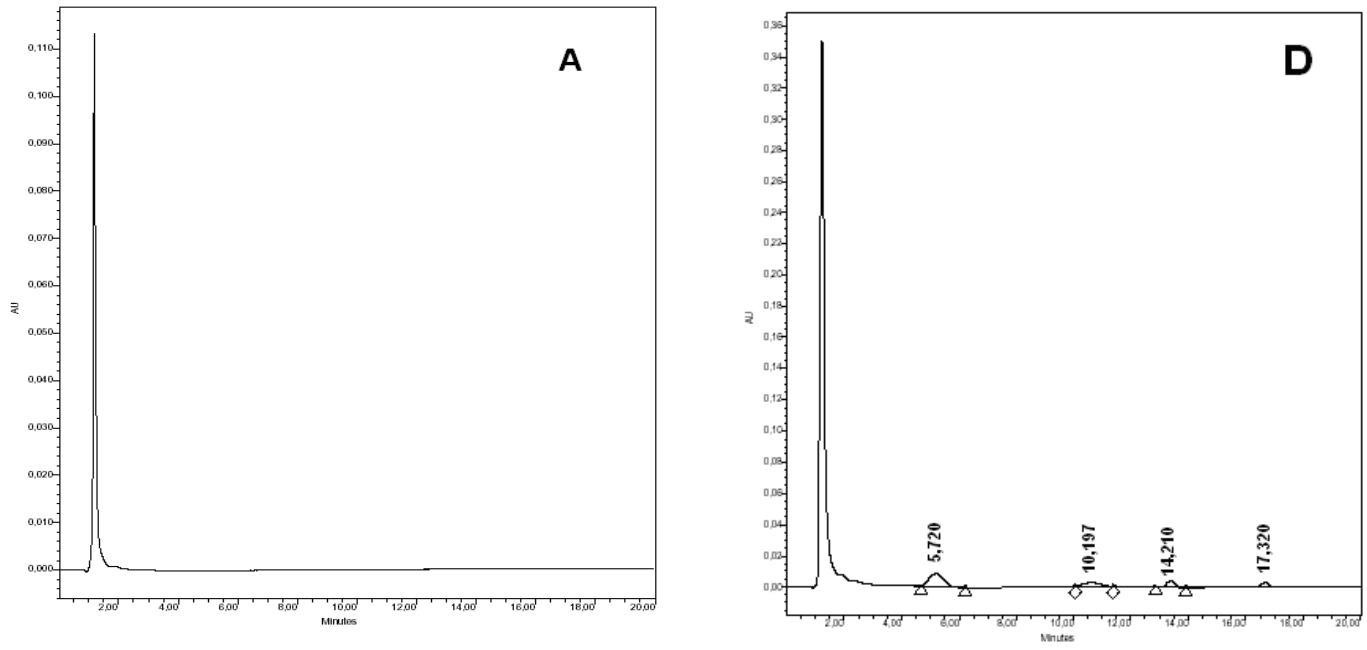

Fig. 5. HPLC chromatogram: A) Blank solvent, B) Peritoneal lavage with drug, C) Peritoneal lavage and drug (Ketoprofen®) and D) Peritoneal lavage with extract of *Pinuspatula* bark

Total phenolsandprocyanidins contents were proportional to the total extract percentage obtained. Studies conducted in Cuba have reported bark extract yields of 8.29% for *P. caribaea* and of 10.19% for *P. cubensis* (Martínez, 1983). For a lower energetic cost, a 250-rpm agitation is recommended. A 1:10 bark:solvent ratio allowed us to obtain 4.77%, which is the largest total extract percentage, and the largest obtained percentage of total phenolic and procyanidin content. With 1:6 and 1:8 bark:solvent ratios, total extract percentages of 3.92 and 4.28% were obtained, respectively. The use of a smaller particle size(1.18 mm) enabled us to obtain the largest total extract percentage(4.77%). Evidently, when the particle size is reduced, the total extract percentage increases because the sample and solvent have greater surface contact.Based on our experiments with extraction conditions, the solvent choice is important. While water (100%) produces the smallest total extract percentage(6.02%), under the same conditions, water obtained 2.23% of the total extract for *P. cooperi*and 10.61% for *P. ayacahuite*, while ethanol extraction (50%) yielded 6.66% for *P.engelmannii* and 19.4% for *P. ayacahuite*. These observations indicate that the performance obtained with 50% ethanol is double or more than that obtained with water (Castro, 2003). Figure 4 shows the levels of procyanidins and total phenols as a function of the solvent water: ethanol ratio and the solvent: bark ratio. Total phenol content was determined for *Pinus bark* extracts using the Folin-Ciocalteu method, using gallic acid as the reference substance. Values in the range of 909.51 and 2013.62 µg/g were obtained, with a mean value of 1610.67 µg/g(%RSD=1.01). Using the vanillin assay (Valls *et al*., 2000), the procyanidin content values were found to bein the range of 353.61 and 691.57 µg/g for *Pinus bark* extracts, with a mean value of 560.82 µg/g (%RSD=1.56). Mexican pine species including *P. cooperi* and *P. durangensis* contained total phenol and procyanidin contents of 491.60 µg/g, 706.00 µg/g and 134.8µg/g, and 782.00 µg/g, respectively (Rosales and Gonzáles, 2003). Total phenolic and procyanidins contents in grape varieties such as Tempranillo and Cabernet Sauvignon are 1939.00µg/g and 783.00 µg/g and 2038.00 µg/g and 698.00 µg/g, respectively (Valls *et al*., 2000), showing a similar content as those found in

the pine bark studied. Rosales *et al*. (2003) found that the procyanidin content is directly proportional to the performance of the total extract, finding the lowest values of procyanidins in the aqueous extracts of *P. cooperi* and *P.engelmannii*and the highest value in the aqueous extract for *P. ayacahuite* with 732.00 µg/g. In ethanol extracts, better yields were observed: 646.00 µg/g for *P. arizonica*to 1618.00 µg/g for *P. leiophylla*. Procyanidins contents of 1050.00 µg/ghavebeen found for aqueous extracts of *P. Radiate* (Inoue *et al*., 1998). Honoratoand Hernández (1998) mentioned that barks with a procyanidin content of 800.00 µg/g can be considered industrially potential as alternative tannin sources, so *P. patula* bark extracts can be considered for this purpose as well. Rosales *et al*.(2003) found that the yield of total phenolics in the extract were smaller in the aqueous than in the ethanol extract, although in some species, the performances were similar, as is the case with *P. leiophylla,* with 722.00 µg/gin the aqueous extract and 785.00 µg/g in the ethanolic extract. These authors found that the lowest yields were those of the aqueous extract of *P. cooperi*, with 375.00 µg/g, and of *P. engelmannii*, with 322.00 µg/g. The spectrophotometric methods used to determine the total phenolics and procyanidins presented good linearity with correlation coefficients of 0.9939 (y = 0.0002x + 0.0025) and 0.9962 (y = 0.0002x – 0.0043), respectively. The methods presented good repetitiveness (n=9) with %RSD values of 1.22 and 1.71, respectively. For the reproducibility, variation coefficients were 1.92 for total phenolics and 2.39 for procyanidins method. Methods also presented good determination coefficients (r^2), all close to 1, showing a good correlation between the concentration and the absorbance, demonstrating the linearity of the studied range of concentrations. The exactitude gave recovery coefficient values R of 98.91(t_{obt}= 1.16) and 99.84($t_{ob\ t}$= 0.26), as well as variation coefficients of 2.82 and 1.89, respectively. The values obtained were within the established criteria of r^2=0.99, repetitiveness(%RSD<5%), reproducibility(%RSD<5%) and exactitude($t_{obt}<t_{tab}$= 2.306 to 95% of confidence) (Gutiérrez *et al*., 2000). According to the total extract percentages and the results obtained with the total phenolics and procyanidins methods, the best phenolic extraction conditions for *P. patula* bark were determined and are presented in Table 2. These conditions allowed us to obtain a total extract of 8.56%.After bark phenolics extraction residues are generated, it is necessary to evaluate their physicochemical characteristics before and after the extraction to identify a way to use them as a fertilizer, food source, or for other uses (Sen *et al*., 2016).

Figure 1 shows the *P. patula* bark proximal analyses results, before and after the extraction. Before the extraction, the sample brute protein percentage was 0.76%, brute fat was 2.54%, ashes were 0.83%, brute fiber was 28.03% and the non-nitrogenous extract was 67.84%. After the extraction, the values were 0.80, 3.49, 0.64, 33.23 and 61.84%, respectively. It can be observed that the phenolic extraction is not representative for brute protein, brute fat and brute fiber content, but there is a diminution of the ashes and non-nitrogenous extract contents, indicating solubilization of minerals and sugars. Pine bark also contains free sugars mainly composed of glucose and fructose. Proximal analyses made on *P. oocarpa* bark showed a constant behavior of brute protein, fat, fiber andnon-nitrogenous extract percentages before and after phenolics extraction and a significant diminution of ashes content due to its solubilization (Gallo and Tandioy, 2005). Fig. 2 shows the behavior of lignin and cellulose content in the *P. patula* bark sample. Before the lixiviation process, the lignin and cellulose contents were 61.48 and 27.87%, respectively. Their percentage increase (68.03 and 43.72%, respectively) could be attributed to the fact that they remained in the extract while levels of minerals and sugars were eliminated; however, a certain quantity of lignin and cellulose is extracted with the phenolics. These lignin and cellulose values are bigger than those found for *P.radiata*, from Chile and *P.radiata*, from New Zealand (Gebert, 1984). The hydroalcoholic extract obtained under the best conditions determined in this study was used in tests of anti-inflammatory activity in an animal model.

Biological assessment of the hydroalcoholic extract of Pinuspatula bark

Lethal dose 50

To determine the LD50, doses of the hydroalcoholic extract obtained under the best extraction conditions(a 30:70 ethanol: water ratio, a particle of size of 1.18 mm, 60 °C and 250 rpm) were tested in a range from 125 to 1000 mg/kg. Groups of six mice were used for each dose, with a range of steps between 31.7 and 37.2, 29.2 and 40.8, 28.7 and 37.0 and 37.3 and 46.7 g for the doses of 125, 250, 500 and 1000 mg/kg of hydroalcoholic extracts, respectively. During the 10 days following the application of the extract, the behavior and symptoms of the rodents were monitored. Most presented digestive alterations, as the feces were noticeably slightly softer. Nevertheless, the administered doses did not result in the death of any of the rodents. As no rodents were found dead at the highest concentration (1000 mg/kg) employed, it can be concluded that the LD50 is greater than this value. These results allowed us to use the highest concentration to test the ability of the extract to inhibit inflammation. There have been no reports of the LD50 of *Pinuspatula* bark, but works on other plants with anti-inflammatory properties were used as reference. In studies carried out with extracts from the bark of *Pinusmaritima* and *Pinuspinaster*, it was found that procyanidinsare non-toxic in doses administered in the short- and long-term (LD50 > 4,000 mg/kg in rats and mice). These concentrations do not cause potential mutations or birth defects and do not present adverse effects on fertility, pregnancy or parenting. Oral administration of the aqueous ethanolic extract of*F. trichopoda*in doses up to 5000 mg/kg body weight did not produce any mortality and any visible signs of toxicity in rats.LD50 values of 3890 mg/kg have been reported for catechinin rats(sciencelab.com). In 1990, Chun-Fu Wu and Quina-Hay, working with the plant *Sambucuswilliamsii* (Caprifoliaceae), obtained an LD50 of 589 mg/kg. In 1995, Tavares and collaborators reported an LD50 of 555 mg/kg for *Croton cajucara* (Euphorbiaceae) (Arce, 2001). In 1994, Adday found an LD50 of 567 mg/kg for *Pteropyrumaucheri* (Polygonaceae) (Adday, 1994). In 2001, Arce found an LD50 of 457.2 mg / kg for *Piper lacunosum* (Piperaceae) (Arce, 2001).

Measuring levels of leukotriene B_4 produced by polymorphonuclear leukocytes as indicators of anti-inflammatory activity

The anti-inflammatory action of the extract of *Pinuspatula* bark was determined according to the procedures described in the methods section. To identify leukotriene B_4 in the peritoneal lavage of rats, the chromatographic method developed by Salari and collaborators (Salari *et al*, 1985) was

used. Identification of leukotriene B_4 was carried out by initially injecting the blank, which contains only buffer pH 7.2 with 1 µg/L ionophore and $CaCl_2$ at a final concentration of 1.75 mM, into the chromatograph. No peak was observed in the blank solvent. Figure 5(A) shows the chromatogram for the blank solvent. Figure 5(B) shows the chromatogram of the control sample that contains the blank solvent and the peritoneal lavage of rats. Four signals were observed at 2.461 min, 5.690 min, 8.783 min and 10.097 min, which were not observed in the blank. The chromatogram in Figure 5(C) presents the chromatogram of the positive control, which contains the rat peritoneal lavage and the drug (Ketoprofen®).

There are signals at 2.416, 5.756, 8.633, 10.209 and 18.576 min. Figure 5(D) shows the chromatogram for the sample containing the blank solvent, rat peritoneal lavage and the hydroalcoholic extract of *Pinuspatula* bark. Signals were observed at 5.720, and 10.197, 14.210 17.320 min. The presence of the same peak is observed in the peritoneal washing, the peritoneal lavage with drug and the peritoneal lavage with *Pinuspatula* bark extract, at 5.690, 5.756 and 5.720 min, respectively. According to the work performed by Arce (Arce, 2001) and using the same conditions reported by Salari and collaborators (Salari *et al*., 1985), the presence of a peak at a retention time of 5.690 min indicates the possible presence of leukotriene B_4. Table 3 shows the peak areas at the retention time of 5.690 min in the control samples, positive control and sample with extract. Average areas for the signal with a retention time of 5.690 min in HPLC. The results shown intable 3 were analyzed by ANOVA to determine if there were significant differences between the treatment groups. Figure 5 C shows the effect of the drug Ketoprofen® on the control. In the chromatogram, there is a clear decrease in the signal at the retention time of 5.690 min, which indicates that the drug has acted on these substances, inhibiting their production by up to 90.58%. The pharmacological activity of Ketoprofen®is carried out on substances of the eicosanoid type; a retention time of 5.690 min corresponds to this class of substances. The chromatogram in Figure 5 D, corresponding to the sample treated with the hydroalcoholic extract of pine bark, also presents a 21.97% decrease in the peak present at 5.690 min, indicating that its action is similar to that exerted by the reference drug, and thus, it possibly affects the production of eicosanoids. According to the chromatographic results obtained, it can be concluded that the hydroalcoholic extract of bark of *Pinuspatula* has anti-inflammatory activity. The factthat oral treatment with *P. patula* bark extract improved and ormaintained the levels of leucocytes suggest that this could be due to improvement in the inflamation state of therats. The seprotective effects are duetosuppression of leucocytestowards inflamedarea, stabilization of reticulo-endothelialsystem and inhibition of release of inflammatorycells (Tatiya *et al*., 2017). The ANOVA results indicated that there are significant differences among the groups of distinct treatments ($p < 0.05$). A variance ratio (14.82) superior to the value critical of F (4.07) was obtained, indicating a statistically significant difference between the treatments made.

Identification of the 5.690 min peak by LC-MS

Considering the injections performed with peritoneal washings, in the samples inhibited by the drug and the ethanolic extract, the fractions corresponding to the 5.690 min were collected to determine their LC-MS spectra and elucidate the type of substance present, which, in this case, was the presence of leukotriene B_4. The samples to be analyzed by electrospray ionization mass spectrometry (ESI-MS)in positive ion mode were injected into an LC-MS Finnigan SSQ 710 C ESI-Instrument. A fused silica capillary column (0.5 m X 50 µm) was used, with a methanol: water (1:1) mobile phase and a flow of 10 µL/min. The spectrum was obtained at 3 s/scan on a mass range m/z of 250 to 500.Figure 6 corresponds to the mass spectrum of the fraction collected at 5.690 min, and one can see that the collected fraction, after separation by HPLC, is composed of various types of substances.

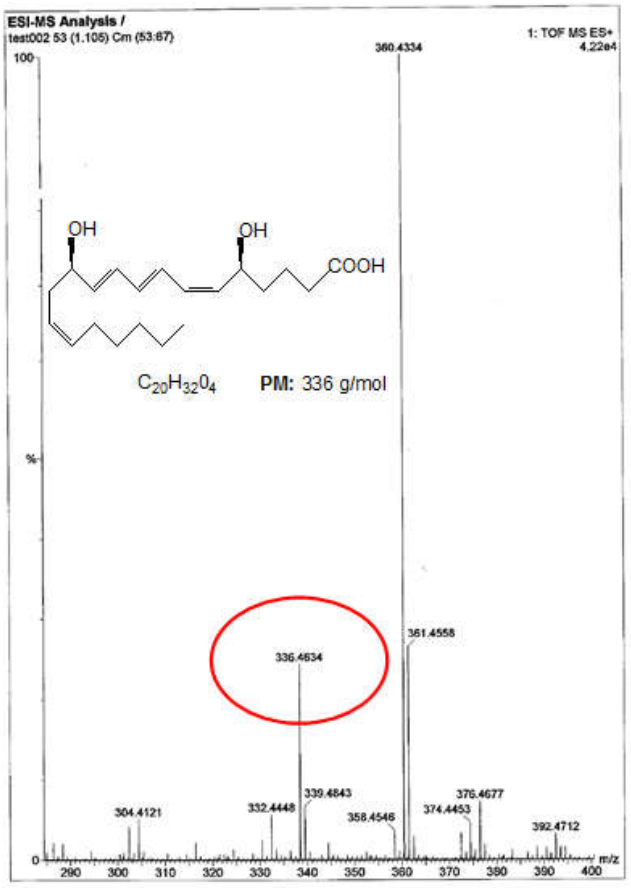

Fig. 6. LC-MS spectrum of the fraction with a retention time of 5.690 min and chemical structure of LTB4

According to the mass spectrometry results in Figure 6, the masses range from 304.41 to 392.47 g/mol. In the spectrum, a mass of 336.46 g/mol is also observed, which could correspond to the ion form of leukotriene B_4 and would confirm the presence of this molecule in the fractions collected at this time of retention. In Figure 6, the chemical structure of the leukotriene B_4 molecule is depicted. In the spectrum, other masses are present at 360.43, 361.45, 376.46 and 392.47 g/mol, which indicate the possible presence of additional substances in the collected fractions. Studies by Wheelan and collaborators (Wheelan, 1996) reported the presence of molecules derived from LTB_4 during the metabolism mediating inflammation, including 14,15-dihydro-LTB_4)([M - $H]^-$, m/z 337), 20-hydroxy - LTB_4) ([M - $H]^-$, m/z 351), 20-carboxy- LTB_4([M - $H]^-$, m/z 365), 16-carboxy- LTB_3)([M - $H]^-$, m/z 311), and 5,12-dihydroxy-6-cysteinylglycyl-7, 9, 14-eicosatrienoic acid (d-LTB_3) ([M-$H]^-$, m/z 513). The presence of 5-hydroxyeicosatetranoic acid (5-HPETE) and LTB_4 and its isomers have also been observed. The presence of LTB_4 and prostaglandin G_2 as mediators of inflammation have also been

reported in fractions separated by HPLC. The molecular weight of prostaglandin G_2 is 366 g/mol (Arce, 2001). Accordingly, it can be deduced that LTB_4 is present in the fraction separated by HPLC; however, because the sample contains others compounds, a more detailed study of the sample is recommended. Taking into account the evidence obtained by HPLC and LC-MS, it can be concluded that the control sample of the rat's peritoneal lavage contains leukotriene B_4in addition to other eicosanoids and that both the drug and the hydroalcoholic extract *Pinuspatula* bark decrease the levels of this component.Oral administration of the ethanol extract ofthe stem bark of Alstoniaboonei to Wistar rats caused a dose-related decrease in the migration of leucocytes in agar-induced inflammation indicating that this is a mechanism of anti-inflammatory effect of the extract (Enechi *et al*., 2013).

Conclusion

Optimal phenolic extraction conditions were determined for *P. patula* bark, optimizing the particle size, bark:solvent ratio, time, agitation, ethanol:water ratio and temperature. Method optimization was reached by using a reduced particle size, a moderate extraction temperature, sufficient extraction time, and optimal solvents ratios for phenolic extraction. The lignin and cellulose *P. patula* bark content make this species an important vegetal source of chemical derivatives such as bioethanol, furfural, and forager yeasts, among others, all with important industrial applications. The best extraction conditions for phenolics present in *P. patula*bark included using a particle size of less than1.18 mm, a 1:10 bark-to-solvent ratio, an extraction time of 6 h, 250 rpm of agitation, a 30:70%water: ethanol solvent, and an extraction temperature of60°C. These conditions resulted in obtaining 8.56% of total extract and total phenolic and procyanidin contents of 1610.67 and 560.82µg/g, respectively. The results obtained for the biological activity of the hydro alcoholic extract of *Pinuspatula* bark indicate that it has the ability to decrease the concentration of eicosanoids produced by thepolymorph nuclear leukocytes of rats, which makes this extract a good therapeutic option. The eicosanoid levels measured by liquid chromatography indicate that the hydro alcoholic extract *Pinuspatula* bark may reduce their production by 21.97%, confirming its anti-inflammatory effects.

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Conflict of interest

We present our original article of investigation titled "Extraction of phenolic compounds from *Pinuspatula* bark using ethanol-water mixtures and anti-inflammatory action of the ethanolic extract". The authors declare that there are no conflicts of interest.

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