

SECONDARY METABOLITES FROM *PACHYSTROMA LONGIFOLIUM* (NEES) I. M. JOHNST (EUPHORBIACEAE) AND EVALUATION OF ITS EXTRACTS AS CATHEPSINS INHIBITORS

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ABSTRACT

In this work, four secondary metabolites were isolated from leaves of *Pachystroma longifolium* (Euphorbiaceae): loliolide, gallic acid, methyl protocatechuate and ethyl gallate. Their structures were established by physical and spectroscopic methods. The extracts of *P. longifolium* and the purified compounds were tested as cathepsins inhibitors and the extracts were active.

Keywords: *Pachystroma longifolium*, secondary metabolites, cathepsins.

INTRODUCTION

Owing to the high diversity of natural products from terrestrial or marine species, secondary metabolites from plants are some of the most successful source of drug leads for the treatment of many diseases and illnesses¹. *Pachystroma longifolium* (Nees) I. M. Johnst, native from Cerrado and Atlantic Forest in Brazil, is an Euphorbiaceae tree popularly known as *canxim*². Euphorbiaceae family has a great economic significance in commercial oil supplying from *Jatropha*, *Croton* and *Ricinus* species as well as significant importance in folk medicine. It shows a widespread traditional use as antipyretic, analgesic, diuretic, antiseptic, laxative and anti-rheumatic³.

As part of an ongoing search for bioactive extracts and purified compounds from Brazilian flora, the leaves of *Pachystroma longifolium* (Nees) I. M. Johnst were submitted to secondary metabolites purification process and biological evaluation as cathepsins inhibitors.

Cysteine cathepsins belong to the papain-like peptidases group and constitute one of the major groups of lysosomal enzymes. Cathepsin L-like peptidases are the largest group and are involved in diverse physiological and pathological processes in animals and numerous non-mammalian organisms⁴. Cathepsin-K (cat-K) is a well-known peptidase involved in bone remodeling and its excessive activity is related to osteoporosis⁵. Cathepsin-L (cat-L) and -V (cat-V) are related to regulation of MHC class II antigen presentation and implicated in many pathological conditions⁴. Cat-V is expressed in thymus, testis and corneal epithelium and its biological role has been associated with cancer progression and atherosclerosis^{6,7}.

This work presents the isolation and the identification of loliolide, gallic acid, methyl protocatechuate and ethyl gallate (figure 1) and the biological evaluation as cathepsins inhibitors of extracts and purified compounds of *P. longifolium*.

EXPERIMENTAL

General procedures. - Column Chromatography (CC) analysis were performed with silica gel 60 (Merck) and Sephadex LH-20 (Sigma-Aldrich). For Vacuum Liquid Chromatography (VLC), silica gel was used as stationary phase and Buchi vacuum pump 700 was used for vacuum. Thin layer chromatography (TLC) was performed with silica gel plates (Merck) and the spots were detected with an ultraviolet lamp (254 and 365 nm), vanillin sulfuric acid solution and iodine vapor. Agilent equipment (G1311C-1260 quaternary pump) coupled to diode array detector (G1315D-1260) was used for High Performance Liquid Chromatography (HPLC). For the analytical system, Phenomenex C18 column (5 microns 4.5 x 250 mm) and flow 1 mL.min⁻¹ of a mixture of H₂O and MeOH - starting at 90:10, after 100% MeOH at 40 min and returning the ratio 90:10 at 46 min to 50 min were employed. In semi-preparative system, it was used Eclipse XDB-C18 column (5 microns 9.4 x 250 mm) under flow 4 mL.min⁻¹. NMR spectra were recorded in chloroform-D₁, acetone-D₆ and methanol-D₄ (Sigma Aldrich), using Varian 400 MHz spectrometer probe,

5mm ATB BroaBand 1H/19F/X and TMS as reference standard. Mass spectra were run in FT-ICR-MS, Solarix- Bruker for HRESIMS and Esquire 3000 Plus equipment for low-resolution spectra.

Plant material. - Leaves of *P. longifolium* were collected in January 2012 at Vila Velha, Espírito Santo, Brazil, and authenticated by Prof. Luciana Dias Thomaz at Institute of Biology at Federal University of Espírito Santo (UFES). A voucher specimen (No. 26618) was deposited in the herbarium (VIES) of UFES.

Extraction. - The leaves of the plants were dried, powdered (1.4 Kg) and extracted by maceration with 95% EtOH (72 h each, 3 times). The crude ethanolic extract was obtained after solvent evaporation to dryness under reduced pressure. Its residues were further subjected to solvent-solvent partitioning using *n*-hexane, EtOAc and a mixture of MeOH/H₂O 1:3. The isolated substances were obtained from the EtOAc fraction.

Isolation procedure. - The EtOAc extract (27.9 g) was initially subjected to VLC, using a gradient of solvents *n*-Hex/EtOAc/MeOH. Among the 10 generated fractions, after analysis by TLC and HPLC, fraction 5 (375.2 mg) was chromatographed on silica gel CC (4.0 x 26.5 cm) using *n*-Hex/EtOAc 4:1, *n*-Hex/EtOAc 3:2, *n*-Hex/EtOAc 1:9, EtOAc/MeOH 9:1, EtOAc/MeOH 7:3 and MeOH. The subfraction 5.7 (94.4 mg) was submitted to a new CC (2.5 x 32.0 cm) using DCM (dichloromethane)/MeOH 9.5:0.5, DCM/MeOH 7:3, DCM/MeOH 1:1 and MeOH. The subfractions 5.7.4 (6.7 mg) and 5.7.5 (26.5 mg), after HPLC purification, afforded compound 1 (2.1 mg). Fraction 7 (4.4 g), which was subjected to CC column (5.5 x 32.0 cm) using *n*-Hex/EtOAc 7:3 EtOAc/MeOH 9:1 EtOAc/MeOH 1:1 and MeOH as mobile phase, afforded the subfraction 7.4 (47.5 mg). The compound 2 (3.1 mg) and 3 (3.9 mg) were obtained through semi-preparative HPLC from subfraction 7.4. Exclusion chromatography, using Sephadex (2.8 x 34.0 cm) as stationary phase and MeOH as mobile phase, was performed for fraction 6 (789.7 mg). The subfraction 6.2 (258.9 mg) was submitted again to Sephadex purification (2.8 x 34.0 cm). Thus, after purification by semi-preparative HPLC, the subfraction 6.2.3 provided compound 4 (1.8 mg).

Cathepsin inhibitory assay. - General procedure adopted for Cathepsin inhibitory assay followed the methodology described by Marques et al, 2012⁸. Recombinant human cathepsins, K, L and V were produced using the *Pichia pastoris* expression system as previously established⁹. A molar concentration of the enzyme was determined by active site titration with E-64 following the conditions previously described¹⁰. All commercially available chemicals and reagents were purchased from Aldrich Chemical Co. and Sigma, and used without further purification. Stock solutions of pure compounds and extracts were prepared at a concentration of 1 mM in DMSO and diluted to 100 µM. The *in vitro* enzyme inhibition experiments were carried out in triplicate (in 96-well black plates). Final volume of the reaction mixture was 200 µL, which was kept under stirring. Each well contained 191 µL of a 100 mM sodium acetate buffer (pH 5.5) containing 5 mM EDTA and 5 mM dithioerythritol (DTE), 2 µL of 1 mM Z-Phe-Arg-MCA dissolved in DMSO, 5 µL of sample, and 1 µL of cat-V 32 (nM), cat-L (20 nM) or cat-K (20 nM). The enzyme was activated

for 5 min with DTE at 27 °C, and then the reaction mixture was incubated for 5 min with the sample. Reactions started by the addition of the fluorogenic substrate 4-methylcoumaryl-7-amide (MCA) and measurements were taken using the Molecular Devices Spectra MAX GEMINI XS (excitation 355 nm, and emission 460 nm). Control assays were performed without an inhibitor (negative control) and in the presence of the irreversible inhibitor for cysteine peptidase, E-64 (positive control). The percentage of inhibition was calculated according to the equation: % inhibition = $100 \times (1 - V_i / V_o)$, where V_i and V_o are initial velocities (enzyme activities) determined in the presence and absence of an inhibitor, respectively.

Loliolide (1): yellow solid; $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ 5.68 (1H, s, H-7), 4.32 (1H, quint, $J = 3.5$ Hz, H-3), 2.46 (1H, dt, $J = 14.0, 2.7$ Hz, H-4), 1.96 (1H, dt, $J = 14.0, 2.7$ Hz, H-2), 1.77 (1H, dd, $J = 14.0, 3.5$ Hz, H-4), 1.77 (3H, s, H-11), 1.52 (1H, dd, $J = 14.0, 3.5$ Hz, H-2), 1.45 (3H, s, H-10), 1.26 (3H, s, H-9); $^{13}\text{C NMR}$ (CDCl_3 , 100 MHz): δ 182.3 (C, C-6), 171.8 (C, C-8), 112.9 (CH, C-7), 86.6 (C, C-5), 66.8 (CH, C-3), 47.3 (CH_2 , C-2), 45.6 (CH_2 , C-4), 35.9 (C, C-1), 30.6 (CH_2 , C-9), 26.9 (CH_2 , C-11), 26.4 (CH_2 , C-10); EIMS m/z $[\text{M-H}]^-$ 195.1 (calcd for $\text{C}_{11}\text{H}_{16}\text{O}_5$, 196.11).

Gallic acid (2): yellow amorphous solid; $^1\text{H NMR}$ (CD_3OD , 400 MHz): δ 6.92 (2H, s, H-2, H-6); EIMS m/z $[\text{M-H}]^-$ 168.8 (calcd for $\text{C}_6\text{H}_6\text{O}_5$, 170.0).

Methyl protocatechuate (3): yellow solid; $^1\text{H NMR}$ (CD_3OD , 400 MHz): δ 7.42 (1H, d, $J = 1.6$ Hz, H-2), 7.40 (1H, dd, $J = 8.0, 1.6$ Hz, H-6), 6.77 (1H, d, $J = 8.0$ Hz, H-5), 3.65 (3H, s, H-2'); $^{13}\text{C NMR}$ (CD_3OD , 100 MHz): δ 175.0 (C, C-1'), 151.3 (C, C-4), 146.0 (C, C-3), 123.8 (C, C-1), 123.7 (CH, C-6), 117.7 (CH, C-2), 115.7 (CH, C-5), 52.1 (CH_3 , C-2'); EIMS m/z $[\text{M-H}]^-$ 169.1 (calcd for $\text{C}_8\text{H}_8\text{O}_4$, 168.0).

Ethyl gallate (4): yellow solid; $^1\text{H NMR}$ (CD_3OD , 400 MHz): δ 7.03 (2H, s, H-2, H-6), 4.26 (2H, q, $J = 7.1$ Hz, H-2'), 1.33 (3H, t, $J = 7.1$ Hz, H-3'); EIMS m/z $[\text{M-H}]^-$ 196.9 (calcd for $\text{C}_9\text{H}_{10}\text{O}_5$, 198.0).

RESULTS AND DISCUSSION

After phytochemical procedure and spectroscopic analysis, four known compounds loliolide (1)¹¹, gallic acid (2)¹², methyl protocatechuate (3)¹³ and ethyl gallate (4)¹⁴ were identified from the EtOAc fraction of the leaves of *P. longifolium* (Figure 1).

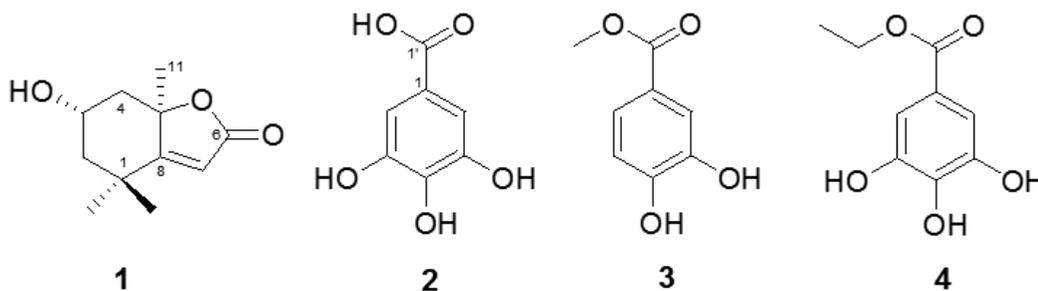


Figure 1: Isolated compounds from leaves of the species *P. longifolium*.

Compound 1, which acts as repellent, also shows immunosuppressive and cytotoxic activity against nasopharyngeal carcinoma and leukemia¹¹, as well as antioxidant and cell protective actions¹⁵. Compounds 2, 3 and 4 are polyphenol derivatives. This group is usually found in Euphorbiaceae species¹⁶.

In biological evaluation, the crude, *n*-Hex and EtOAc extracts from leaves of *P. longifolium* were tested as cat-K, -L and -V inhibitors at different concentrations (5 to 500 mg.mL⁻¹). Concerning the inhibition of cat-L, the most significant results came from the EtOAc fraction. It caused a 70% inhibition of the enzyme at 125 mg.mL⁻¹. In turn, the most significant results for the inhibition of the cat-K came from the *n*-Hex and EtOAc extracts, remarkably

the latter (Table 1).

It was noted that most promising results have been obtained with cathepsin V inhibition. The crude extract showed a significant rate of inhibition at concentrations of 500, 250 and 125 mg.mL⁻¹. Besides, the results obtained with *n*-Hex and EtOAc extracts were quite satisfactory at lower concentrations, such as 50 mg.mL⁻¹ and 25 mg.mL⁻¹. Notably, EtOAc fraction showed the highest inhibitory activity percentages at almost all concentrations tested (Table 1). However, isolated compounds did not show activity as cat-K, -L and -V inhibitors.

Table 1: Percentage of inhibition of Cat-K, -L and -V of main extracts from *P. longifolium*.

Cat-K (%)						
	500 mg mL ⁻¹	250 mg mL ⁻¹	125 mg mL ⁻¹	50 mg mL ⁻¹	25 mg mL ⁻¹	5 mg mL ⁻¹
<i>n</i> -Hex	96	56	74	8	26	nd
EtOAc	90	69	63	27	40	10
Crude	75	23	47	nd	43	nd
Cat-L (%)						
	500 mg mL ⁻¹	250 mg mL ⁻¹	125 mg mL ⁻¹	50 mg mL ⁻¹	25 mg mL ⁻¹	5 mg mL ⁻¹
<i>n</i> -Hex	62	33	22	nd	nd	nd
EtOAc	86	81	70	38	37	24
Crude	29	4	nd	nd	nd	nd
Cat-V (%)						
	500 mg mL ⁻¹	250 mg mL ⁻¹	125 mg mL ⁻¹	50 mg mL ⁻¹	25 mg mL ⁻¹	5 mg mL ⁻¹
<i>n</i> -Hex	100	95	86	71	66	13
EtOAc	96	95	92	85	79	45
Crude	97	87	75	50	49	nd

nd: not detected

Although the inhibition of cathepsins by extracts and pure compounds constitutes a promising enzyme target for new drugs research, development and innovation (R, D & I), there are few studies in the literature. For example, agathisflavone, tetrahydrobustaflavone, 3-oxo-urs-12-en-28-oic acid, and quercetin showed significant inhibitory activity on cathepsin L-like rCPB2.8 with IC_{50} values ranging from 0.43 to 18.03 μM ¹⁷. Rhynedulins A-C and rhynedulinal showed weak inhibitory activity towards rhodesain, the major cathepsin-L like protease in *Trypanosoma brucei*¹⁸. Four dihydrochalcones were tested against cathepsins B and L and these compounds presented good inhibitory activity with IC_{50} values ranging from 1.0 to 14.9 μM . These compounds also showed good selectivity in their inhibitory activities against the cysteine proteases¹⁹.

Both crude extract and active fractions provided by leaves of *Pachystroma longifolium* were able to inhibit cat-K, -L and -V in higher concentrations. Hence, this work contributes to reducing the gap in the area by providing data of phytochemical analysis and biological assays of leaves of *Pachystroma longifolium* (Euphorbiaceae).

CONCLUSIONS

In summary, typical polyphenols variants and monoterpene lactone loliolide were isolated from *P. longifolium* (Euphorbiaceae). Concerning biological findings, the EtOAc fraction showed the greatest activity as cathepsin inhibitor and all three extracts displayed a particularly notable activity as cat-V inhibitor. Cathepsin inhibition studies are a promising investigation area in therapy and combating diseases, since they are considered as potential therapeutic targets, gathering large investments in the pharmaceutical companies²⁰.

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