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Original Article

Isovitexin as marker and bioactive compound in the antinociceptive activity of the Brazilian crude drug extracts of *Echinodorus scaber* and *E. grandiflorus*

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ABSTRACT

Echinodorus scaber Rataj and *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli, Alismataceae, are popularly used to relieve inflammatory complaints and as diuretic. A study on the antinociceptive effect and selected marker compounds in eleven extracts from different locations was undertaken and their antinociceptive effect was assessed. The fingerprints were compared by HPLC-DAD and the content of vitexin, isovitexin, isoorientin and vitexin-2-*O*-rhamnoside were determined. All samples presented antinociceptive activity reducing the writhes by 36.4–62.5% and 47.4–79.8% at 10 and 50 mg/kg, respectively; indomethacin (5 mg/kg) reduced writhes by 82.6–90.1%. The content of the flavonoids *C*-glycosides, however, presented a strong variation. Isovitexin and isoorientin were found in all the samples, with content ranging from traces to 14.70 μg/mg and 2.12–84.27 μg/mg extract, respectively, while vitexin-2-*O*-rhamnoside occurred in quantifiable amounts only in 3 out of 11 samples ranging from 5.43 to 33.13 μg/mg extract; vitexin was not detected at all or detected in trace amounts. According to the fingerprints, the samples could be arranged in four main groups. All eleven extracts showed antinociceptive activity. Isovitexin was the only flavonoid present in all samples and can be regarded, acting in synergy with the other compounds or not, as the responsible for the antinociceptive activity. Therefore, isovitexin is a good choice as chemical marker when the antinociceptive activity of *E. scaber* and *E. grandiflorus* is investigated.

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Introduction

The genus *Echinodorus*, Alismataceae, comprises about 45 species, occurring mainly in tropical areas and about 25 of them have been described in Brazil (Lehtonen, 2008; Matias et al., 2015). The best-known *Echinodorus* species used as medicinal plant in Brazil is *Echinodorus scaber* Rataj, heterotypic synonym of *Echinodorus macrophyllus* subsp. *scaber* (Rataj) R. R. Haynes and Holm-Niels, but also *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli is mistakenly collected and used as a substitute of the official crude drug. Both plants are known as "chapéu-de-couro", "chá-mineiro", "erva-de-pântano", "erva-de-brejo", among others. A leaf infusion or decoction is used as anti-inflammatory, antinociceptive, diuretic, hypotensive and antirheumatic (Brandão et al., 2009).

Previous chemical studies in *E. scaber* led to unusual nitrogen-containing clerodane diterpenes (Kobayashi et al., 2000a,b,c). From the methanolic leaf extract several cembrane diterpenes (Manns and Hartmannn, 1993; Tanaka et al., 1997; Shigemori et al., 2002), phenolics (Tanus-Rangel et al., 2010), flavonoids and caffeic acid derivatives (Schnitzler et al., 2007) were reported. A comparative study of five *Echinodorus* species was undertaken by Tanaka (2000) using hyphenated methods. Diterpenes, steroids and fatty acids were used as chemical markers.

The anti-inflammatory activity of *E. scaber* (*E. macrophyllus*) leaf extract in acute and sub-chronic models of inflammation

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Table 1
Botanical identification, voucher specimen number, collection place, geographic coordinates and extraction yields of *Echinodorus scaber* and *E. grandiflorus* samples investigated.

Plant species and sample	Collection place and state	Geographical coordinates (GPS)	Voucher herbarium number	w/w extraction yield %
E. grandiflorus – L_1	Juína, MT	15°56′70″S 56°36′07″W	31645	18.50
E. scaber – L_2	Poconé, MT	16°31′40″S 56°43′53″W	33635	12.70
E. grandiflorus – L_3	Chapada dos Guimarães, MT	15°36′30″S 56°03′44″W	33637	13.80
E. grandiflorus – L_4	Dom Aquino, MT	15°48′40″S 54°55′02″W	33638	20.60
E. scaber – L_5	Cuiabá, MT	15°42′80″S 55°53′20″W	33639	18.70
Echinodorus sp. – L_6	Comercial sample, Chapada dos Guimarães, MT	–	–	41.50
E. scaber – L_7	Campo Grande, MS	20°30′79″S 54°34′87″W	33665	47.38
E. scaber – L_8	Campo Grande, MS	20°30′79″S 54°34′87″W	33665	41.31
E. scaber – L_9	Campo Grande, MS	20°28′28″S 54°34′06″W	33665	50.21
E. scaber – L_{10}	Campo Grande, MS	20°29′84″S 54°35′52″W	33665	48.95
E. scaber – L_{11}	Campo Grande, MS	20°30′02″S 54°36′52″W	33665	43.32

States of Mato Grosso (MT) and Mato Grosso do Sul (MS).

was assessed by Tanus-Rangel et al. (2010) using hydroethanolic extract in mice and rats. Potent acute, systemic and topic anti-inflammatory activity was found. The same authors reported the isolation of isovitexin from the ethyl acetate fraction of the extract and the detection of the close related vitexin in the sample.

The immunosuppressive effect of *E. scaber* aqueous extract was described by Pinto et al. (2007).

A toxicological study on aqueous extract of *E. scaber* was undertaken by Lopes et al. (2000). The authors found minor hepatic toxicity but no genotoxic effect at the daily dose recommended in traditional medicine. However, Vidal et al. (2010) using a bacterial assay, found mutagenic effect in the aqueous extract and solvent partition fractions of the plant.

Therefore, considering the importance of "chapéu-de-couro" to the local folk medicine and the several scientific studies reported above that seems to confirm its traditional use, it is important to develop a simple and cheap method for the quality control of a possible "chapéu-de-couro" phytotherapic medicine. So, the aim of this work is to perform HPLC-DAD fingerprint analyses in "chapéu-de-couro" samples collected in different sites from Mato Grosso and Mato Grosso do Sul states, and to investigate the possible use of vitexin, vitexin-2-*O*-rhaminoside, isovitexin and isoorientin as chemical markers in the crude drugs presenting antinociceptive activity.

Materials and methods

Chemicals

The solvents used were of ACS grade from Vetec (Rio de Janeiro, Brazil), and for HPLC analysis were of spectroscopic grade from Tedia (Fairfield, OH, USA). Ultrapure water was obtained by the Milli-Q system (Waters, Darmstadt, Germany). Acetic acid from Merck (Darmstadt, Germany) and NaCl Proquimios (Rio de Janeiro, RJ, Brazil). Indomethacin, sodium bicarbonate and standard compounds for the chromatographic analyses from Sigma–Aldrich (St. Louis, MO, USA). The purity of the standards used in the HPLC analysis was as follows: vitexin (>95%), isovitexin (98%), vitexin-2-*O*-rhamnoside (>98%), catechin (>98%). Isoorientin (≥97%) was isolated from leaves of *E. scaber*, and its identity was confirmed by ^{1}H and ^{13}C NMR analyses on a Varian Mercury 300 (Palo Alto, CA, USA), in agreement with the literature (Peng et al., 2005).

Plant material

The plants were collected in May 2011 in eleven different locations from the Mato Grosso and Mato Grosso do Sul states, Brazil and were identified by MSc. Vali Joana Pott. Voucher herbarium specimens have been deposited at the Central Herbarium of UFMS (Table 1). The aerial parts of the air-dried plant material were used for the analyses, according to the traditional use. The powdered dry material (20–25 mesh; 5 g) was extracted with EtOH–H_2O (100 ml; 70:30, v/v) at r.t. during 4 h under stirring. After filtration, the solvent was evaporated to dryness under reduced pressure, and the remaining syrup was lyophilized. The extractions were carried out in triplicate.

Animals

The antinociceptive assays were carried out using Swiss albino mice (20–35 g) from the Central Animal House of UFMT. Animals were kept in polypropylene cages with free access to water and commercial Purina® (Labina, São Paulo, Brazil) at 25 ± 1 °C and 12 h (light)/12 h (darkness) cycles. Experiments were carried out according to the ethical principles on Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA). The experiments were allowed by the Animal Use Ethics Committee (CEUA) of the UFMT under authorization number 23.108043016/10-6.

Total phenolic content

The total phenolic content (TP) was determined by the Folin–Ciocalteu method. All samples and catechin were dissolved in 50% (v/v) aqueous methanol. Samples (50 μl) were placed into

test tubes and 250 μl Folin–Ciocalteu reagent were added. The mixture was left to stand for 5 min, and 750 μl of 20% sodium carbonate solution and 5 ml distilled water were added. After 30 min of incubation at room temperature (20 °C) the resulting absorbance was measured at 765 nm. The calibration curve was performed with catechin (concentrations ranging from 4 mg/ml to 15 mg/ml) and the results were expressed as mg of catechin equivalents per 100 g of dry plant material.

Total flavonoids content

Determination of the total flavonoids content (TF) of the hydroalcoholic extract was performed as reported previously, using the $AlCl_3$ colorimetric method (Simirgiotis et al., 2008). Quantification was expressed by reporting the absorbance in the calibration graph of vitexin used as standard flavonoid (from 1 to 29 μg/μl; $R^2 = 0.992$). Results are expressed as mg vitexin equivalents/g dry mass.

HPLC analyses of marker compounds

HPLC analyses were carried out using Varian Pro Star 5.5 (Palo Alto, CA, USA) equipment with a quaternary pump (model 240), autosampler (model 410) and UV-DAD detector (model 330). The conditions were as follows: column HiChrom C-18 [250 mm × 4.6 mm × 5 μm] (Reading, UK), pre-column Kromasil C-18 [3.0 mm × 4.6 mm] (Bohus, Sweden), mobile phase CH_3CN (solvent A), MeOH (solvent B) and 0.05% (v/v) aqueous trifluoroacetic acid (TFA) solution (solvent C); flow rate, 1 ml/min; injection volume, 4 μl. The gradient program used was as follows: 0–20 min (75% C:15% B:10% A); 20–25 min (65% C:20% B:15% A); 20–25 min (55% C:25% B:20% A); 25–30 min (75% C:15% B:10% A). The analyses were monitored by the ProStar 5.5 software and the spectra were detected using the PoliView 2000 program. The chromatograms were obtained at 270 nm, and spectra from the extract constituents were measured in the range 218–400 nm. Under our experimental conditions, the retention time of the reference compounds were: isoorientin (8.6 min), vitexin-2-*O*-ramnoside (11.2 min), vitexin (11.6 min) and isovitexin (12.4 min). Calibration curves for the reference compounds were built using the internal standard method (catechin as internal standard), in eight concentrations for vitexin, isovitexin and vitexin-2-*O*-rhamnoside (0.25–20 μg/ml), and in ten concentrations for isoorientin (7–100 μg/ml), injecting each concentration in triplicate. The internal standard and the analyzed compounds were dissolved (1 mg) separately in CH_3CN:0.05% TFA (10 ml, 1:1, v/v), the injection volume was 4 μl. The linearity of the detector for vitexin, vitexin-2-*O*-rhamnoside, isoorientin and isovitexin was obtained from linear plots of curves, built-up using the Microsoft Office Excel 2007 program. The concentrations of vitexin, vitexin-2-*O*-rhamnoside, isovitexin, and isoorientin in leaves of *E. scaber* and *E. grandiflorus* were calculated from the peak areas using the equation for linear regression obtained from the calibration curve. The detection limit and quantification limit for the compounds were as follow in μg/μl, respectively: vitexin (0.29 and 0.9), vitexin-2-*O*-rhamnoside (0.68 and 2.05), isovitexin (0.25 and 0.75) and isoorientin (1.55 and 4.7). The method was validated (specificity, linearity, detection and quantification limits, accuracy and precision), according to the literature (Anvisa, 2003).

Principal Component Analysis

Unsupervised Principal Component Analysis (PCA) was performed with selects peaks obtained by matching from HPLC-DAD date, aligned by Chromaligner platform (Wang et al., 2010). The aligned data were analyzed in MetaboAnalyst platform (Xia et al., 2012). Samples were clustered into two groups based on their geographical location: Mato Grosso (MT) and Mato Grosso do Sul (MS) states. PCA was performed with the log-transformed and autoscaled data, at UFMS.

Antinociceptive activity

The antinociceptive activity was determined in mice as described by Koster et al. (1959). The experiments were carried out employing 6–8 animals per treatment group. The extracts were administered orally at doses of 10 or 50 mg/kg body weight, 60 min before intraperitoneal injection of 0.6% acetic acid in 0.9% normal saline (0.1 ml/10 g body weight) to induce the characteristic writhings. Vehicle (10 ml/kg body weight, *p.o.*) and indomethacin (5 mg/kg body weight, *p.o.*) were dissolved in 2% $NaHCO_3$ solution and were given to mice in the control and reference groups, respectively. The mice were observed and counted for the number of abdominal writhes and stretchings in a period of 0–30 min. The responses in the treated groups were compared with those of animals in the control group and reference compound. The percentage of inhibition of the number of writhings was calculated and results are presented as percent inhibition.

Statistical analyses

The results are expressed as means ± S.E.M. One way analysis of variance (ANOVA) was used for the comparison of more than two means followed by Student–Newman–Keuls test; p values <0.05 were considered significant. Graph Pad InStat® version 2.01 was used for data analysis.

Results

Taxonomic authentication

Eleven samples (L_1–L_{11}) from "chapéu-de-couro" were collected or purchased in different locations of Mato Grosso (MT) and Mato Grosso do Sul (MS) states in 2011, during the flowering time. From the eleven samples, seven were confirmed as *E. scaber*, while three turned out to be the close related species *E. grandiflorus* and a commercial sample remains as *Echinodorus* sp. The w/w extraction yields for the *E. scaber* samples ranged between 12.70 and 50.21% while for *E. grandiflorus* the soluble content was 13.80 and 20.60%, respectively and 41.50% for the *Echinodorus* sp. sample. The best extraction yields (41.31–50.21%) were observed from the samples collected in MS sites (Table 1).

The total phenolic, flavonoid and C-glycoside flavones quantification

The total phenols and flavonoid content, as well as the *C*-glycosyl flavonoid content of hydroethanolic extracts from "chapéu-de-couro" leaves are given in Table 2. Table 3 provides calibration curve data, obtained using standard *C*-glycoside flavones. The total phenolic concentration did not vary appreciably among the eleven analyzed samples, ranging from 283.25 mg/g (*Echinodorus* sp., from MT) to 330.00 mg/g (*E. scaber* from MS). However, great variation was observed regarding the total flavonoid concentration, ranging from 3 mg/g (*E. scaber*, from MS) to 233.75 mg/g (*E. scaber* from MT).

The content of the *C*-glycoside flavones also presented great variation (Table 2). Vitexin was not detected in any but one of the eleven analyzed samples, whereas vitexin-2-*O*-rhamnoside was quantified in only three sites (all from MT), ranging from 5.43 to 33.13 μg/mg, and isovitexin and isoorientin were detected in all samples with concentration ranging from traces 14.70 μg/mg for the former and 2.12–84.27 μg/mg for the latter. An interesting picture, however, arises from the flavone content found in

Table 2
Total phenolic (TP), total flavonoid (TF) (mg of catechin and vitexin equivalents per g of dry plant material, respectively), isovitexin, vitexin-2-*O*-rhamnoside and isoorientin (μg per mg of hydroethanolic extracts of *Echinodorus scaber* and *E. grandiflorus*).

Plant species and sample	TP (mg/g)	TF (mg/g)	Isovitexin (μg/mg)	Vitexin-2-*O*-rhamnoside (μg/mg)	Isoorientin (μg/mg)
E. grandiflorus – L_1	290.00	25.50	nq	nd	2.12
E. scaber – L_2	292.50	198.00	0.72	13.46	21.82
E. grandiflorus – L_3	296.75	39.25	1.06	nd	22.24
E. grandiflorus – L_4	296.00	145.5	0.62	nq	21.54
E. scaber – L_5	324.00	233.75	nq	5.43	14.34
Echinodorus sp. – L_6	283.25	95.00	2.44	33.13	84.27
E. scaber – L_7	308.75	141.75	6.75	nq	12.22
E. scaber – L_8	298.50	61.25	14.70	nq	34.27
E. scaber – L_9	287.50	100.50	9.22	nq	18.10
E. scaber – L_{10}	330.00	123.00	6.99	nq	35.54
E. scaber – L_{11}	308.75	3.00	5.71	nq	16.89

Collection places L_1–L_5 and L_7–L_{11} are from the Mato Grosso (MT) and Mato Grosso do Sul (MS) states, respectively; L_6 refers to a commercial sample (please see Table 1); nq, detected, but not quantified (below quantification limit); nd, not detected.

Table 3
Linear regression equation, correlation coefficient, detection and quantification limits for vitexin-2-*O*-rhamnoside, vitexin, isovitexin and isoorientin.

Standard	Conc. range (μg/ml)	Linear regression ($y = a + bx$)	Correlation coefficient (r^2)	Detection limit (μg/ml)	Quantification limit (μg/ml)
Vitexin-2-*O*-rhamnoside	0.50–8.00 8.00–20.00	$y = 0.0280 + 0.1051x$ $y = 0.1651 + 0.0877x$	0.9967 1.0000	0.68	2.05
Vitexin	0.25–8.00 8.00–20.00	$y = 0.0136 + 0.2242x$ $y = 0.574 + 0.1562x$	0.9994 1.0000	0.29	0.90
Isovitexin	0.25–8.00 8.00–20.00	$y = 0.0072 + 0.2512x$ $y = 0.3473 + 0.2073x$	0.9996 0.9997	0.25	0.75
Isoorientin	7.00–40.00 40.00–100.00	$y = 8.3363 + 6.1229x$ $y = 7.109x - 36{,}274$	0.9982 0.9997	1.55	4.70

the *Echinodurus* sp. analyzed; while *E. grandiflorus* samples presented only trace amounts or none of vitexin-2-*O*-rhamnoside and very small amounts of isovitexin (traces – 1.06 μg/mg) but appreciable content of isoorientin (2.12–22.24 μg/mg), a different pattern can be observed to *E. scaber*, also presenting trace amounts of vitexin-2-*O*-rhaminoside (except sites 2 and 5; 13.46 and 5.43 μg/mg respectively) along with higher concentrations of isovitexin (traces – 14.70 μg/mg) and high concentrations of isoorientin (12.22–35.54 μg/mg). The commercial *Echinodorus* sp. sample presented very high concentration of isoorientin and vitexin-2-*O*-rhaminoside (84.27 and 33.13 μg/mg respectively) and a moderate amount of isovitexin (2.44 μg/mg).

Antinociceptive activity

Oral administration of the extracts elicited antinociception in the animals at both selected doses of 10 and 50 mg/kg. In vehicle treated groups, injection of acetic acid caused an intense nociceptive response in mice, ranging from 38.9 ± 2.5 to 40.4 ± 4.0 writhings (Table 4).

Pretreatment of the animals with hydroethanolic extracts of "chapéu-de-couro" (10 mg/kg), resulted in inhibition of writhings, attaining the lowest value (36.4%, $p < 0.001$) with *E. scaber* – L_5 and highest value (62.5%, $p < 0.001$) with *E. scaber* – L_{10}, when compared to the vehicle group. When a 50 mg/kg extract dose was employed, reduction in number of writhings was greater than at the lower dose, reaching the lowest value (47.4%, $p < 0.001$) with *E. scaber* – L_1, and the highest value (79.8%; $p < 0.001$) with *E. scaber* – L_7, when compared to the vehicle group.

Animals that received 5 mg/kg indomethacin, the standard drug for this test, had the highest percentage inhibition of nociceptive response, with values ranging from 82.6% to 90.1% ($p < 0.001$).

Discussion

This work performed fingerprint analyses, as well as flavonoid quantification on samples of the popular phytotherapic drug named as "chapéu-de-couro". The samples were collected in different places from Mato Grosso and Mato Grosso do Sul States in Brazil, to search for phytochemical differences, and for chemical markers in the crude drugs presenting antinociceptive activity.

The doses of *E. scaber* and *E. grandiflorus* extracts in antinociceptive tests models were chosen based on murine pilot experiments of our lab with the acetic acid test, and from previous literature reports on other inflammatory or antinociceptive models. The classical method of Whittle (1964), using acetic acid, was chosen because it is a simple, rapid and cheap test, being a non-selective model that serves for both peripheric and central analgesia, without altering consciousness. Indomethacin was used as a positive control since it is a classical standard non-steroidal drug that assures its specificity as an inhibitor of cyclooxygenase (Whittle, 1964; Cabral-Silva, 2013).

Despite the distinct profile of the two studied species and *Echinodorus* sp. (commercial sample) regarding flavonoid content, the amount of the *C*-glycosylated flavonoids can be successfully used in the quality control if a "chapéu-de-couro" drug is to be commercialized. The PCA (Fig. 1) shows that the geographical location of the species is the main differentiating factor in the polar secondary metabolism. In the last years several authors showed that edaphoclimatic factors may substantially change metabolomics composition (Gobbo-Neto and Lopes, 2007).

Leaf extracts from "chapéu-de-couro", corresponding to the crude drug were orally administered at the doses of 10 and 50 mg/kg using for comparative purposes the acetic acid writhing assay. The best effect (Table 4) was associated with the sample origin, with a clear dose–response effect for the MS plants. Taken together, higher extraction yields with good antinociceptive

Table 4
Effect of oral administration of *Echinodorus scaber* and *E. grandiflorus* hydroethanolic extracts on acetic acid-induced writhes in mice.

Extracts	Dose (mg/kg)	No. of writhes	% inhibition
Vehicle	–	38.9 ± 2.5	–
E. grandiflorus – L_1	10	21.0 ± 2.2[b]	46.0
	50	20.4 ± 2.6[b]	47.4
E. grandiflorus – L_3	10	15.9 ± 1.7[b]	59.2
	50	13.0 ± 3.6[b]	66.5
E. scaber – L_{10}	10	14.6 ± 2.6[b]	62.5
	50	9.4 ± 3.3[b]	75.7
Indomethacin	5	5.3 ± 0.9[b]	86.3
Vehicle	–	40.1 ± 3.0[b]	–
E. scaber – L_2	10	19.0 ± 2.7[b]	52.7
	50	14.1 ± 2.1[b]	64.8
Echinodorus sp. – L_6	10	21.3 ± 4.2[b]	46.9
	50	18.1 ± 3.6[b]	54.8
Indomethacin	5	4.8 ± 1.1[b]	88.0
Vehicle	–	40.4 ± 4.0	–
E. scaber – L_7	10	23.4 ± 3.9[a]	42.1
	50	8.2 ± 1.7[b]	79.8
E. scaber – L_8	10	18.7 ± 4.7[b]	53.8
	50	8.6 ± 2.5[b]	78.6
E. scaber – L_9	10	19.8 ± 2.3[b]	51.0
	50	12.6 ± 1.6[b]	68.9
Indomethacin	5	4.0 ± 1.2[b]	90.1
Vehicle	–	40.3 ± 1.7[b]	–
E. grandiflorus – L_4	10	19.0 ± 2.8[b]	52.9
	50	15.7 ± 3.0[b]	61.1
E. scaber – L_5	10	25.7 ± 4.1[b]	36.4
	50	15.0 ± 2.0[b]	62.8
E. scaber – L_{11}	10	22.8 ± 2.5[b]	43.4
	50	10.3 ± 1.8[b]	74.5
Indomethacin	5	7.0 ± 1.1[b]	82.6

Results are presented as mean values ± S.E.M. for $n = 6–8$ animals/group. One way ANOVA followed by the Student–Newman–Keuls test.
[a] $p < 0.01$ vs vehicle.
[b] $p < 0.001$ vs vehicle.

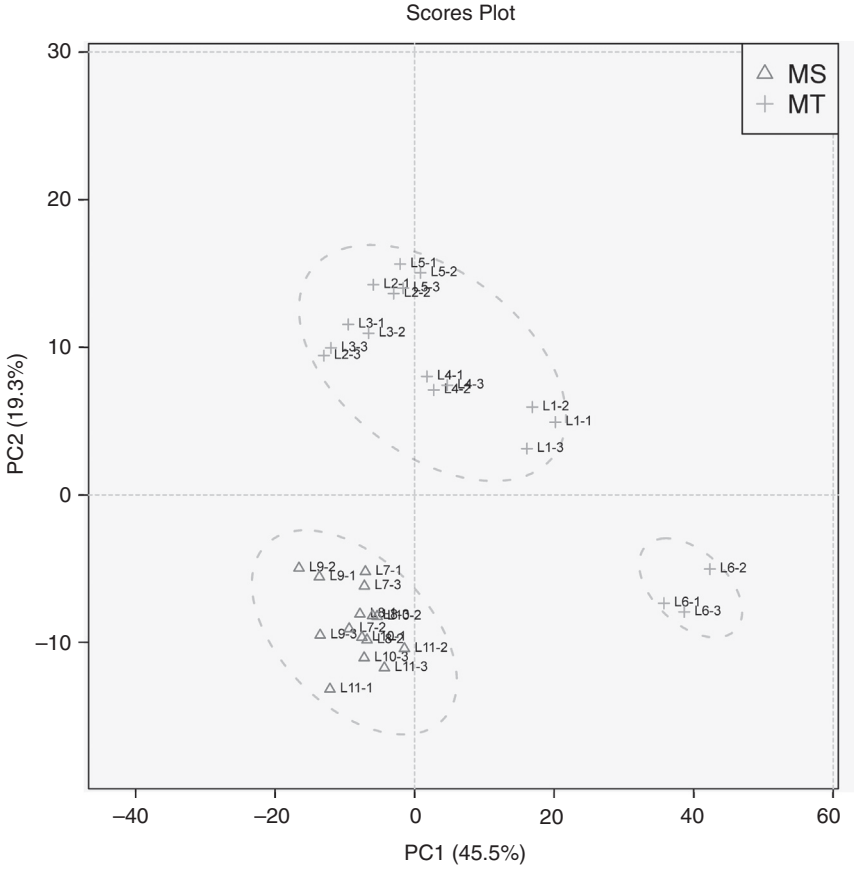

Fig. 1. Distribution of *Echinodorus scaber*, *E. grandiflorus* and *E.* sp. on the plan of the first two principal components PC1 and PC2 (64.8% variance explained).

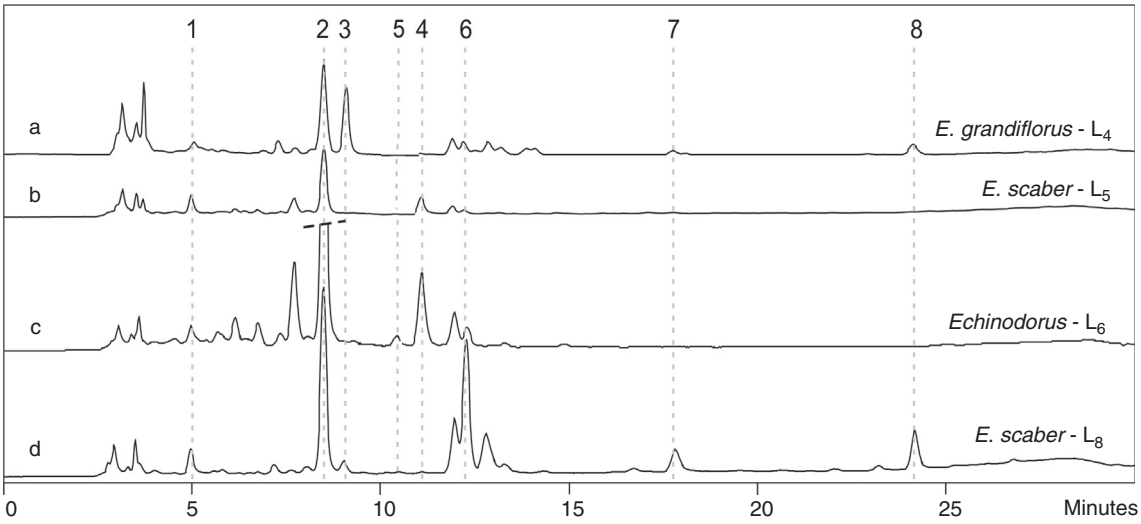

Fig. 2. Representative HPLC chromatograms of the *Echinodorus scaber* and *E. grandiflorus* samples from the Brazilian Pantanal. (A) *E. grandifloras*: L_4 (representative from collection places L_1, L_3, and L_4 from MT state); (B) *E. scaber*: L_5 (representative from places L_2 and L_5 – MT state); (C) *Echinodorus* sp. – L_6 (commercial sample); (D) *E. scaber*: L_8 (representative from collection places L_7 to L_{11} from MS state). Peak identities: (1) catechin (internal standard), retention time: 5 min, λ_{max} 324 and 279 nm; (2) isoorientin*, retention time: 8.6 min, λ_{max} 348 and 269 nm; (3) swertiajaponin**, retention time: 9.2 min, λ_{max} 344 and 269 nm; (4) vitexin-2-*O*-rhamnoside, retention time: 11.2 min, λ_{max} 334 and 269 nm; (5) vitexin*, retention time: 11.6 min, λ_{max} 335 and 269 nm; (6) isovitexin*, retention time: 12.4 min, λ_{max} 335 and 269 nm; (7) caffeoyl/feruloyl acid derivatives, retention time: 18.2 min, λ_{max} 327, 290*sh* and 245 nm; (8) caffeoyl/feruloyl acid derivative, retention time: 25.1 min, λ_{max} 328, 290*sh* and 243 nm. *Identity confirmed by co-injection of standards. **Identity confirmed by NMR data, UV spectrum and comparison with literature (Schnitzler et al., 2007; Peng et al., 2005).

effect suggest that the crude drug from MS present a better profile regarding the traditional indications of use. The related species, *E. grandiflorus*, with the same common name and use, showed similar antinociceptive effect than that observed for the MT collections of *E. scaber*.

The analgesic and anti-inflammatory activity of extracts obtained from the rhizomes of *E. grandiflorus* was reported by Dutra et al. (2006) and the hypotensive effect of the leaf ethanol extract on spontaneous hypertensive rats was also described (Lessa et al., 2008).

Garcia et al. (2010) published a combined phytochemical and anti-inflammatory study of *E. grandiflorus* leaves extract and found activity at the highest dose of 1 g/kg, too high to explain the traditional indications of use.

The main differences of the present work with previous studies is the ethnopharmacological approach, using the crude drug following the traditional indications of use and the doses used which are lower than in the study of Dutra et al. (2006). In addition, our work was undertaken using a larger number of plant samples, including material from different locations and purchased at local markets. In this way, a closer picture of the crude drug "chapéu-de-couro" is obtained.

All extracts were compared by HPLC looking for characteristic compounds in the polar extracts. According to the fingerprints, our samples can be arranged in four main groups, two of them representative of *E. scaber* from MT and MS and a third set in agreement with *E. grandiflorus*, being the fourth sample the commercial *Echinodorus* sp. The chromatograms representing the different profiles are presented in Fig. 2. Table 5 presents the retention time, the UV data and the tentative identification of the main phenolic compounds found in the analyzed "chapéu-de-couro" leaf extracts.

The HPLC chromatograms representative of all of our *E. scaber* samples from MS shows two main compounds identified as isoorientin and isovitexin (Fig. 2, trace D; peaks 2 and 6, respectively). However, a subset from the samples can be recognized by comparing the occurrence of two compounds eluting at retention time (Rt) of 18.2 and 25.1 min (peaks 7 and 8), respectively, both presenting UV spectra with maxima at 327, 290*sh* and 245 nm, in agreement with caffeoyl/feruloyl acid derivatives (Schnitzler et al., 2007). Fig. 2, trace B, representing samples L_2 and L_5 from MT shows isoorientin (peak 2) as the main constituent and vitexin-2-*O*-rhamnoside (peak 4) as secondary compound, differently from the MS plants, which show very low content of this compound. The unidentified crude drug sample of *Echinodorus* sp. purchased in MT (L_6 in Fig. 2, trace C) is close to *E. scaber* from MT according to the phenolic compounds pattern and showed the highest content in vitexin-2-*O*-rhamnoside from all the samples investigated.

Table 5
Identification of phenolic compounds in *Echinodorus scaber* and *E. grandiflorus* hydroethanolic" extracts by HPLC-DAD.

Peak	Rt (min)	λ_{max} (nm)	Tentative identificaction
1	5.0	324, 279	Catechin (internal standard)
2	8.6	348, 269	Isoorientin[a]
3	9.2	344, 269	Swertiajaponin[b]
4	11.2	334, 269	Vitexin-2-*O*-rhamnoside[a]
5	11.6	335, 269	Vitexin[a]
6	12.4	335, 269	Isovitexin[a]
7	18.2	327, 290*sh*, 245	Caffeoyl/feruloyl acid derivative[b]
8	25.1	328, 290*sh*, 243	Caffeoyl/feruloyl acid derivative[b]

Rt: Retention time; HPLC-DAD: High Performance Liquid Chromatography with Diode Array Detector.
[a] Identity confirmed by coinjection of standards.
[b] Identity confirmed by UV spectrum and comparison to the literature (Schnitzler et al., 2007).

The HPLC profile of the *E. grandiflorus* extracts corresponding to "chapéu-de-couro" (L_1, L_3 and L_4 – Fig. 2, trace A) were similar to those reported by Garcia et al. (2010) for a sample from the same species collected in the Paraná state in southern Brazil. Peaks 2 and 3 from our sample show the same UV spectrum suggesting that they are flavonoids from the same structural group, namely isoorientin and apparently swertiajaponin.

In a study of the phenolic constituents from *E. grandiflorus* ssp. *aureus*, Schnitzler et al. (2007) carried out a fractionation of the plant extract and unambiguously identified their main compounds by spectroscopic and spectrometric means. The sample reported, however, correspond to a variety of *E. grandiflorus* that can be easily recognized by the main peak of swertiajaponin (peak 3). The plant was collected in the Santa Catarina state, southern Brazil.

The main flavonoids occurring in the polar hydroethanolic extracts of "chapéu-de-couro" are *C*-glycosyl flavonoids differing in the number of free hydroxy and/or methoxy groups in the flavonoid ring, namely isoorientin, swertiajaponin, vitexin-2-*O*-rhamnoside and isovitexin.

Vitexin is a *C*-glycosyl flavone used as a chemical marker for *Passiflora* species (Dhawan et al., 2004). The *C*-glycosyl flavonoids found in the plant have been associated with antiinflammatory (Sridhar et al., 2006) and antinociceptive effect (Kumar et al., 2012).

Isoorientin is present in high concentrations in all extracts of "chapéu-de-couro" (Table 2); this flavone is also found in species of *Passiflora* and is frequently associated with anti-inflammatory and antinociceptive activities. However, this compound cannot be considered the main responsible for the antinociceptive activity of "chapéu-de-couro", since the antinociceptive tests responded randomly to isoorientin concentrations. Swertiajaponin was tentatively identified (Table 5) only in *E. grandiflorus* extracts and, therefore, cannot be the main responsible for the antinociceptive activity.

Vitexin-2-*O*-rhaminoside is often found in *Passiflora* species. In the present work this compound was quantified only in extracts of *E. scaber* from MT as well as the commercial sample (Table 2). In extracts where this flavone was not detected, however, there was considerable percentage of pain inhibition, indicating that vitexin-2-*O*-rhaminoside cannot be the main responsible by the analgesic activity presented by "chapéu-de-couro" samples analyzed in this work.

Isovitexin was detected in all extracts from *E. scaber*, in concentration ranging from traces to 14.60 μg/mg; higher amounts were detected in samples from MS (Table 2). A dose-dependent antinociceptive activity could be observed, when comparing isovitexin concentration and the percentage of inhibition of *E. scaber* extracts (Table 4). The same trend was observed when analyzing *E. grandiflorus* samples. As commercial *Echinodorus* and *Passiflora* species share the same *C*-glycosylflavonoid constituents, new studies on the anxiolytic effect of "chapéu-de-couro" are of interest. Recent reports on the effect of *Passiflora* extracts on the central nervous system include the isoorientin and isovitexin (Li et al., 2011).

Overall the HPLC method presented allowed a clear and rapid differentiation of *E. scaber* and its substitute *E. grandiflorus* and is suitable for comparative analyses of *Echinodorus* samples.

The collections displaying better antinociceptive effect shows the higher isovitexin content and also better w/w yields, making the MS samples the better source for the crude drug. Further studies should be carried out, including the harvesting sources of the crude drug for commercial purposes to provide recommendations on quality control based on the active constituents and extraction yields. Thus, isovitexin can be regarded, at least in part, as responsible for the antinociceptive activity and seems to be a suitable chemical marker for "chapéu-de-couro" crude drug.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CLE, KCL and ELD participated in phytochemical analysis. VCS participated in interpretation of NMR spectral data. EFGCD participated in HPLC analysis, acquisition and interpretation of data. RVR and DTOM participated in the evaluation of antinociceptive activity, acquisition and interpretation of data. CAC participated in PCA analysis, acquisition and interpretation of data. PTSJ authored the project and supervised its execution and the drafting of this paper and GSH participated in the discussion of the data presented herein as well as the drafting of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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