

Chemodiversity and trypanocidal activity of the essential oils of tropical black sage

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Research Article

Keywords: Varronia curassavica, Cordia verbenacea, volatile, genetic resources, chemical diversity.

Posted Date: February 6th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3843210/v1>

Abstract

Tropical black sage (*Varronia curassavica* Jacq.) is a species native to ecosystems subject to strong anthropic action, and thus is at risk of genetic erosion. However, little is known about this important genetic resource, with possible uses in the aromas, fragrances and pharmaceutical sectors. The objective of this work was to prospect and study native specimens of tropical black sage regarding the concentration, chemical profile and trypanocidal activity of the essential oils (EOs). For this purpose, we collected *in situ* 47 specimens. The EOs were obtained by distillation from leaves, quantified, and analyzed by GC-FID and GC-MS. The resulting data were submitted to descriptive statistical and multivariate analyses. The concentration of EOs ranged from 0.3 to 4.2% based on dry leaves. The EOs had average contents of 33.4 and 44.9% of oxygenated and non-oxygenated sesquiterpenes, respectively. The concentration of the substance α -humulene varied from 0.3 to 11.1%, with frequency of 100%. Cluster analysis indicated the formation of 10 groups with 50% similarity among the EOs samples. The sample with the best trypanocidal activity was ESB45, with $IC_{50\%}$ of 74.6 $\mu\text{g/mL}$ and 100% inhibition of the viability of the epimastigote form of *Trypanosoma cruzi* at the concentration of 300 $\mu\text{g/mL}$. Tropical black sage is a source of oxygenated sesquiterpenes, an important chemical class for synthesis and development of medicines. For this reason, efforts are necessary to preserve the genetic information about the species.

1 INTRODUCTION

Restinga is common ecosystem in the Atlantic Forest biome, with specific morphoclimatic conditions due to marine and estuarine influences, causing the formation of habitats with great diversity (Marques et al., 2011). It is estimated that some 370 species of plants are endemic to restinga, many needing scientific analysis (Costa et al., 2018; Zappi et al., 2015). The expanding urbanization of the Brazilian coastline, including irregular and disorderly occupation of areas reserved for permanent preservation, such as vegetation near beaches, rivers and wetlands, is causing genetic erosion of sensitive species such as tropical black sage (Cunha et al., 2020; Dos Santos and Pedroso Junior, 2021; Ribeiro et al., 2011)

Tropical black sage [*Varronia curassavica* Jacq. (Cordiaceae) synonyms: *Cordia verbenacea* DC.; *Cordia curassavica* (Jacq.) Roem. & Schult] is a shrub species with a strong odor, similar to chicken seasoning. The essential oils of tropical black sage (henceforth just “black sage”) have been traditionally used to treat muscle pains, although there are reports of their use for treatment of rheumatism and ulcers, and as antiallergics and analgesics, among others (Bristot et al., 2021; El Toghlobi et al., 2022). Besides this, these essential oils have toxic activity against various insects, mites, protozoa and microorganisms (Carvalho Castro et al., 2019; Hernández et al., 2014; Oliveira et al., 2019; Pereira et al., 2021).

Based on a public-private partnership, a phytotherapeutic product developed integrally in Brazil was launched utilizing an essential oil of black sage at a concentration of 5.0 mg/g (cream or spray), equivalent to 0.130 mg of α -humulene, a marker used in the quality control of the active principal (Ache,

2023; Calixto, 2005), with anti-inflammatory action. However, other studies have also found analgesic and antiallergic action of the sesquiterpene β -caryophyllene (Bakir et al., 2008; Fernandes et al., 2007).

In light of the importance of the essential oils from black sage for the production of pharmaceuticals and aromas/fragrances, we report here the chemical diversity of these oils, obtained from leaves collected *in situ*. We also investigated the toxic activity of the oils against the epimastigote form of *Trypanosoma cruzi*, to demonstrate the biotechnological potential and opportunities that can be explored from this Brazilian native plant, to highlight the need for preservation.

2. EXPERIMENTAL

2.1 Regularization of the Study.

We enrolled this study with the National System for Management of Genetic Heritage and Associated Traditional Knowledge – SisGen (code A6985DE) and requested authorization for the field collection activities from the Rio de Janeiro State Environmental Institute – INEA (authorization 046/2020, SEI-070002/006020/2020, verification code 8762851, CRC 0AE0F68F) and the Chico Mendes Institute for Conservation of Biodiversity – ICMBio (authorization code 75906-1, 13/10/2020; 0759060120201013).

2.2 Prospection and Collection of Specimens.

We initially defined the collection places based on a cursory analysis of the terrain and vegetation using the Google Earth application. The collection periods were defined in the months of October and November, as follows: **Collection A** – 16 plants between the municipalities of Armação de Búzios and Cabo Frio in the state of Rio de Janeiro, on October 6, 2017; **Collection B** – 8 plants from the Vitória Metropolitan Region of the state of Espírito Santo, in the coastal area of the municipalities between Guapimirim and Fundão, on October 23, 2018; **Collection C** – 12 plants in the North Coast Region, municipalities from Rio das Ostras to Campos dos Goytacazes, in the state of Rio de Janeiro, on October 23, 2020; and **Collection D** – 12 plants in the Coastal Lowlands Region, between the municipalities of Maricá and Cabo Frio, in the state of Rio de Janeiro, on November 27, 2020 (Fig. 1).

All the materials collected (branches containing leaves, inflorescences and fruits) were in the vegetative/reproductive stage and received an identification code, as follows: ES or RJ (respectively denoting Espírito Santo and Rio de Janeiro, the states where the collection occurred), followed by the letter A, B, C or D (representing the respective collection dates of October 6, 2017, October 23, 2018, October 23, 2020 and November 27, 2020), and a number between 1 and 48 (identifying the particular plant sampled in the field). All the plants collected were georeferenced and the information is available in the supplementary material (**Table S1**).

2.3 Stabilization and Registration of the Plant Material.

The branches containing flowers and/or fruits were taken to the herbarium of UFRRJ, for preparation of exsicates, registration and obtainment of listing numbers (**Table S1**). The leaves used for extraction of

the essential oils were dried in a forced-air oven (model Solab/SL 102) in paper bags for 48 hours as 36°C. The average moisture after drying varied from 13 to 17%. The dried materials were placed in paper bags protected from light and moisture until the moment of essential oil extraction.

2.4 Distillation and Content (% w/w) of the Essential Oils.

The distillation of the essential oils was carried out in the Laboratory of Aromatic and Medicinal Plants of Rio de Janeiro Federal Rural University, located in the city of Seropédica. The potential oils were obtained by hydrodistillation using a modified Clevenger apparatus, with 30 g of dried leaf material (without grinding) in a round-bottom flask (1 L) containing 400 mL of distilled water. The distillation time was 2 hours, with a constant steam flow of 3 mL/min (Nascimento et al., 2020). The essential oils were separated by phase difference and a small quantity of dichloromethane was used to wash and recover all the essential oil remaining in each flask. Then the mixture was filtered and dried with anhydrous sodium sulfate and the solvent was eliminated under constant nitrogen gas flow.

The concentration (% w/w) of essential oil was calculated based on the dried leaf matter (DLM). A few minutes before distillation, a 2 g sample of the dried leaves of each of the plants was submitted to total drying in an oven until reaching constant weight, determined by an analytic balance (Unibloc AUW-320, Shimadzu). The weight of the water of each sample was subtracted to obtain the DLM. The essential oil content (EOC) was calculated by the equation $EOC = wOE \times 100 / DLM$, where: DLM is the dry leaf material used in the distillation (g); and wOE is the essential oil weight (g).

2.5 Sampling and analysis by GC-FID and GC-MS

The samples were prepared by diluting each essential oil sample in absolute ethanol at concentration of 1.0% (m/v). Then, 1.0 µL of each sample was injected in a gas chromatograph (5890 Series II, Hewlett-Packard, USA) equipped with a flame ionization detector (GC-FID) operating in split mode (1:20), to separate and quantify the constituents present in the essential oils. The substances found were separated in a fused silica capillary column, with stationary phase consisting of 5% phenyl and 95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm ID). The carrier gas was helium, at a flow of 1.0 mL/min. The temperature program of the column was 50°C for 5 min, followed by an increase of 3°C/min until 120°C, and then a further increase of 5°C/min until 250°C, and finally an increase of 15°C/min to 290°C, which was maintained for 20 min. The temperatures of the injector and detector were 250 and 290°C, respectively. The same sample and volume (1.0 µL) were injected into a gas chromatograph-mass spectrometer (GC-MS) QP-2010 Plus (Shimadzu, Japan), used to separate and identify the substances composing the essential oil. The column and program were the same as used in the GC-FID analysis. The injector and interface temperatures were 220 and 250 °C, respectively. The mass spectrum was obtained in a quadrupole detector operating at 70 eV, with mass interval between 40 and 4100 m/z and ratio of 0.5 scan/s. The substances found in the essential oil were quantified based on the area under the GC-FID peaks and converted into percent values. The identification of each substance in the essential oil was performed based on the GC-MS analysis, considering the calculation of the retention index (RI), based on a homologous series of n-alkanes (C7-C30) injected in the same condition as the sample. The data were

processed with the GC-MS Solution v.2.53 software (Shimadzu), and each substance in the essential oil sample was identified by comparison of the mass spectra against a database (NIST, 2008) and the literature (Adams, 2007; Sciarrone et al., 2017).

2.6 Construction of the Data Matrix Referring to the Essential Oils.

Based on the plants collected along the coastline of Rio de Janeiro and Espírito Santo, we organized a data matrix with 47 essential oils from those plants (independent variables – rows) and 42 chemical substances identified in all the essential oil samples evaluated (dependent variables – columns). We proposed some restrictions with the goal of emphasizing the dependent variables with greatest contribution to the total variance of the data, namely the substances with concentration greater than or equal to 9% in at least one of the essential oils and/or frequency ($f\%$) higher than 50%, with removal of the compounds co-eluted and those not identified. The matrix was composed of percent values of the areas under each peak (substances) in the chromatograms of the essential oils (Table S2).

2.7 Proposed Characterization of Chemotypes (CT)

For the purpose of standardizing the denomination of chemotypes, we propose a classification key that considers the chemical dominance among the substances present with highest concentration in an essential oil sample, based on dominance of one (A), two (A/B) or three (A/B/C) majority substances in relation to the others, according to the following key:

1st) CT: A, if $A \geq 25\%$ and $A \geq 2B$;

2nd) CT: A/B, if $B \geq 15\%$ and $B \geq 2C$;

3rd) CT: A/B/C, if $C \geq 10\%$ and $C \geq 2D$;

4th) CT: undefined or mixed, if none of the previous options are satisfied.

Remark

We only considered integral values without rounding.

2.8 Trypanocidal Activity

The antiparasitic test was performed against the epimastigote form of the protozoan *Trypanosoma cruzi* (strain Tulahuen C2C4-LacZ) at the Laboratory of Veterinary Immunology and Virology of UFRRJ. For the treatments, we used essential oils representing the groups formed by hierarchical cluster analysis, namely: RJA38 from Group 2 (G2); RJC23 representing Group 7 (G7); ESB45 from Group 9 (G9); RJC18 from Group 10 (G10); and ESB44 from Group 13 (G13).

The parasites in epimastigote form were incubated for 7 days in a transparent 96-well plate at concentration of 1×10^6 parasites/mL in LIT medium supplemented with hemin (25 mg/L) and 10% fetal

bovine serum, at a final volume of 150 µL/well. The treatments were administered with serial dilutions of the essential oils (concentrations of 19.2; 48; 120 and 300 µg/mL), with benznidazol (BZD) as the reference drug. Parasites without treatment (live control), treated with 0.5% v/v DMSO (vehicle) and samples without parasites (blank control) served as experimental controls. After the incubation period, the plate was centrifuged (1800 rpm for 10 min at 4°C) and the supernatant from each well was removed, followed by addition of 100 µL of PBS solution, and then 30 µL (0.5 mM) of the red substrate chlorophenol β-galactopyranoside (CPRG) in PBS, with 0.9% v/v Igepal CA-630. After incubation for 1.5 h, the absorbance was measured at $\lambda = 570$ nm in a plate reader. The entire experiment was conducted in triplicate. The absorbance data were converted into viability values relative to the average of the vehicle control, minus the average of the blank control, according to the formula $V(\%) = (Abs-MB)/(MV-MB)$, where $V(\%)$ = viability in %, Abs = absorbance reading in each well, MB = mean absorbance of the triplicates of the blank control, and MV = mean absorbance of the triplicate of the vehicle control.

2.9 Statistical Analyses.

2.9.1 Descriptive Statistics and Univariate Analysis.

The minimum, maximum, mean and frequency related to the concentrations and chemical characterization of the essential oils were organized and are presented in tables and boxplots. For visual analysis, we also prepared colored donut charts and chemoarrays, also in the form of donuts, as described by Alves et al. (2019), Singh et al. (2005) and Gimenes et al. (2021), considering the 14 substances with highest concentration in the data matrix. These tables and graphs were prepared using Microsoft Excel and GraphPad Prism 9.

The concentration inhibiting the activity of the epimastigote form of the protozoan *T. cruzi* by 50% ($IC_{50\%}$) was calculated based on a nonlinear regression model (concentration of inhibitor versus normalized slope response variable) and the $IC_{50\%}$ values obtained from the mean of at least three independent experiments. To test the statistical significance, we used one-way analysis of variance (ANOVA) and the Tukey test of the means, at 5% significance. This step was performed using the GraphPad Prism 9 software.

2.9.2 Multivariate Analysis .

The matrices were submitted to the unweighted pair group method with arithmetic mean (UPGMA) and Pearson correlation as distance to construct dendrograms. To verify the goodness of fit between the phenetic and cophenetic matrices, we calculated the cophenetic correlation coefficient (Sokal & Rohlf, 1962), with the optimal number of groups of the dendrogram being defined as 50% similarity between the samples. The hierarchical grouping describe above was performed with the Origin v.2022b software (OriginLab Corporation, Northampton, MA, USA).

3 RESULTS AND DISCUSSION

3.1 Content of the Essential Oil.

We collected 48 specimens of black sage in their natural environment (Fig. 1), duly identified, georeferenced and registered with the herbarium of UFRRJ (**Table S1**). One of the samples was lost after the initial steps (RJD10), leaving 47 that were used to extract the essential oils by hydrodistillation. The values were converted into concentration (% m/m) based on dry matter (Table 1).

Table 1
Description of the concentration (%) of the essential oils obtained from the leaves of
specimens of tropical black sage collected in different places.

Region/State	Municipality	ID	Concentration (%)
Coastal Lowlands Region/ RJ	Maricá	RJD01	3.7
		RJD02	1.0
		RJD03	2.1
		RJD04	1.9
	Saquarema	RJD05	3.6
		RJD06	3.9
	Arraial do Cabo	RJD07	0.4
		RJD08	2.4
		RJD09	4.2
	Cabo Frio ^a	RJD10	-
		RJD11	1.7
		RJD12	3.4
	Armação de Búzios	RJA33	0.5
		RJA34	0.7
		RJA25	0.3
		RJA26	2.6
		RJA27	0.5
		RJA28	1.2
		RJA29	0.6
		RJA30	0.8
		RJA31	0.7
		RJA32	1.7
		RJA35	0.7
		RJA36	0.4
		RJA37	0.8

a – the essential oil was distilled by the data were lost.

Region/State	Municipality	ID	Concentration (%)
		RJA38	1.4
		RJA39	1.1
		RJA40	0.8
	Rio das Ostras	RJC13	1.1
		RJC14	1.1
		RJC15	0.5
		RJC16	1.1
North Coast Region/ RJ	Macaé	RJC17	1.4
		RJC18	2.0
	Carapebus	RJC19	1.2
	Quissamã	RJC20	1.6
		RJC21	1.5
	Campos dos Goytacazes	RJC22	2.4
		RJC23	1.8
		RJC24	4.2
Vitória Metropolitan Region/ ES	Vila Velha	ESB41	3.9
		ESB42	0.7
	Guarapari	ESB43	2.7
		ESB44	1.7
		ESB45	1.5
	Serra	ESB46	0.5
		ESB47	0.9
	Fundão	ESB48	1.9
a – the essential oil was distilled by the data were lost.			

We found variation from 0.3 to 4.2% in the concentration of the essential oils of the 47 black sage specimens. The mean and median values were 1.6 and 1.4%, respectively (Fig. 2). Specimens RJD09 from Arraial do Cabo (Coastal Lowlands Region) and RJC24 from Campos dos Goytacazes (North Coast Region), both in the state of Rio de Janeiro, contained the highest essential oil concentration (4.2%), while

the minimum value was 0.3% in specimen RJA25, collected in Armação de Búzios (Coastal Lowlands Region), also in the state of Rio de Janeiro (Table 1).

3.2 Chemical Profile, Diversity and Classification of the Essential Oils.

The essential oils of the 47 black sage specimens were analyzed by GC-MS and GC-FID for the purpose of chemically characterizing them and defining the concentrations (%) of the substances in the respective essential oils. A table reporting the chemical analysis of the substances with greater relevance in this study can be consulted in the supplementary material (**Table S2**). With the objective of summarizing and facilitating observation of the data obtained from the 47 samples, we prepared a table with information on the main substances found in the essential oils in terms of frequency, mean and median concentration (%) as well as the interval containing the lowest and highest concentrations observed (%) (Table 2).

Table 2

Description of the chemical profile of the essential oils of the leaves of tropical black sage specimens employing GC-FID and GC-MS.

Substances ^a	f ^b	Min	Max	Mean	Median
	————— % —————				
Tricyclene	30	1.3	17.8	8.1	7.4
Thujene < α->	49	0.3	9.2	2.9	2.0
Pinene < α->	83	0.8	23.1	6.9	7.1
Sabinene	62	0.2	2.5	0.9	0.9
Pinene < β->	51	0.2	4.4	1.8	1.3
Myrcene	57	0.2	1.1	0.5	0.5
1,8-Cineole	68	0.2	3.1	1.4	1.1
Bornyl Acetate	68	0.3	1.4	0.7	0.7
Elemene < β->	66	0.1	6.1	0.9	0.5
Sesquithujene	51	0.4	3.5	2.3	2.4
Bergamotene <(Z)-α>	53	0.3	3.5	1.9	1.8
Santalene < α->	47	5.2	22.0	13.4	13.8
Caryophyllene < β->	74	0.4	21.8	9.0	7.6
Bergamotene <(E)-α>	60	0.1	6.2	1.4	0.3
Farnesene <(Z)-β->	51	0.3	1.8	0.6	0.5
Humulene < α->	100	0.3	11.1	2.4	1.9
Santalene < β->	62	0.4	5.0	2.0	1.5
Caryophyllene < 9-epi-(E)->	19	0.8	9.6	3.5	3.1
Dauca-5,8-diene	26	0.6	13.0	6.3	6.5
Germacrene D	94	0.2	13.4	3.0	2.3
Bicyclogermacrene	47	0.3	12.2	2.9	1.3
Bisabolene < β->	70	0.2	7.6	3.8	4.5
Cadinene < γ->	55	0.3	13.4	4.0	1.6

a – The table presents the 42 substances that contributed the most to the total variation of the data observed based on the concentration ($\geq 9\%$ in at least one of the essential oils) and/or frequency ($\geq 50\%$). b – frequency (%).

Substances ^a	f ^b	Min	Max	Mean	Median
Cubebol	11	0.8	12.3	5.2	3.5
Sesquiphellandrene < β->	64	0.3	1.6	0.9	0.9
Elemol	13	0.2	16.7	3.1	0.3
Nerolidol <(E)->	55	0.2	1.8	0.7	0.5
Germacrene D-4-ol	28	0.4	17.3	7.2	7.3
Caryophyllene oxide	94	0.2	11.8	2.2	1.4
Carotol	23	0.3	9.1	6.0	7.2
Humulene epoxide II	53	0.2	3.1	0.6	0.3
Cubenol < 1,10-di-epi->	36	0.2	20.0	7.7	8.7
Santalol <(Z)-α->	51	0.4	3.0	1.0	0.8
Santalal <(E)-α->	45	0.1	10.7	5.9	5.9
Farnesol < 2,3-dihydro->	13	0.2	23.7	4.5	0.8
Bisabolol < α->	17	0.5	24.3	4.9	1.7
Bisabolol < epi-α->	9	1.3	9.1	3.6	1.9
Shyobunol	19	0.9	31.7	18.5	17.3
Farnesol <(2E,6Z)->	4	1.5	32.9	17.2	17.2
Farnesol <(2Z,6E)->	6	2.0	15.5	7.1	3.9
Santalol acetate <(Z)-α->	43	0.3	30.3	2.4	0.9
Methyl farnesoate <(2E,6E)->	13	0.4	23.1	5.1	0.9
a – The table presents the 42 substances that contributed the most to the total variation of the data observed based on the concentration (≥ 9% in at least one of the essential oils) and/or frequency (≥ 50%). b – frequency (%).					

The substances with the highest frequencies in the black sage essential oils were α-humulene with 100% (0.3–11.1%) frequency, followed by germacrene D (0.2–13.4%) and caryophyllene oxide (0.2–11.8%), both with 94%, α-pinene with 83% (0.8–23.1%), β-caryophyllene with 74% (0.4–21.8%) and β-bisabolene with 70% (0.2–7.6%) frequency (Table 2). The substances with highest concentration in the essential oils were (2E,6Z)-farnesol (32.9%), shyobunol (31.7%), (Z)-α-santalol acetate (30.0%), α-bisabolol (24.0%), 2,3-dihydro-farnesol (23.7%), α-pinene (23.0%), (2E,6E)-methyl farnesoate (23.1%) and α-santalene (22.0%) (Table 2).

The chemical analysis of the samples revealed that the predominant terpenoid classes were: sesquiterpene hydrocarbons, with average of 44.9% (17.3–69.0%), followed by oxygenated sesquiterpenes with average of 33.4% (1.9–66.2%) and monoterpene hydrocarbons with average of 12.5% (0–46.7%), while the lowest was oxygenated monoterpenes, with average of 2.3% (0–13.0%) (Fig. 3).

The chromatographic analyses of the essential oil samples allowed constructing a data matrix (47 essential oils and 42 substances), which was submitted to hierarchical cluster analysis (Fig. 4). The cophenetic correlation was 0.8544 and we considered 50% similarity as ideal for the formation of distinct groups, resulting in the formation of 10 groups: group 1 with RJD01; group 2 (the largest), with RJD02, RJD03, RJA32, RJA33, RJA35, RJA39, RJA25, RJA36, RJA37, RJD12, RJA38, RJA29, RJA34, RJD11; RJD04, RJD05 and RJD09; group 3 with RJD08; group 4 with RJD06, RJA30, RJA27, ESB43, RJC15, RJD07, ESB46 and ESB47; group 5 with RJC19, RJC20, RJA31, RJC23 and RJA28; group 6 with RJA40; group 7 with RJC22; group 8 with ESB41, ESB48, ESB42 and ESB44; group 9 with ESB45; and group 10 with RJC13, RJC14, RJC17, RJC18, RJC21, RJC24, RJA26 and RJC16 (Fig. 4).

Based on interpretation of the donut charts constructed according to the modifications proposed by Alves et al. (2019) and Singh et al. (2005), it was possible to observe with greater clarity the contribution of the 14 substances with highest concentrations in the essential oils for the formation of the groups in the dendrogram (Fig. 4). All told, there were 10 groups composed of different substances, considering 50% similarity.

In general, the essential oils of groups 1, 2 and 3 had the substance α -santalene (5.2–22.0) in common. Specifically, these were α -bisabolol (24.3%) and tricyclene (8.1%) in group 1; α -pinene (1.2–12.0%), 1,10-di-epi-cubenol (0.8–15.4%) and tricyclene (1.3–17.8%) in group 2; and (Z)- α -santalol acetate (30.3%) and α -pinene (13.1%) in group 3. On the other hand, β -caryophyllene (1.4–21.8%) was the substance in common with the essential oils of groups 4, 5, 6, 7 and 8. However, each of these groups also contained other substances in common, such as α -pinene (3.0–23.1%) in group 4; 1,10-di-epi-cubenol (9.3–20.0%); α -pinene (1.3–10.7%) in group 5; 2,3-dihydro-farnesol (23.7%) and tricyclene (8.6%) in group 6; elemol (16.7%) in group 7; and α -pinene (1.4–9.7%) in group 8. Group 9 stood out from the others by containing the majority substances (2E,6E)-methyl farnesoate (23.1%) and (2E,6Z)-farnesol (32.9%). Finally, the essential oils of group 10 contained the substances shyobunol (6.2–31.7%) and germacrene D-4-ol (7.3–17.3%) in common (Fig. 4).

We propose the classification of the essential oils based on the predominance of the majority substances with the greatest quantitative contributions in the chemical profiles. This proposal consists basically of applying a very simple dominance key (previously described in the methodology section), which enables including the name of up to three substances to classify the chemotype. The results are presented below (Table 3).

Table 3

Characterization of the chemotypes in function of the criteria of dominance of the majority substances present in the essential oils of tropical black sage from plants collected *in situ*.

Chemotypes	Type	ID ^a (% substance)
Shyobunol	A	RJC18 (31%)
Santalol acetate <(Z)-α->	A	RJD08 (30%)
Pinene < α->/ Caryophyllene < β->	A/B	RJD06 (23%/ 21%)
Shyobunol/ Germacrene D-4-ol	A/B	RJC17 (30%/ 17%)
Farnesol <(2E,6Z)->/ Methyl farnesoate <(2E,6E)->	A/B	ESB45 (32%/ 23%)
Shyobunol/ Cubebol/ Germacrene D-4-ol	A/B/C	RJC21 (23%/ 12%/ 11%)
Mixed	M	RJD01, RJD02, RJD03, RJD04, RJD05, RJD07, RJD09, RJD11, RJD12, RJC13, RJC14, RJC15, RJC16, RJC19, RJC20, RJC22, RJC23, RJC24, RJA25, RJA26, RJA27, RJA28, RJA29, RJA30, RJA31, RJA32, RJA33, RJA34, RJA35, RJA36, RJA37, RJA38, RJA39, RJA40, ESB41, ESB42, ESB43, ESB44, ESB46, ESB47 e ESB48
a – ID referring to Table S2.		

With application of the criteria, it was possible to confirm that the majority of the essential oils of the plants collected did not have a defined chemotype. Of the 47 chemically characterized essential oils, only six presented dominance of one, two or three substances over the others composing the respective essential oils (Table 3). We found the presence of the chemotypes shyobunol and (Z)-α-santalol acetate, with only one majority substance in the essential oils RJC18 and RJD08, respectively. We also observed the presence of chemotypes with two majority substances, in the essential oils RJD06 (pinene < α->/ caryophyllene < β->), RJC17 (shyobunol/ germacrene D-4-ol) and ESB45 (farnesol <(2E,6Z)->/ methyl farnesoate <(2E,6E)->). The essential oil of plant RJC21 was the only one containing three majority substances defining its chemotype: shyobunol/ cubebol/ germacrene D-4-ol (Table 3).

3.3 Trypanocidal Activity.

Of the 47 black sage essential oils, we prospected the trypanocidal activity of those with distinct chemical profiles, namely RJA38, RJC23, ESB45, RJC18 and ESB44 (Fig. 4), against the epimastigote

form of *T. cruzi*. To carry out the *in vitro* biological tests we exposed the protozoa to a medium containing rising concentrations (0-300µg/mL) of the essential oils (Fig. 5). This enabled estimating the concentration that inhibited the biological process by 50%, the IC_{50%} (Table 4).

Table 4

Trypanocidal activity of the essential oils of *Varronia curassavica* against epimastigote forms of *T. cruzi*.

Samples	Substances ^b	IC _{50%} ^c (µg/mL) ± SD ^d
ESB45	(2E,6Z)-Farnesol (32%), (2E,6E)-Methyl farnesoate (23%)	74.6 ± 4.8
RJC18	Shyobunol (31%)	101.4 ± 12.0
RJA38	α-Santaleno (15%), α-Pineno (10%)	> 300.0
ESB44	(2Z,6E)-Farnesol (15%)	129.6 ± 3.0
RJC23	1,10-di-epi-cubenol (14%), β-caryophyllene (11%)	> 300.0
BZN ^a	Positive control	6.4 ± 0.3
a – Reference drug benznidazole. b – Substances with greatest concentration in the essential oils. c – inhibitory concentration of the epimastigote form activity of the protozoan <i>T. cruzi</i> by 50%. d – standard deviation		

The tests confirmed that the most effective essential oils, i.e., those with IC_{50%} lower than 300 µg/mL, were ESB45, RJC18 and ESB44, with IC_{50%} values of 74.6, 101.4 and 129.6 µg/mL respectively (Table 4). We also calculated the relative viability of the epimastigote form de *T. cruzi* after exposure to the essential oils from samples ESB45, RJC18 and ESB44, i.e., those with IC_{50%} lower than 300 µg/mL. The results confirmed that ESB45 at concentration of 300 µg/mL caused zero viability, and the concentration of 118.6 µg/mL caused 80% inhibition of the epimastigote forms (Fig. 5). On the other hand, there were some viable epimastigote forms after exposure to the essential oils of samples RJC18 and ESB44 at concentration of 300 µg/mL, i.e., the inhibition of the protozoa was not total at the highest concentration tested (Fig. 5).

4 DISCUSSION

The variation in the concentration of an essential oil is only one of many quantitative factors that should be investigated to support genetic improvement programs seeking to obtain specimens with greater agronomic value (Jannuzzi et al., 2011, 2010). However, quantitative factors such as biomass production and the effect of essential oil exposure should not be evaluated in isolation. It is necessary to determine high performance by aggregating qualitative aspects such as the chemical profile of the essential oil, as well as the stability of the profile independently of the season of the year (Soares et al., 2019) or another edaphoclimatic variable.

In this work, we observed variations from 0.3 to 4.2% in the essential oils' concentration, with average of 1.6% in the 47 accessions of black sage studied (Fig. 2 and Table 1). We found various reports in the literature indicating a wide range of essential oil contents. For example, there were scientific reports of black sage plants containing concentrations from 0.1 to 0.2% (Carvalho et al., 2004; Santos et al., 2013) and 3.3% (Nizio et al., 2018). Other authors have reported intermediate values, such as 1.2% (Queiroz et al., 2016), 1.3% (Alves et al., 2015a) and 1.8% (Carvalho et al., 2017), near the average found by us.

The surviving Atlantic Forest areas are noteworthy for their high floristic diversity and potential, which need to be studied and reported in benefit to society and for preservation and valorization of this biome. We conducted an *in situ* study of the essential oils of black sage, a native species that served as an example to discuss the intraspecific diversity (chemodiversity) and impact on total biodiversity.

We identified and selected the 42 substances with greatest relevance regarding the essential oils from the 47 specimens of black sage investigated (**Table S2**). These substances were mainly oxygenated and non-oxygenated terpenoids (Fig. 3), a similar result to those observed by other authors regarding populations of black sage (Nizio et al., 2015; Oliveira et al., 2020; Queiroz et al., 2019). The substances found with greatest frequency were α -humulene (100% frequency) and germacrene D and caryophyllene oxide (both with 94% frequency). Nizio et al. (2015) reported that β -caryophyllene was the substance with greatest frequency (100%), while Oliveira et al. (2019) and Queiroz et al. (2019) reported 100% frequency of α -humulene and β -caryophyllene. We stress that we studied plants found along the southeastern Brazilian coastline, unlike the regions studied by the other authors mentioned above.

α -Humulene is considered a chemical marker of black sage essential oil (Gilbert & Favoreto, 2012), and its concentration varies from 0.3% (Marques et al., 2019) to 7.0% (Silva et al., 2014) according data in the literature. In the present study, plant ESB43, collected in the municipality of Guarapari-ES, contained 11% α -humulene in the essential oil, 54% higher than found previously (Nascimento, 2021). Germacrene D and caryophyllene oxide also are substances often found in black sage essential oils, ranging from 70 to 80% respectively (Nascimento, 2021).

Our results demonstrate that black sage has intraspecific diversity regarding the chemical profile of its essential oils. The multivariate analysis confirmed the presence of 10 groups with similarity of at least 50% within each group (Fig. 4). The standouts were the oils with codes RJD01, RJD08, RJA40 and ESB45, which had high levels of the oxygenated sesquiterpenes α -bisabolol (24.3%), (Z)- α -santalol acetate (30.3%), 2,3-dihydro-farnesol (23.7%) and (2E,6Z)-farnesol (32.9%) in their compositions, respectively (**Table S2**). We did not find in the literature reports of concentrations of these substances higher than 5% in black sage essential oils. The substances shyobunol, α -pinene, (2E,6E)-methyl farnesoate and α -santalene, found in the essential oils analyzed by us, have been reported in concentrations above 20% in other studies of the plant (Alves et al., 2015b; Castro et al., 2013; de Castro Nizio et al., 2018; Nizio et al., 2018).

By applying a key involving the dominance between one, two or three majority substances in the essential oils, we found that the great majority of the plants collected in natural growth conditions contained

essential oils with mixed chemical composition. All told, 41 of the 47 plants did not contain majority substances that distinguished them from the others, i.e., mixed chemotype (CT) (Table 3). On the other hand, we observed the following chemotypes: 1) shyobunol, 2) (Z)- α -santalol acetate, 3) α -pinene/ β -caryophyllene, 4) shyobunol/ germacrene D-4-ol, 5) (2E,6Z)-farnesol/ (2E,6E)-methyl farnesoate and 6) shyobunol/ cubebol/ germacrene D (Table 3).

Our results demonstrate a potentially attractive trypanocidal activity of three of the essential oils (ESB45, RJC18 and ESB44) versus the epimastigote form of *T. cruzi* (strain Tulahuen C2C4-LacZ) out of a total of six tested at the highest concentration analyzed, of 300 μ g/mL. This was chosen since it was the limit for the control DMSO not to surpass 0.5% in the culture medium (Fig. 4). These results can be related to the chemical composition of the majority components of these essential oils, with highlight on ESB45, which had the majority component (2E,6Z)-farnesol (32.9%). This was possibly directly related to the promising antiparasitic activity, since the terpene portion present in the structure is responsible for the interesting antiparasitic activities (Reis, 2017). Pereira et al. (2021) also reported activity of black sage essential oil against the protozoa *Leishmania brasiliensis* and *Trypanosoma cruzi*. However, the essential oils in this study and theirs had different chemical compositions.

5 CONCLUSION

The results reported here confirm the existence of intraspecific diversity of the populations of *Varronia curassavica* investigated along the southeastern coastline of Brazil, organized into 10 groups presenting 50% similarity in the chemical profile and six chemotypes, namely: shyobunol, (Z)- α -santalol acetate, α -pinene/ β -caryophyllene, shyobunol/germacrene D-4-ol, (2E,6Z)-farnesol/(2E,6E)-methyl farnesoate and shyobunol/cubebol/ germacrene D-4-ol. Besides this, the essential oils of some of the tropical black sage specimens have pharmacological potential for treatment of Chagas disease, which has largely been neglected by pharmaceutical companies, especially accession ESB45, which totally inhibited the viability of the amastigote form at a concentration of 300 μ g/mL, with IC_{50%} equal to 74.6 μ g/mL.

Declarations

Ethics approval –

Not applicable.

Competing interests –

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding –

This study was financed in part by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) - Finance Codes: E-26/211374/2021 and E-26/211.547/2021 and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Finance Code 001.

Authors' contributions –

Elisabeth Medeiros, Rayssa Nascimento: conceptualization, formal analysis, supervision, writing - original draft. Eduardo Junior, Camila Pereira, Ygor Moreira, Afonso Velez: investigation, formal analysis. Debora Decote-Ricardo, Marco Lima, Andre Santos, Pedro Damasceno-Junior, Marco Souza: supervision. Andre Santos, Pedro Damasceno-Junior, Marco Souza: funding acquisition, writing - review & editing. Marco Souza: conceptualization, project administration, funding acquisition, writing - review & editing.

ACKNOWLEDGEMENTS

We gratefully acknowledge support from Professor Rosane Nora Castro (IQ-UFRRJ), the Postgraduate Program in Chemistry (PPFQ-IQ-UFRRJ), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Figures

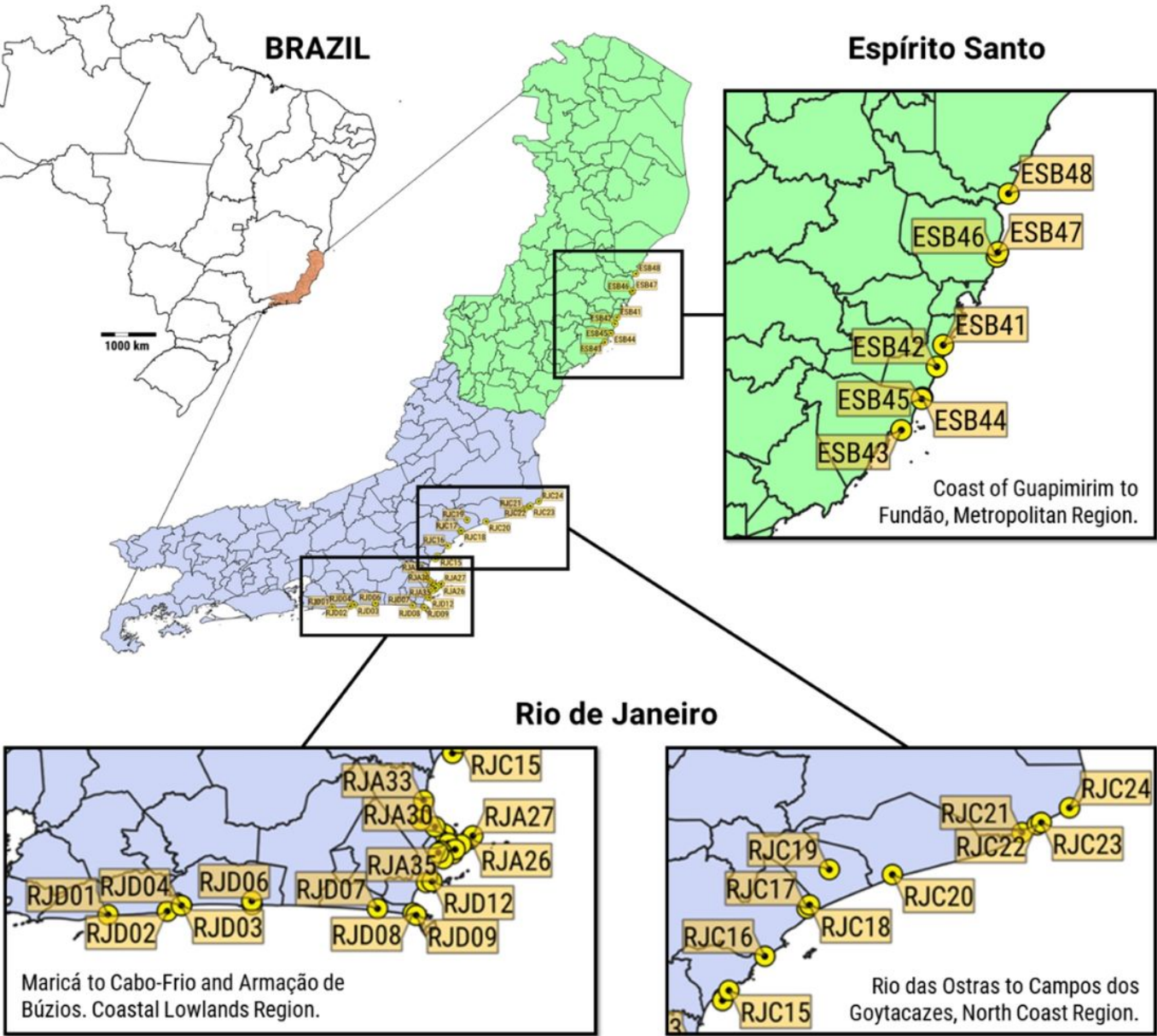


Figure 1

Collection sites: 40 plants in the state of Rio de Janeiro (28 in the Coastal Lowlands Region and 12 in the North Coast Region) and 8 plants in the state of Espirito Santo, in the Vitória Metropolitan Region. The detailed description of the geographic coordinates of each sample is presented in Table S1.

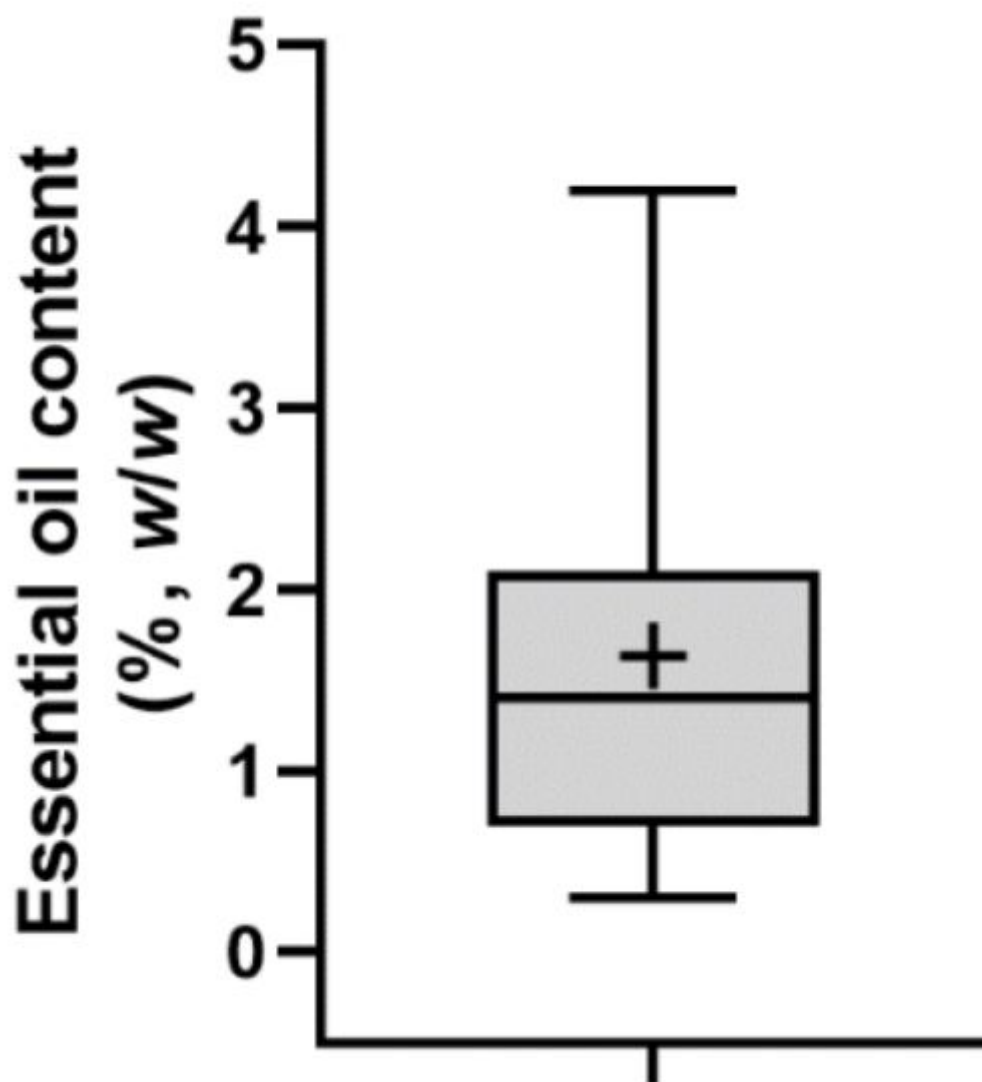


Figure 2

Concentration (%) of essential oil from the leaves of tropical black sage collected *in situ*. Boxplot (quartiles), central line (median) and cross (mean).

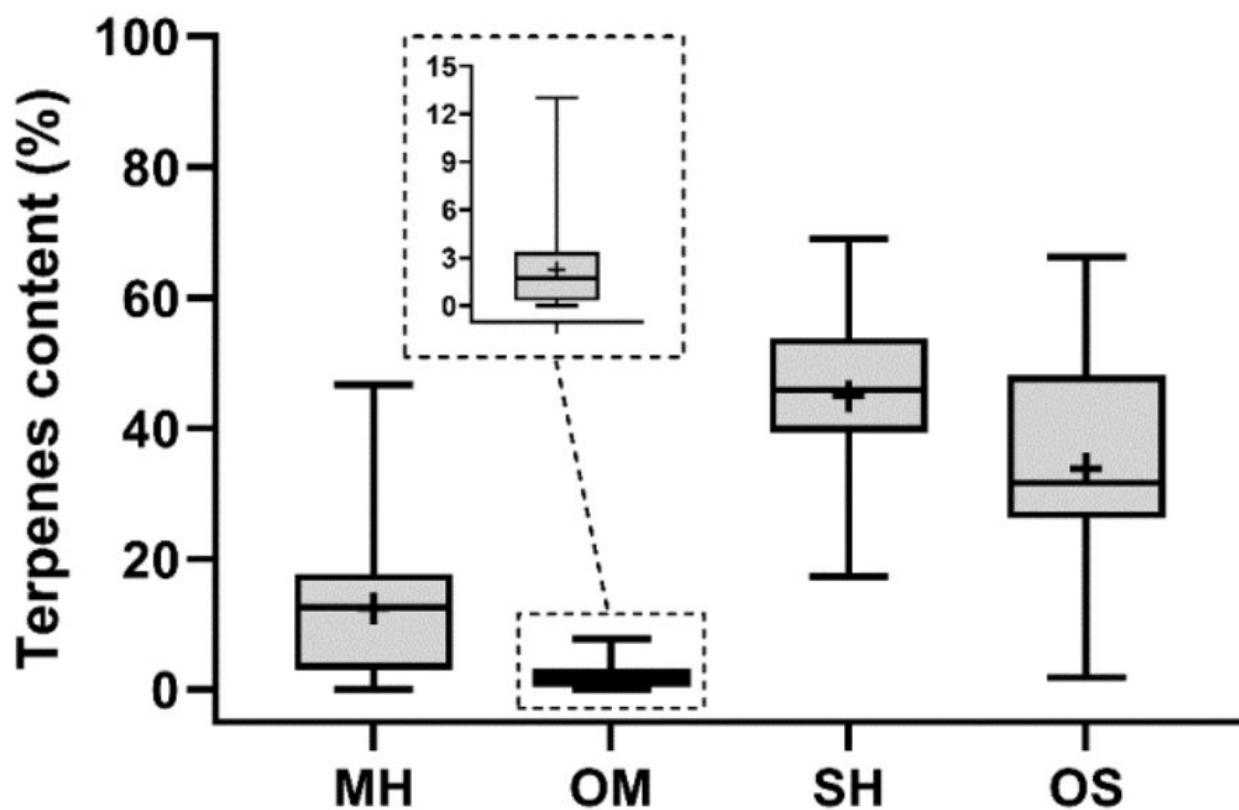


Figure 3

Description of the concentrations of monoterpene hydrocarbons (MH), oxygenated monoterpenes (OM), sesquiterpene hydrocarbons (SH) and oxygenated sesquiterpenes (OS) in the essential oil samples obtained from the leaves of tropical black sage collected *in situ*. Boxplot (quartiles), central line (median) and cross (mean).

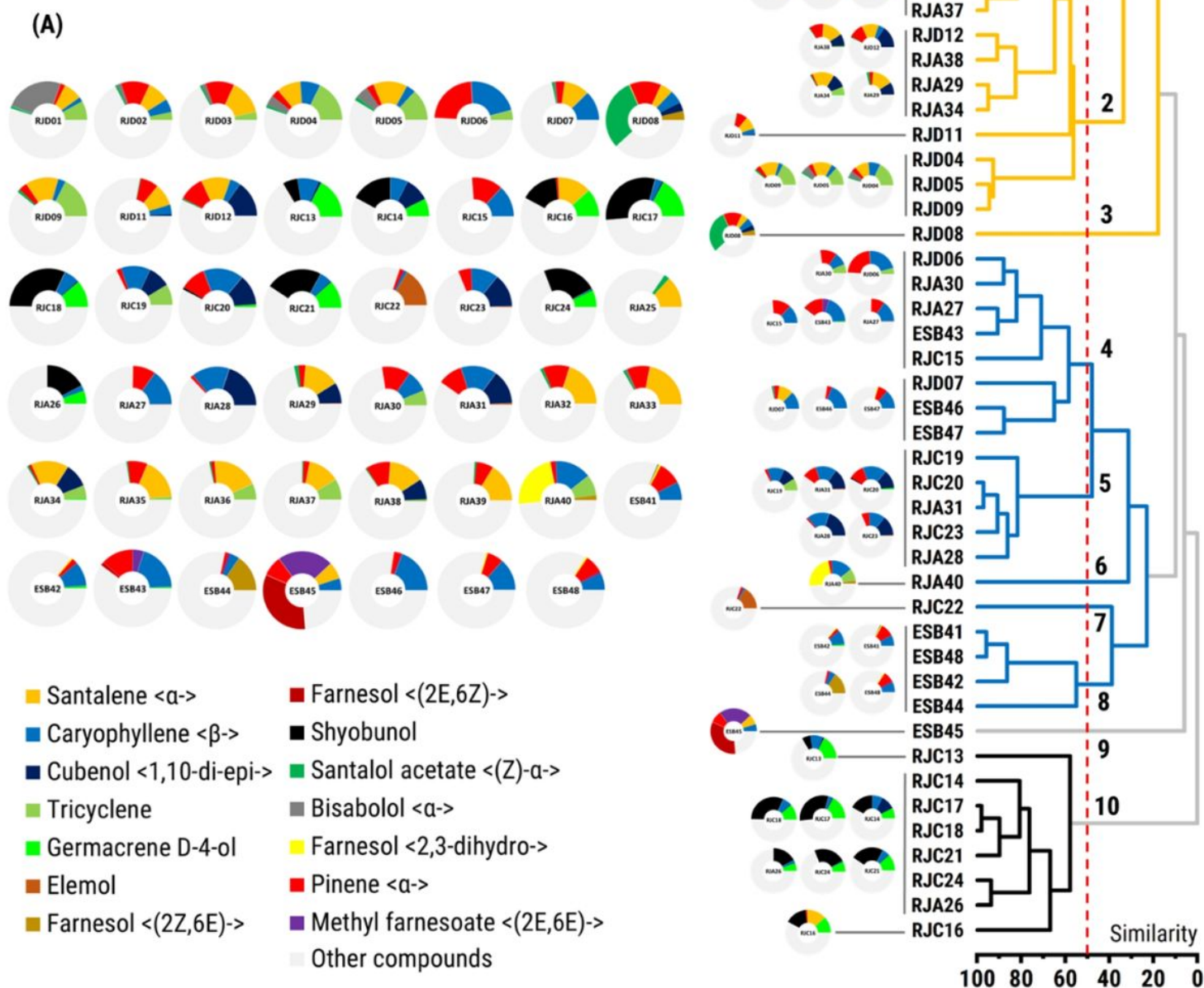


Figure 4

Graphic representation of the essential oils, chemoarrays (A). A total of 14 substances with great relevance were defined to construct the donut charts. The colored donut charts represent the essential oils of each of the 47 plants collected *in situ* and the colors denote the proportion of each one of the 14 substances in relation to the total. Hierarchical cluster analysis (B). The dendrogram was constructed based on the UPGMA and Pearson correlation coefficient estimated between the independent variables (47 essential oils) and dependent variables (42 chemical substances).

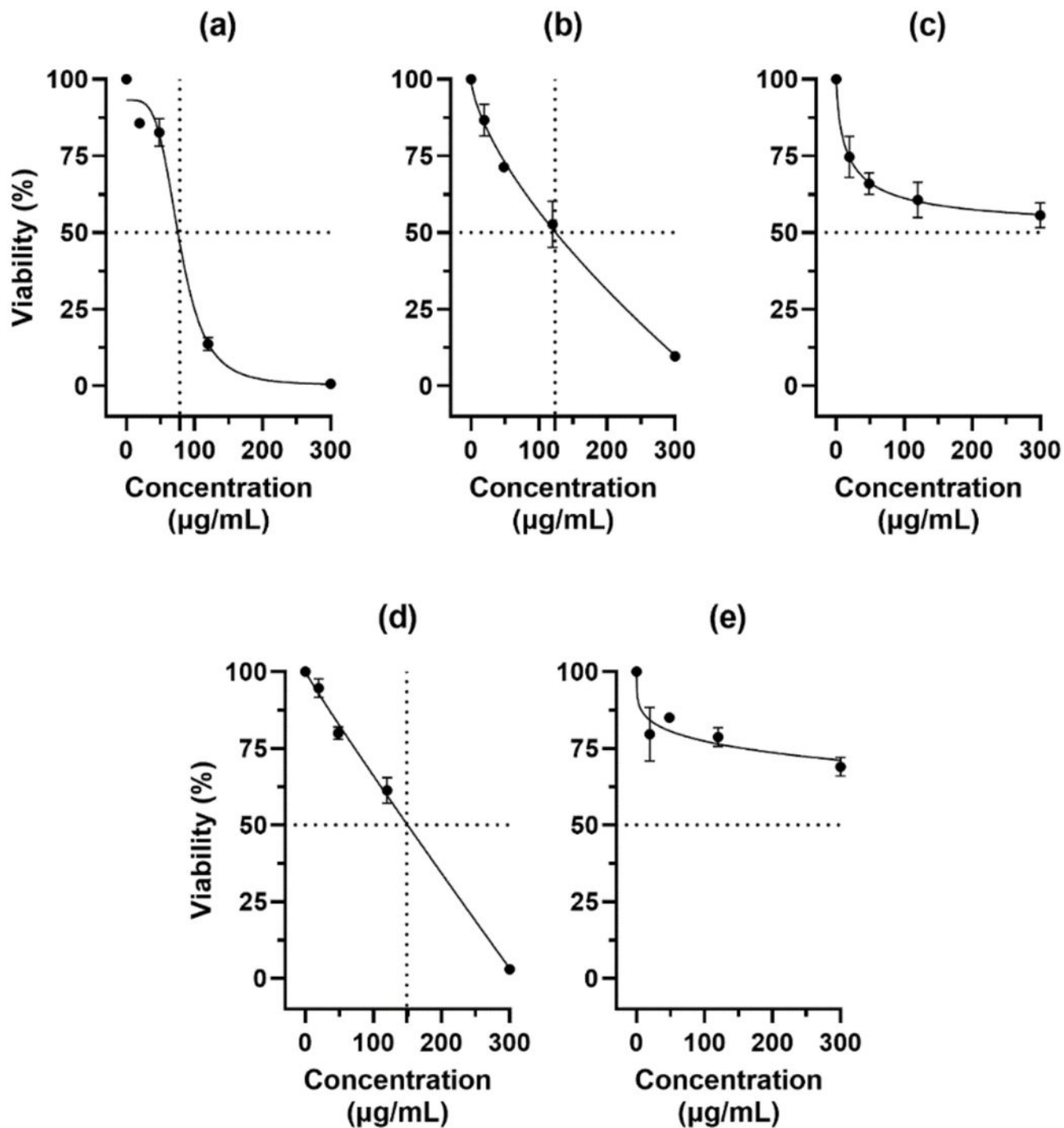


Figure 5

Activity and viability of the epimastigote form of *T. cruzi* under the effect of the essential oils of tropical black sage of the plants ESB45 (a), RJC18 (b), RJA38 (c), ESB44 (d) and RJC23 (e).

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