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Coumarin isolation and comparative study of biological activities of *Pterocaulon alopecuroides* DC and *Pterocaulon lorentzii* Malme

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Abstract

5-(2,3-Dihidroxy-3-methylbuthyloxy)-6,7-methylenedioxycoumarin was isolated from the chloroform extract of the two Asteraceae species *Pterocaulon alopecuroides* DC. and *Pterocaulon lorentzii* Malme. The structure was elucidated through IR and ^{1}H and ^{13}C NMR analyses. The extracts and the isolated compound did not exhibit toxic activity, as determined through the brine shrimp lethality method, and did not interfere with the integrity of erythrocytes, as demonstrated through a hemolytic assay. The antioxidant activities were investigated through three methods. In the phosphomolybdenum test, the ethyl acetate fraction of *P. alopecuroides* exhibited an antioxidant activity of 137.7% compared with rutin (positive control), and the ethyl acetate fraction of *P. lorentzii* exhibited an antioxidant activity of 101.7% compared with vitamin C (positive control). The two ethyl acetate fraction also exhibited excellent activity through the DPPH assay: *P. alopecuroides* and *P. lorentzii* exhibited IC_{50} values of 10.74 μg/ml and 7.63 μg/ml, respectively. In the TBARS bioassay, the crude extracts showed the more significant results: IA% 0.419 ± 0.0517 for *P. alopecuroides* and IA% 0.213 ± 0.0094 for *P. lorentzii.*

Keywords: *Pterocaulon*, Asteraceae, Phytotherapy, Antioxidant, Toxicity, Coumarin

Introduction

The species of the Asteraceae family are known to produce polyacetylenes, essential oils, terpenes, and sesquiterpene lactones. Previous studies have shown that the plants of the *Pterocaulon* genus exhibit antifungal activities due to the presence of coumarins [1]. *Pterocaulon lorentzii* Malme and *Pterocaulon alopecuroides* DC. are species of the Asteraceae family and are scattered from the southern United States to central Argentina. *Pterocaulon lorentzii* Malme, which is known as Yerba Infidel or Tuya, is used in folk medicine as a decoction in bath accents to relieve the symptoms of hemorrhoids. Its features include congested ears, bright leaves, and adaxial revolute at the margins [2]. In folk medicine, *Pterocaulon alopecuroides* is applied in the treatment of skin diseases, such as mycoses, in humans and in animals [3]. This species is characterized by apical leaves that are lanceolate to oblong-lanceolate in shape and by flowering and fructification in the summer months [2]. The objective of this work was to study the toxic, hemolytic, and antioxidant activities of the extracts, fractions, and compounds isolated from these two species.

Materials and Methods

General Experimental Procedures

IR (Infrared): Bomem-Hartmann & Braun MB Series. NMR (Nuclear magnetic resonance): 1D Bruker® 200 MHz.

Plant material

During the month of January from 2005 to 2007, the aerial parts of *Pterocaulon lorentzii* Malme and *Pterocaulon alopecuroides* DC. were collected in the city of Piçarras, which is located in the coast of Santa Catarina in Brazil. The determination was performed in a botanical herbarium of the Botanical Museum in the City of Curitiba, State of Parana, Brazil, by the curator, Osmar Santos Ribas. The herbarium specimens deposited at this site are registered under numbers 323 448 (*P. lorentzii*), 266 085, and 266 086 (*P. alopecuroides*).

Extraction and isolation

The plant material was dried, ground, and subjected to exhaustive alcoholic extraction in a Soxhlet apparatus. The alcoholic extract was concentrated to 1/3 of the initial volume and partitioned. The produced hexane, chloroform, ethyl acetate, and the remaining extracts were used in the biological assays. The chloroform fractions from both species were submitted to liquid column chromatography using silica gel and subsequently Sephadex LH20 to isolate compound 1.

Toxicity evaluation with *Artemia salina*

The toxic potential of the fractions and the isolated compound was evaluated against *Artemia salina* [4]. The lethal dose 50% (LC_{50}) was determined by counting the dead nauplii after a 24-h incubation in the presence of the tested samples. The data were analyzed with the Probits statistical program using a 5% confidence interval.

Hemolysis assays

Dilution method

Sheep blood (Newprov®) was washed once with a phosphate buffer solution and resuspended to obtain a 2% diluted solution. A 1-mL aliquot of the blood suspension was mixed with the sample solution (0.1, 0.2, 0.5, and 1.0 mL) and 2 mL of phosphate buffer, pH 7.4. The solutions were shaken, allowed to rest for 30 min, and shaken again. After resting for 130 min, the solutions were centrifuged at 3,000 rpm for 5 min, and the hemolysis verification was conducted. Saponin R was used as the positive control, and distilled water was used as the negative control [5].

Blood agar plates method

Sterile paper discs impregnated with 1,000 µg of the samples were placed on blood agar plates (Newprov®). After incubation at 36ºC for 24 hours, the verification of the hemolysis halo was conducted. Saponin R and Triton solutions were used as the positive controls [6].

Determination of antioxidant potential

Phosphomolybdenum spectrophotometric method

The coumarin 5-(2,3-dihidroxy-3-methylbuthyloxy)-6,7-methylenedioxycoumarin, the crude extracts, and the fractions of both species were subjected to analysis through the phosphomolybdenum spectrophotometric method [7]. The results are expressed as the percentage of antioxidant activity in relation to that of Vitamin C and rutin.

DPPH assay

The coumarin 5-(2,3-dihidroxy-3-methylbuthyloxy)-6,7-methylenedioxycoumarin, the crude extracts, and the fractions of both species were analyzed through the DPPH assay. Test tubes containing dilutions of the samples and 300 µM DPPH were maintained at room temperature for 30 min. The absorbance was recorded at 518 nm. The solvent without DPPH was used as the blank. The results were expressed as IC50 values, which represent the concentration of the sample required to scavenge 50% of DPPH free radicals [8].

Determination of the antioxidant activity through the thiobarbituric acid reactive substances (TBARS) test

The antioxidant activity of the crude extracts, fractions, and coumarin isolated from *P. lorentzii* and *P. alopecuroides* was evaluated based on the reaction with thiobarbituric acid (TBARS), as described by Morais et al. (2006) [9]. An aqueous solution of chicken egg yolk (10% w/v) was used as the source of lipids, and the samples tested were dissolved in methanol at three different concentrations (100, 500, and 1000 ppm w/v) in quintuplicate. Chloride 2,2 '-azobis (2-amidinopropane) dihydrochloride (ABAP, 0.07 mol / L) was used to induce lipid peroxidation in a mixture of 20% acetic acid (pH 3.5), 0.8% w/v thiobarbituric acid (TBA), and 1.1% w/v sodium dodecyl sulfate (SDS). For the reaction to occur, the test tubes were maintained at 95ºC for one hour. After the 1-h incubation, 1-butanol was added, and the organic portion of the reaction was analyzed with a spectrophotometer at a wavelength of 532 nm. The antioxidant content was calculated by the equation AI% = (1 - A / C) x 100, where C is the absorbance of the fully oxidized control and A is the arithmetic mean of the absorbance of the samples. The results are expressed as the mean and the standard deviation. The data were subjected to analysis of variance (ANOVA), and the differences were considered statistically significant if $p < 0.05$.

Results and Discussion

The chloroform fraction of the two species analyzed in the study was subjected to liquid column chromatography to yield the spontaneous precipitation of a yellowish crystal, which was identified through IR, ^{1}H and, ^{13}C NMR experiments.

The IR spectrum showed bands at 3,400 cm^{-1}, which correspond to a stretch O-H binding and thus indicate the presence of alcohols associated by a hydrogen bond. A band at 1,620 cm^{-1} corresponds to a C=O binding stretch of carbonyl compounds. The presence of a band at 1,450 cm^{-1} characterizes the C=C binding stretch, which is indicative of an aromatic alkene group. This function can also be observed by the presence of bands between 1,000 and 650 cm^{-1}, which correspond to the angular deformation of the C-H bond plane of aromatic alkenes. The presence of an absorption band at 1,250 cm^{-1} indicates the C-O bond stretch, which is typical of esters and anhydrides, and confirms the presence of a carbonyl function [10].

The ^{1}H and ^{13}C NMR data are shown in Table 1. The two singlets at 1.23 and 1.31 ppm correspond to the two methyl groups at C4 'and C3', respectively, and the singlet at 6.06 ppm characterizes the methylenedioxy grouping. These data are in agreement with those already described by Debenedetti et al. (1997) [11] and Heemann (2006) [12]. Compound 1 was identified as 5-(2,3-dihidroxy-3-methylbuthyloxy)-6,7-methylenedioxycoumarin (Figure 1).

Table 1. NMR spectroscopic data for 5-(2,3-dihidroxy-3-methylbuthyloxy)-6,7-methylenedioxycoumarin (1).

Position	$_C$, mult.	$_H$ (*J* in Hz)
2	161,7	
3	112,3	6,23, d
4	139,8	7,96, d
4a	107,2	
5	136,9	
6	132,6	
7	151,8	
8	93,3	6,57, d
8a	152,7	
O-CH_2-O	102,3	6,06, s
1'	73,9	4,51 – 4,37, dd
2'	77,2	3,82, dd
3'	71,8	1,31, s
4'	27.0	1,23, s
3'-CH_3	25,0	

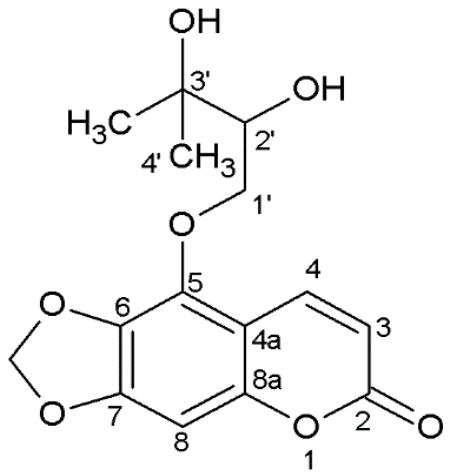

Figure 1. Chemical structure of compound 1.

The extracts and fractions of the two species, as well as the isolated coumarin, were tested in a lethality test against brine shrimp. No toxicity was detected because all of the samples exhibited an LC_{50} higher than 1,000 μg/mL.

In the hemolysis evaluation, the ethanol extracts and the fractions obtained from both species exhibited no ability to cause hemolysis, as shown by both methods used. This is an important finding because these extracts and fractions do not have the ability to damage red cells.

The antioxidant potential of the extracts, fractions, and isolated coumarin was evaluated using the DPPH method and the phosphomolybdenum spectrophotometric method using vitamin C and rutin as the positive controls. The antioxidant potential was also evaluated using the thiobarbituric acid method (TBARS) using butylated hydroxytoluene (BHT) and -tocopherol as the positive controls.

The results of the phosphomolybdenum test are expressed as the percentage of relative antioxidant activity (RAA%) compared to the positive controls. Of the *P. alopecuroides* samples, the most significant was the ethyl acetate fraction, which exhibited an activity of 36.4% compared to vitamin C and an activity of 137.7% compared to rutin. The other samples exhibited significant activities only when compared to rutin. The extracts of *P. lorentzii* gave better results. Of these samples, the ethyl acetate fraction also exhibited higher activity compared with the other samples tested with an RAA% of 101.7% compared to vitamin C. This expressive result can be attributed to unidentified secondary metabolites that are present in the fraction. As a result, this fraction shows considerable antioxidant capacity, whereas the highest activity exhibited by the other extracts and fractions was 33.6%, and this value was obtained for the crude extract.

The scavenge rate (IC_{50}) of DPPH was calculated through linear regression of the graphs of the activity percentage as a function of the concentration of antioxidant sample. In this assay, the ethyl acetate fractions of both species exhibited significantly improved activities (the IC_{50} values of *P. alopecuroides* and *P. lorentzii* were 10.74 μg/ml and 7.63 μg/ml, respectively) compared with the IC_{50} values of vitamin C of 2.48 μg/ml and rutin of 9.43 μg/ml, as determined by Tukey's test ($p < 0.05$). The activity exhibited by the remaining samples, including the crude extract, the hexane fraction, the chloroform fraction, and isolated coumarin, was not significantly different compared with the positive controls. In the thiobarbituric acid reaction (TBARS) test, the crude extracts of the two species exhibited the most significant results compared with the positive control BHT (0.169 ± 0.0087% IA). The IA of the crude extract of *P. alopecuroides* was 0.419 ± 0.0517%, and the IA of the crude extract of *P. lorentzii* was 0.213 ± 0.0094%. In contrast, the AI of 5-(2,3-dihydroxy-3-methylbutyloxy)-6,7-metilenedioxicumarina was low at 0.462 ± 0.0184%.

Conclusions

The remarkable results observed in the scavenging activity of the ethyl acetate fractions of both species (*Pterocaulon alopecuroides* DC. and *Pterocaulon lorentzii* Malme) can be attributed to secondary metabolites present in the extract fraction. The negative results obtained for the hemolytic capacity and toxicity toward *Artemia salina* ensure the nontoxic nature of the compounds. The results from this study encourage the identification of novel bioactive compounds from these two species and the further analysis of the bioactivities of these novel compounds as well as those of 5-(2,3-dihydroxy-3-methylbutyloxy)-6,7-metilenedioxicumarina, which was isolated in this study.

Author's contributions

CMSM performed the biological assays, the isolation of the compound, and the statistical analysis, and wrote the manuscript. BCKH helped draft and translate the manuscript. LCP assisted in the interpretation of the RMN data. MCV performed the hemolysis assays. CSP and SCC helped with the biological assays. PMPN and MDM revised and helped draft the manuscript. OGM helped

with the isolation and identification of the compound, conceived the study, and participated in its design and coordination.

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