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GEOSCIENCES

Phytotoxic bioassays and Fingerprinting by HPLC-DAD of *Eragrostis plana* Nees Root Extracts – application of chemometrics

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Abstract: *Eragrostis plana* (Nees) (Tough Lovegrass) shows ability to interfere with other plants, a phenomenon known as allelopathy. This chemical interaction between plants occurs due to the release of compounds into the environment. Thus, a phytotoxicity study was carried out with *E. plana* roots collected during each season throughout the year, and the compounds were extracted with solvents of increasing polarity. The data from the bioassays were analyzed by GLM and PCA. In addition, a fingerprint of these extracts was obtained by HPLC-DAD. The extracts in petroleum ether from roots collected in the winter and summer showed greater phytotoxicity on *Ipomoea grandifolia* germination and growth. The PCA obtained from the chromatogram of the crude extract showed that the extracts in petroleum ether were chemically different from the extracts in ethyl acetate and methanol. Thus, continuing this study in order to develop a new generation of bio-herbicides is essential.

Key words: allelopathy, capim annoni-2, chemometric methods, *Eragrostis plana*, fingerprinting.

INTRODUCTION

Weeds are unwanted plant species that negatively interfere with plants of human interest by reducing agricultural yield, increasing production costs and decreasing product quality, which makes weed control indispensable to farming productivity (de Lima et al. 2011, Duke 2012, Vats 2015).

The use of synthetic herbicides has been the main tool for weed control worldwide. Over the years, the lack of planning for herbicide employment and its indiscriminate utilization has made weed species increasingly resistant to them (Knezevic et al. 2016, Maxwell & Mortimer 1994). Herbicide-resistant weeds are currently one of the main problems facing farmers and their presence threatens the food security of the world's population. Worldwide, there are currently 262 known species (152 dicotyledonous and 110 monocotyledonous) of weeds that are resistant to herbicides. They have developed resistance to 23 of the 26 known mechanisms of action of herbicides, and they are resistance to 167 different herbicides. Herbicide-resistant species have been reported in 93 crops across 70 countries (Heap 2019).

Furthermore, due to the overall success of crops with engineered tolerance to herbicides such as glyphosate, efforts to find new herbicidals have been reduced dramatically (Perotti et al. 2020). This trend is reflected in the decline in annual herbicide patent applications

from approximately 250 in 1990 to a current average of 54 annual applications over the past 10 years (Peters & Strek 2018).

Due to new regulatory requirements and market demands, the discovery of a new herbicide is difficult and the finding of a new MoA is rare. The uncovering of a new allelochemical with herbicidal potential greatly increases the chances of introducing a new herbicidal mechanism of action because the overlap with ever existing mechanisms is considered low (Dayan & Duke 2014). Therefore, the study of new substances with potential herbicidal activity and new mechanisms of action such as allelopathic compounds may be used to overcome the problem of resistant weeds (Huang et al. 2010, Duke 2012, Travaini et al. 2016).

Plants may release specialized metabolites, known as allelochemicals, into the environment, which influence the regular metabolic processes of other plants, including breathing, cellular division, growth, development, productivity, and enzymatic activity (Carmo et al. 2007, Li et al. 2011, Dastan et al. 2014). Environmental and geographical conditions, such as hydrical stress, season, and year of collection, influence production and the release of these specialized compounds (Tavares et al. 2013, Botha et al. 2018, Dalmagro et al. 2018, Caser et al. 2019).

Eragrostis plana (Nees) is a perennial and exotic South African Poaceae that was accidentally introduced in Brazil in 1950. It is a species that is well known for its ability to compete with other plants, including species of economic interest (Ferreira et al. 2008). Therefore, the study of this plant, aimed at obtaining metabolites with allelopathic activity, which could be potential herbicides, is of great interest.

Previous studies of allelochemicals of *E. plana* have already led to the identification of several compounds, such as pimarane, cassane (Nishiya et al. 1991), norlabdane (Sebastião et al. 2010), labdane (Sebastião et al. 2012) and neocassane (Favaretto et al. 2019). However, species synthetize and release a variety of components which are influenced by environmental factors and determine modifications in the spectrum of produced substances. In addition, appropriated isolation techniques are crucial to the efficiency of isolation and identification of allelopathic compounds, which could contribute to advances in the discovery of new bioherbicides.

In this sense, the aim of this work was to evaluate the seasonal allelopathic potential of the crude extracts of roots from *E. plana* and to perform a chromatographic fingerprinting by high performance liquid chromatography - diode array detector (HPLC-DAD) to evaluate the influence of solvent polarity and seasonality on the extractions, which was aided by principal component analysis (PCA).

MATERIALS AND METHODS

Plant material and extraction procedures

Roots of *E. plana* were collected in November 2016 (spring), February 2017 (summer), June 2017 (autumn), and September 2017 (winter) in Pacheco Farm (coordinates of –26.527820 and–52.255808; average altitude of 850 m) in Abelardo Luz city, Santa Catarina State, Brazil. A voucher specimen (HPB1102) was deposited at the Herbarium of Universidade Tecnológica Federal do Paraná (UTFPR) by Dra G. F. Pereira. The roots were dried in an oven at 40°C with air circulation, brushed to remove excess soil, and crushed in a Wiley mill. The dried and crushed roots (200.89 g) were extracted with the following solvents of increasing polarity: petroleum ether (PE), ethyl acetate (EA), and methanol (M). After the extraction, the extracts were concentrated

in a rotary evaporator (Fisatom 801) at reduced pressure and a temperature of 40°C.

Phytotoxicity bioassays of crude extracts

Three-milliliters aqueous solutions of each extract, at concentrations ranging from 100 to 500 mg/L, were added to Petri dishes. Water was used as the control 1, and a solution containing water, extraction solvent and Tween 80 was used as control 2. All assays were performed in triplicate.

Osmotic potential test using the schardakow method

Methylene blue was added to the aqueous solutions of the crude extract. One drop (10 µL) of each extract solution was added to aqueous solutions of sucrose prepared at concentrations ranging from 0.00 to 0.50 mol/L, and the movement of the drop was observed by evaluating the possible osmotic interference of the crude extract solutions (Maestri et al. 1998).

Plant test

Ipomoea grandifolia seeds were used in the bioassays for the plant test. The seeds were acquired from a seed production company, and they were multiplied in the Departamento de Agronomia – Universidade Tecnológica Federal do Paraná (Pato Branco city – Paraná State), where they went through cleaning and dormancy breaking.

Germination bioassay with *Ipomoea grandifolia*

The bioassay was conducted in a BOD incubator under controlled conditions at 25°C (nighttime) and 30°C (daytime), with a photoperiod of 12 hours, and it was monitored daily for a period of 7 days (Souza Filho et al. 2010). Petri dishes (9 cm) lined with qualitative filter paper contained 10 seeds from the plant test and 3 mL of the aqueous solutions of the crude extract and controls 1 and 2. The seeds of the plant test were considered germinated when their root extensions were equal to or greater than 2 mm (Junttila 1973). On the last day of the bioassay (7^{th} day), the radicle and shoot length of the seedlings of the plant test were measured (Souza Filho et al. 2010). The germination mean time (GMT), germination percentage (GP), and germination speed index (GSI) were calculated (Maguire 1962, Nery et al. 2007). The bioassays were performed in triplicate.

Chromatographic analysis conditions and sample preparation

The crude extracts of the roots were analyzed by HPLC, which was carried out in a Waters chromatograph equipped with a 717 plus autosampler, two 600E high pressure pumps, a 2998 photodiode array detector (DAD), and a KinetexXB-C18 100 Å column (4.6 × 250 mm, 5 µm; Phenomenex, USA), with a mobile phase consisting of water (A) and methanol (B). The gradient elution for all extracts was 50–100% B for 50 min and 100% B for 10 min. The flow rate of the mobile phase was 0.8 mL/min, and the injection volume was 10 µL. Monitoring was performed at 254 nm. The data were collected using Empower 2 software. The samples were prepared by dissolving 5.2 mg of crude extract in 1.0 mL of spectroscopic grade methanol. Ultrapure water was obtained with Purelab Classic equipment, and the methanol was HPLC grade (SK Chemicals, Korea).

Statistical analysis

The variables calculated in the bioassays were submitted to the generalized linear model with 95% significance using IBM SPSS Statistics 2.0. The phytotoxicity bioassay variables and HPLC data were submitted to PCA, which was carried out using PLS_toolbox 3.0 (Eigenvector Research,

Inc.) in MATLAB 7.0.1 software (MathWorks®). The data were preprocessed (auto-scaling for the variables and mean centering for HPLC data) prior to PCA. HPLC data were pretreated (peak alignment) using Icoshift tool 1.1 in MATLAB 7.0.1 software (MathWorks®). All graphs were generated using Origin 8.5 software.

Phytotoxicity bioassay with *I. grandifolia*

E. plana roots collected during the four seasons of the year and subjected to extraction with different solvents resulted in the following extracts and yields: PE_{spring} (1.30 g), PE_{summer} (1.80 g), PE_{autumn} (1.60 g), PE_{winter} (1.90 g), EA_{spring} (2.50 g), EA_{summer} (3.10 g), EA_{autumn} (2.20 g), EA_{winter} (1.90 g), M_{spring} (4.20 g), M_{summer} (5.10 g), M_{autumn} (7.20 g), and M_{winter} (6.00 g). These crude extracts were used in phytotoxicity bioassays.

The dependent variables of the bioassays were the radicle length (RL), the shoot length (SL), the germination percentage (GP), the germination speed index (GSI) and the germination mean time (GMT). The factors considered in the experiment were the harvest seasons, the solvent type, and the extract concentration. Generalized linear models (GLMs) were used to analyze the data set in the experimental design. The germination variables (GP, GSI and GMT) were adjusted to a gamma distribution with the log link function. The growth variables (RL and SL) were adjusted to a normal distribution. In this work, only the main factors and interactions that showed significant statistical differences within a 95% confidence interval were analyzed (Table I).

Root extract effects on the growth of *I. grandifolia*

The GLM for the SL (Equation 1) and RL (Equation 2) presented an Akaike Information Criterion (AIC) measure fit equal to 781.84 and 721.52, respectively.

$$\Delta = e^{3.459+2.299*\boldsymbol{autumn}+2.000*\boldsymbol{spring}+1.067*\boldsymbol{Control}1-1.447*\boldsymbol{EA*Control}1-1.373*\boldsymbol{PE*C}500} \quad (1)$$

$$\Delta = e^{3.365-0.631*winter+1.921*autumn+1.738*spring-0.691*C100-0.636*C200-0.842*C300-0.786*C400-1.207*C500 \\ -1.430*autumn*PE-1.216*spring*PE} \quad (2)$$

The season of the year in which *E. plana* roots were collected and the extract concentration significantly affected (p<0.5%) the SL and RL of *I. grandifolia*. The lowest SL values were observed in the summer, which were significantly different from the autumn and spring seasons. The partial effect of each factor was measured by calculating the odds ratio for each coefficient of the selected model (Equations 1 and 2). Equation 1, generated by the model, predicted that the extracts from spring and autumn increases the odds of an increase in the SL by 6- and 9-fold, respectively, when compared to extracts from the summer.

Filep et al. (2016) observed that the allelopathic effects of *Helianthus tuberosus L.* were the strongest in the early summer and late autumn when allelochemicals accumulate in the rhizosphere. In another study, it was noted that root extracts of *Ludwigia hexapetala* obtained during autumn positively affected the SL of *Egeria densa* (Thiébaut et al. 2018).

The RL of *I. grandifolia* was lower with the winter season extracts, and it was statistically different from the RL with the extracts from the other seasons of the year. Equation 2, generated by the model, predicted that the extracts from the spring and autumn increases the odds of an increase in the RL by 5- and 6-fold, respectively, when compared to extracts from the summer, while extracts from the winter decrease the odds of an increase in the RL by 47% when compared to extracts from the summer.

Therefore, it may be observed that the application of root extracts collected in the summer and winter resulted in the lowest

Table I. Factors that presented significant statistical differences for the dependent growth variables and germination variables of the plant test, *Ipomoea grandifolia.*

Factors	Wald chi-square	*Degrees of freedom*	*p*-value
Radicle Length			
season	220.350	3	0.000
solvent	26.102	2	0.000
concentration	34.091	6	0.000
season*solvent	15.804	6	0.015
Shoot Lenght			
season	180.038	3	0.000
concentration	30.060	6	0.000
season*solvent	16.339	6	0.012
solvent*concentration	22.480	12	0.032
Germination Percentage			
season	28.368	3	0.000
solvent	7.017	2	0.030
season*solvent	14.633	6	0.023
Germination Speed Index			
season	226.875	3	0.000
Germination Mean Time			
season	389.783	3	0.000

values of the growth variables. Considering that both seasons usually present extreme weather conditions, these results indicate that abiotic environmental factors are capable of inducing changes in the production of different classes of specialized metabolites, which was also noted in the work by Sampaio et al. (2016)

The negative effect caused by *E. plana* on the RL and on the hypocotyl length of other seedlings was also reported in the study by Bittencourt et al. (2018) In another study, it was observed that the aqueous extracts of *E. plana* caused greater inhibition of the recipient species roots (Favaretto et al. 2017).

Franco et al. (2016) studied the seasonal variation in the allelochemical concentration in dry and rainy seasons and the allelopathic potential of *Copaifera langsdorff* leaves, confirming the influence of seasonal variation on the composition and phytotoxic effects. In

another study, anthraquinones were found in *Luehea candicans* samples, but only in the plant collected during the dry season, while saponins were detected only in the samples collected in the rainy season (Pinto & Kolb 2016).

The lowest values of the RL and SL were observed with *E. plana* root extract at a concentration of 500 mg/L, which were statistically different from controls 1 and 2 for both variables. Equation 1 predicted that control 1 increase the odds of an increase in the SL by 2-fold when compared to control 2. It was also noted that the SL of control 1 was statistically different than the SL of concentrations of 300 mg/L and 500 mg/L. According to Equation 2, extracts with concentrations of 100 mg/L, 200 mg/L, 300 mg/L, and 400 mg/L decrease the odds of an increase in the RL by approximately 50%, while extracts with a concentration of 500 mg/L decrease the odds of an increase in the RL by 70%,when compared to control 2.

The petroleum ether extract of *E. plana* roots presented the highest inhibition of the RL of *I. grandifolia*, which was statistically different from the RL resulting from the ethyl acetate and methanol extracts.

Regarding the interaction between the solvent and the concentration it was observed that the petroleum ether extract, with a concentration of 500 mg/L, decreased the odds of an increase in the SL by approximately 75%, compared to control 2 (Equation 1). The SL observed at a concentration of 500 mg/L also differed from the values obtained with control 1, 100 and 200 mg/L for the petroleum ether extract. Concentrations of methanol extract of 300 mg/L and 400 mg/L resulted in lower SL values compared to those obtained with control 1.

Equation 1 predicted that for the ethyl acetate extract, the control 1 would decrease the odds of an increase in the SL by 76% when compared to control 2. It is believed that the addition of Tween 80 and ethyl acetate in control 2 may exert a stimulatory effect on the SL of *I. grandifolia* plants.

Root extracts effects on the germination of *I. grandifolia*

The GLM for the GP (Equation 3), GSI (Equation 4), and GMT (Equation 5) resulted in an AIC adjustment measure equal to 2008.92, 1348.37, and 169.97, respectively.

$$\Delta = e^{4.255+0.207*autumn+0.229*spring+0.127*EA+0.139*PE-0.178*autumn*PE-0.166*spring*PE} \quad (3)$$

$$\Delta = e^{2.864-0.307*winter+0.212*autumn+0.111*spring} \quad (4)$$

$$\Delta = e^{0.381+0.473*winter-0.295*autumn} \quad (5)$$

The season in which *E. plana* roots were collected significantly affected the germination variables of *I. grandifolia*. The lowest GP was obtained with the extract from the summer, which was statistically different from the GP obtained with the extracts from the autumn and spring.

The partial effect of each factor was measured by calculating the odds ratio for each coefficient of the selected model (Equations 3, 4, and 5). Equation 3, generated by the model, predicted that the extracts from the spring and autumn increases the odds of an increase in the GP by approximately 1.24-fold, when compared to extracts from the summer.

The lowest GSI values for *I. grandifolia* were observed in the extract from the winter. All seasons had GSIs that were statistically different from each other. Equation 4 shows that extracts from the winter decrease the odds of an increase in the GSI by 26%, while extracts from the autumn and spring increases the odds of an increase in the GSI by 1.24 and 1.12-fold, respectively, when compared to extracts from the summer.

Finally, it was observed that the highest values of the GMT were obtained with *E. plana* roots collected in winter, which were different from other seasons. Equation 5 shows extracts from the winter increase the odds of an increase in the GMT by 1.6-fold, while extracts from the autumn decrease the odds of an increase in the GMT by 26%, when compared to extracts from the summer.

Pinto & Kolb (2016) observed that the *L. candicans* extracts obtained during the rainy season decreased the germination capacity and the germination speed of the plant test, confirming that the phytotoxic effect, as well as the amount and composition of the secondary metabolites, differ seasonally.

The GP also showed significant statistical differences in the solvent used in the preparation of the extracts. In general, the methanol extract presented the lowest GP values. Using Equation 3, it can be verified that the extracts in ethyl acetate and petroleum ether increased the odds of an increase in the GP of *I. grandifolia* by, approximately 1.14-fold when compared to the methanol extract.

However, the interactions between the season and the solvent showed that petroleum ether extracts in the autumn and the spring decreased the odds of an increase in the GP by 15% compared to the methanol extracts for both seasons.

For *E. plana* roots collected in the winter, the GP values obtained with the methanol extract were statistically lower than those obtained with the petroleum ether extract. The lowest values for the GP were observed when using the methanol extract from the summer, which in turn differed from the extracts in ethyl acetate and petroleum ether (at a significance level of 5%). Thus, it is evident that the influence of the solvent used in the extraction depends on the season in which *E. plana* roots are collected.

In another study, the aqueous extracts from the aerial parts of *E. plana* affected the GP and GSI of corn, white oats, annual ryegrass, red clover, and birdsfoot trefoil seeds (Fiorenza et al. 2016). Similarly, it was observed that the higher the cover level during the decomposition of *E. plana* the smaller the germination and the GSI of the *Paspalum notatum* (Alain ex Flüggé) seeds (Ferreira et al. 2008), which confirms the allelopathic potential of *E. plana*.

Plant development is regulated by six main types of hormones: auxins, gibberellins, cytokinins, ethylene, abscisic acid, and brassinosteroids, which can be negatively affected by the presence of allelochemicals. Changes in these growth regulators may influence seed germination and seedling growth (Taiz & Zeiger 2009, Cheng & Cheng 2015).

Allelochemicals have different effects on the synthesis, functions, and activities of various enzymes. Chlorogenic acid, caffeic acid, and catechol, for example, may inhibit the enzyme λ-phosphorylase, which is involved in seed germination (Cheng & Cheng 2015).

In relation to osmotic effects, according to the Schardakow method, the crude extracts aqueous solutions do not harm seed germination (Souza Filho et al. 2010).

Analysis of the variables calculated in the bioassays by PCA

PCA is a very useful chemometric tool for experiments with various information because it reduces the dimensionality of the data without losing relevant information, promoting, at the same time, the analysis of the results in a more efficient and objective form (Jesus et al. 2018). The chromatographic data were organized and then submitted to PCA, providing a reduction in dimensionality for two new coordinates, generating the score graph PC1 *vs.* PC2 (Figure 1). PC1 represents 72.19% of the data variance,

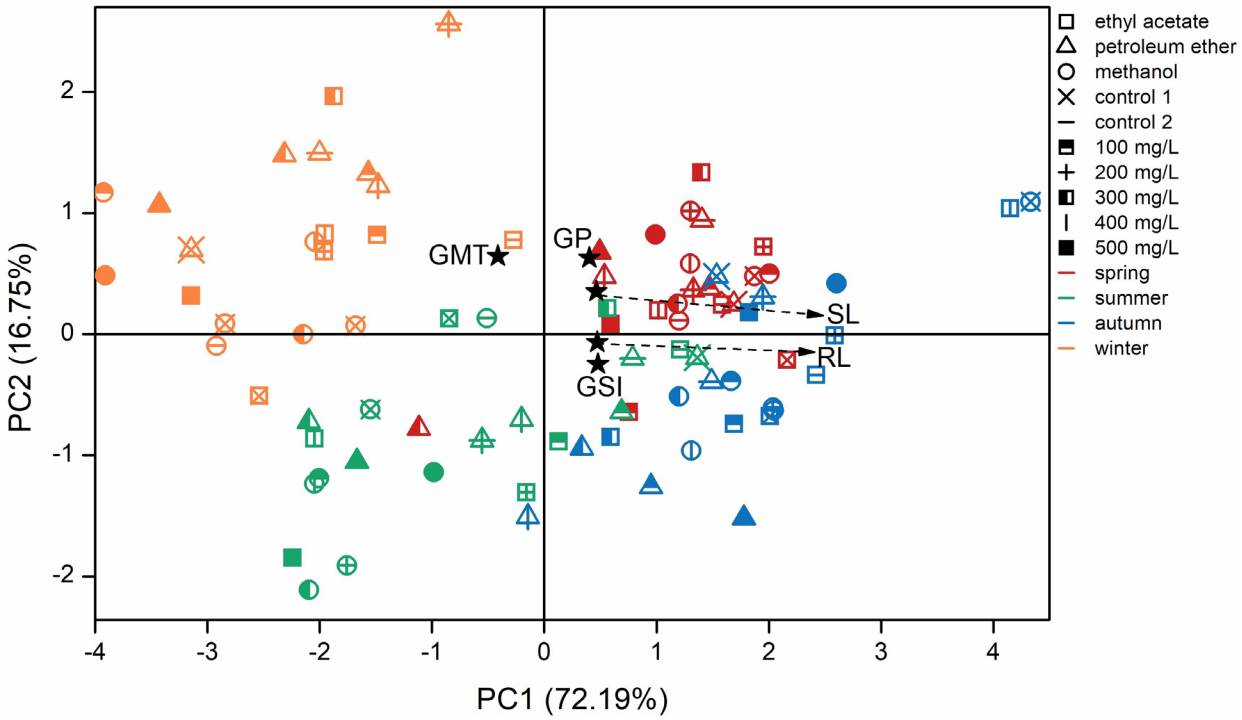

Figure 1. Biplot of Scores and Loadings obtained by principal component analysis of the phytotoxicity bioassay date of the crude extract of the root of *E. plana*.

while PC2 represents 16.75% of the data variance (together, they explain 88.94% of the total data). It was possible with PC1 to observe separations between the seasons since the samples from the spring and autumn were on the positive side of PC1, while all samples from the winter and most summer samples were on the negative side of PC1.

PC2 also showed that there was a difference between the seasons, where spring and winter samples were on the positive side of PC2, and autumn and summer samples were on the negative side of PC2.

The loadings, according to PC1, show that the highest values for the variables GP, GSI, SL, and RL were observed in spring and autumn extracts. The lowest values for these variables were obtained with winter and summer extracts. This statement is consistent with the results observed with the GLM, where the SL and GP were lower with the summer extracts and the RL and GSI were lower with the winter extracts.

This statement was also confirmed by the loading analysis, according to PC2, since it was observed that the lowest values for the GP and SL were obtained with summer extracts, while the GSI and RL were lower with the winter extracts.

PC1 shows that the winter extracts caused the highest values of the GMT, and autumn and spring extracts resulted in the lowest values for this variable. The results, once more, corroborate those observed with the GLM; thus, both the GLM and PCA analysis showed that summer and winter root extracts have higher phytotoxic potential, although these extracts did not affect the same variables of the growth and germination in the plant test.

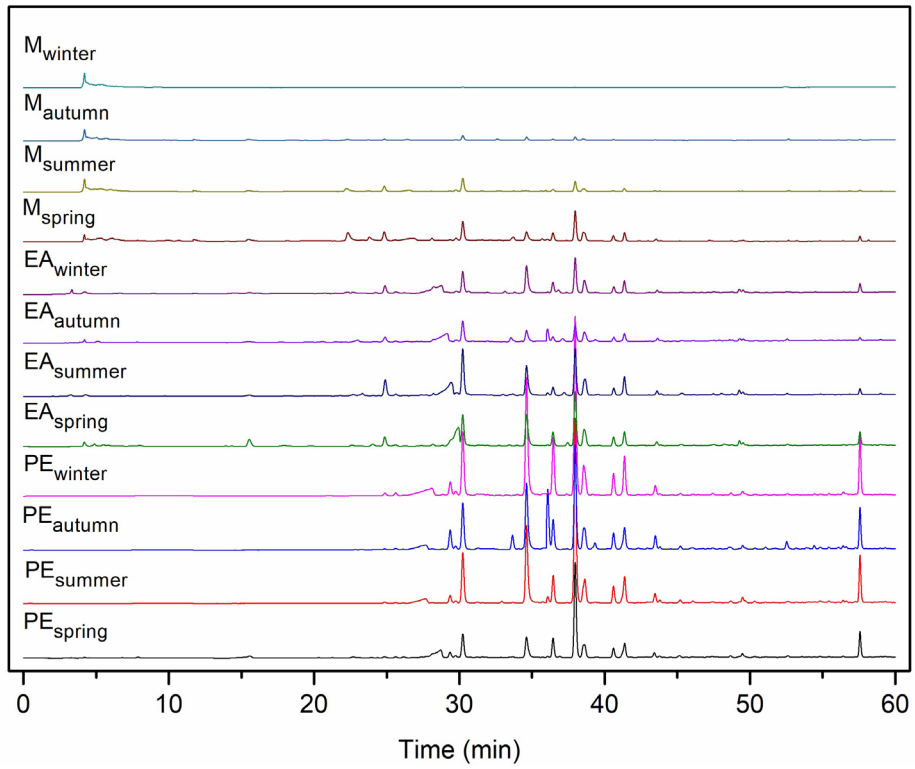

Figure 2. Fingerprint chromatograms of *E. plana* root extracts collected during different seasons and obtained with different solvents [petroleum ether (PE), ethyl acetate (EA), and methanol (M)] using detection at 254 nm.

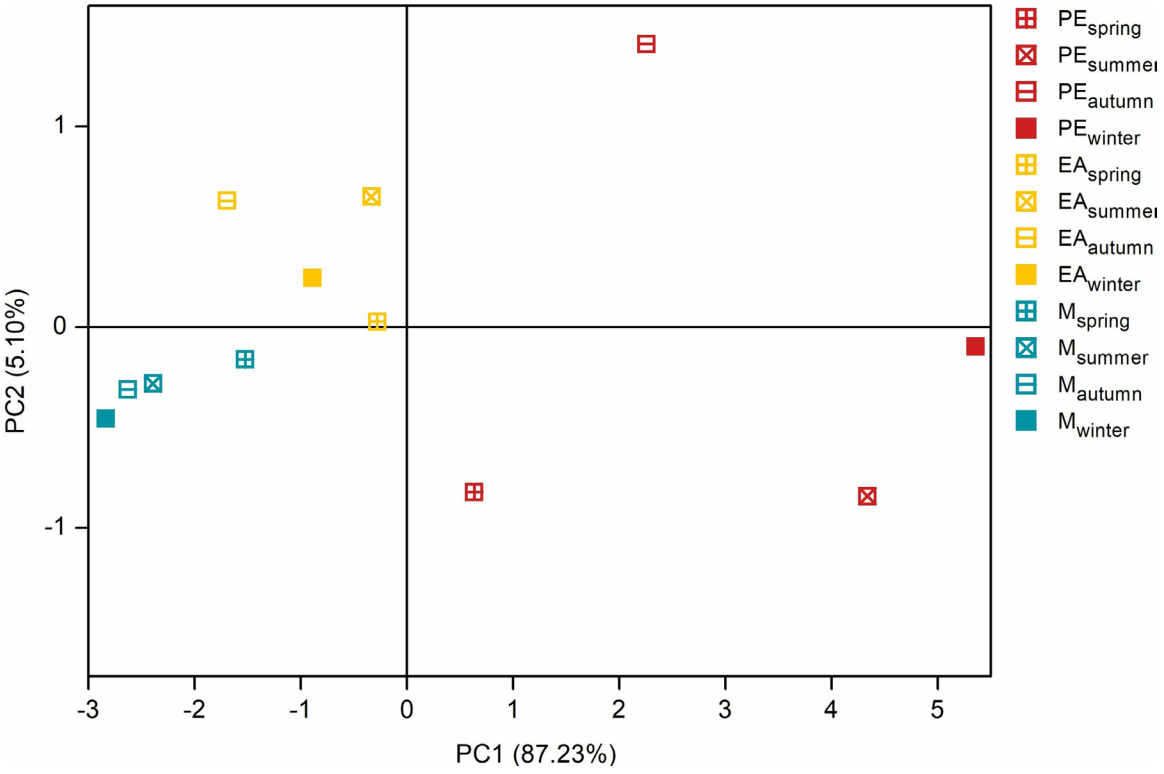

Figure 3. Scores plot of the *E. plana* root extracts [PE - petroleum ether, EA - ethyl acetate, and M - methanol].

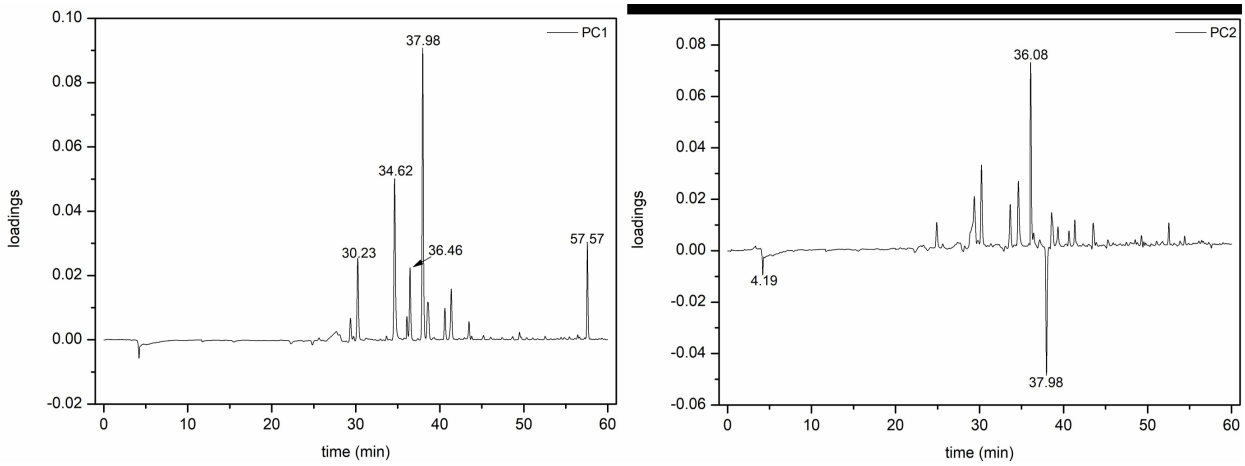

Figure 4. Loadings plot of *E. plana* root extracts. PC1 (left) and PC2 (right).

Chromatographic analysis of the crude extracts and PCA

The crude extracts of different solvents from the roots collected in the four seasons were analyzed by HPLC-DAD (Figure 2).

It is difficult to confirm whether the chemical profiles of the extracts differ as a function of the extraction solvents and/or root collection season, and, in these cases, PCA is very important.

The chromatographic data were organized into a matrix containing 12 lines (samples) and 36000 columns (variables: retention time, 60 min). The first two PCs explain 92.33% of the total data variance (PC1, 87.23%; PC2, 5.10%); therefore, they were chosen to demonstrate correlations between the samples (Figure 3).

It was possible with PC1 to observe a separation between the extracts obtained with the different solvents. The petroleum ether extracts are in the positive quadrant of PC1, while the ethyl acetate and methanol extracts are in the negative quadrant of PC1, suggesting diversity between these extracts in terms of the secondary metabolites. Thus, it can be suggested that the phytotoxicity bioassay results are related to the difference in the fingerprint of the crude extracts since the petroleum ether extracts that presented higher phytotoxicity are chemically different from the ethyl acetate and methanol extracts.

The analysis of PC2 shows that there is a difference between the solvents used in the extraction since it is noted that the ethyl acetate extracts are different from the methanol extracts in this PC. According to PC2, there is a separation between the seasons when using petroleum ether extracts since autumn is in the positive quadrant of PC2 and spring, summer, and winter are in the negative quadrant of this PC.

Even in PC1 it is observed that the petroleum ether extracts are far from each other, suggesting a difference in the chemical composition of the extracts as a function of the season during which the root was collected. In the phytotoxicity bioassay, it was observed that the best results were obtained with petroleum ether extracts from roots collected in the summer and winter, depending on the variable studied. For the other solvents, there was not much difference in seasonality.

The analysis of the loading plot showed which variables had the greatest influence on the projection of the extracts in each PC. This graph presents variables with positive and negative values. Petroleum ether extracts on the positive side of PC1 are mainly characterized by

chromatographic bands with retention times of 30.23, 34.62, 37.98, 36.46, and 57.57 min (Figure 4).

The compound in 37.98 min on the negative side of PC2 is responsible for the differentiation among spring, summer, and winter petroleum ether extracts and methanol extracts from the ethyl acetate extracts and the autumn petroleum ether extract. Similarly the compound in 36.08 min on the positive side of PC2 also contributed for this differentiation (Figure 4).

In this study, both the GLM and PCA analysis showed that summer and winter root extracts have higher phytotoxic potential since they are able to increase the GMT and decrease the GP, GSI, RL, and SL of the plant test, confirming that abiotic environmental factors are capable of inducing changes in the production of different classes of specialized metabolites. It was also observed that the influence of the solvent used in the extraction depends on the season of the year in which *E. plana* roots are collected; however, in general, the extracts obtained with petroleum ether had higher phytotoxic activity.

In this sense, our promising results of the crude extracts of *E. plana* bioassays encourage further studies of identification of compounds with phytotoxic properties by bioassay-guided fractionation.

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