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Pattern of allelochemical distribution in leaves and roots of tough lovegrass (*Eragrostis plana* Nees.)

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Abstract

This is the first report on chemical compounds in tough lovegrass, an allelopathic plant considered to be the most abundant invasive plant in the rangelands of Southern Brazil. This study aimed to evaluate the distribution and concentration of allelochemicals in the leaves and roots of tough lovegrass. Plant material was collected from an area of secondary vegetation in April 2013. Aqueous extracts (leaves and roots) were prepared and subjected to turboextraction and subsequent lyophilization. The extracts were subjected to qualitative chemical tests in order to evaluate their alkaloids, anthraquinones, flavonoids, tannins, and saponins content. Post-validation high-performance liquid chromatography (HPLC) assay was used to evaluate the allelochemicals caffeic, ferulic, p-coumaric, vanillic acids, coumarin, catechin, epicatechin, rutin, quercetin, gallic acid, and kaempferol. Qualitative chemical tests detected the presence of saponins, alkaloids, and flavonoids in the leaves and roots of tough lovegrass and tannins in only leaves. HPLC assay verified the presence of coumarin in the roots and leaves in equal amounts; ferulic acid in higher quantities in roots; caffeic, p-coumaric, and vanillic acids in higher quantities in leaves; and catechin and epicatechin in leaves only. The compounds rutin, quercetin, gallic acid, and kaempferol were not detected in the extracts. The presence of secondary metabolites varied in different plant organs. Our results demonstrated greater amount of compounds in the leaves; therefore, this organ should be prioritized in the future with isolation of compounds. The presence of allelochemicals indicates that the compounds of this species can be potentially used as bioherbicides.

Keywords: allelopathy; *Eragrostis plana;* weed; phenolics; phytochemical.
Abbreviations: CRS_chemical reference substance; DAD_photo diode array detector; DM_dry matter; HPLC_high-performance liquid chromatography; IAA_indole acetic acid; LOD_limit of detection; LOQ_limit of quantitation; RSD_relative standard deviation

Introduction

In the recent years, with increasing human activity and the strengthening of international trade, the transfer of biological species from different habitats has become more frequent. Some of these species demonstrate strong environmental adaptability and can grow and spread rapidly in new environments, which may lead to adverse consequence on the economy and ecology of a society. From an ecological perspective, allelopathy is an important factor that influences the invasion and spread of exotic plants (Chengxu et al., 2011). This process contributes to the ability of particular exotic species to become dominant in plant-invaded communities (Ridenour and Callaway, 2001). Besides aiding in the study of biological invasion processes, allelopathic plants have received attention from chemists in order to identify and isolate allelochemicals with the potential of bioherbicides. Natural compounds are more environmentally friendly than most synthetic pesticides; furthermore, their environmental half-lives is shorter and they act in a large number of local unexplored by herbicides. This fact and the necessity of new target sites suggest concentration of more efforts on natural products (Duke et al., 2000). The *Eragrostis* genus has the highest number of weed species in the Brazilian, Uruguayan, and Argentinean Pampas (Fonseca et al., 2013). In the natural grassland of Southern Brazil, an estimated more than 2 million hectares of land area was invaded by tough lovegrass (*Eragrostis plana* Nees) (Medeiros and Focht, 2007). This alien African grass is the most abundant and aggressive invasive plant in the Pampa Biome, responsible for causing widespread economic impacts on the livestock by modifying the structure of plant communities and by changing the ecological balances. The allelopathic effect of tough lovegrass on forage plants (Coelho, 1986; Ferreira et al., 2008; Favaretto et al., 2011) is an important decisive factor for the success of the invasion (Medeiros and Focht, 2007), since it affects the growth of neighboring plants. Our investigations revealed that the leaf extracts are more heterotoxic on germination and seedlings of white clover (*Trifolium repens* L.) in relation to roots extracts (Favaretto et al., 2011), indicating a possible difference in the allelochemical composition between these structures. The difference in the allelopathy between plant organs has been reported in alfalfa (*Medicago sativa* L.) (Chon and Kim, 2002), *Eucalyptus* spp. (Zhang and Fu, 2010), sun spurge (*Euphorbia helioscopia* L.) (Tanveer et al., 2010), thousand-men (*Aristolochia esperanza* O. Kuntze) (Gatti et al., 2004), alligator weed (*Alternanthera philoxeroides* (Mart. Griseb),

and sessile joyweed (*A. sessilis* L.) (Mehmood et al., 2014), where leaves extracts are more potentially allelopathic than the roots, stems, flowers, fruits, and seeds. We evaluated the distribution and concentration of allelochemicals in the leaves and roots of tough lovegrass. This first of its kind study developed and validated analytical methods by HPLC for the determination of p-coumaric, caffeic, ferulic, and vanillic phenolic acids, as well as of coumarin (1.2-benzopyrone).

Results

Qualitative chemical tests

The first characterization of allelochemicals in tough lovegrass revealed qualitative and quantitative differences between the contents of leaves and roots. The leaf extracts, which are more allelopathic than roots (Favaretto et al., 2011), showed higher concentrations of allelochemicals and presented compounds that are not present in the roots. Moreover, we performed validation of a new method involving HPLC identification and quantification of some allelochemicals belonging to the phenolic group. The preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, and saponins in the leaves and roots; but, tannins were detected only in the leaves (Table 1).

While no studies exist on the chemical groups in the species of interest, qualitative phytochemical screening can help understand a variety of chemical compounds produced by plants, and quantification of these metabolites can help extract, purify, and identify the bioactive compounds (Geetha and Geetha, 2014). The compounds detected in this study are potentially allelochemicals (Rice, 1984). Among them, phenolic compounds are the most important class, the most common allelochemicals in plants (Li et al., 2010) and the main compounds in weed plants (El-Khatib et al., 2004). This class includes flavonoids, tannins, coumarins, and phenolic acids. Thus, we selected some of the main allelochemicals from the phenolic group found in grasses and submitted the extracts of the tough lovegrass to HPLC investigation. Moreover, we performed validation of the chromatography methods for detecting these compounds for enhanced accuracy.

HPLC method validation

The elution system that allowed the best resolution of the peaks referent to phenolic acids (for which we developed a simultaneous detection system) were acetonitrile and water (pH 3.4) (25:75, v/v). For coumarin, the most appropriate mobile phase was a mixture of acetonitrile and water (pH 3.4) (55:45, v/v). The results from peak purity ensured the selectivity of the method, considering that all results were between 1 and 1.5, in accordance with the Chromera Flexar software. The regression equations confirmed the existence of a linear relationship between the areas and predetermined concentrations, with a correlation coefficient of >0.99 (Table 2) (ICH, 2005). The LOD and LOQ indicated the adequate sensitivity of the method (Table 2).

The accuracy test showed values with percentage recovery suitable for the study (nearly 100%). Similarly, the intra-day precision revealed values of RSD within the acceptance criteria of up to 5% (ICH, 2005). These values were 0.91, 1.06, 1.03, 1.88, and 0.38%, respectively, for the acids caffeic, ferulic, p-coumaric, vanillic, and coumarin. Thus, the developed methodologies were found to be accurate and precise. Moreover, small changes introduced in the chromatographic conditions of the pH and mobile phase flow did not change the method and concentrations of CRSs, demonstrating the robustness of the method.

HPLC analysis

By HPLC extracts analysis, the flavonoids rutin, kaempferol, and quercetin were not found in the extracts, although qualitative chemical tests revealed positive results, indicating that the plant possessed other flavonoid compounds not detected by HPLC. Tannin gallic acid was also not detected in the extracts.On the other hand, the presence of caffeic, ferulic, vanillic, and p-coumaric acids was verified in the leaves and roots of tough lovegrass (Fig 1). Except for ferulic acid, the other acids were detected in greater quantities in the leaves (Table 3). The chromatograms indicated the presence of two major peaks with polar characteristics in the leaves and roots, with the retention times of 2.18 and 3.37 min, respectively (Fig 1). The peaks were larger for the leaf extract, indicating greater amount of polar compounds in these organs. Coumarin was detected in the leaves and roots (Fig 2) in equal concentrations (Table 3). Catechin and epicatechin were observed only in the leaves (Fig 3; Table 3).

Discussion

Phenolic acids, identified in tough lovegrass, have already been deemed responsible for the allelopathic potential of other grasses such as *Brachiaria mutica* (Forssk.) Stapf., rice (*Oriza sativa* L.), kikuio grass (*Pennisetum clandestinum* Hochst) (Chou, 1989), and sorghum (*Sorghum bicolor* L.) (Guenzi and McCalla, 1966). The phenolic acids, especially p-coumaric acid, inhibit the germination and growth of plants (Blum, 1996). These compounds decrease the membrane permeability; inhibit the absorption of nutrients, root elongation, cell division, ultrastructural cellular changes, and lipid metabolism (Baleroni et al., 2000); reduce chlorophyll content and photosynthetic rates (Li et al., 2010); and alter the level of indole acetic acid (IAA) (Einhellig, 1986). The ferulic and p-coumaric acids increase the level of hydrogen peroxide and peroxidase activity (Politycka et al., 2004).

The amount of phenolic acids in tough lovegrass (Table 3) is equal to or less than the amounts of the same compounds in black mustard (*Brassica nigra* L.), which is an allelopathic plant (Al-Sherif et al., 2013). Thus, the quantities observed here were sufficient to cause a heterotoxic effect in seeds and seedlings. The HPLC analysis revealed the presence of the monomers of procyanidins, catechin, and epicatechin in the leaves, but not in the roots, of tough lovegrass. The qualitative chemical tests also detected the presence of these compounds only in the leaves (Table 1). The catechin inhibits the germination of seeds; however, in greater quantities, it impairs the development of roots and hypocotyls, while epicatechin inhibits the germination of the plants more than the development process (Lôbo et al., 2008). In addition to inhibiting the germination and growth process, catechin stimulates the production of reactive oxygen species and can cause oxidative stress, damage in the molecules (DNA), and the oxidation of proteins (Bais et al., 2003). Lôbo et al. (2008) reported that only 5 mg of catechin and epicatechin was sufficient to cause allelopathic effects in *Mimosa pudica* L. In the present study, a similar concentration was observed in the leaf extract for catechin (4.22 $mg.g^{-1}$ DM) and a higher concentration for epicatechin (9.56 $mg.g^{-1}$ DM).

The coumarins inhibit germination (Rice, 1984), the growth of roots and shoots (Hierro and Callaway, 2003), and

Table 1. Qualitative chemical tests in the leaves and roots of tough lovegrass.

Chemical group	Reaction	Leaf	Root
Alkaloids	Dragendorff	+	+
	Mayer	+	+
	Bertrand	+	+
	Bouchardat	+	+
Anthraquinones	Borntraeger	-	-
Flavonoids	Oxalo-boric	+	+
Tanins	Gelatin	+	-
	Iron salts	+	-
Saponins	Foaming index	+	+

(+) positive reaction; (−) negative reaction

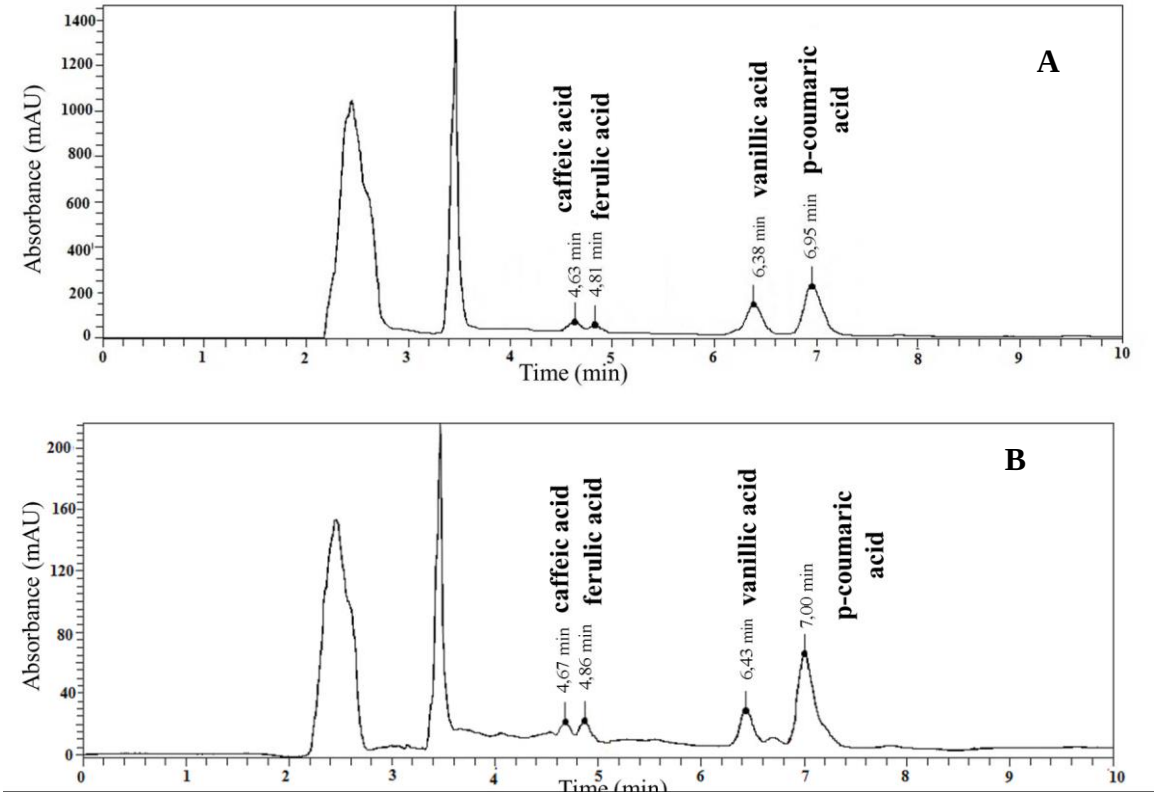

Fig 1. Chromatographic profile of leaf (A) and root (B) extracts of tough lovegrass (50 mg/mL) for the detection of phenolic acids.

oxidative phosphorylation (Razavi, 2011), as well as affect ATP synthesis (Mata et al., 1998). Moreover, they limit the production of the bristle root, which consequently changes the plant water status and thereby affects the leaf area and the opening and closing of stomata (Colpas et al., 2003). In extracts of *Annona glabra* L., 0.039 $mg.g^{-1}$ DM of coumarin inhibited wheat coleoptile length (*Triticum aestivum* L.) (Matsumoto et al., 2010). As the concentration of coumarin described in *A. glabra* was lower than that detected in the present work (0.09 $mg.g^{-1}$ DM), it is possible that the amount of this substance in tough lovegrass can contribute to the allelopathic potential of this species. Until date, there has been numerous reports on phytotoxic, fungitoxic, insecticide, antibacterial, and nematocidal activity of different coumarins. This study demonstrated that some coumarins such as imperatorin and psoralen exhibited considerable allochemical potential. Therefore, this compounds can be used to generate a new generation of bioherbicides and other pesticide chemicals that are more ecologically friendly (Razavi, 2011). On the other hand, coumarin possesses appetite-suppressing properties, which may discourage animals from eating plants containing it. Although this compound has a pleasant sweet odor, it has a bitter taste, which animals tend to avoid (Quick, 1942). This may be one factor that decreases the tough lovegrass consumption by animals. The findings of this study may be related to the differences in the allelopathic activity of the leaves and roots of tough lovegrass. Except for the concentration of ferulic acid, which is greater in the roots than in leaves, and the coumarin, which is present in equal amounts in both the organs, other compounds occur in greater quantities or are present only in the leaves of tough lovegrass, justifying their greater allelopathic effect (Favaretto et al., 2011). Similarly, some previous works have reported higher amounts of phenolic compounds in leaves than in the extracts of roots, stems, flowers, and fruits (Ali et al., 2013; Mehmood et al., 2014). The higher amount of phenolic compounds in the leaves of *R. capitata* (Ali et al., 2013) and in the species of *Alternanthera* spp. can be correlated with the greater allelopathic effect of this plant organ. The distribution of the allelochemicals in the plant organs is not uniform, both qualitatively and quantitatively, in space and time (Harborne, 1972). These metabolites can be found in the

Table 2. Linear equations, correlation coefficients, limit of detection (LOD), and limit of quantitation (LOQ) of chemical reference substances (CRSs).

CRS	Linear equation	Correlation coefficient	LOD	LOQ
			($\mu g.mL^{-1}$)	
Caffeic acid	y = 53.947x – 142.669	0.9998*	2.96	8.97
Ferulic acid	y = 34.188x – 19.192	0.9996*	6.82	20.67
p-Coumaric acid	y = 54.331x – 210.570	0.9992*	1.92	5.8
vanillic acid	y = 78.391x – 11.883	0.9998*	0.49	1.47
coumarin	y = 76.336x + 393.699	0.9981*	6.47	19.60

*Indicates significance.

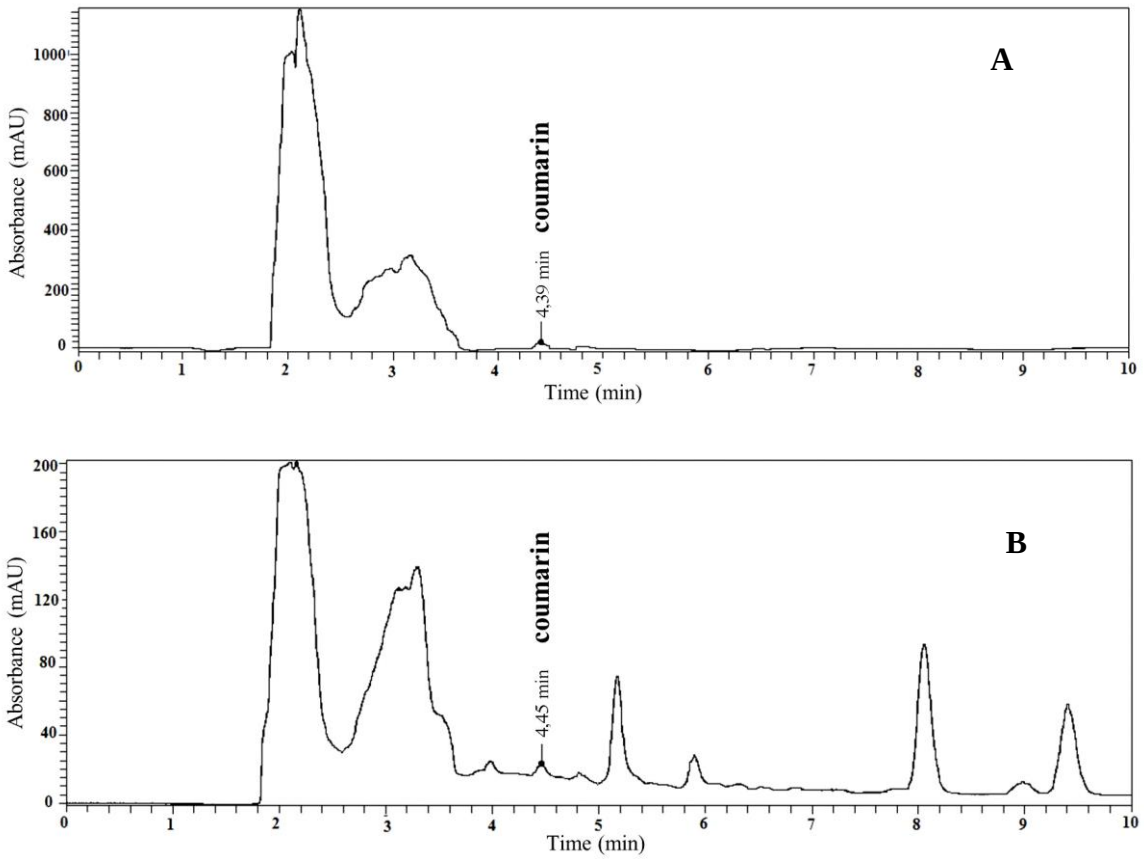

Fig 2. Chromatographic profile of leaves (A) and roots (B) of tough lovegrass for coumarin detection.

leaves, stems, roots, pollen, flowers, fruits, and seeds in varying amounts, depending on aspects such as the age and development stage (Bourgaud et al., 2001). Nevertheless, leaves seem to be the most consistent sources of chemicals involved in phytotoxicity (Reinhardt and Bezuidenhout, 2001). As the leaf is the most metabolically active plant body, it is reasonable to believe that it introduces a greater diversity of allelochemicals and, hence, greater allelopathic effects (Ribeiro et al., 2009). Whereas one of the main advantages of allelochemicals is the discovery of new modes of action for the development of bioherbicides (Macías et al., 2007). In the case of tough lovegrass, this investigation should be focused on the leaf, which is the most allelopathic body in the plant and possesses greater concentration and diverse variety of secondary compounds.

Materials and methods

Plant material

Plant material was collected from an area of secondary vegetation in Passo Fundo (28°15′S, 52°24′W), Brazil, in April 2013. After the separation of leaves and roots, the material was dried at 40°C and then ground for the preparation of extracts.

Qualitative chemical tests

Chemical qualitative tests for detecting the presence of alkaloids, anthraquinones, flavonoids, tannins, and saponins was performed. For these analyses, we used dried and ground plant materials. After acid–base extraction, the presence of alkaloids was determined by reactions of precipitation with several reagents, including Dragendorff, Mayer, Bertrand, and Bouchardat. The Borntraeger reaction was used to investigate the presence of anthraquinones; to detect flavonoids, the reaction was performed using a solution of 3% boric acid and 10% oxalic acid in 75% ethanol, followed by the observation of fluorescence under UV light (365 nm). To determine the presence of tannins, the reactions were performed using 5% ferric chloride solution in methanol, 10% aqueous lead acetate, and 1% gelatin in acidic media; an index of foam was used to indicate the presence of saponins (Harborne, 1998).

Table 3. Concentration of chemical reference substances (CRSs) found in the leaves and roots of tough lovegrass.

CRS	Leaf	Root
	----------mg.g^{-1} DM*----------	
Caffeic acid	0.19 A	0.07 B
Ferulic acid	0.06 B	0.18 A
p-Coumaric acid	0.94 A	0.34 B
Vanillic acid	0.42 A	0.05 B
Catechin	9.56 A	0.00 B
Eepicatechin	4.22 A	0.00 B
Coumarin	0.09 A	0.09 A

* DM: Dry material. Means followed by the same letter in the row do not differ (P > 0.05) by Tukey's test.

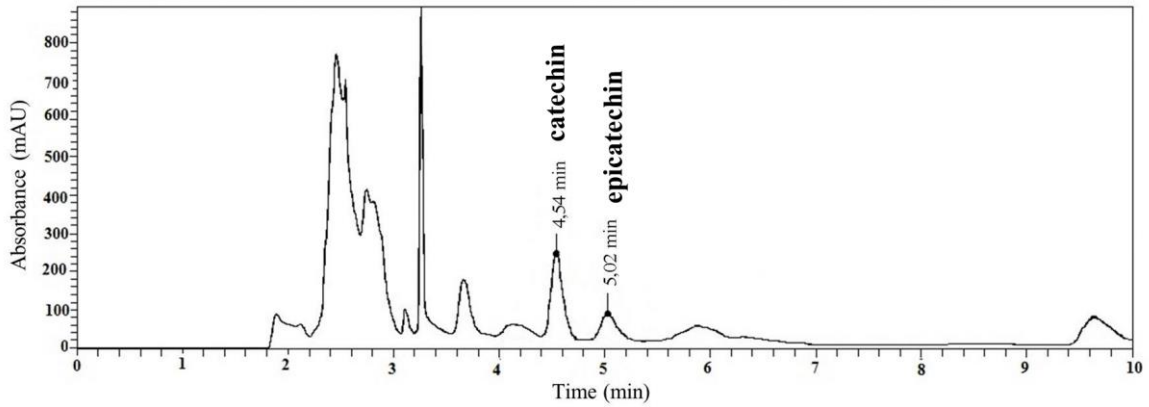

Fig 3. Chromatographic profile of tough lovegrass leaves for catechin and epicatechin detection.

High-Performance Liquid Chromatography

Two chromatographic methods were developed and validated by HPLC. For the detection of phenolic acids and for the detection of coumarin by using CRSs: catechin (99%), epicatechin (99%), gallic acid (99%), rutin (99%), quercetin (99%), kaempferol (99%), coumarin (>98%), vanillic acid (>97%), ferulic acid (99%), p-coumaric acid (>98%), and caffeic acid (>98%) (Sigma Aldrich, St. Louis, MO, USA). For the preparation of mobile phases, ultrapure water was obtained from the Direct-Q System (Millipore Corporation (EUA); acetonitrile and methanol were of HPLC-grade; and phosphoric and acetic acid were of analytical grade. Stock solutions of CRSs were prepared in methanol at 1000 µg/mL^{-1}. An HPLC Flexar LC Perkin Elmer (Burnsville, MN, USA) was used for the validation and analysis of the extracts. It was fitted with an RP column C18 ACE (250 × 4.6 mm, 5 µm), binary pump, photo diode array detector (DAD) in 280 nm and 274 nm, and auto-sampler with a 20-µL loop. Peak areas were integrated into Chromera Workstation software. Several combinations of the mobile phase (composed of water and acetonitrile) were tested to determine the best mobile phase to CRS at a flow rate of 1 ml/min.

Method validation

For the CRSs catechin, epicatechin, gallic acid, rutin, quercetin, and kaempferol, we used a method previously developed and validated by Chini (2013). For the caffeic, ferulic, and vanillic acids and p-coumaric and coumarin, we developed analytical methods, which was validated in accordance with ICH (2005) and Brazil (2003). The parameters evaluated in this test were specificity, linearity, limit of quantitation (LOQ), limit of detection (LOD), accuracy, precision, and robustness. Specificity was verified from the peak purity determined by the photo DAD. Linearity was determined by preparing three calibration curves containing five concentrations each for CRS in the range 40–200 µg.mL^{-1}, diluted in methanol. Three replicates of injections were prepared for each solution to verify the repeatability of the detector response. The peak areas of the chromatograms were plotted against the concentration to obtain a calibration curve. The linearity was expressed as a correlation coefficient by linear regression analysis. The curves were subjected to analysis of variance (ANOVA) ($p \leq 0.05$), and the linear regression and linearity deviation were observed. The LOD and LOQ were calculated from the slope and standard deviation of the intercept of the mean of the three calibration curves. The accuracy was evaluated by a recovering test. Nine solutions of CRS were prepared, contemplating three concentrations—low (100 µg.mL^{-1}), medium (140 µg.mL^{-1}), and high (180 µg.mL^{-1})—in triplicate. The percent recovery was calculated according to the equation:

% Recovery = (found concentration)/(theoretical concentration) × 100

The repeatability was evaluated by analyzing six sample solutions of each CRS at 200 µg.mL^{-1} during the same day under the same experimental conditions. Precision was expressed as a percentage relative standard deviation (RSD) of the peak areas. The robustness was established by introducing small changes in the chromatographic system, such as in the flow rate (0.98 mL.min^{-1} and 1.02 mL.min^{-1}) and the pH of the mobile phase (3.37 and 3.43) of the CRS solution at 40 µg.mL^{-1}, in triplicate.

Plant extracts

After validation, the aqueous extracts of leaves and roots (separately) were obtained with the mixture of 1:10 plant:distilled water, followed by subjecting to turboextraction and subsequent lyophilization (lyophilizer Interprise K2504/Terroni). The powders obtained were resuspended in methanol at a concentration of 50 mg.mL^{-1}

and filtered through a 0.45-µm nylon membrane. Aliquots of 20 µL were injected into the HPLC system in triplicate.
The data relating to injections of the extracts of leaves and roots of tough lovegrass were subjected to ANOVA, followed by comparison of means by Tukey's test ($p \leq 0.05$).

Conclusions

The preliminary phytochemical screening indicated the presence of alkaloids, flavonoids, and saponins in the leaves and roots, but tannins were detected only in the leaves of tough lovegrass. The HPLC analysis indicated the presence of the phenolic acids: p-coumaric, caffeic, ferulic, and vanillic; coumarin; and catechin and epicatechin, in amounts varying with the organ of the plant. The analytical method proposed for the determination of acids caffeic, ferulic, p-coumaric, vanillic, and coumarin by HPLC is selective, linear, accurate, precise, and robust. Our results showed greater amount of compounds in the leaves; therefore, this organ should be prioritized in the future work on isolation of compounds. The presence of allelochemicals indicated that the compounds of this species can be potentially used as bioherbicides.

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