

Anti-inflammatory activity and identification of the *Verbena litoralis* Kunth crude extract constituents

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Verbena litoralis is a plant popularly known as “gervãozinho-do-campo” in Portuguese. It is traditionally used for stomach, liver and gallbladder problems, and as an anti-inflammatory and anthelmintic. The goal of this study was to determine the chemical composition of the crude extract obtained from the aerial parts of *V. litoralis* by Ultra High Performance Liquid chromatography coupled with Mass Spectrometry in Tandem (UHPLC-MS/MS); assess the anti-inflammatory activity of ethyl acetate fraction and of crude extract; and verify liver, kidney and pancreas damage. In this study, the chemical composition of the extract was identified via UHPLC/MS/MS, assessing the anti-inflammatory activity of the crude extract and the acetate fraction in an induction model of the granulomatous tissue, as well as given liver, kidney and pancreas damage markers. Chlorogenic acid, luteolin, caffeic acid, apigenin, p-coumaric acid, vanillic acid, ferulic acid and quercetin were quantified in the extract. After the seven-day treatment, the granuloma of the animals treated with the plant extract and fraction presented values very close to the positive control (nimesulide). The *V. litoralis* crude extract and ethyl acetate fraction show anti-inflammatory activity similar to the nimesulide without evidence of liver, kidney and pancreas damage, which attributes the plant’s pharmacological action to the flavonoids found.

Keywords: *Verbena litoralis*. UHPLC/MS. Flavonoids. Anti-inflammatory.

INTRODUCTION

Vegetables produce a wide variety of metabolites and are considered the oldest source of medicine for men. As potential sources of natural bioactive and antioxidant compounds, they have been investigated for their characteristics and effects on health (Brandão *et al.*, 2010; Ghasemzadeh, Ghasemzadeh, 2011).

Verbena litoralis is a species of the Verbenaceae family, which occurs in tropical and subtropical regions, mainly in temperate regions of the Southern Hemisphere and, to a lesser extent, in temperate regions of the Northern Hemisphere (Barroso, 2004; Souza, Lorenzi, 2005).

This plant, commonly known as “gervãozinho-do-campo” or “erva-de-pai-caetano” in Portuguese, is used

ethnopharmacologically in popular medicine for stomach, liver, gallbladder, tonsillitis, bladder and kidney problems, as well as for uterus and ovary inflammations, spine affections, colic, and as an anthelmintic (Garlet, 2000; Marodin, 2000; Sebold, 2003). Studies with *V. litoralis* extracts show the presence of flavonoids, anthracenosides, cardioactives, hemolytic saponins, catechinic tannins and carotenoids in this plant. Volatile oils, coumarins and mucilages were present in a smaller quantity (Souza *et al.*, 2005).

A drug’s anti-inflammatory activity can be assessed through granuloma assay, which consists in the implementation of cotton pellets on the back of rats, causing the formation of a vascularized fibrous capsule, fibroblasts and infiltrating mononuclear cells. The drug’s effect is evaluated by the size of the fibrous capsule developed. The smaller the capsule, the bigger the anti-inflammatory effect produced by the tested drug (Bailey, Sturm, Lopez-Ramos, 1982; Dalmora, 1996).

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Flavonoids, which occur normally in plants, are water-soluble pigments that act as potent antioxidants able to eliminate hydroxyl radicals and superoxide anions (Dunne, 2009). Flavonoids are known to present anti-inflammatory properties, an important characteristic, since the anti-inflammatory processes are involved in the development of cancer, so this confers to them a significant pharmacological importance (Seelinger *et al.*, 2008).

This study's goal was to identify the chemical constituents of the crude extract, assess the crude extract and the acetate fraction anti-inflammatory activity of the aerial parts of *V. littoralis* via induction method of the granulomatous tissue, as well as assess if there was kidney, liver and pancreas damage by the determination of urea, creatinine, aspartate aminotransferase, alanine aminotransferase, amylase and lipase.

MATERIAL AND METHODS

Botanic material collection and extract procurement

Verbena littoralis was collected at the Federal University of Santa Maria (UFSM) campus (coordinates S 29°42'56.1276" e W 53°43'13.6632") in the City of Santa Maria, RS, Brazil. The plant was identified by Professor Ph.D. Thais Dorow of the Biology Department of UFSM. One exsiccate sample of the species was deposited in the Department of CCNE herbarium of the UFSM under the registry SMDB 13095.

Extract preparation

The crude extract of the aerial part pulverized was obtained by cold maceration with daily agitation and solvent renovation every 7 days for 30 days at room temperature. The solvent used was alcohol 70%, which evaporated and the extract was freeze-dried and portioned by organic solvents of crescent polarity with n-hexane, chloroform, ethyl acetate and butanol originating the respective fractions (Simões *et al.*, 2017).

Polyphenol identification and quantification by UHPLC-ESI-MS/MS

The analytical grade standards used with 95% purity were (+)-catechin, 3-acetyl coumarin, 3,6-dihydroxyflavone, 4-hydroxycoumarin, 6-hydroxycoumarin, apigenin, chlorogenic acid, chrysin, fisetin, galangin, gallic acid, kaempferol, luteolin, myricetin, p-coumaric acid, quercetin, quercitrin,

resveratrol, rosmarinic acid, rutin, trans-cinnamic acid and vanillic acid obtained from Sigma-Aldrich (St. Louis, MO, USA). Caffeic and ferulic acids were obtained from Fluka Analytical (Buchs, Switzerland).

Ultrapure water was obtained from a Milli-Q Synergy UV system (Merck Millipore, Darmstadt, Germany). Methanol and acetonitrile (LC-MS grade) were obtained from Panreac (Castellar del Vallès, Spain). Acetic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the standards (1000 mg L⁻¹) were prepared by the dissolution of appropriate amounts of substances in LC-MS grade methanol. All the solutions were stored in amber glass flasks at -30 °C until used. Working solutions of the standards were prepared by the dilution of the stock solutions according to the desired concentrations (Faccin *et al.*, 2016).

UHPLC-ESI-MS/MS conditions

Following Faccin *et al.* (2016), these separations were carried out on a UHPLC 1260 Infinity Binary system (Agilent, Santa Clara, CA, USA), which was able to operate at pressures up to 600 bar. A Zorbax SB-C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 µm, Agilent) was used at a temperature of 40 °C. The injection volume was 5 µL, and the injected aliquots were acidified to a final concentration of 0.1% acetic acid (v/v). The phenolic compounds were separated using a gradient elution composed of 0.1% acetic acid in water (A) and acetonitrile (B) as the mobile phase at a constant flow rate (0.8 mL min⁻¹) according to the following elution program: 8.0% B (0.00–0.10 min); 8.0–25.8% B (0.10–3.45 min); 25.8–54.0% B (3.45–6.90 min); 54.0–100.0% B (6.90–7.00 min); and 100.0% B (7.00–9.00 min).

An electrospray ionization source (ESI) was used to ionize the chromatographic effluent generated up to 7.0min. The parameters for ESI were optimized to give the best response for the analytes especially for intensity and signal stability. The final optimized parameters were a gas flow of 11L min⁻¹, a nebulizer of 30psi, a capillary voltage of ± 2.4 kV and a gas temperature of 250 °C, with nitrogen used as the drying gas. The compounds were subsequently analyzed in an Agilent 6430 Triple Quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) mode with a resolution of 0.7 m/z (FWHM). The quantification transitions were divided into three temporal segments of acquisition and the dwell time for each transition was optimized to 20ms. High purity nitrogen (99.999%) obtained from Linde (Munich, Germany) was used as the gas for inducing collision (Faccin *et al.*, 2016).

Sample analysis

Around 0.06g of the freeze-dried crude extract was re-suspended in 3mL of methanol, sonicated until complete dissolution, and diluted with 12 mL of ultrapure water. Next, acetic acid was added to a final proportion of 0.1% (v/v). Then, the extracts were filtered through hydrophilic PTFE membranes with a 0.2 μm pore size.

The sample was submitted to a cleanup step using solid phase extraction (SPE). The Strata C18-E cartridges (Phenomenex, Torrance, USA), 500 mg, 3 mL, were conditioned with 6mL of methanol:0.2% acetic acid (1:1, v/v) and equilibrated with 6 mL of 0.1% acetic acid (v/v) in water. A fixed volume of 2 mL of the obtained extracts with a final methanol:water:acetic acid composition of 20:80:0.1 (v/v) was percolated with a 2 mL min^{-1} flow rate, followed by washing with 2 mL of 0.1% acetic acid. Lastly, the cartridge was eluted with 2 mL of methanol.

Just before the chromatographic analysis, the eluate obtained from the SPE procedure was diluted in a MeOH:0.2% CH_3COOH (1:1, v/v) solution.

Anti-inflammatory activity

Animals

Fasted male Wistar rats weighing between 180 and 200 g, divided into 5 groups of 6 animals each were housed in air-conditioned, controlled conditions, with room temperature 22 ± 2 °C, relative humidity of 65%, and artificial illumination 12h per day. They were given free access to food and water. The assays were conducted in accordance with the national protection laws on animal welfare, as registered in the Ethical Committee of the Federal University of Santa Maria, number 009/2012.

In vivo anti-inflammatory effect

The anti-inflammatory effect of the formulations was assessed by the cotton pellet granuloma method, as described by Meier, Schuller and Desaulles (1975). The animals were divided into groups of six rats and anesthetized. Sterilized cotton dental roll pellets weighing 40 mg each were implanted subcutaneously in four symmetrical positions at dorsal sites of each animal. *V. litoralis* crude extract and the acetate fraction, at a dose of 300 mg/kg/day, were divided into two administrations with a volume of 0.5 mL and given orally at intervals of 6 hours during 7 consecutive days. Control groups received a mixture of propylene glycol/water (20:80 v/v) and only water. As a reference, nimesulide, a non-steroidal anti-inflammatory drug at a concentration of

5 mg/kg/day was administered twice a day at intervals of 6 hours.

On the eighth day, the granulomas were removed, and the weights were determined after drying in an incubator at 60 °C for 24 h. The difference between the final and initial weight of the granuloma was considered as the weight of the granulomatous tissue produced.

Hematological assessment

At the end of the treatment, approximately 5 mL of blood was collected in sterile bottles with and without anticoagulant ethylenediamine tetraacetic acid (EDTA). The blood samples for the hemogram were collected in bottles of 0.5 mL of blood containing 10 μL of EDTA. This material was appropriately homogenized and refrigerated by a period no longer than two hours and then sent to the *Laboratório de Análises Clínicas Veterinárias* (LacVet) of UFSM to be processed. The serum was immediately separated from the blood portion and kept frozen for posterior use in biochemical assays.

A hematological assessment was performed as per the methods described by Schalm (1970), including global leukometry, hematimetry performed in electronic counter (BC-2800 Vet – Auto Hematology Analyzer, Mindray), and globular volume determination by the microhematocrit method. The cell differential count was determined after coloration with the hematological coloring (panoptical) of blood smear and microscopic revision. With the electronic counter help, total erythrocyte concentration ($/\mu\text{L}$), hemoglobin concentration (g/dL), RDW (%), platelet concentration ($/\mu\text{L}$) and total leukocyte concentration ($/\mu\text{L}$) were determined. The mean corpuscular volume (MCV) and concentration of mean corpuscular hemoglobin (MCHC) values were calculated as per classic formulas recommended by Jain (1993). The total plasma proteins concentration ([PTref]) was obtained by manual refractometry (Instrutherm, Brazil).

Biochemical evaluation

A part of the collected blood samples was immediately separated to obtain the serum for later biochemical evaluation. These serum were stored at a temperature of -20 °C. Biochemical analysis of serum samples was performed to estimate the enzymes aspartate transaminase ([AST]) and alanine aminotransferase ([ALT]), the metabolic products urea ([urea]) and creatinine ([Crt]), as well as glucose ([glucose]) and cholesterol ([Cholesterol]) using commercial kits (Labtest®) in a semi-automatic analyzer (Bioclin

Mindray BA 88A®). This analyzer supports bi-chromatic tests for endpoint, fixed-time and kinetics methods. The principles of these determination methods are described below.

The [AST] determination is performed through catalyzation of a reversible reaction involved in aspartate deamination to form oxaloacetate. On the other hand, [ALT] mensuration is based on reversible catalyzation involved in alanine deamination to form pyruvate (Stockham, Scott, 2011). Both deamination methods are kinect and the results are express in units per liter (U/L).

[Crt] determination is performed via the kinect reaction of creatinine with alkaline picrate. Reaction measurement in the first minutes allows for [Crt] determination. The system is colorimetric by end-point reaction. Urea is determined through enzymatic hydrolysis by urease-producing ammonia and CO₂. The ammonia formed reacts with 2-ketoglutarate and NADH in a reaction catalyzed by glutamate dehydrogenase causing oxidation of NADH to NAD. The consequent reduction of absorbance is proportional to the [urea] in the sample. The results of both products were expressed in milligrams per deciliter (mg/dL).

Glucose was determined based on the quantity of the hydrogen peroxide (H₂O₂) formed in the catalyzed reaction by glucose oxidase with 4-aminoantipyrine and phenol, under peroxidase catalyst action forming a red antipyrilquinonimine, which has a color intensity proportional to the [glucose] in the sample (Labtest Diagnóstica S.A.). The enzymatic system used in the reaction was measured by the colorimetric method of endpoint and the results are expressed in mg/dL.

For the total cholesterol dosage, the enzymatic colorimetric method based on the hydrolysis of cholesterol

esters was used, forming free cholesterol and fatty acids. Free cholesterol is oxidized forming (H₂O₂) and cholest-4-en-one. In the presence of peroxidase and H₂O₂, phenol and 4-aminoantipyrine are oxidized forming, which has a maximum absorptivity in 500nm. The intensity of the color red formed in the final reaction is proportional to the [cholesterol] in the sample (Labtest Diagnóstica S.A.), results are expressed in mg/dL.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by the Tukey Test ($p < 0.05$) using the GraphPad Prism software.

RESULTS

Polyphenol identification and quantification by UHPLC-MS/MS

With the Ultra High Efficiency Liquid Chromatography Coupled to Mass Spectrometry the Multiple reaction monitoring (MRM), chromatogram of calibration patterns in a 0.5 µg/mL in MeOH/CH₃COOH a 0.2% (1:1, v/v) concentration and Multiple reaction monitoring (MRM) chromatogram of the *V. littoralis* crude extract (0.4%, m/v) were obtained (Figure 1).

According to the results presented in Table I, the predominant phenolic compound was chlorogenic acid at a concentration of 1484.5 ± 24.2 µg/g, followed by luteolin (177.7 ± 4.7 µg/g), caffeic acid (41.8 ± 2.9 µg/g), apigenin (37.9 ± 0.8 µg/g), *p*-coumaric acid (33.1 ± 0.4 µg/g), vanillic acid (18.1 ± 3.0 µg/g) and ferulic acid (10.7 ± 0.6 µg/g).

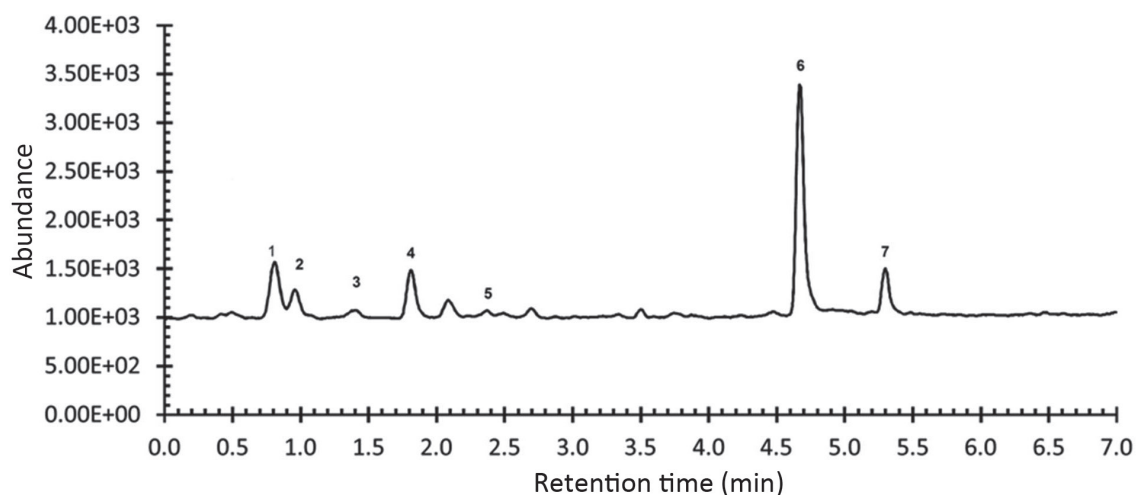


FIGURE 1 - UHPLC/MS chromatogram of *V. littoralis* crude extract: 1-chlorogenic acid; 2-vanillic acid; 3-caffeic acid; 4-*p*-coumaric acid; 5-ferulic acid; 6-luteolin; 7-apigenin.

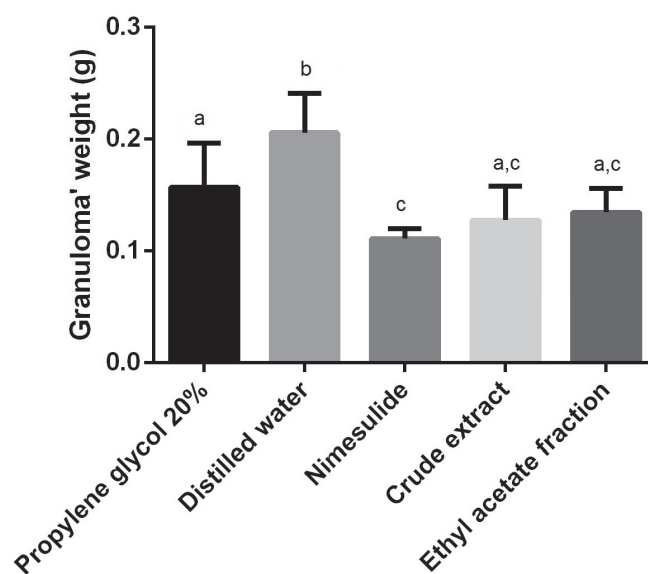
TABLE I - Compounds of *V. litoralis* crude extract identified and quantified by Ultra High Performance Liquid chromatography coupled with Mass Spectrometry in Tandem (UHPLC-MS/MS)

Peak	Retention time (min)	Quantification transition	Confirmation transition	Compound	Concentration $\mu\text{g g}^{-1}$ of plant
1	0.61-0.94	353.1>191.1(5)	-	chlorogenic acid	1484.5 \pm 24.2
2	0.87-1.13	167.0>152.2(10)	167.0>152.2	vanillic acid	18.1 \pm 3.0
3	0.89-1.10	179.0>135.1(10)	-	caffeic acid	41.8 \pm 2.9
4	1.70-2.01	163.0>119.1 (9)	-	<i>p</i> -coumaric acid	33.1 \pm 0.4
5	2.40-2.66	193.1>134.1 (9)	193.1>178.1(7)	ferulic acid	10.7 \pm 0.6
6	4.37-4.97	285.0>133.2 (27)	285.0>151.1 (27)	luteolin	177.7 \pm 4.7
7	5.19-5.53	269.0>117.1(29)	269.0>149.0(20)	apigenin	37.9 \pm 0.8
8	4.26-4.54	147.0>103.2(5)	147.0>77.1(15)	<i>trans</i> -cinnamic acid	<ILOQ
9	4.37-4.96	301.0>151.1(17)	301.0>179.0(15)	quercetin	<ILOQ

Anti-inflammatory activity

Treatment of animals during seven consecutive days with the crude extract and the acetate fraction of *V. litoralis* induced a significant reduction of the granuloma weight as shown in Figure 2. Animals treated with nimesulide showed the granuloma weight of 0.11 ± 0.01 g. The hydroalcoholic extract and the acetate fraction resulted in the granuloma weight of 0.12 ± 0.03 g and 0.13 ± 0.02 g, respectively, comparable to the reference substance. The ethyl acetate fraction was selected for the analysis based on the literature, which shows that it contains higher concentrations of phenolic compounds (Rosa *et al.*, 2010; Silva *et al.*, 2012).

Administration of the crude extract and the acetate fraction of *V. litoralis*, together with nimesulide, was compared to the control group of propylene glycol/water, demonstrating that there was a significant difference in lipase and alanine aminotransferase (ALT) between groups (Table II). Hemograms and leukograms of the animals

**FIGURE 2** - Effect of daily oral administration of extracts for 7 days on the granulomatous tissue formation. The bar represents the mean \pm S.D. of six rats. Means identified by the same letter do not differ statistically.**TABLE II** - Liver, kidney and pancreas damage markers obtained from animal serum after treatment through the administration of the crude extract and the ethyl acetate fraction of *V. litoralis*

	PR	H ₂ O	N	EB	FA
AST (U/L)	236.3 \pm 38.99 ^a	271.8 \pm 67.53 ^a	247.5 \pm 75.40 ^a	239.3 \pm 57.93 ^a	241.5 \pm 53.18 ^a
ALT (U/L)	54.84 \pm 9.10 ^c	43.40 \pm 13.13 ^{a,c}	51.82 \pm 6.30 ^{b,c}	37.98 \pm 5.32 ^{a,b}	32.88 \pm 8.67 ^a
Urea (mg/dL)	51.48 \pm 9.97 ^a	57.80 \pm 14.30 ^a	53.57 \pm 7.96 ^a	49.90 \pm 11.12 ^a	50.61 \pm 7.83 ^a
Amylase (U/L)	1305 \pm 195.6 ^a	1306 \pm 279.5 ^a	1084 \pm 214.4 ^a	1490 \pm 347.1 ^a	1295 \pm 190.1 ^a
Lipase (U/L)	8.80 \pm 1.30 ^{a,c}	9.40 \pm 1.82 ^{a,c}	5.80 \pm 2.16 ^a	12.20 \pm 2.28 ^{b,c}	15.00 \pm 4.95 ^b
Creatinine (mg/dL)	0.55 \pm 0.04 ^a	0.54 \pm 0.06 ^a	0.56 \pm 0.03 ^a	0.55 \pm 0.06 ^a	0.58 \pm 0.09 ^a

PR: animals treated with propylene glycol 20%; H₂O: animals treated with distilled water; N: animals treated with nimesulide; EB: animals treated with crude extract; FA: animals treated with acetate fraction. Data represent mean and standard deviation. Means followed by the same letter do not differ statistically (horizontal). ANOVA was applied followed by Tukey Test ($p < 0.05$).

showed no significant differences between the groups with and without treatment.

DISCUSSION

Data obtained in the literature reveal that many plant species possessing analgesic and antipyretic activities are able to interfere with the proliferative components present in the inflammatory process, thus effectively inhibiting the granuloma formation (Xu *et al.*, 2014; Kumar *et al.*, 2016; Ghosh *et al.*, 2013). Since the aerial parts of *Verbena litoralis* are traditionally employed in the treatment of inflammation, fever and pain (Cáceres, 1999; Li *et al.*, 2003; Castro-Gamboa, Castro, 2004), this study was performed in order to assess the potential anti-inflammatory activity of this plant through the inhibition of granulomatous tissue formation.

In this study, both the crude extract and the ethyl acetate fraction showed significant inhibitory activity in the formation of the granulomatous tissue, indicating a potential anti-inflammatory action of *Verbena litoralis*. This activity can be related to the chemical compounds identified in the phytochemical analysis (UHPLC/MS). Chlorogenic acid, a naturally occurring phenolic acid, exerts a positive impact on the organism due to its wide range of biological actions such as antioxidant, antibacterial, hypoglycemic, anti-inflammatory, antifungal and anticancer properties (Kujawa, Mikolajczyk, Kleszczynska, 2015). A recent study realized by Shin *et al.* (2015) showed the effects of chlorogenic acid on inflammation (colitis) induced by dextran sodium sulfate (DSS) in mice. It was observed that the supplementation with chlorogenic acid orally administered by gavage was able to cause an attenuation of the symptoms related to the inflammatory process. Another compound identified in *Verbena litoralis* and with proven anti-inflammatory activity is the caffeic acid. Zhang *et al.* (2014) reported its anti-inflammatory activity in mice that had a contact dermatitis (edema and local inflammation) induced by the application of 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) to the ears. The topical application of caffeic acid significantly reduced the ear thickening, thus indicating its therapeutic effect. Among the phenolic compounds found in the crude extract of *V. litoralis*, we can emphasize luteolin. Luteolin is reported to exert its anti-inflammatory activity by inhibiting COX-2, leukotrienes and arachidonic acid enzymes. When testing cotton pellet-induced granuloma, Gosh *et al.* (2013) found that luteolin significantly decreased the weight of the cotton pellet compared to indomethacin. Another important phenolic compound identified in *V. litoralis* was apigenin.

Chen *et al.* (2017) in their studies found that levels of IL-2, IL-4 and IL-10 increased significantly after isoflurane exposure in rats and treatment with 100 mg/kg of apigenin suppressed isoflurane-induced neuroinflammation.

The hepato- and nephrotoxicities of plant extracts are frequently accessed by the dosages of AST, ALT, BUN and CRE, respectively (Brondani *et al.*, 2017). Moreover, lipase and amylase determinations are used as markers of pancreatic injuries (Steinberg *et al.*, 2017). In this study, although lipase had an increase in crude extract and ethyl acetate fraction, Muniraj *et al.* (2015) indicate that, for a diagnosis of acute pancreatitis, the amylase and/or lipase must be three times higher than the physiological limit. Therefore, the elevation in this parameter may have occurred as a reflex of a non-pancreatic disease, indicating that both the crude extract and the ethyl acetate fraction may affect the metabolism and excretion of lipase from the peripheral kidney or tissues, causing its increase in blood (Steinberg *et al.*, 2017).

Moreover, the alanine aminotransferase (ALT) enzyme was significantly decreased in the groups treated with the crude extract and the ethyl acetate fraction. It is well known that ALT is primarily limited to the hepatocyte cytosol and considered a highly sensitive indicator of hepatocellular damage. Within certain limits, this parameter may also provide a quantitative rate of damage to the liver (Al-Habori *et al.*, 2002). The reduction of ALT suggests that *V. litoralis* has some hepatoprotective properties that may be related to its chemical composition identified by UHPLC/MS, since luteolin, chlorogenic acid, caffeic acid and apigenin have already been proved to possess such activity. Corroborating with our findings, other studies have also reported similar hepatoprotective effects of plants (Brondani *et al.*, 2017; Adewale *et al.*, 2016; Pereira, Barros, Ferreira, 2016).

CONCLUSION

The flavonoids identified in *V. litoralis* are of great importance since they were found for the first time in the plant and anti-inflammatory action was attributed mainly to compounds chlorogenic acid, caffeic acid, luteolin and apigenin. As demonstrated, treatment with crude extract and acetate fraction inhibited the formation of granulomatous tissue with no evidence of damage to the liver, kidneys and pancreas.

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