



# Tissue culture and metabolome investigation of a wild endangered medicinal plant using high definition mass spectrometry

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## Abstract

An increasing effort is dedicated to investigate the potential of native plants used in traditional medicine as a source of bioactive compounds for numerous industries. The bioprospection of the metabolome of medicinal and/or endangered plants has two important merits: confirming or revealing the biotechnological potential of that species, and assisting in its conservation. In addition, biotechnological techniques, such as tissue culture, are key strategies in conservation and multiplication of medicinal plants. This is the first in vitro development and non-targeted metabolome study by UPLC–QTOF–MS<sup>E</sup> of extracts from *C. menthoides*, an endangered medicinal plant. In vitro development investigation with a wide range of plant growth regulators resulted in maximum survival rate (81%) and the highest growth rate (1.74 cm ± 0.36) for plantlets cultured on Murashige and Skoog medium, supplemented with 1 μM gibberellic acid. Maximum rooting occurred on medium supplemented with 4.4 μM 6-benzyladenine, which also resulted in more leaves per plantlet (10.16 ± 1.7). We developed a protocol that can be used for the clonal propagation and ex situ conservation of this species. In terms of metabolome analysis, a total of 107 metabolites from several classes were detected and identified in its hydrophilic extract (HE), including organic acids and derivatives, glucosinolates, terpenes, phenolic compounds as well as other polar metabolites. The metabolites in HE with the greatest signal intensity included the isoquinoline alkaloid magnoflorine; the coumaric acid rosmarinic acid; the steroid-cardanolide convallatoxin; two anthraquinones including the poorly investigated ventinone A. Several molecules identified here carry potential pharmacological benefits such as anti-inflammatory and anticancer applications.

**Keywords** *C. menthoides* · In vitro · Growth regulators · Metabolomics · UPLC–MS · Non-targeted

## Abbreviations

PGR	Plant growth regulator
IAA	Indole-3-acetic acid
BAP	8-Benzylaminopurine
TDZ	Thidiazuron

GA3	Gibberellic acid
UPLC	Ultra-performance liquid chromatography
Qq-TOF-MS	Quadrupole time of flight mass spectrometry
ESI	Electrospray ionization
LE	Lipophilic extract
HE	Hydrophilic extract

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## Introduction

In the last two decades several drugs approved by the US Food and Drug Administration (FDA) are small natural molecules or their direct derivatives. With recent advances in plant biochemistry and due to the enormous Brazilian biodiversity, native species already used in the traditional medicine can generate innovative bioproducts for vast industrial sectors (Agostini et al. 2014; Isah et al. 2017). However, many of these plants, which include endangered species, have no in vitro regeneration studies and are poorly chemically characterized (Xu et al. 2016).

The *Cunila* D. Royen Ex. L. genus belongs to the *Lamiaceae* family, a family used in traditional medicine due to several of its aromatic species. Due to their high content of phenolic compounds, the *Lamiaceae* family members have always been considered as a valuable source of eco-friendly natural substances for the healthcare, food and pesticide industries (Trivellini et al. 2016).

*Cunila* spp. production by tissue culture methods has been poorly studied. There have only been two reports of the extremely recalcitrant *Cunila* tissue culture: with *C. galioides* (Fracaro and Echeverrigaray 2001) and *C. incisa* (Agostini and Echeverrigaray 2006). Although the composition of its oils has attracted the interest of the pharmaceutical and cosmetic industries, there are no *Cunila*-derived commercial products available to date. Specifically for the rare *C. menthoides* Benth, a xylopodiferous tall subshrub spread in the southernmost tip of Brazil and in Uruguay (Agostini et al. 2010), no tissue culture nor metabolome study have been performed to date.

In terms of the metabolome, only a few non-targeted metabolomic studies of other members of the *Lamiaceae* family have been performed to date (Silva and Câmara 2013; Mišić et al. 2015; Scognamiglio et al. 2015), with chief constituents identified including terpenoids and phenols, mainly flavonoids, many with economic and medicinal value (Agostini et al. 2009; Silva and Câmara 2013).

In the present study, a more extensive characterisation was undertaken, intending to extract not only previously reported terpenes, but also a wide range of other terpenes, alkaloids, and phenolic compounds. This was performed in order to provide a more comprehensive chemical profile of this important and endangered aromatic species from the neo-tropical biodiversity. Terpenes and phenolic compounds were identified and relatively quantified using a high sensitivity and confidence method, an Ultra Performance Liquid Chromatography–Mass Spectrometry<sup>Elevated Energy</sup> (UPLC–MS<sup>E</sup>) approach with data independent acquisition (DIA) (Souza et al. 2017). MS<sup>E</sup> methods that provide precursor mass as well as molecular structure information on the LC scale of all eluting species

are attractive since all information is obtained in a single experiment (Cramer et al. 2017). Therefore, this technique enables a more accurate identification of unknown molecules in the context of untargeted analyses (Zhao and Lin 2014).

Furthermore, this study evaluated and compared the growth and development of *C. menthoides* plant tissue cultures on media containing different plant growth regulators (PGRs), thus providing a tool for its optimal growth for further investigation.

## Materials and methods

### Plant material

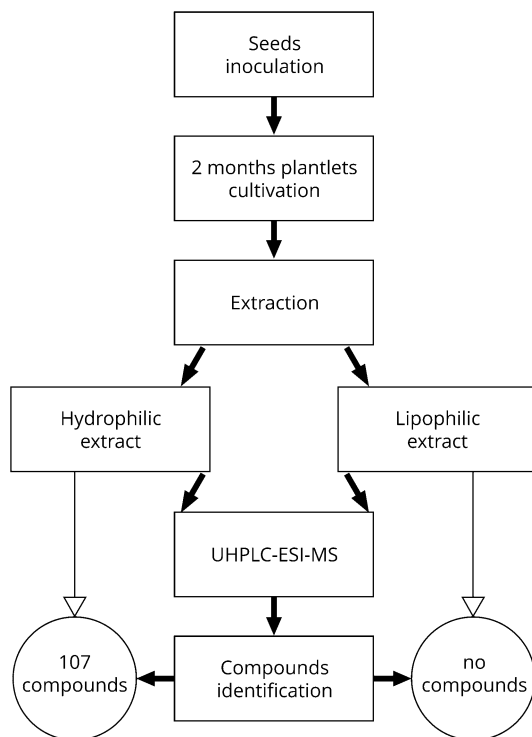
*C. menthoides* seeds used in this study were donated from the personal collection of Prof. Dr. Gustavo Agostini. Seeds were collected and catalogued as previously described (Agostini et al. 2010). Briefly, *C. menthoides* seeds were harvested in different cities in the state of Rio Grande do Sul, Brazil, using specific geographical coordinates. Representative samples were identified botanically and duly filed, cataloged and recorded in the Herbarium-accredited ICN (183282) located at the Institute of Biosciences, Federal University of Rio Grande do Sul (UFRGS).

### Ex vitro cultivation

Seeds were sown on a mixture of expanded clay pallets rocks (1506, granulometry of 6/15 mm, 600 kg m<sup>-3</sup> density, Cinexpan, São Paulo, Brazil), earthworm humus (Cia da Minhoca, Paraná, Brazil) and thin sand (AB Areias, São Paulo, Brazil). Regular plant plastic vases of 500 mL were used for germination and the plantlets were watered three times a week (Fig. 1).

### In vitro development responses to PGRs assays

Seeds surface were sterilized by a wash using a 50% (v/v) bleach solution for 12 min, rinsed three times in sterile distilled water for 5 min each time, immersed in 70% ethanol for 5 min, and once again rinsed three times in sterile distilled water for 5 min each time. Surface-sterilized seeds were placed in contact with the medium in culture flasks. The sterile MS growth medium (Murashige and Skoog 1962) was supplemented with 30 g/L sucrose and 7 g/L agar, without growth regulators (MS0) (Macedo et al. 1999). pH was adjusted to 5.7–5.8, followed by autoclave procedures (1 atm, 127 °C, 20 min). The same culture conditions were maintained throughout the process of germination, plantlet multiplication and development. All experiments were performed in a climate-controlled room equipped with white



**Fig. 1** Fluxogram showing the experimental design used (figure created with Micromedia Fireworks)

fluorescent lamps (Osram F20T12/CW) (approximately  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation, PAR). A 16-h photoperiod was used for all treatments. Cultures were maintained at  $25 \pm 1 \text{ }^\circ\text{C}$ .

After 9 weeks, germinated plantlets had nodes removed to evaluate if the explants would respond to medium supplemented with one PGR at a time or combinations thereof. Nodal explants were placed in the same culture conditions cited above, but with medium supplemented or not with a different PGR.

In total, 30 growth medium formulations were created by combining one or more PGRs: indole acetic acid (IAA) (1; 6.7 or  $13.4 \mu\text{M}$ ); gibberellic acid (GA3) (1; 6.7 or  $13.4 \mu\text{M}$ ); 6-benzylaminopurine (BA) (0.44; 2.2; 4.4; 6.7; 8.8 or  $17.6 \mu\text{M}$ ); and thidiazuron (TDZ) (0.44; 2.2; 4.4; 6.7; 8.8 or  $17.6 \mu\text{M}$ ). Assays with combinations of PGRs were: BA + IAA (0.44 +  $1 \mu\text{M}$ ; 0.44 +  $6.7 \mu\text{M}$ ; 0.44 +  $13.4 \mu\text{M}$ ;  $6.7 + 1 \mu\text{M}$ ;  $6.7 + 6.7 \mu\text{M}$  or  $6.7 + 13.4 \mu\text{M}$ , respectively); BA + GA3 (0.44 +  $1 \mu\text{M}$ ; 0.44 +  $6.7 \mu\text{M}$ ; 0.44 +  $13.4 \mu\text{M}$ ;  $6.7 + 1 \mu\text{M}$ ;  $6.7 + 6.7 \mu\text{M}$  or  $6.7 + 13.4 \mu\text{M}$ , respectively). BA concentrations were decided after weighing all previously reported data for the *Cunila* species (Fracaro and Echeverrigaray 2001; Agostini and Echeverrigaray 2006). Finally, a concentration of  $6.7 \mu\text{M}$  BA was chosen based on Leng and Lai-Keng (2004), as it was shown to support a higher number of shoots.

Each treatment consisted of 12 axillary buds and was repeated three times. Four nodes were placed in each glass flask. After 60 days of culture, the evaluated parameters were: number of shoots per explant, number of leaves, percentage of rooted plantlets, callus formation and shoot viability. We recorded multiple shoot formation when more than ten shoots/explant were produced. Statistical differences were calculated using ONE WAY ANOVA, and means were compared using the Tukey's test.

## Metabolomics study of *C. menthoides*

### Preparation of standard solution

Stock solution of 31 reference compounds were individually prepared by dissolving standards in aqueous methanol (LC-MS Ultra CHROMASOLV®, Fluka® Analytical). An aliquot of each stock solution was mixed at a concentration of 10 ppm for each compound and diluted in the initial mobile phase. All standard chemicals were HPLC grade purchased from Sigma-Aldrich (St. Louis, USA). In all experimental steps, ultrapure water (resistivity  $> 18.2 \text{ M}\Omega \text{ cm}$ ) (Barnstead Smart2Pure, Thermo Fisher Scientific, USA) was used.

### Sample preparation, extraction and UPLC-Qq-oeTOF-MS acquisition

For the extraction of lipophilic and hydrophilic compounds, an adapted Bligh-Dyer (Bligh and Dyer 1959) methodology was applied. A pool of three different 2 months old ex vitro plantlets of *C. menthoides* weighing 1.6 g in total were finely ground in liquid nitrogen. The combined powder was then extracted by vortexing with chloroform (B'Herzog, Rio de Janeiro, Brazil) and methanol (LC-MS Ultra CHROMASOLV®, Fluka® Analytical) (1:2, v/v) solution according Bligh-Dyer methodology (Bligh and Dyer 1959). After centrifugations at  $14,000\times g$ , for 10 min, at  $4 \text{ }^\circ\text{C}$  (Megafuge 16R Centrifuge, Thermo Fisher Scientific, USA), the final solutions presented two different layers, corresponding to the hydrophilic and lipophilic extracts (HE and LE, respectively) (Fig. 1). The remaining solvents were removed by rotary vacuum concentrator (RVT400, Savant, Thermo Scientific, USA). Equal volumes of dried HE and LE samples were pooled, vortexed, sonicated using an ultrasonic probe (DESRUPTOR 500W, Ultrasonic) and centrifuged as mentioned above. Supernatants were filtered (PTFE Hydrophilic, 13 mm diameter,  $0.22 \mu\text{m}$ , Analítica, Brazil) and prepared for UPLC-Qq-oeTOF-MS analysis in a UPLC system coupled to a hybrid quadrupole orthogonal time-of-flight mass spectrometer, Xevo G2-S QToF (Waters Corporation, Milford, USA).

Chromatographic separation was performed on an Acquity I-Class UPLC (Waters Corporation, Milford, USA) using an ACQUITY UPLC® HSS T3 C<sub>18</sub> column (2.1 × 100 mm, 1.8 μm particle size). The mobile phase (A) was 0.3% formic acid in ultrapure water and phase (B) was 0.3% formic acid and 5 mM ammonium formate in acetonitrile. Gradient method followed the steps: 97% A and 3% B at 0 min; 50% A and 50% B at 11.8 min; 15% A and 85% B at 12.38–13.53 min and equilibration with 97% A and 3% B from 14.11 to 16.99 min. The flow rate was 0.6 mL min<sup>-1</sup>, the column was maintained at 35 °C. The injection volume of each sample and standard mix solution was 2 μL. Each batch of samples was injected with randomized sequence in triplicate.

The mass data were acquired in negative electrospray ion mode ESI (-). The scan range was selected from m/z 50 to 1000. Data were acquired using a multiplexed MS/MS acquisition with simultaneous application of low and high energy acquisition (MS<sup>E</sup>) on centroid mode. MS<sup>E</sup> experiments were performed with a collision energy range from 15 to 55 eV. Leucine-enkephalin (Waters Corporation, Milford, USA), C<sub>28</sub>H<sub>37</sub>N<sub>5</sub>O<sub>7</sub>, ([M-H]<sup>-</sup> = 554.2615 m/z) at concentration of 0.4 ng L<sup>-1</sup> was used as lockmass reference for negative ion mode. Lock-mass scan time was set to 0.3 s, with intervals of 15 s and 3 scans to average with a mass window of ±0.3 Da.

### Data processing and compound identification

Progenesis QI 2.1.v for metabolome (Nonlinear Dynamics, Waters Corporation, UK) software was used to process raw

data. The conditions selected for data processing were: centroid data full width resolution at half maximum (FWHM) of 50,000, ionization negative ion mode and adducts [M - H<sub>2</sub>O - H]<sup>-</sup>, [M - H]<sup>-</sup>, [M + Cl]<sup>-</sup> and [M + FA - H]<sup>-</sup>. For compound identification analytical standards and database platforms such as KEGG (<http://www.genome.jp/kegg/>), LIPID MAPS (<http://www.lipidmaps.org/>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) were used. For identification beyond compounds database, we used precursor mass error ≤ 5 ppm and fragment tolerance ≤ 10 ppm. Non-targeted selection parameters were established as: Anova (*p*) ≤ 0.5, minimum coefficient of variance < 30, score > 30 and isotope similarity > 80. Only compounds present in at least two of the three technical replicates that have CV ≤ 0.3 were considered as identified.

## Results

### Developmental responses to PGRs

The effect of various concentrations of synthetic cytokinins BA and TDZ, GA3 and IAA were analyzed here for micro-propagation through direct organogenesis from intact nodal segments of *C. menthoides*. Positive results for shoot elongation were obtained with plantlets developed in medium with BA or GA3 (Table 1) but not with medium supplemented with TDZ, IAA or combinations of PGRs (Table 1 and Online Resource 1). Among all of the PGRs, gibberellin was essential for plant growth and shoot regeneration of *C. menthoides*. When compared with the control and other

**Table 1** Shoot elongation in cm of *C. menthoides* grown on MS medium supplemented with BAP or GA3 with different concentrations

Medium	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
MS0	0.43 ± 0.02*ab	0.49 ± 0.04ab**	0.43 ± 0.04ab	0.62 ± 0.05abc	0.63 ± 0.07bcde	0.52 ± 0.09cde	0.51 ± 0.11cde	0.44 ± 0.01bcd	0.40 ± 0.02bcd
BAP 0.44 μM	0.38 ± 0.03ab	0.45 ± 0.05ab	0.52 ± 0.05ab	0.39 ± 0.06c	0.21 ± 0.07f	0.18 ± 0.07de	0.02 ± 0.02e	0.00 ± 0.00d	0.00 ± 0.00d
BAP 2.2 μM	0.36 ± 0.04ab	0.44 ± 0.04ab	0.47 ± 0.04ab	0.38 ± 0.05c	0.28 ± 0.06ef	0.11 ± 0.06e	0.05 ± 0.06de	0.05 ± 0.06 cd	0.05 ± 0.06bcd
BAP 4.4 μM	0.35 ± 0.03ab	0.45 ± 0.04ab	0.58 ± 0.08ab	0.59 ± 0.11abc	0.63 ± 0.15abc-def	0.43 ± 0.21cde	0.28 ± 0.26cde	0.30 ± 0.28bcd	0.42 ± 0.29bcd
BAP 6.7 μM	0.50 ± 0.05ab	0.64 ± 0.05ab	0.76 ± 0.08a	0.92 ± 0.01a	1.03 ± 0.15ac	1.18 ± 0.18ab	1.88 ± 0.25ab	1.22 ± 0.31a	0.94 ± 0.35ab
BAP 8.8 μM	0.38 ± 0.03ab	0.52 ± 0.04ab	0.61 ± 0.06ab	0.71 ± 0.06abc	0.63 ± 0.10abc-def	0.53 ± 0.13bcde	0.48 ± 0.14bcde	0.48 ± 0.14bcd	0.49 ± 0.15bcd
BAP 17.6 μM	0.42 ± 0.02ab	0.49 ± 0.03ab	0.58 ± 0.04ab	0.65 ± 0.03abc	0.69 ± 0.05abc-def	0.69 ± 0.07bcde	0.55 ± 0.11bcde	0.51 ± 0.16bcd	0.24 ± 0.14bcd
GA3 1 μM	0.38 ± 0.02ab	0.45 ± 0.03ab	0.66 ± 0.04ab	0.92 ± 0.07a	1.15 ± 0.09a	1.43 ± 0.18a	1.60 ± 0.20a	1.90 ± 0.24a	1.74 ± 0.37a
GA3 6.7 μM	0.39 ± 0.02ab	0.33 ± 0.04b	0.33 ± 0.07b	0.42 ± 0.09c	0.48 ± 0.10bcdef	0.61 ± 0.14bcde	0.61 ± 0.16bcde	0.61 ± 0.17bcd	0.50 ± 0.20bcd
GA3 13.4 μM	0.37 ± 0.02ab	0.38 ± 0.02ab	0.43 ± 0.04ab	0.42 ± 0.06c	0.30 ± 0.07def	0.25 ± 0.07de	0.18 ± 0.09cde	0.14 ± 0.08cd	0.21 ± 0.11bcd

Evaluations were performed weekly for 9 weeks. All the shoots produced from explants were recorded, even those that died during the experiment, but not explants that had developed multiple shoots (figure created with Microsoft Excel)

\*Standard error (SE) error bar represents the standard error of each average

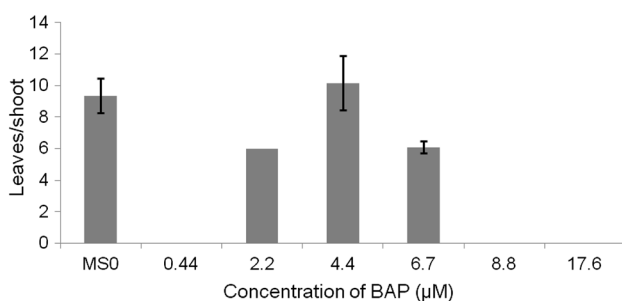
\*\*Mean values with the same letter within a column are not significantly different based on analysis of variance (ANOVA) followed by Tukey's test at *p* ≤ 0.05

concentrations of GA3, and all concentrations of auxin and cytokinins tested, combined or not, the greatest height ( $1.90 \text{ cm} \pm 0.24$ ), as well as the maximum regeneration rate of plants (81% viability) (Table 1 and Online Resource 2), was achieved with  $1 \mu\text{M}$  GA3. All plantlets developed in medium with TDZ, or BA + GA3 and BA + IAA combinations, displayed shoot length statistically similar to those plantlets grown in MS0 with less than 0.6 cm (Table 1 and Online Resource 1). Plantlets cultured in media with different concentrations of BA displayed multiple shoots and a smaller size (Online Resource 2).

The highest shoot proliferation (more than ten shoots/explant) was recorded in media with  $8.8 \mu\text{M}$  BA and with  $0.44 \mu\text{M}$  BA +  $6.7 \mu\text{M}$  GA3 (Online Resource 2). Media supplemented with different concentrations of IAA and GA3 did not trigger multiple shoots (Online Resource 2). All medium supplemented with auxin regulator produced brown callus with the highest percentages observed (Online Resource 2). More leaves per plantlet ( $10.16 \pm 1.7$ ) (Fig. 2) and higher % of root formation (Online Resource 2) were observed in medium supplemented with  $4.4 \text{ BA}$ , compared with the control and the other culture 3rd internode ( $0.63 \text{ cm} \pm 0.04$ ) conditions.

## Metabolome

Using a UHPLC–ESI–MS<sup>E</sup> method, the phytochemical composition of the plantlets of *C. menthoides*, with focus on the phenolic fraction, were investigated, providing comprehensive screening of its potential (Fig. 1). Using the analytical method detailed here, we did not detect any compounds in the lipophilic extract, probably because it contained mainly non-polar compounds that need to be analysed using a more appropriate chromatographic technique, such as gas chromatography or normal phase liquid chromatography. In



**Fig. 2** Number of leaves per shoots from *C. menthoides* grown on MS medium supplemented with benzyladenine. Error bar represents the standard error of each average. Means with the same letter are not statistically different, based on Tukey test by ANOVA  $p \leq 0.05$ . Evaluations were performed after 60 days. Only viable explants that had developed shoots were recorded. We did not record explants that had developed multiple shoots (figure created with Microsoft Excel)

the hydrophilic fraction, however, a total of 107 different molecules were identified (Fig. 1 and Online Resource 3). Of these, four were fully validated using a mix of standard phenols (Online Resource 3).

The first set of most representative chemical class consisted of phenols (Fig. 3a). A total of 73 different phenolic compounds were identified. The phenols with the most intense ions were rosmarinic acid, question, (2S)-2'-methoxykumarinone, vertinone A and savianolic acid B (SAB) (Fig. 4b and Online Resource 3). The largest group of compounds were flavones, cinnamic/hydroxycinnamic acids, flavonols and isoflavanone with 13, 9, 8 and 8 different molecules, respectively, that were dominated by isoswertisin 2''-rhamnoside, rosmarinic acid, phellamurin and glycitin, the most abundant flavone, cinnamic/hydroxycinnamic acids, flavonols and isoflavanone, respectively (Fig. 3b and Online Resource 3).

The second most represented chemical class was the terpenes. The analysis revealed that the extracts contained 21 terpenes, mainly monoterpenes and triterpenes (five different molecules each) (Fig. 3b and Online Resource 3). The steroid convallatoxin ( $595.2790$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ), the apocarotenoid picrocrocin ( $375.1649$   $[\text{M} - \text{H}]^-$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ), the monoterpene menthol glucuronide ( $377.1818$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ) and the triterpenoid–dammarane, sergeolide ( $549.1656$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ) were the major relative components in HE (Fig. 4a and Online Resource 3).

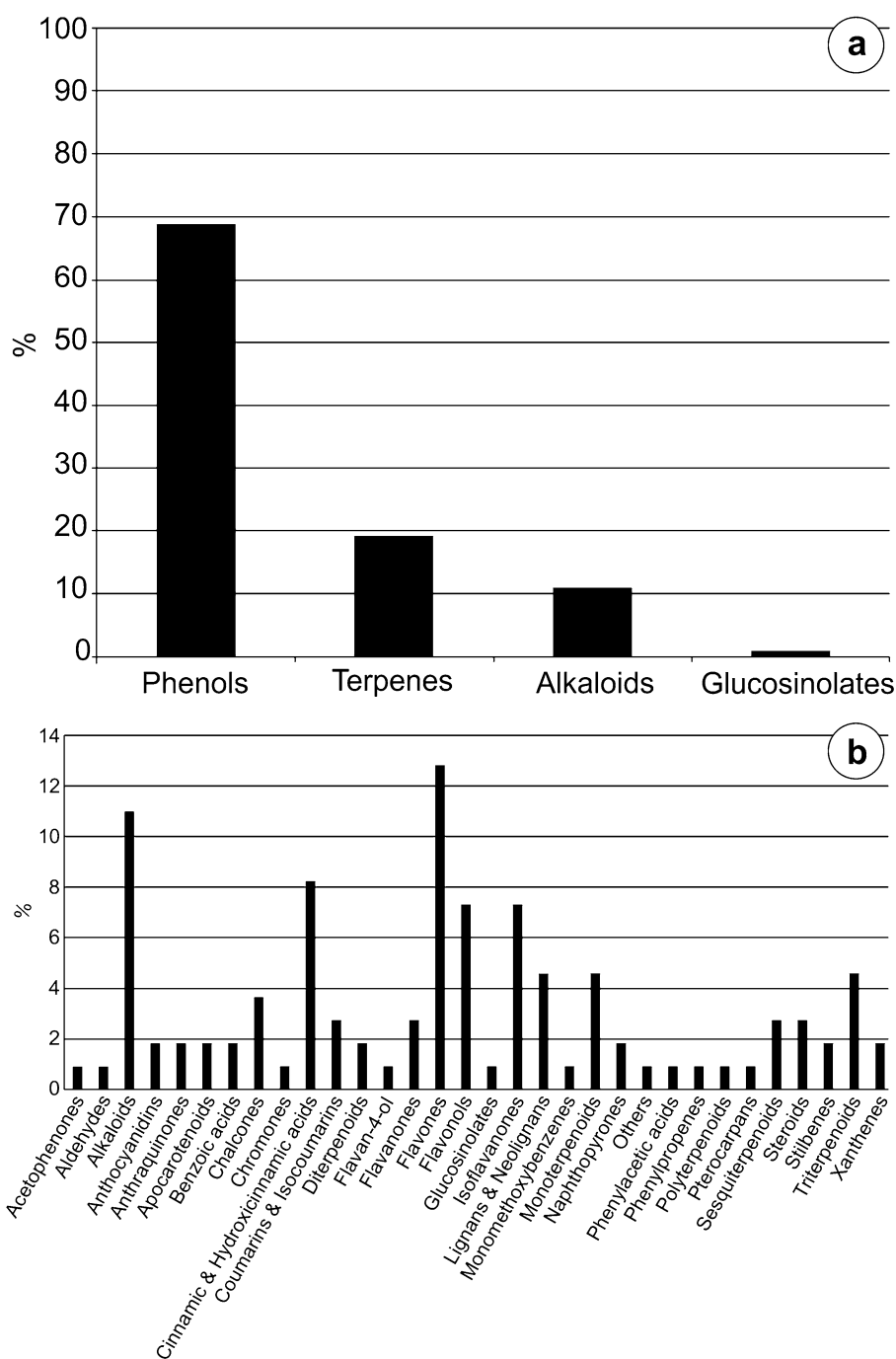
The third most diverse group of compounds was alkaloids with 12 compounds (Online Resource 3). The alkaloids with most intense ions were magnoflorine, bebeerine, macarpine, vincristine and methyllycaontine (Fig. 4c and Online Resource 3). One glucosinolate was also detected, neoglucobrassicin ( $477.0611$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ) (Online Resource 3).

Among the ten compounds with the highest signal intensity we identified the isoquinoline alkaloid magnoflorine ( $387.1658$   $\text{m/z}$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ); the coumaric acid rosmarinic acid ( $359.0767$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ); the steroid-cardanolide convallatoxin ( $595.2790$   $\text{m/z}$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ), two anthraquinones: question ( $329.0670$   $\text{m/z}$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ) and the poorly investigated ventinone A ( $313.0714$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ), the flavanone (2S)-2'-methoxykumarinone ( $497.2228$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ,  $[\text{M} + \text{FA} - \text{H}]^-$ ); and the stilbene salvianolic acid B ( $717.1453$   $\text{m/z}$ ,  $[\text{M} - \text{H}]^-$ ) (Fig. 4 and Online Resource 3).

## Discussion

The purpose of this study was to develop a method for optimal in vitro culture of *C. menthoides*, an endangered and recalcitrant medicinal species and to characterize its metabolome. Metabolomic results show many compounds with

**Fig. 3** Normalized expression of different chemical classes (a) and subclasses (b) on hydrophilic extracts of *C. menthoides* (figure created with Microsoft Excel)

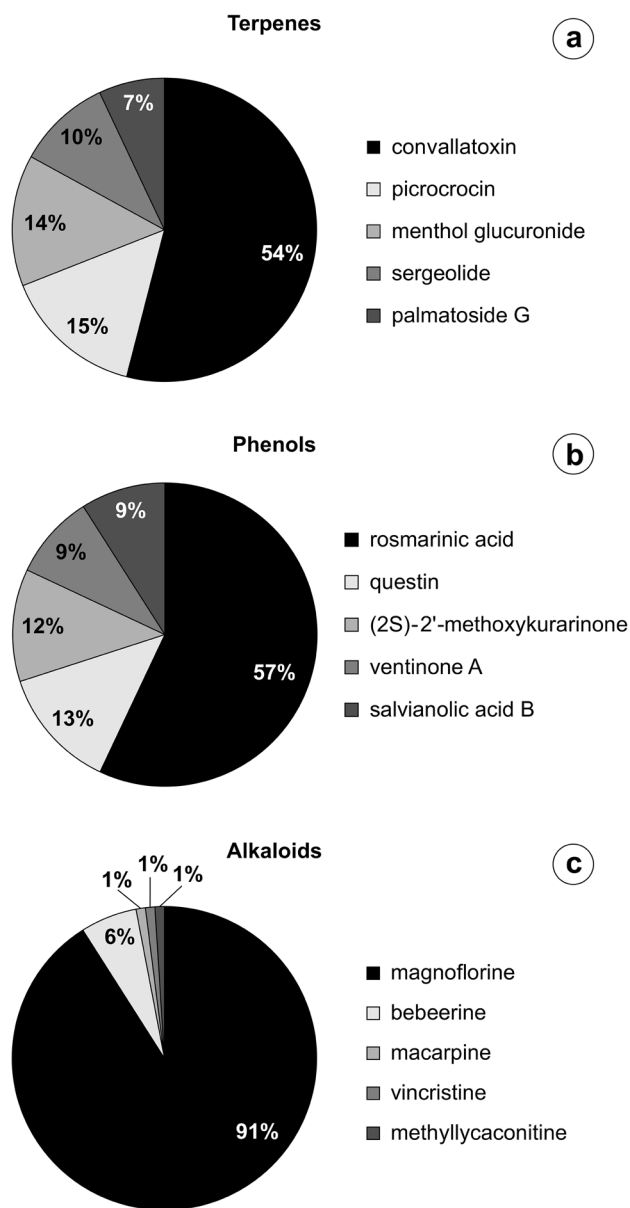


significant biotechnological potential including phenols, alkaloids and terpenes.

### Developmental responses to PGRs

The best results for plantlet in vitro production was obtained with GA3 (1  $\mu$ M) and BAP (6.7  $\mu$ M). Gibberellins, especially GA3, are commonly used to increase growth of aerial parts (Erland et al. 2017). Separately, BA and GA3 fulfilled their roles during the in vitro vegetative propagation of *C.*

*menthoides*, but when combined they did not achieve success. The combination of BA and GA3 inhibited shoot viability compared with GA3 and MS0 (Online Resource 2). At high concentrations a high mortality rate was observed (up to 97%), as well as the appearance of oxidated callus (Online Resource 2). Musembi et al. (2015) also reported that cytokinins and gibberellins are known to interact negatively. On the other hand, a higher plant viability rate was observed when cultured in media with GA3 (Online Resource 2), although the height of the produced shoots decreased as



**Fig. 4** Proportions of the five most abundant molecules in *C. menthoides* hydrophilic extract. **a** Terpenes, **b** phenols and **c** alkaloids (figure created with Microsoft Excel)

the GA3 concentration increased (Online Resource 2). In an experiment with *Mentha arvensis* under GA3 treatment, Bose et al. (2013) found similar results. The increase in plant height for treatments with gibberellic acid in plants of the *Lamiaceae* family was also previously reported (Haider et al. 2009).

A stimulatory effect of BA on bud break and multiple shoot formation was observed. The percentage of multiple shoots increased with the increase in the concentration of BA from 2.2 to 8.8  $\mu\text{M}$  (multiple shoots) (Online Resource 2). Fracaro and Echeverrigaray (2001) found a similar high

rate of multiple shoot formation for *C. galioides* with 8.8  $\mu\text{M}$  BA. Nonetheless, 0.44 and 17.6  $\mu\text{M}$  of BA suppressed the development, indicating there is an optimum range for the regeneration of *C. menthoides* (Table 1). Similar results were reported by Agostini and Echeverrigaray (2006).

Another cytokinin (TDZ) was tested with the same concentrations used with BA. According to growth analyzes, no statistical difference was found among the explants treated with six concentrations of TDZ and the MS control (Online Resource 1). After the fifth week, the beginning of mortality was noticed (Online Resource 1). Based on results from other members of the *Lamiaceae* family, the biological activity of TDZ is known to be generally higher than that of adenin-type cytokinins. Low concentrations of TDZ produce significant effects and high concentrations may have inhibitory effects (Bhattacharyya et al. 2018). Most other studies used lower concentrations than those used in this study. Pourebad et al. (2015) used the same lower concentrations and found similar results in *Lallemantia iberica*, reporting no significant difference on shoot production with the 0.22 and 0.44  $\mu\text{M}$  TDZ concentrations compared to control (MS0). Among the two cytokinins tested (BA and TDZ), the best response for plant growth was obtained in the presence of BA, so this cytokinin was chosen for further tests, combining it with other PGRs (Table 1, Online Resource 1).

Usually, for the success of in vitro shoot proliferation of the *Lamiaceae* family, it is effective to use a combination of PGRs, like cytokinin-auxin or cytokinin-gibberellin, to regulate the plant growth (Evans et al. 1981). However, in our results, combinations did not further improve the regeneration capacity of the explant; instead, it resulted in adverse effects, not optimizing all the parameters evaluated. Similar findings have also been reported with auxins/cytokinins combinations in *C. galioides* (Fracaro and Echeverrigaray 2001) and *C. incisa* (Agostini and Echeverrigaray 2006).

Auxin gave rise to oxidated callus at the basal ends of the plantlets. This callus formation was abundant and undesired, being incompetent to support efficient in vitro shoot proliferation (Online Resource 2). Consequently, this PGR failed to produce further shoot regeneration. Karam et al. (2003) observed 100% of callus induction in a medium containing TDZ and IAA, either singly or in combination in *Salvia* species.

## Metabolome

The vast majority of *Cunila* spp., phytochemical studies to date used steam distillation extraction and GC and GC–MS analytical techniques, with two studies restricted only to its essential oil. Here, a hydrophilic extraction followed by MS analysis produced a comprehensive list of compounds from a species poorly characterized before. Several groups

of compounds have been identified here, and in particular phenols and terpenes.

Results presented here show that *C. menthoides* is rich in a wide variety of phenols compounds that can be used as antioxidants or as pro-oxidants (Quideau et al. 2011) adding further to its potential as a source of valuable natural substances for healthcare. Several of the phenols detected in the present work have been previously detected in other *Lamiaceae* species: rosmarinic acid, lithospermic acid (519.0935 m/z,  $[M - H_2O - H]^-$ ), caffeic acid (179.0351 m/z,  $[M - H]^-$ ), ferulic acid (239.056 m/z,  $[M + FA - H]^-$ ), and derivatives from luteolin (669.1271 m/z,  $[M + FA - H]^-$ ), kaempferol (541.0298 m/z,  $[M - H]^-$ ) and quercetin (487.0900 m/z,  $[M - H_2O - H]^-$  and 711.2181 m/z,  $[M - H]^-$ ,  $[M + FA - H]^-$ ) (Online Resource 3) (Mišić et al. 2015; Scognamiglio et al. 2015).

Probably the most significant phenol identified here was rosmarinic acid. It is a major phenolic compound contained in the tissues of several plant species belonging to the *Lamiaceae*. Its presence is associated with antioxidant activity, and has been related to anticancer, neuroprotective, antiatherogenic, antibacterial, antiviral, antidiabetic and other properties (Link et al. 2010; Stansbury 2014; Gavarić et al. 2015; Kim et al. 2015).

SAB was also one of the most abundant metabolites detected in the present work. It is known to have strong pharmacological activities and has been used to treat cardiovascular diseases (Huang et al. 2015), ameliorate hepatic fibrosis (Xu et al. 2012), inhibit oxidative stress (Tang et al. 2014) and prevent cancer (Wang et al. 2013).

Despite the widespread occurrence of flavonoids in *Lamiaceae*, there are only two reports of their occurrence in *Cunila* species (Delgado et al. 1989; Bordignon et al. 2003). Although ten different phenol compounds have been previously identified by Bordignon et al. (2003), none of them has been detected in our work, so all phenols identified here are new for *Cunila* spp.

According to Bordignon et al. (1997), the main terpenes in the essential oil of *C. menthoides* are the monoterpenes isomenthone, menthone and pulegone. However, Agostini et al. (2010) investigated essential oils from four populations of *C. menthoides*, and has found mainly pulegone and linalool, and low concentrations of isomenthone. Here we did not identify the terpenoids menthone, isomenthone or pulegone. We identified conjugate/derivate molecules in HE from linalool: linalool 3,6-oxide 6-*O*-xylopyranosylglucopyranoside (509.2242 m/z,  $[M - H]$ ,  $[M + FA - H]^-$ ) and linalool-3-rutinoside (461.2389 m/z,  $[M - H]^-$ ). Linalool has substantial commercial value, as it has antimicrobial, anti-inflammatory, anticancer, anti-oxidant properties and several in vivo studies have confirmed various effects of linalool on the central nervous system (Kamatou and Vijojoen 2008). Besides linalool derivatives, several

terpenoids were identified here for the first time, including convallatoxin, with the highest ion intensity, and the antimalarial quassinoid sergeolide (Online Resource 3). Convallatoxin (Fig. 4 and Online Resource 3), a cardenolide with one l-rhamnose sugar, has been investigated in vitro against different cancer cells, with promising results (Schneider et al. 2017). Sergeolide has a proven strong antiplasmodial activity in vitro and in vivo (Fandeur et al. 1985), adding potential commercial value.

Besides the molecules cited above, magnoflorine, the most abundant molecule in the hydrophilic extract, can justify *C. menthoides* popular use and potential future applications as an important bioproduct. This quaternary benzyloquinoline alkaloid has been related to diverse pharmacological properties that include potential anti-diabetic, anti-inflammatory, antimicrobial, antitumor, sedative, and anxiolytic applications (Morris and Facchini 2016). The anthraquinone question, one of the ten major metabolites detected here, has been positively evaluated as a preventive agent for Alzheimer's disease (Fig. 4b) (Jung et al. 2016).

In conclusion, this study included two distinct sections. The first was an establishment of in vitro culture and efficient plant regeneration protocol as a crucial initial step for an ex situ conservation strategy. In this work we developed various supplemented media and obtained the best developed plantlets in medium supplemented with GA3. The higher viability rate (twice, compared to plants treated with BA) observed in this medium demonstrated that gibberellin interacted better with *C. menthoides* than other PGRs. It is valuable for enhancing the clonal propagation and the productivity for application in the pharmaceutical, food and cosmetic industries, besides the ex situ conservation of this species.

The second section included an extraction and analysis of the metabolome of *C. menthoides* using UHPLC–ESI–MS<sup>E</sup>. The method was established to produce a comprehensive list of compounds and investigate the presence and relative abundance of phenols, alkaloids and terpenes not yet described. The present work is the first metabolome characterization of this endangered neotropical medicinal plant. Using this method, 107 compounds were identified, including several highly abundant and interesting molecules: magnoflorine, rosmarinic acid, convallatoxin, question, ventinone A, methoxykuraridinone and salvianolic acid. This study shows that *C. menthoides* differs from other members of its genus in South America by the presence of flavones and flavanones not described to date, cinnamic and hydroxycinnamic acids and alkaloids. For the first time, we describe the presence of specific compounds with potential biotechnological application- giving further motivation to preserve it.

Studies such as this have the potential to explore and unravel biodiversity of native plants, and future work will focus on the effects of PGRs on the metabolome of



*C. menthoides* in vitro and improving of yield for desired compounds.

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**Authors contribution** JPSO—performed tissue culture, extraction, identification and acquisition experiments; analysed data; prepared all figures and wrote the manuscript. OH—advise; helped in experimental design; manuscript correction and final approval. MM—advise for compounds identification; manuscript correction and final approval. MGBK—advise; helped in experimental design; planned and performed extraction experiments funding of the project; manuscript correction and final approval. MSLF—planned and performed experiments of sample acquisition for metabolomics; helped in experimental design; project's funding; manuscript correction and final approval. LCC—mass spectrometry equipments funding and final approval. AFM—planned and performed experiments of plant material cultivation and extraction assays; helped in sample preparation for metabolomics, sample acquisition for metabolomics, compounds identification; project's funding; manuscript correction and final approval.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interests regarding the publication of this paper.

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