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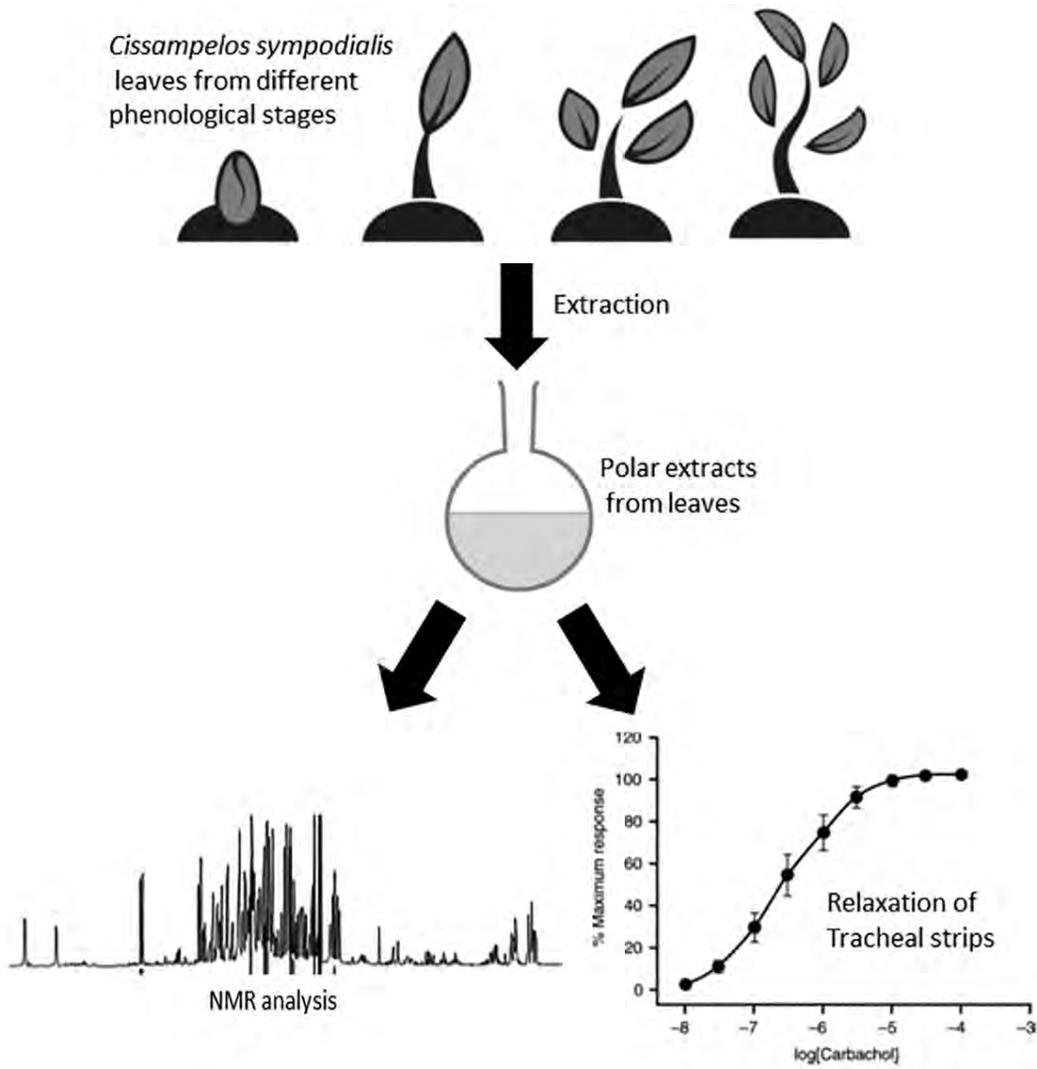
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**METABOLOMIC FINGERPRINTING OF *Cissampelos sympodialis* Eichler LEAF EXTRACT AND CORRELATION WITH ITS SPASMOLYTIC ACTIVITY**

**SHORT TITLE: “Metabolomic Fingerprinting and bioactivity of *C. sympodialis*”**

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**LIST OF ABBREVIATIONS:**

PCA = Principal Component Analysis.

PLS = Partial Least Square Regression.

TSP= 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt.

HPLC = High performance liquid chromatography.

CCh = Carbachol (carbamylcholine).

NMR = Nuclear Magnetic Resonance Spectroscopy

**ABSTRACT**

**Ethnopharmacological Relevance:** *Cissampelos sympodialis* Eichler (Menispermaceae) is popularly used in northeastern Brazil for the treatment of respiratory diseases such as bronchitis and asthma. Despite many pre-clinical pharmacological studies, the compounds mediating the anti-asthma activity of polar extracts of *Cissampelos sympodialis* leaves have not been definitively identified.

**Aim of the study:** The aim of the study was to investigate the correlation between the bioactivity of polar extracts prepared from the leaves of *C. sympodialis* and the chemical composition using a <sup>1</sup>H-NMR-based metabolomics approach.

**Material and Methods:** The metabolic profile of the leaf polar extract during different phenological stages of the plant was investigated using <sup>1</sup>H NMR spectroscopy while simultaneously screening for spasmolytic activity using guinea-pig tracheal preparations. The content of the alkaloids previously implicated in the bioactivity of *Cissampelos sympodialis* was determined by HPLC.

**Results:** PCA analysis of the <sup>1</sup>H NMR data discriminated the extracts from different plant phenological stages. The contents of the major alkaloids decrease (from  $2 \pm 0.32$   $\mu\text{g/mL}$  for warifteine and  $1 \pm 0.14$   $\mu\text{g/mL}$  for methylwarifteine) to undetectable levels from 90 (CsL<sub>90</sub> extract) and 120 (CsL<sub>120</sub>) days onwards for warifteine and methylwarifteine, respectively. All six extracts relaxed the trachea pre-contracted with carbachol, but the CsF<sub>210</sub> extract was more potent ( $\text{EC}_{50} = 74.6 \pm 7.9$   $\mu\text{g/mL}$ ) compared to both CsL<sub>90</sub> extracts and CsL<sub>180</sub> in the presence of functional epithelium. PLS regression analysis of <sup>1</sup>H-NMR spectral data demonstrated that the spasmolytic activity was better correlated with signals for flavonol derivatives.

**Conclusions:** Our data challenge the idea that warifteine and methylwarifteine mediate the spasmolytic activity of the polar extract of *C. sympodialis* leaves.

**Keywords:** *Metabolomics; Cissampelos sympodialis; Asthma; Spasmolytic activity; Bisbenzylisoquinoline alkaloids.*

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## 1. Introduction

*Cissampelos sympodialis* Eichler (Menispermaceae) occurs mainly in the northeastern states of Brazil and is used in traditional medicine as a tea of its leaves (either from the wild or as cultivated plant) for the treatment of inflammatory conditions such as bronchitis, asthma and arthritis (Agra *et al.*, 2007). The genus *Cissampelos* is historically known for its use in the preparation of Curare, the arrow poison and its extracts have exhibited diverse pharmacological actions (Semwal *et al.*, 2014). The aqueous fraction of the hydroalcoholic leaf extract of *C. sympodialis* has shown a number of interesting pre-clinical pharmacological actions related to the pathophysiological process of asthma using both *in vitro* and *in vivo* approaches. Its actions involve multiple targets including a direct bronchodilator effect equipotent to aminophylline (Thomas *et al.*, 1995), the decrease of leucocyte infiltration in lung tissue by eotaxin inhibition (Bezerra-Santos *et al.*, 2006), the decrease in the production of antigen-specific IgE (Bezerra-Santos *et al.*, 2004), decrease of mucus production (Bezerra-Santos *et al.*, 2012) and an increase in the production of anti-inflammatory cytokines such as IL-4 and IL-10 (Piuvezam *et al.*, 1999).

Warifteine, a bisbenzylisoquinoline alkaloid present in the root and leaves of the plant has been implicated as the main bioactive substance of this species and isolated warifteine was indeed shown to possess some of the effects reported for the extracts, such as inhibition of eosinophil infiltration in ovalbumin-sensitized mice (Bezerra-Santos *et al.*, 2006), spasmolytic activity in guinea pig trachea (Cortes *et al.*, 1995) and decrease of ovalbumin-specific IgE titers (Costa *et al.*, 2008).

Our group has previously demonstrated that the concentrations of warifteine, methylwarifteine and milonine (a dihydromorphinandienone-type alkaloid) in the hydroalcoholic extract of the leaves of *C. sympodialis* vary according to the

phenological stage of the plant, reaching a minimum during fructification (Marinho *et al.*, 2012). However, given its low solubility in polar solvents, it is unlikely that appreciable concentrations of warifteine are present in the bioactive polar fractions, such as the aqueous fraction of the hydroalcoholic extract of leaves used in all of the pharmacological assays to date. Thus, the true nature of the bioactive compounds responsible for mediating the effects of the aqueous fraction remains inconclusive.

It is well known that metabolite levels in a plant species are affected by both abiotic (environmental) and biotic factors, such as phenology (Pavarini *et al.*, 2012). Metabolomic approaches are well suited for the qualitative and quantitative study of variations in the profile of primary and secondary metabolites in response to these factors, since multivariate data analysis can detect minute variations in the levels of key metabolites.

Untargeted metabolomics using NMR-based profiling has the advantage of being a rapid and efficient technique for studying major components in plant extracts without the need for time-consuming purification and isolation of individual components and has found different uses in the study of medicinal plants such as quality control (Heyman & Meyer, 2012), detecting chemical variation on extracts from different origins (Wang *et al.*, 2004), to determine authenticity of plant material (Nguyen *et al.*, 2016) and to search for extracts with promising pharmacological activities (Graziani *et al.*, 2018).

The aim of our study was to investigate the relationship between chemical composition of polar extracts prepared from leaves of cultivated *C. sympodialis* collected at different phenological stages and the spasmolytic activity of *C. sympodialis* using guinea-pig tracheal strips. Thus, we used HPLC, 1D and 2D  $^1\text{H}$  NMR spectroscopy and multivariate data analysis to investigate qualitative and quantitative

variations in the chemical composition of the extracts while simultaneously screening the extracts for their potency in relaxing pre-contracted guinea pig tracheal strips in order to determine the class of metabolites that better correlated with the biological activity of the extract. Spasmolytic activity using guinea pig tracheal strips was used as a simple, convenient and quantitative *in vitro* proxy assay for the bronchodilator activity of the extracts and can thus be considered related to the anti-asthma effect of the plant.

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## 2. Material and Methods

### 2.1 Materials

Deuterated and HPLC grade solvents were purchased from Tedia (Tedia Brazil). The internal standard used in NMR spectroscopy experiments, 2,2,3,3-d<sub>4</sub>-3-(trimethylsilyl)propionic acid sodium salt (TSP) was acquired from Sigma-Aldrich (Sigma-Aldrich Brazil). Salts used for preparing the buffers used in the pharmacology experiments were purchased from Vetec (Vetec, Brasil) and Sigma-Aldrich (Sigma-Aldrich Brazil). Cremophor<sup>®</sup> EL, used for dissolving the extracts for the pharmacological assays was purchased from Sigma-Aldrich (Sigma-Aldrich Brazil). Warifteine and methylwarifteine were previously isolated from the rhizomes of *C. sympodialis* and its identity and purity (>98%) assessed by HPLC, <sup>1</sup>H and <sup>13</sup>C NMR and High Resolution Mass Spectrometry+ as described elsewhere (Marinho, 2012).

### 2.1 Plant material

Leaves of *C. sympodialis* Eichl were collected from cultivated specimens from the Medicinal Herb Garden of the Biotechnology Center (CBiotec), Federal University of Paraíba (UFPB) at João Pessoa, Paraíba Brazil (7.141632S, 34.846290W). A voucher specimen (AGRA 1476) was deposited at the Lauro Pires Xavier Herbarium, UFPB. Six different pooled samples of leaves (consisting of 15 leaves from 8 different plant specimens, amounting to 60-80g of fresh plant material) were collected monthly from March to August 2012, with 30 days' interval from each other at the same time of day (10 am). Whole leaves were manually collected. The first harvesting was initiated 60 days after seeding and the last after the first fruits appeared (210 days after seeding).

## 2.2 Extract preparation

Collected leaves were immediately crushed using liquid nitrogen and then lyophilized and kept in a freezer (-80 °C). A sample of the lyophilized vegetable material (100 mg) was vortexed for 1 minute in a mixture of 750 µL of methanol-d4 and 750 µL of 90 mM phosphate buffer (pH 6,0) containing 0,1% (w/w) of 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. The mixture was then sonicated for 20 minutes and centrifuged at 5000 rpm for 30 minutes. The supernatant was filtered through a nylon syringe filter (0,45 µM) and 600 µL transferred to an NMR tube. The same extraction procedure was adopted for extracts used in the pharmacological experiments except that TSP was not added and HPLC-grade solvents were used instead of deuterated ones. For the pharmacological tests the final extract was further evaporated using a rotary evaporator (40-45 °C) to remove the methanol and the residue was lyophilized. The lyophilized residue was dissolved in Cremophor® (3%, v/v) and diluted in distilled water to a final concentration of 10,0 mg/mL. Five different samples from each collection time point were separately processed and the extracts were coded according to the period of harvesting (number of days after seeding) as: CsL<sub>60</sub>, CsL<sub>90</sub>, CsL<sub>120</sub>, CsL<sub>150</sub>, CsL<sub>180</sub> e CsL<sub>210</sub>.

## 2.3 Qualitative and quantitative NMR spectroscopy analysis

<sup>1</sup>H NMR spectra were obtained using a Bruker Avance III NMR spectrometer with an ASCEND 500 MHz magnet. A total of 256 scans were obtained with pre-saturation of the water signal. A spectral width of 20 ppm and a relaxation delay of 5 s were used; 64K datapoints were recorded. For processing, zerofilling of a factor 4 was applied and the FID was multiplied with an exponential function with a lb of 0.2 Hz. To aid in the

assignment of signals, both homonuclear and heteronuclear 2D experiments such as  $^1\text{H}$ - $^1\text{H}$  correlation spectroscopy (COSY) and heteronuclear multiple bond correlation spectroscopy (HMBC) were used. For quantitative determination of compounds using NMR data, the peak area of each identified signal was measured against the peak area of the 3-(trimethylsilyl)-propionic acid signal used as internal standard, as described elsewhere (Kim *et al.*, 2010).

#### 2.4 Spectroscopic data analysis

Phase and baseline correction of  $^1\text{H}$  NMR spectra was done using Mestrenova (Mestrenova, version 6.1.0-6224). The TSP signal was used for chemical shift referencing in all spectra. NMR spectral data was extracted using the binning function of Mestrenova and signal intensity was reduced to integrated regions of same width (0.04 ppm) from  $\delta$  -0.06 to 8.7 ppm. The spectra were reduced to ASCII files generating a data matrix of 218 variables for each spectrum.

#### 2.5 Quantification of warifteine and methylwarifteine by HPLC

The content of the major bisbenzylisoquinoline alkaloids warifteine and methylwarifteine was determined in the same extracts used for measuring the spasmolytic activity. The HPLC determination was done in triplicate using a previously validated method (Marinho *et al.*, 2012). The HPLC system consisted of a Shimadzu Class VP chromatograph composed of a low pressure gradient elution system and a UV detector set at 278nm. Separation was achieved using a C-18 column (Phenomenex Luna<sup>®</sup>, 250 x 4,6 mm; 5 $\mu\text{m}$ ) and a mobile phase consisting of 0.05% aqueous triethylamine (A) and methanol (B) delivered at 1 ml/min. A gradient elution was used

for separation: 0-5 min, 60%B, 5-15 min, 72%B, 15-25 min, 80%B, 25-30 min 60%B. A calibration curve for warifteine and methylwarifteine (in the range 2 to 30  $\mu\text{g/mL}$ ) was used for quantification.

## 2.6 Animals

Adult guinea pigs of both sexes (*Cavia porcellus*), weighting between 300-500 g were obtained from the Prof. Thomas George Animal Housing Unit from the Federal University of Paraiba (UFPB, João Pessoa, Paraíba, Brazil). Animals were given free access to water and food, kept at a constant temperature of  $21 \pm 1$  °C, and submitted to a 12h:12h light-dark cycle. The animal handling and experimental procedures were approved by the University's animal ethics committee (CEUA, CBIotec/UFPB) under the protocol 0705/12.

## 2.7 Determination of spasmolytic activity of extracts using guinea-pig tracheal rings

Animals were euthanized by an overdose of sodium pentobarbital ( $30 \text{ mg kg}^{-1}$ ) and exsanguinated. Trachea was removed and cleaned of all connective and adipose tissue and then divided into segments containing 3-4 cartilage rings. Segments were suspended in isolated organ baths containing Krebs buffer adjusted to pH 7.4 (NaCl 118.0 mM, KCl 4.55 mM,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  5.70 mM,  $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  1.10 mM,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  2.52 mM,  $\text{NaHCO}_3$  25.0 mM and glucose 11.0 mM). The solution was kept at 37 °C and gassed with a carbogenic mixture (95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ). Tracheal strips were stabilized for 1 h with buffer replaced every 15 minutes. Isometric contractions were recorded with a force transducer coupled to a digital acquisition system (ANCAD, AVS Projetos, São Paulo, SP, Brasil). After the stabilization period a first contraction

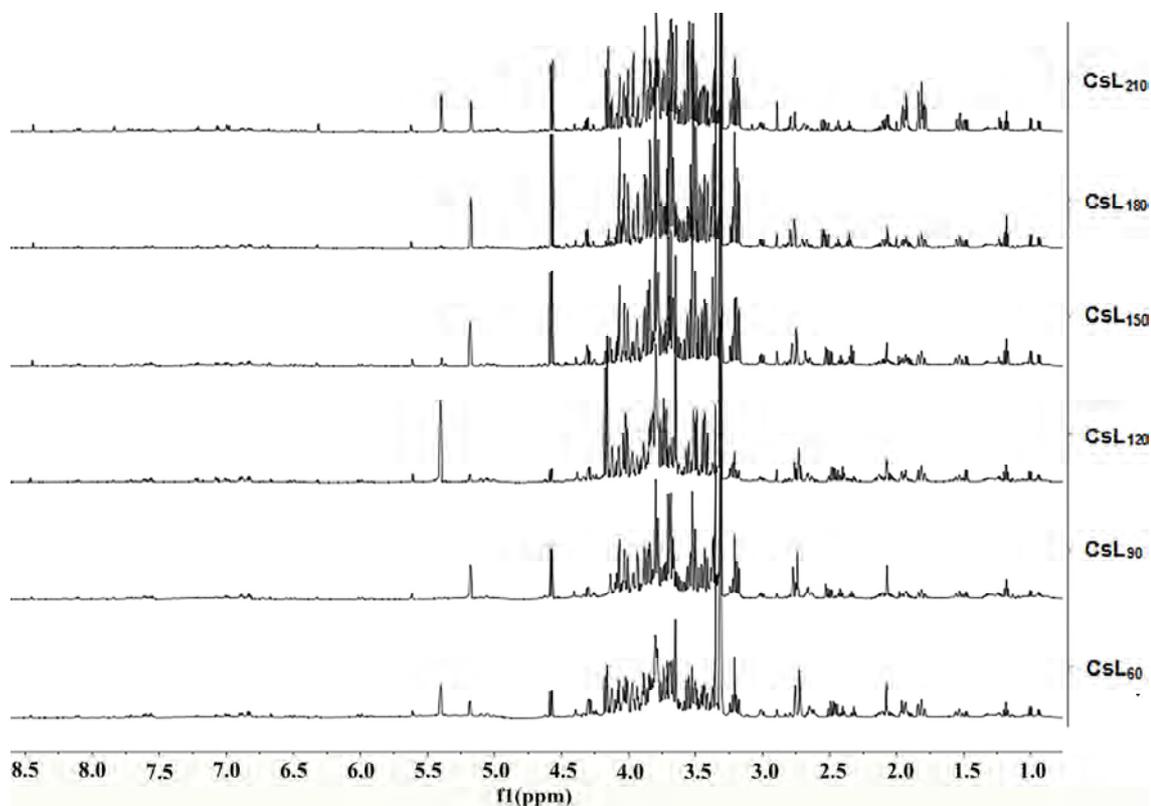
was induced with the addition of  $10^{-6}$  M carbachol (CCh) to the bath. Epithelium-free tracheal strips were prepared by rubbing the intimal surface with a cotton swab moistened in Krebs buffer. The presence of functional epithelium was verified by adding  $10^{-4}$  M arachidonic acid to the bath during the tonic phase of CCh-induced contraction. Tracheal strips with a relaxation degree of 50% or higher (calculated in relation to the initial contraction force) were considered to have functional epithelium, while those with relaxation of 10% or less were considered devoid of epithelium (Tschirhart *et al.*, 1987). After confirmation of epithelium status the preparations were washed every 15 minutes with Krebs buffer for 30 minutes. Then, a new contraction was induced with  $10^{-6}$  M CCh and cumulative concentrations of the extracts (CsL<sub>60</sub>, CsL<sub>90</sub>, CsL<sub>120</sub>, CsL<sub>150</sub>, CsL<sub>180</sub> or CsL<sub>210</sub>) were added to the bath (0.1-1000  $\mu$ g/mL). The relaxation induced by the extracts was expressed as the inverse percentage of the initial CCh contraction.

## 2.8 Statistical data analysis

Results were expressed as mean  $\pm$  standard error of mean and analyzed using Student's *t*-test for 2 group comparison or one-way ANOVA followed by Bonferroni post-test for multiple groups. Differences were considered significant when  $p < 0.05$ . The concentration of extract that caused a relaxation of 50% ( $EC_{50}$ ) was calculated using non-linear regression analysis of concentration-effect curves (Neubig *et al.*, 2003). Data were analyzed using GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego CA, U.S.A.). Principal component analysis (PCA) and partial least squares regression of  $^1H$  NMR data was done using the software Unscrambler version 9.7 (CAMO software, Woodbridge, NJ, USA) and data were area normalized. PLS was performed using values of  $pEC_{50}$  ( $-\log EC_{50}$  (g/l)) generated with presence of epithelium (E+).

### 3. Results

The superimposed  $^1\text{H}$  NMR spectra of the MeOH: water leaf extracts of *Cissampelos sympodialis* prepared from plant material collected at different phenological stages are shown in Figure 1.

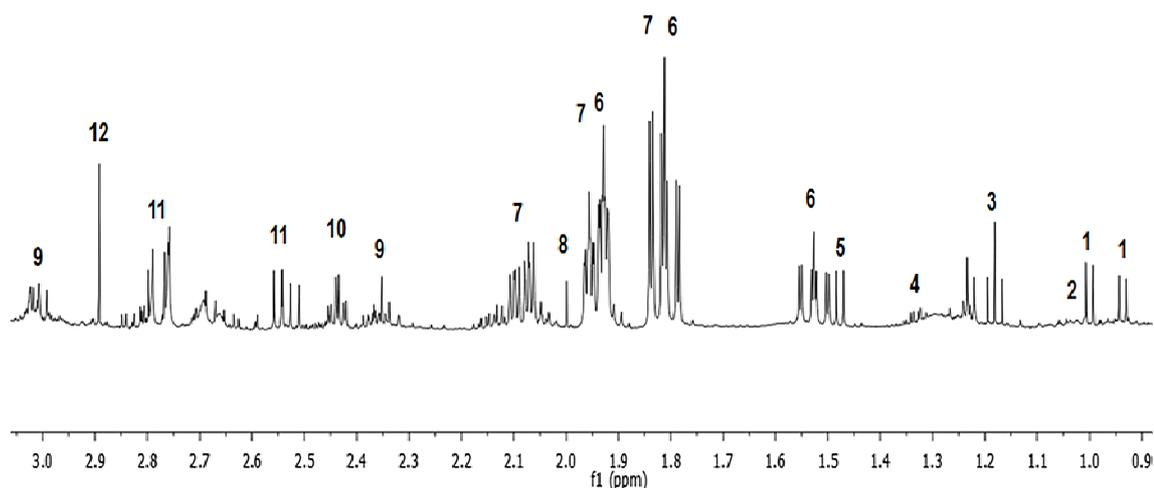


**Figure 1.** Representative  $^1\text{H}$  NMR spectra of polar extracts prepared from leaves of *C. sympodialis* collected at different phenological stages.

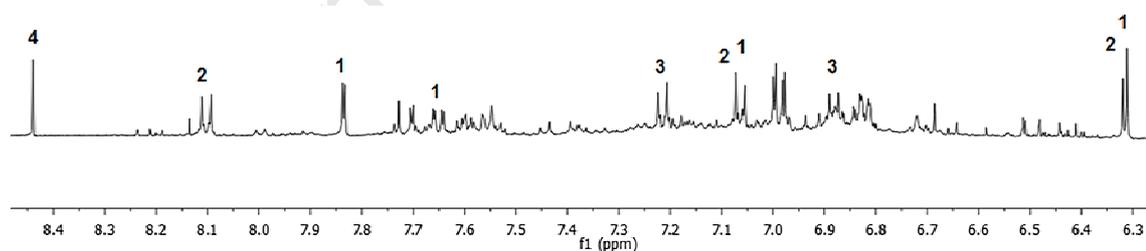
A few diagnostic signals typical of common plant metabolites can be identified by visual inspection and are detailed in Table 1 and in the expansions of the  $^1\text{H}$  NMR spectra in the aliphatic (Figure 2) and aromatic regions (Figure 3).

**Table 1** –  $^1\text{H}$  chemical shifts and coupling constants used for identification of the main metabolites detected in the leaf polar extracts of *C. sympodialis*.

Metabolite	Chemical shift (ppm) and coupling constants (Hz)
alanine	1.48 (d)
valine	1.01 (d); 1.06 (d)
leucine	0.94 (d, $J = 5$ Hz), 1.0 (d, $J = 5$ Hz)
threonine	1.34 (d)
glutamine	2.09 (m); 2.42 (m)
tyrosine	6.88 (d, $J = 8.5$ Hz); 7.22 (d, $J = 8.5$ Hz)
GABA	1.93 (m); 2.35 (dd); 3.01(m)
dimethylamine	2.89 (s)
quinic acid derivative 1	1.53 (m); 1.82 (dd, $J = 3.0; 11.5$ ); 1.94 (dddd)
quinic acid derivative 2	1.82 (dd, $J = 3.0; 11.5$ ); 1.94 (dddd); 2.10 (m)
$\alpha$ -glucose	5.18 (d)
$\beta$ -glucose	4.57 (d)
fructose	4.12 (d)
saccharose	4.16 (d); 5.42 (d)
acetic acid	1.98 (s)
formic acid	8.45 (s)
malic acid	2.55 (dd); 2.75 (dd); 4.31 (dd)
ethanol	1.18 (t)
kaempferol	6.31 (sl); 6,32 (sl); 7,06 (d, $J = 8.5$ Hz); 8,10 (d, $J = 8.5$ Hz)
quercetin	6.31 (sl); 6,32 (sl); 7,06 (d, $J = 8.5$ Hz); 7.66 (dd, $J = 8.5; 2.5$ Hz); 7.84 (d, $J = 2.5$ Hz)

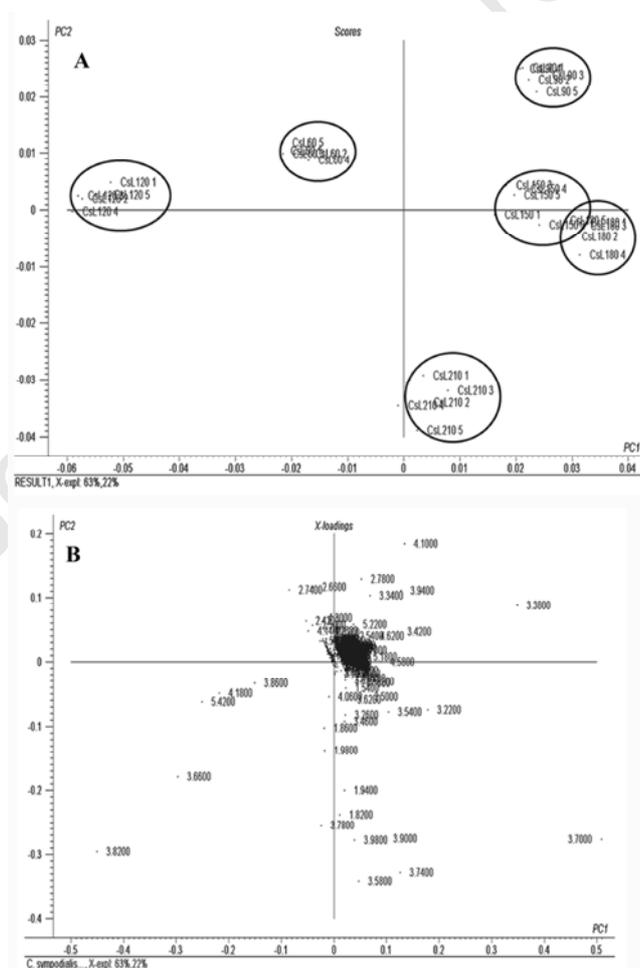


**Figure 2.** Expansion of the  $^1\text{H}$  NMR spectrum of *C. sympodialis* showing diagnostic signals in aliphatic proton region: 1. leucine (0.94 d; 1.0 d), 2. valine (1.05 d), 3. ethanol (1.18 t); 4. threonine (1.34 d); 5. alanine (1.48 d), 6. quinic acid derivative 1 (1.53 m; 1.82 dd; 1.94 dddd), 7. quinic acid derivative 2 (1.82 dd; 1.94 dddd; 2.10 m); 8. acetic acid (1.98 s); 9.  $\gamma$ -aminobutyric acid (2.35 dd, 3.01m), 10. glutamine (2.42 m); 11. malic acid (2.55 dd; 2.75 dd), 12. diethylamine (2.89 s).



**Figure 3.** Expansion of the  $^1\text{H}$  NMR spectrum of *C. sympodialis* showing diagnostic signals in the aromatic proton region: 1. quercetin (6.31 sl; 6.32 sl; 7.06 d; 7.66 dd; 7.84 d); 2. kaempferol (6.31sl; 6.32 sl; 7.06 d; 8.10 d); 3. tyrosine (6.88 d; 7.22 d); 4. formic acid (8.45 s).

On visual inspection the  $^1\text{H-NMR}$  spectra of the samples reveal a similar metabolic profile irrespective of the phenological stage. However, principal component analysis of the NMR data (Figure 4) using the full spectrum width (0.06-8.7 ppm) is able to discriminate samples from all phenological stages, with PC1 and PC2 accounting for 63 and 22% of the data variance respectively (Figure 4A). The distinctly separate clusters show that the phenological stages are associated with a characteristic chemical fingerprinting with samples CsL<sub>120</sub> and CsL<sub>210</sub> being the most distinct of all in terms of chemical composition. The small variation of score values between replicate samples demonstrates the robustness of the sample preparation methodology.



The loadings plot (Figure 4B) reveals that extracts from young leaves (CsL<sub>60</sub>) are marked by signals (bin size was 0.04 ppm) from saccharose (4.16, 5.42 ppm) and fructose (4.12 ppm), a result that is in agreement with quantification of these compounds for extracts from young leaves (up to CsL<sub>120</sub>) that tended towards higher concentration of these compounds than in extracts prepared from older leaves (Table 2). Signals of quinic acid derivatives at 3.58, 3.98, 1.82 and 1.94 ppm contribute negatively to PC2, therefore CsL<sub>210</sub> samples have higher area values for these bins.

The concentration of detected amino acids is higher in extracts from young leaves (up to 120 days after seeding). This behavior is shared by leucine, valine, threonine, glutamine and tyrosine. However, the non-proteic amino acid GABA reaches its maximal concentration during fructification (CsL<sub>210</sub> = 13.2 mmol/L  $\pm$  0.79,  $p < 0.001$ ) and is the most abundant amino acid followed by glutamine. Volatile compounds, such as ethanol, formic acid and acetic acid were detected at low concentrations in all samples (Table 2). Malic acid was the most abundant organic acid found in the samples, with extracts from young leaves with significantly higher values (CsL<sub>60</sub> = 22.0  $\pm$  0.82 mmol/L) than those found in older ones (CsL<sub>210</sub> = 4.9  $\pm$  0.64 mmol/L,  $p < 0,001$ ). Quinic acid and flavonol derivatives showed an opposite trend. Signals from flavonol derivatives (quercetin and kaempferol) were most abundant ( $p < 0.001$ ) in extracts from older leaves (CsL<sub>210</sub>: quercetin = 1.0  $\pm$  0.12 mmol/L and kaempferol = 1,0  $\pm$  0,16 mmol/L) compared to young ones (CsL<sub>60</sub>: quercetin = 0.1  $\pm$  0.05 mmol/L and kaempferol = 0.4  $\pm$  0.12 mmol/L). Other metabolites that deserve mention are derivatives of quinic acid that reached considerable levels in the extracts, varying from 8.3 to 35.2 mmol/L, with the higher concentration occurring in the CsL<sub>210</sub> samples.

**Table 2.** Concentration (mM/L) of the main metabolites identified on the leaf polar extracts of *C. sympodialis*. Values are mean  $\pm$  SD (n=5).<sup>a</sup>total phenolics as determined by Folin-Ciocalteu method is expressed here as mg of gallic acid equivalent/100 mg

Metabolite	Extract					
	CsL <sub>60</sub>	CsL <sub>90</sub>	CsL <sub>120</sub>	CsL <sub>150</sub>	CsL <sub>180</sub>	CsL <sub>210</sub>
alanine	0.7 $\pm$ 0.06	0.8 $\pm$ 0.05	0.5 $\pm$ 0.04	0.8 $\pm$ 0.06	0.9 $\pm$ 0.04	1.0 $\pm$ 0.05
valine	0.4 $\pm$ 0.03	0.9 $\pm$ 0.05	0.7 $\pm$ 0.06	0.7 $\pm$ 0.06	0.6 $\pm$ 0.07	0.4 $\pm$ 0.02
leucine	1.3 $\pm$ 0.11	1.6 $\pm$ 0.16	1.3 $\pm$ 0.10	1.4 $\pm$ 0.08	1.2 $\pm$ 0.10	0.9 $\pm$ 0.04
tyrosine	0.6 $\pm$ 0.09	0.9 $\pm$ 0.06	1.2 $\pm$ 0.17	0.9 $\pm$ 0.11	0.5 $\pm$ 0.05	1.0 $\pm$ 0.10
GABA	5.9 $\pm$ 0.10	4.2 $\pm$ 0.23	5.1 $\pm$ 0.43	5.1 $\pm$ 0.25	4.8 $\pm$ 0.22	13.2 $\pm$ 0.79
threonine	0.4 $\pm$ 0.05	0.9 $\pm$ 0.09	0.5 $\pm$ 0.08	0.4 $\pm$ 0.02	0.2 $\pm$ 0.04	0.3 $\pm$ 0.05
glutamine	8.8 $\pm$ 0.34	6.2 $\pm$ 0.34	7.3 $\pm$ 0.74	4.6 $\pm$ 0.37	4.5 $\pm$ 0.24	2.7 $\pm$ 0.22
$\alpha$ -glucose	7.3 $\pm$ 0.35	11.5 $\pm$ 0.61	2.6 $\pm$ 0.34	11.4 $\pm$ 0.74	15.9 $\pm$ 0.59	9.0 $\pm$ 0.74
$\beta$ -glucose	8.5 $\pm$ 0.20	20.9 $\pm$ 0.33	3.2 $\pm$ 0.24	20.6 $\pm$ 0.29	23.0 $\pm$ 0.73	12.7 $\pm$ 0.63
fructose	9.7 $\pm$ 0.52	10.7 $\pm$ 0.70	9.5 $\pm$ 0.86	6.3 $\pm$ 0.70	1.0 $\pm$ 0.10	7.5 $\pm$ 0.93
saccharose	17.7 $\pm$ 0.77	0.3 $\pm$ 0.16	36.5 $\pm$ 1.2	4.4 $\pm$ 0.59	0.6 $\pm$ 0.04	13.9 $\pm$ 0.91
acetic acid	0.1 $\pm$ 0.02	0.4 $\pm$ 0.07	0.1 $\pm$ 0.02	0.3 $\pm$ 0.05	0.2 $\pm$ 0.17	0.2 $\pm$ 0.03
formic acid	0.2 $\pm$ 0.06	0.3 $\pm$ 0.07	0.4 $\pm$ 0.07	0.4 $\pm$ 0.05	0.3 $\pm$ 0.03	0.4 $\pm$ 0.03
malic acid	22.0 $\pm$ 0.82	18.1 $\pm$ 1.05	10.2 $\pm$ 0.78	11.1 $\pm$ 0.97	11.3 $\pm$ 1.40	4.9 $\pm$ 0.64
quercetin	0.1 $\pm$ 0.05	0.6 $\pm$ 0.09	0.4 $\pm$ 0.1	0.4 $\pm$ 0.08	0.2 $\pm$ 0.09	1.0 $\pm$ 0.12
kaempferol	0.4 $\pm$ 0.12	1.0 $\pm$ 0.30	0.7 $\pm$ 0.24	0.8 $\pm$ 0.20	0.3 $\pm$ 0.05	1.0 $\pm$ 0.16
ethanol	1.5 $\pm$ 0.13	3.3 $\pm$ 0.21	2.2 $\pm$ 0.10	2.1 $\pm$ 0.17	1.5 $\pm$ 0.15	1.4 $\pm$ 0.05
dimethylamine	0.3 $\pm$ 0.02	0.4 $\pm$ 0.04	0.4 $\pm$ 0.03	0.3 $\pm$ 0.02	0.4 $\pm$ 0.02	0.6 $\pm$ 0.05
quinic acid derivative 1	8.3 $\pm$ 0.08	9.5 $\pm$ 0.07	7.7 $\pm$ 0.09	9.6 $\pm$ 0.06	10.5 $\pm$ 0.07	12.5 $\pm$ 0.12
quinic acid derivative 2	15.7 $\pm$ 0.05	10.2 $\pm$ 0.09	13.9 $\pm$ 0.07	11.3 $\pm$ 0.08	11.9 $\pm$ 0.09	35.2 $\pm$ 0.28
warifteine <sup>b</sup>	2 $\pm$ 0.32	2 $\pm$ 0.24	ND	ND	ND	ND
methylwarifteine <sup>b</sup>	1 $\pm$ 0.05	1 $\pm$ 0.14	1 $\pm$ 0.03	ND	ND	ND
total phenolics <sup>a</sup>	0.73 $\pm$ 0.03	0.81 $\pm$ 0.03	0.83 $\pm$ 0.08	0.85 $\pm$ 0.04	0.63 $\pm$ 0.03	0.98 $\pm$ 0.01

<sup>a</sup>plant.<sup>b</sup>These alkaloids were quantified using HPLC and are expressed here as  $\mu$ g/mL. ND=not detected

All extracts were able to relax tracheal rings pre-contracted with  $10^{-6}$  M CCh in a concentration-dependent manner (see Figures 1 and 2, supplementary material), both in the presence as well as in the absence of functional epithelium (Table 3). However, the order of potency as measured by  $EC_{50}$  values was different according to the presence or absence of functional epithelium. In rings with intact epithelium, a preparation that resembles a more physiological condition, the most potent extract was the one prepared from leaves collected during the fructification period (CsL<sub>210</sub>,  $EC_{50} = 74.6 \pm 7.9$   $\mu$ g/mL) that was significantly ( $p < 0.05$ ) more potent than extracts prepared from young leaves (CsL<sub>60</sub>,  $EC_{50} = 231.6 \pm 38.9$ ).

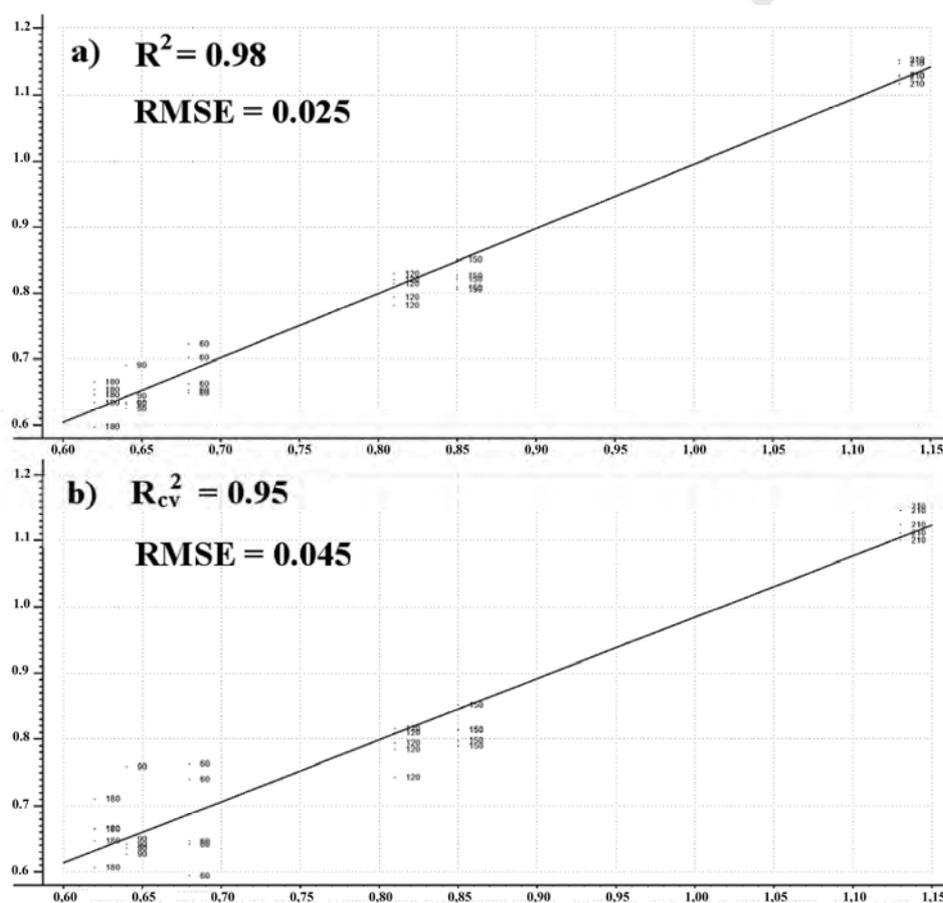
**Table 3.**  $EC_{50}$  values for extracts of *Cissampelos sympodialis* leaves in relaxing  $10^{-6}$  M carbachol-induced tonic contractions of guinea-pig tracheal rings.

Extract	$EC_{50}$ ( $\mu$ g/mL)	
	E+	E-
CsL <sub>60</sub>	207.0 $\pm$ 22.4*	114.0 $\pm$ 18.7
CsL <sub>90</sub>	231.6 $\pm$ 38.9** <sup><math>\delta</math></sup>	70.3 $\pm$ 15.0
CsL <sub>120</sub>	154.3 $\pm$ 29.0	114.9 $\pm$ 21.2
CsL <sub>150</sub>	141.0 $\pm$ 13.0	167.4 $\pm$ 56.8
CsL <sub>180</sub>	239.7 $\pm$ 15.1*** <sup><math>\delta\delta</math></sup>	87.8 $\pm$ 16.9
CsL <sub>210</sub>	74.6 $\pm$ 7.9*	173.3 $\pm$ 33.9

Student's t test, \* $p < 0,05$ , \*\* $p < 0,01$ , \*\*\* $p < 0,001$  (E+ vs. E-). ANOVA "one-way" followed by Bonferroni, post test  <sup>$\delta$</sup>  $p < 0.05$  (CsL<sub>210</sub> vs CsL<sub>90</sub>);  <sup>$\delta\delta$</sup>  $p < 0.01$  (CsL<sub>210</sub> vs CsL<sub>180</sub>). E+ = presence of epithelium; E- = absence of epithelium.

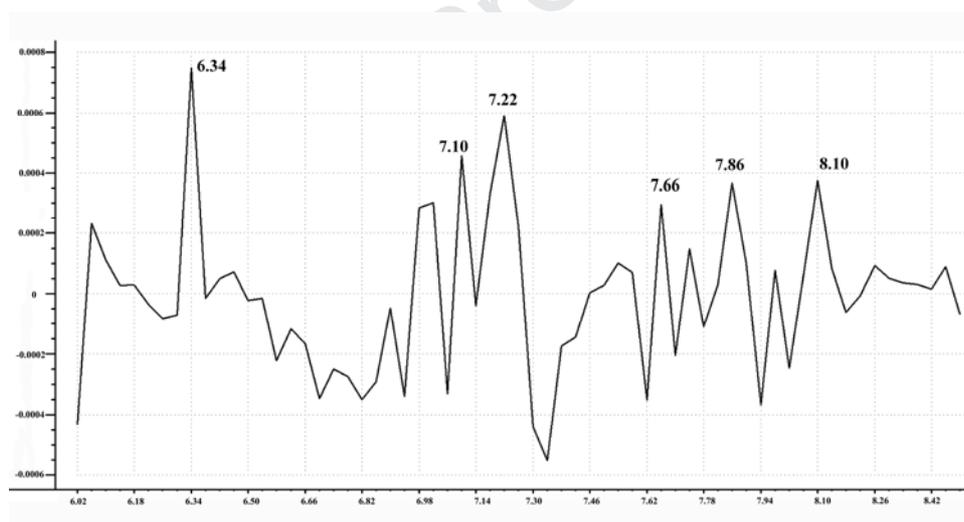
All extracts were equi-efficient with an  $E_{max}$  of 1000  $\mu$ g/mL (see Supplementary material, Figure 1). Additionally, we can suggest that CsL<sub>60</sub>, CsL<sub>90</sub> e CsL<sub>180</sub> exert its relaxing effect by acting directly on the smooth muscle and that they might induce the release of endothelium-derived contractile factors since the presence of endothelium decreases their relaxing potency. On the other hand it might be inferred that CsL<sub>210</sub> induce the release of endothelium-derived relaxing factors since its potency was higher

in the presence of functional endothelium. We investigated the correlation of the relaxing potency of extracts with the chemical profile using partial least squares analysis (Figure 5 and figure 6), using 25 samples, five different samples from each collection time point. The plot of calculated versus experimental values of pEC<sub>50</sub> (Figure 5a and Figure 5b) showed a high value for the coefficient of determination ( $R^2 = 0.98$ ) and for the coefficient of internal prediction (cross-validation leave-one-out,  $R_{cv}^2 = 0.95$ ) and low root mean square errors (RMSE): 0.025 and 0.045 respectively.



**Figure 5.** Correlation between the relaxing potency of extracts (as pEC<sub>50</sub> values) and chemical composition. a) Plot of calculated versus experimental values. b) cross validation leave-one-out prediction.

The signals that showed a positive correlation with pEC<sub>50</sub> values of extracts were the ones from flavonol derivatives of quercetin and kaempferol (6.34 ppm, 7.22 ppm, 7.66 ppm, 7.86 ppm, 8.10 ppm, Figure 6). This result is in agreement with the significant positive correlation found between the content of total phenolics and relaxation potency of the extracts (linear regression coefficient,  $r^2=0.78$ ). No correlation was found between EC<sub>50</sub> and concentration of other metabolites. In particular, no correlation was found between the EC<sub>50</sub> and the concentration of the bisbenzylisoquinoline alkaloids warifteine and methylwarifteine as determined by HPLC. The concentration of these alkaloids decrease (from  $2 \pm 0.32 \mu\text{g/mL}$  for warifteine and  $1 \pm 0.14 \mu\text{g/mL}$  for methylwarifteine) to undetectable levels from 90 and 120 days onwards for warifteine and methylwarifteine, respectively.



**Figure 6.** Partial least squares correlation analysis between relaxation potency of extracts (as determined by pEC<sub>50</sub> values) and chemical composition as determined by the chemical shifts (in ppm) of diagnostic signals. Correlation values are displayed in the y axis and chemical shifts in the x axis.

#### 4. Discussion

The results presented here, together with all accumulated evidence of the use of extracts of *Cissampelos sympodialis* for the treatment of asthma (Cavalcanti *et al.*, 2013) could help in establishing a monograph for a herbal medicine. Our results have shown that there are marked differences associated with the maturation of *Cissampelos sympodialis* leaves reflected in the discrimination observed when using principal component analysis of  $^1\text{H}$  NMR data, mainly due to signals in the region of sugars and amino acids. GABA was the most abundant amino acid found in the samples. Although the definite role of GABA in plants is not fully established, it has been suggested that it may participate in the plant development, nitrogen metabolism, cell signaling and defense such as against hydric stress (Lancien & Roberts, 2006; Roberts, 2007). Other metabolites that showed high levels in the extracts were derivatives of quinic acid. Quinic acid is considered one of the most important metabolites exclusive of plants and is derived from the shikimate pathway. Quinic acid participates in the biosynthesis of all aromatic amino acids such as tryptophan, phenylalanine, and tyrosine (Bhatia *et al.*, 2015) and is thus indirectly associated with the synthesis of bisbenzylisoquinoline alkaloids in *Cissampelos* species. It is associated with several biological activities such as anti-inflammatory, immunomodulatory (Beara *et al.*, 2015; Pero & Lund, 2011), antioxidant and neurogenic effects (Lee *et al.*, 2013). In the present work the concentration of quinic acid derivatives varied from 8.3 to 35.2 mmol/L, with significantly higher concentrations in the CsL<sub>210</sub> samples.

All extracts were able to relax guinea-pig tracheal rings pre-contracted with carbachol both in preparations with intact epithelium as well as in rings devoid of it. However, in rings with epithelium the most potent extract was CsL<sub>210</sub>. Since no warifteine nor methylwarifteine was detected in this extract by HPLC analysis, and no

correlation between alkaloid content and relaxation potency could be established, our results indicate that these alkaloids do not participate in the relaxation of tracheal smooth muscle promoted by these polar extracts, even though they have been implicated in this effect in the past, since isolated warifteine has been shown to relax guinea-pig tracheal strips (Cavalcante *et al.*, 2011; Thomas *et al.*, 1997).

PCA analysis using  $^1\text{H}$  NMR data aids to visualize chemical differences of the extracts of leaves according to the period of harvesting. Except for the samples CsL<sub>150</sub> and CsL<sub>180</sub> that are more similar, with bins of 3.4 and 3.7 ppm that are characteristic of aliphatic and aromatic methoxyl groups respectively, all samples differentiate significantly according the time of harvesting. This should reflect differences in the metabolite profile of these samples as the plant grows. Indeed, in a previous study our group demonstrated that the concentration of the main bisbenzylisoquinoline alkaloids present in the ethanolic extract of *C. sympodialis* leaves, namely warifteine, milonine and methylwarifteine varies with the phenological stage of the plant, reaching low levels as the fruits develop (Marinho *et al.*, 2012). In the leaf polar extracts used in this study that reflect more closely the composition of aqueous extracts used in traditional medicine, alkaloid levels decreased to undetectable concentrations from 90 days onwards for warifteine and 120 days onwards for methylwarifteine, and PCA data shows that extracts prepared from leaves of older plants are richer in flavonoids and quinic acid derivatives. In a previous clinical toxicology study of *Cissampelos sympodialis* leaf tea (Mangueira *et al.*, 2015), the authors did not detect warifteine or methylwarifteine in their tea samples, a result that is in agreement with our findings here. NMR-based metabolomics is an interesting technique for discovering metabolites with a particular biological activity, and this approach has recently been applied for the discovery of

cytotoxic triterpene glycosides and steroidal saponin from Fabaceae species (Graziani *et al.*, 2018).

PLS analysis (Figure 5 and 6) of aromatic region (5.5 – 8.7 ppm) have revealed that bins assigned to quercetin and kaempferol show positive contribution to the values of pEC<sub>50</sub>, and are thus related to the relaxing potency. This result corroborates with the regression analysis obtained using total phenolics (Table 2) and relaxing potency (Table 3). Analysis of both methodologies demonstrate the contribution of flavonol derivatives, such as those of quercetin and kaempferol to spasmolytic activity.

The spasmolytic activity of flavonoids on tracheal smooth muscle is well known (Lemmens-Gruber *et al.*, 2006; Sadraei *et al.*, 2018). Hispidulin, a naturally-occurring flavone was implicated in the bronchodilator effect of *Clerodendrum petasites* ethanolic extract (Hazekamp, *et al.*, 2001). Also, the flavonoid-rich fraction of *Allium cepa* exerts its bronchodilatory effect through Ca<sup>+2</sup> channel inhibition and interference with phosphodiesterase mediated mechanisms (Mandhukail, *et al.*, 2014). In our case, the positive correlation of pEC<sub>50</sub> values calculated for the spasmolytic effect of extracts and characteristic flavonoid <sup>1</sup>H NMR signals suggests that flavonoids in these extracts contribute to the pharmacological effect of the extracts.

Taken together our results contribute to elucidate the chemical composition of *C. sympodialis* polar extracts and challenge the idea that the bisbenzylisoquinoline alkaloids are the major biomarkers of the plant, pointing to an important contribution of phenolic compounds to the spasmolytic activity of the plant's polar extracts. Further studies should be aimed at isolating the phenolic compounds responsible for the spasmolytic activity of the extracts.

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The authors have no conflicts of interest to disclose.

Journal Pre-proof

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## 8. Figure Legends

**Figure 1.** Representative  $^1\text{H}$  NMR spectra of polar extracts prepared from leaves of *C. sympodialis* collected at different phenological stages.

**Figure 2.** Expansion of the  $^1\text{H}$  NMR spectrum of *C. sympodialis* showing diagnostic signals in aliphatic proton region: 1. leucine (0.94 d; 1.0 d), 2. valine (1.05 d), 3. ethanol (1.18 t); 4. threonine (1.34 d); 5. alanine (1.48 d), 6. quinic acid derivative 1 (1.53 m; 1.82 dd; 1.94 dddd), 7. quinic acid derivative 2 (1.82 dd; 1.94 dddd; 2.10 m); 8. acetic acid (1.98 s); 9.  $\gamma$ -aminobutyric acid (2.35 dd, 3.01m), 10. glutamine (2.42 m); 11. malic acid (2.55 dd; 2.75 dd), 12. diethylamine (2.89 s).

**Figure 3.** Expansion of the  $^1\text{H}$  NMR spectrum of *C. sympodialis* showing diagnostic signals in the aromatic proton region: 1. quercetin (6.31 sl; 6.32 sl; 7.06 d; 7.66 dd; 7.84 d); 2. kaempferol (6.31sl; 6.32 sl; 7.06 d; 8.10 d); 3. tyrosine (6.88 d; 7.22 d); 4. formic acid (8.45 s).

**Figure 4.** Principal component analysis score (A) and loadings plot (B) of  $^1\text{H}$ -NMR data for *C. sympodialis* extracts.

**Figure 5.** Correlation between the relaxing potency of extracts (as  $\text{pEC}_{50}$  values) and chemical composition. a) Plot of calculated versus experimental values. b) cross validation leave-one-out prediction.

**Figure 6.** Partial least squares correlation analysis between relaxation potency of extracts (as determined by  $\text{pEC}_{50}$  values) and chemical composition as determined by the chemical shifts (in ppm) of diagnostic signals. Correlation values are displayed in the y axis and chemical shifts in the x axis.

**Figure 1, Supplementary Files.** Cumulative concentration-response curves of the effect of extracts in relaxing  $10^{-6}$  M carbachol-induced tonic contractions of guinea-pig tracheal rings in the presence of functional epithelium. (Data are expressed as mean  $\pm$  SEM. n=5).

**Figure 2, Supplementary Files.** Cumulative concentration-response curves of the effect of extracts in relaxing  $10^{-6}$  M carbachol-induced tonic contractions of guinea-pig tracheal rings in the absence of functional epithelium. (Data are expressed as mean  $\pm$  SEM. n=5).

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