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Research Article

Extraction, chemical characterization and biological evaluation of the essential oil of *Campovassouria cruciata* (Vell.) R. M. King & H. Rob.

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Abstract: The Atlantic Forest, one of the most biodiverse biomes in the world, moving from the Coast to High Fields in the State of Santa Catarina (SC), is an important source of natural products, highlighting the aromatic plants, which can provide a variety of essential oils. *Campovassouria cruciata* (Vell.) R. M. King & H. Rob. (Asteraceae), is a shrubby plant widely spread in Brazil and in some other South American countries. In the literature, there are few reports on the phytochemistry and biological activity of this species. Therefore, this work aims the chemical characterization of the essential oil from flowers, fresh and dried leaves of *C. cruciata* and the evaluation of the antimicrobial and anticholinesterase activity of the essential oils obtained. The plant material of *C. cruciata* was collected in the city of Lebon Régis, SC. The essential oils were obtained by hydrodistillation in a modified Clevenger apparatus and their analysis performed by infrared, GC-MS and GC-FID techniques. The chemical characterization of the oils identified 20 compounds in the fresh flowers, 33 in the fresh leaves and 23 in the dried leaves of *C. cruciata*. Most of these compounds were terpene: α-tujene, α-pinene, β-pinene, germacrene D, bicyclogermacrene, limonene, viridiflorol, and β-caryophyllene. In the antibacterial assays *Mycoplasma pneumonia* and *Pseudomonas aeruginosa* were the most susceptible microorganisms towards the essential oils tested, with MIC values ranging from 125-500 μg mL^{-1}. *Staphylococcus aureus* also showed moderate susceptibility but just for the oil from fresh flowers (MIC of 125 μg mL^{-1}). For acetylcholinesterase inhibiting activity it was significant for the essential oil from fresh flowers, and this promising effect is related to synergistic action of the various monoterpene components of the EO.

Keywords: chemical composition; acetylcholinesterase, *Mycoplasma spp.*, antibacterial assays.

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1. Introduction

Essential oils are defined as complex mixtures of volatile compounds, lipophilic and usually liquid and odoriferous. The odors are generally related to their main constituents, a determining property for their economic value [1]. The volatile compounds

can be in several parts of the plant providing essential oils with different composition and concentrations according to the part of the plant used. Essential oils from distinct populations of the same species can also present significant variations in the chemical composition, odors and physicochemical properties. These variations are justified by some factors, such as the time of collection, stage of the plant development, weather conditions and soil besides biotic interference [2,3,4]. One of the families that stands out in this field is the Asteraceae family. Its species have therapeutic, aromatic, and cosmetic properties, besides having great importance for the environment and for the economic point of view [5]. Many studies have focused on the species of this family for presenting biological potential as anti-inflammatory, antimicrobial, anticancer, antisyphilitic, antigonorrhoeic and insecticidal [6]. In view of this, this work seeks to promote the valorization of Brazilian biodiversity, specifically in the state of Santa Catarina, through the identification of secondary metabolites with biological potential of little explored species, aiming to obtain, chemically characterize and evaluate the biological activities of the essential oils from flowers and leaves of *Campovassouria cruciate*.

2. Materials and Methods

2.1 Plant Material

Flowers and leaves of *Campovassouria cruciata* were collected in the city of Lebon Régis - SC, at coordinates 26°51'14'' South Latitude, 50°50'44'' West Latitude and elevation: 1062 m, during the afternoon period. The identification was performed by the botanist André Luís de Gasper and an exsiccate was deposited in the Dr. Roberto Miguel Klein Herbarium of the Blumenau Regional University - FURB under the registration No. 51717.

2.2 Essential Oil Extraction

The plant material of *Campovassouria cruciata* was selected, cleaned and the leaves separated from the stems. Subsequently, 876.40 g of flowers and 537.20 g of fresh leaves of *Campovassouria cruciata* were stored in plastic bags under refrigeration for a period of three days until the essential oil extraction was performed. Another portion of 537.20 g of *C. cruciata* leaves was dried in the shade, at room temperature (± 25 °C), for a period of 13 days, until constant mass. The essential oil was obtained by hydrodistillation in a modified Clevenger apparatus for a period of 4 hours, using a 6:1 proportion of distilled water and plant material, respectively.

The hydrolate was extracted with three 5 mL portions of bidistilled dichloromethane in a separating funnel. To the combined organic phase was added anhydrous magnesium sulfate, to absorb any water that may have remained in the solution. Afterwards the solution was filtered to remove the drying agent, in a round bottom flask with a capacity of 50 mL and the solvent was concentrated in a rotatory evaporator at a temperature of 40-50 ºC under reduced pressure. The essential oil yield was determined by the gravimetric method (m/m) according to the mass of plant material used in the process and the mass of essential oil obtained. The essential oils obtained from the samples were stored in appropriate amber flasks under nitrogen atmosphere, stored in freezers and wrapped with aluminum foil until the chemical characterization and biological activity analyses were performed.

2.3 Density Determination

The density of *C. cruciata* essential oil was determined by the Drummond method [7], which consists in obtaining the capillary volume by a liquid of known density at a given temperature. The liquid used was water at a temperature of 26 °C, where the volume of the capillary was determined by the mass, first of the empty capillary and then the capillary filled with water. After determining the volume of the capillary, it was dried with compressed air, filled with the essential oil, and then its mass was determined. The analysis was performed in triplicate.

2.4 Chemical Characterization

2.4.1 Chemical Characterization of the Essential Oil by Gas Chromatography with Flame Ionization Detection (GC-FID)

The analyses were performed on a gas chromatograph. The samples were previously diluted in 1 % (m/m) dichloromethane and manually injected three times to a volume of 0.2 μL in split mode to check the repeatability of the instrument. A Supelco fused silica capillary column (DB5/5% diphenyl and 95% dimethylpolysiloxane) with 30 m length, 0.25 mm diameter and 0.25 μm thickness of the stationary phase, and He gas as mobile phase was used. The analyses conditions were as follows: split ratio 1:20; drag gas flow rate of 1 mL min^{-1}; Injector and detector temperature, 250 and 280 °C, respectively; initial column temperature, 60 °C with a heating rate of 3 °C min^{-1} until it reached 240 °C, remaining at this temperature for 2 minutes. The total analyses time was 62 minutes.

2.4.2 Chemical Characterization of the Essential Oil by Gas Chromatography Coupled with Mass Spectrometry (GC-MS)

The analyses were performed in a gas chromatograph coupled to a quadrupole mass spectrometer. Samples were previously diluted in 0.5% (m/m) bidistilled dichloromethane and automatically injected to a volume of 0.1 μL in split mode. The detector operated in electron impact mode at 70 eV. A Perkin Elmer DB5 capillary column (30 m x 0.25 mm x 0.25 μm) and He gas as mobile phase was used. The analysis conditions were as follows: Split ratio 1:20; drag gas flow rate of 1.02 mL min^{-1}; injector temperature 220 °C; source temperature 250 °C; transfer line 240 °C; initial column temperature of 60 °C, with a heating rate of 3 °C min^{-1} until 246 °C was reached. The total analyses time was 62 min. The identification of the essential oil constituents by GC-MS was determined by comparing the mass spectra produced with the spectra available in the NIST mass library, by visual comparative analysis of the mass spectra obtained with those available in the Adams (2007) literature, and by the retention index (IR), experimentally determined by analyzing a homologous series of hydrocarbons under the same conditions as for the sample.

2.4.3 Retention Index

To perform the retention index (RI) calculation, a homologous series of saturated linear hydrocarbons from C8 to C19 was employed. The Van den Dool and Kratz equation [8] was then applied to calculate the retention index for each constituent.

2.4.4 Chemical Characterization of Essential Oil by Infrared (IR) Spectroscopy

The IR analysis of the essential oils were performed in the Chemistry Department of FURB, where a drop of pure sample was placed in the spectrophotometer, Vertex 70 model in platinum ATR (attenuated total reflectance) and read to obtain the spectra.

2.5 Evaluation of Biological Activity

2.5.1 Antimollicute Activity

The microorganisms used in this assay were strains of *Mycoplasma capricolum* and *Mycoplasma pneumoniae* strain 129. Replicates of the cultures were taken from 0.2 mL of inoculum, which were added to a test tube with 2.0 mL of culture medium and inoculated at 36 °C +/- 1 °C for 48 hours in microaerophilic conditions (2-3 % CO_2). After this period, the strains were used for the minimum inhibitory concentration (MIC) tests. The MIC tests were performed by the standardized broth microdilution method in 96-well plates [9]. The essential oil was diluted to 40 mg mL^{-1} in 100% dimethyl sulfoxide (DMSO). 190 μL of SP4 culture medium was added and 10 μL of essential oil to be tested was added to the first wells. Then, from the medium containing sample, 100 μL were transferred to adjacent wells, which already contained 100 μL of specific culture medium (SP4), to obtain a serial dilution of order two. The mollicutes culture inoculum (100 μL) in log growth phase, (10^3 microorganisms mL^{-1}), was added to all microwells. As a negative control, a serial dilution of the solvent itself (DMSO 100%) was performed without the presence of essential oil; as a control of culture medium, only medium; as a growth control, a serial dilution of the microorganism culture, without the addition of solvent or essential oil. As a positive control, the antibiotic erythromycin (0.5 μg mL^{-1}) was used. Finally, 2 to 3 drops of liquid vaseline were added in all cavities to isolate each

cavity from the external environment and create a microaerophilic environment. The plate with *M. pneumoniae* was incubated at 37 °C for 30 days, while the plate with *M. capricolum* was incubated at 37 °C for 24 hours and growth was observed from the change in color of the culture medium due to the presence of the indicator phenol red. All the experiments were conducted in triplicate.

2.5.2 Antibacterial Activity

Flowers, fresh leaves, and dried leaves of *C. cruciata* were used for the test to determine the antibacterial activity of the essential oil. To perform the antibacterial tests, it was necessary to dilute the sample to a standard concentration of 40 mg mL^{-1} with 10% dimethyl sulfoxide (DMSO). The antibacterial activity was evaluated against Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and Gram-positive *Staphylococcus aureus* (ATCC 25923), by the broth microdilution method, with 96 microwell plates. Once dilution was performed, the sample was added to the first well of the microplate and transferred to the adjacent well, which already contained Mueller Hinton (MH) broth, in a serial dilution of order two, with sample concentrations ranging from 1000 μg mL^{-1} to 7.81 μg mL^{-1}. To enable species growth, the microorganisms were seeded on Mueller-Hinton agar (24 h at 37 °C) for reactivation of the bacterial strains. The bacterial inoculum was prepared on McFarland 0.5 scale and analyzed at 620 nm absorbance, ranging from 0.08 - 0.1 nm, and added (10 μL) to each microwell. Some wells of each microplate were reserved for performing the negative control (broth (MH) + study bacteria) and for sterility control of the culture medium (MH broth). As a positive control, the study bacteria were incubated with the antibiotic gentamicin at concentrations ranging from 40 to 0.31 μg mL^{-1}. The entire procedure was performed in duplicate and in a sterile chapel. The microplates were incubated aerobically at 37 ± 1°C for 24 hours. After incubation, bacterial growth was checked by adding 10 μL of a methanolic solution of 2,3,5-triphenyltetrazolium chloride (5 mg mL^{-1}) in each microwell. After one hour, a color change of the culture medium was observed. The minimum inhibitory concentration (MIC) was defined as the last concentration of the sample capable of inhibiting bacterial growth. To classify the antibacterial activity, it was used the following range described in the literature [10] :lower than 10 μg mL^{-1} was considered to have excellent antibacterial activity; a value between 10 and 100 μg mL^{-1} was considered good; a value between 100 and 500 μg mL^{-1} was considered to have moderate activity; a value between 500 and 1000 μg mL^{-1} and for a MIC value superior to 1000 μg mL^{-1} the sample was considered inactive.

2.5.3 Anticholinesterase activity

The assay for anticholinesterase activity was conducted *in vitro* using the Ellman method [11]. Solutions of each test sample were prepared in methanol at a concentration of 1 mg mL^{-1}. In each test tube, 325 μL of Tris-HCl buffer (pH 8), 100 μL of sample at different concentrations and 25 μL of acetylcholinesterase enzyme solution (0.28 U mL^{-1}) were added, and this mixture was incubated at room temperature for 15 minutes. Then, 75 μL of acetylcholine iodide solution (0.023 mg mL^{-1}) and 475 μL of Ellman's Reagent [5,5-dithiobis-(2- nitrobenzoic acid)] were added. The tubes were homogenized and incubated in the shade for 30 minutes. After 30 minutes, absorbance reading of the solution was performed in a spectrophotometer at 405 nm. As positive control was used a solution of neostigmine bromide diluted in 100 μg mL^{-1} of methanol and as negative control only the solvent used for dilution of samples. The enzyme inhibition was calculated by the equation:

$$\text{Inhibitory Activity (\%)} = 100 - \frac{(\text{Sample Abs} - \text{White Abs}) * 100}{\text{Average Negative C. Abs}}$$

The test was performed in triplicate, and the results were expressed as % inhibition in the form of mean ± standard deviation.

3. Results

3.1 Essential Oil Yield and Density

The essential oil yield results were obtained from six hydrodistillations of the fresh and dried plant material. The yield data can be seen in Table 1.

Table 1. Essential oil yield of flowers, fresh and dried leaves of *C. cruciata*.

Type of plant material	Plant material (g)	Essential Oil (g)	Yield (%)
Flowers	876.40	1.71	0.19
Fresh Leaves	537.20	1.08	0.20
Dried Leaves*	537.20	1.60	0.29

From the data described in Table 1, a difference in essential oil yield is observed, with the lowest value associated to the dried leaves. This fact occurred, most likely, due to the loss of water from the leaves during the drying process.

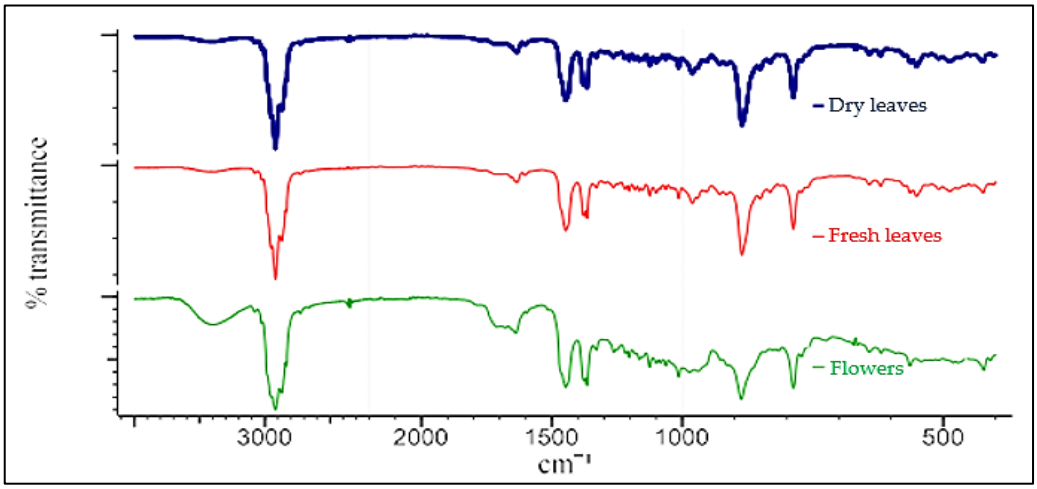
The densities of essential oils from flowers, fresh leaves, and dried leaves of *C. cruciata* obtained experimentally by the Dummond method [7] at 26 °C [12] were, respectively, 0.9415 ± 0.0021 g mL^{-1}, 0.8751 ± 0.0040 g mL-1 and 0.9059 ± 0.0013 g mL^{-1}.

3.2 Chemical Characterization of the Essential Oils

3.2.1 Infrared Spectroscopy (IR) Analysis

To investigate the functional groups in the essential oils obtained from the flowers, fresh and dried leaves of *C. cruciata*, the samples were submitted to IR spectroscopy in platinum ATR (attenuated total reflectance). In the IR spectra of the essential oils from flowers, fresh and dried leaves the following main absorption bands can be seen (Figure 1): -OH axial deformation at 3396 cm^{-1} with greater intensity in the flowers; =CH axial deformation at approximately 3050 cm^{-1}; aliphatic C-H axial deformation at 2918 cm^{-1}; C=C axial deformation at ~1600 cm^{-1}; -CH_2 angular deformation at 1446 cm^{-1}; -CH_3 angular deformation at ~1365 cm^{-1}; =CH angular deformation at 870 cm^{-1}. Based on the information obtained, Lopes and Fascio (2004) suggest the following explanations for the interpretation of spectra of organic substances in the infrared region: The absence of absorption between 1820 and 1630 cm^{-1} excludes all carbonylated functions. Absorptions at 1068 cm^{-1} (νC-O) and 3396 cm^{-1} (νO-H) are compatible with the alcohol function. Absorptions between 3000 and 2800 cm^{-1} (C-H) are compatible with the presence of carbon with sp3 hybridization. Absorptions between 3100 and 3000 cm^{-1} (Csp2 -H) and between 1680 and 1629 cm^{-1} (νC=C) are characteristic for olefins. The absorption at ~1380 cm^{-1} (δC-H) indicates that the sample has a methyl group. The spectrum obtained was characteristic of a mixture of saturated and unsaturated hydrocarbons with the presence of alcohol function.

Figure 1. Infrared spectrum (FTIR/ATR) of the essential oil obtained from flowers, fresh and dried leaves of *C. cruciata*.



3.2.2 Identification and Quantification of Essential Oils by GC-MS and GC-FID

A homologous series of saturated linear hydrocarbons C8 - C19 was used under the same analytical conditions used for GC-FID analysis of the essential oils. The retention times of these hydrocarbons were used in the arithmetic index calculations accordingly to Van den Dool and Kratz equation [8]. The combination of the mass spectra and the arithmetic index values allowed the chemical characterization of 98.98% of the essential oil from fresh flowers, 95.61% from fresh leaves, and 99.87% from dried leaves. By means of GC-FID it was possible to quantify the characterized volatile constituents of *C. cruciata.* Table 2 presents the chemical composition of the essential oils from flowers, fresh leaves, and dried leaves of *C. cruciata*, with the percentage concentration of each constituent, the experimentally calculated retention index (RI), and RI from the literature.

Table 2. Percent chemical composition of compounds from the essential oils of flowers, fresh leaves, and dried leaves of *Campovassouria cruciata*.

Entry	Constituent	RI_{Lit}	RI_{Exp}	Fresh Flowers [%]	Fresh leaves [%]	Dry leaves [%]
1	α-thujene	924	-	tr	Tr	-
2	α-pinene	932	933	57.26 ± 0.5	49.15 ± 2,1	47.61 ± 0.7
3	Sabinene	969	970	3.58 ± 0.01	1.66 ± 0.02	1.68 ± 0.1
4	β-pinene	974	973	3.76 ± 0.04	1.55 ± 0.02	1.25 ± 0.01
5	Myrcene	988	986	2.61 ± 0.02	2.09 ± 0.01	1.55 ± 0.04
6	δ-2-carene	1001	-	2.15 ± 0.03	-	-
7	α-terpinene	1014	-	-	tr	-
8	*p*-cymene	1020	-	-	tr	-
9	Limonene	1024	1024	6,93 ± 0,2	4,76 ± 0,2	4,24 ± 0,3
10	β-phellandrene	1025	-	tr	-	-
11	(*Z*)-β-ocimene	1032	-	-	tr	-
12	(*E*)-β-ocimene	1044	1043	tr	2,65 ± 0,1	1,42 ± 0,08
13	γ-terpinene	1054	1055	-	0,34 ± 0,02	-
14	*p*-menthene-2,4(8)-diene	1085	-	-	tr	-
15	mint 1,5-dien-8- ol	1166	-	-	tr	-
16	terpinen-4-ol	1174	1176	1,63 ± 0,03	0,73 ± 0,03	0,38 ± 0,02

17	α-terpineol	1186	1191	0,87 ± 0,03	0,43 ± 0,01	0,31 ± 0,0
18	(3Z)-hexenyl-2-methylbutanoate	1229	-	-	tr	
19	α-copaene	1376	1376	-	-	0,30 ± 0,0
20	β-bourbonene	1387	1387	-	-	0,45 ± 0,03
21	*E*-caryophyllene	1417	1415	3,80 ± 0,2	3,02 ± 0,1	6,90 ± 0,3
22	α-caryophyllene	1452	1454	-	-	0,38 ± 0,02
23	germacrene D	1484	1482	1,52 ± 0,02	7,50 ± 0,1	12,59 ± 0,3
24	γ-amorphene	1495	-	-	tr	-
25	bicyclogermacrene	1500	1497	-	6,20 ± 0,1	8,29 ± 0,2
26	α-muurolene	1500	-	-	tr	-
27	δ-cadinene	1522	1519	tr	1,29 ± 0,07	1,19 ± 0,01
28	Spatulenol	1577	1578	4,90 ± 0,03	2,42 ± 0,1	3,60 ± 0,08
29	caryophyllene oxide	1582	1581	4,80 ± 0,1	1,09 ± 0,3	1,45 ± 0,01
30	tujopsan-2-α-ol	1586	1587	-	1,76 ± 0,3	0,81 ± 0,02
31	β-copaen-4-α-ol	1590	-	tr	-	-
32	Viridiflorol	1592	1591	2,81 ± 0,04	4,56 ± 0,4	3,33 ± 0,1
33	1-*epi*-cubenol	1627	1630	-	0,31 ± 0,03	-
34	allo-aromadendrene epoxide	1639	1638	-	0,46 ± 0,03	-
35	α-muurolol	1644	1644	0,74 ± 0,01	0,43 ± 0,1	0,42 ± 0,01
36	Cubenol	1645	-	-	tr	0.36 ± 0.03
37	α-cadinol	1652	1652	1.62 ± 0.02	2.89 ± 0.2	1.36 ± 0.1
38	eudesma-4(15)-7-dien-1β-ol	1687	1686	-	0.32 ± 0.02	-

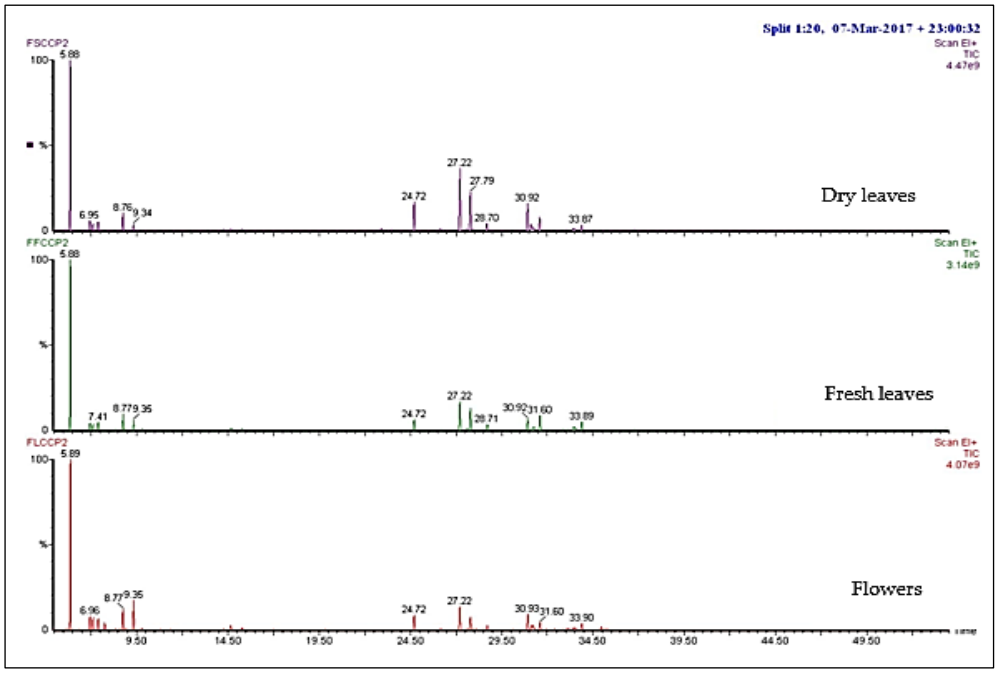
39	mint sulfide	1740	-	-	tr	-
	Total			98.98	95.61	99.87
	MH			79.29 ± 0.01	62.2 ± 0.7	57.75 ± 0.20
	OM			2.51 ± 0.10	1.16 ± 0.01	0.69 ± 0.01
	SH			5.32 ± 0.01	18.01 ± 0.05	30.1 ± 0.01
	OS			14.87 ± 0.05	14.24 ± 0.10	11.33 ± 0.06

RI_{Lit}: literature retention index [8]; **RI_{Exp}:** retention index determined experimentally; **tr:** constituents characterized only by GC-MS; **MH:** monoterpene hydrocarbons; **OM:** oxigenated monoterpenes; **HS:** sesquiterpene hydrocarbons; **OS:** oxygenated sesquiterpenes.

The characterization comprises 20 constituents of the essential oil from flowers, 33 constituents of the essential oil from fresh leaves and 23 constituents of the dried leaves. The oil from the flowers of *C. cruciata* showed as major constituents α-pinene (57.3 %), limonene (6.9 %), spatulenol (4.9 %), caryophyllene oxide (4.8 %), β-caryophyllene (3.8 %), β-pinene (3.76 %) and sabinene (3.58 %). The oil from fresh and dried leaves showed the same majority constituents, differing only quantitatively, they are: α-pinene (49.2 and 47.6 %), germacrene D (7.5 and 12.6 %), bicyclogermacrene (6.2 and 8.3 %), limonene (4.8 and 4.2 %), viridiflorol (4.6 and 3.3 %), β-caryophyllene (3.0 and 6.9 %) and spatulenol (2.4 and 3.6 %), respectively.

Some representative chromatograms have been selected and are presented in Figure 2. There are two distinct regions, the monoterpenes and sesquiterpenes, below and above 17 min, respectively. Furthermore, based on the major constituents the 3 samples showed very high similarity on a simple visual analysis.

Figure 2. GC-MS chromatograms of the essential oil from flowers, fresh and dried leaves of *Campovassouria cruciata*.



Finally, it is significant the reduction in monoterpene concentration, 63.36% in fresh leaves to 58.44% in dried leaves, as well as the high concentration associated to the sesquiterpene hydrocarbons in dried leaves, which reached 30.1%.

3.3 Biological Activities

3.3.1 Antimicrobial Activity

Table 3 presents the results of the antimicrobial assays of the essential oils from *C. cruciata* flowers, fresh and dried leaves against *Mycoplasma capricolum and Mycoplasma pneumoniae* strains.

Table 3. Effect of essential oil from fresh flowers, fresh leaves and dried leaves of *Campovassouria cruciata* on antimycoplasmatic activity.

Bacteria	Fresh flowers MIC (μg mL-1)	Fresh leaves MIC (μg mL-1)	Dried leaves MIC (μg mL-1)	Erythromycin MIC (μg mL-1)
Mycoplasma capricolum	500	500	1000	0.5
Mycoplasma pneumoniae	125	250	250	0.5

In the tests performed against the *M. pneumoniae* strain, only the flowers presented MIC of 125 μg mL-1. This result may be associated with a higher concentration and rich monoterpene constitution (79.29%). The fresh and dried leaves showed MIC of 250 μg mL-1. This result can be related to the lower monoterpene concentration of the fresh and dried leaves (62.2 and 57.75%), respectively, compared to the result obtained from the essential oil of the flowers. However, it was possible to verify that the three samples have antimicrobial activity against *M. pneumoniae* strains. The same antimicrobial activity was observed against *M. capricolum*, with an MIC of 500 μg mL-1 for fresh flowers and leaves, and 1000 μg mL-1 for dried leaves. This indicates that the activity of the essential oil may be related to a synergism between the monoterpenes.

3.3.2 Antibacterial Activity

The results of the antibacterial activity are presented in Table 4. It can be observed that the essential oil from flowers has a moderate and promising activity against *S. aureus*. However, the dried leaves of *C. cruciata* were inactive against this bacterium. All the samples were moderately active against *Pseudomonas aeruginosa*. *Escherichia coli* showed to be the less susceptible to the samples tested. The best result was a weak activity related to the sample from dried leaves.

Table 4. Effect of essential oil from fresh flowers, fresh leaves and dried leaves of *Campovassouria cruciata* on antibacterial activity.

Bacteria	Fresh Flowers MIC (μg mL-1)	Fresh leaves MIC (μg mL-1)	Dried leaves MIC (μg mL-1)	Gentamicin MIC (μg mL-1)
Pseudomonas aeruginosa	500	500	250	2.5
Staphylococcus aureus	125	1000	Inactive	2.5
Escherichia coli	1000	1000	500	2.5

3.3.3 Anticholinesterase Activity

The acetylcholinesterase enzyme inhibition and IC_{50} assays are presented in Table 5. A sample capable of inhibiting 50% of enzyme activity is considered promising, as described by a study conducted in 2015 [13].

Table 5. Acetylcholinesterase inhibitory activity and IC_{50} of essential oils from flowers, fresh leaves and dried leaves of *Campovassouria cruciata*.

	Fresh flowers (%)	Fresh leaves (%)	Dried leaves (%)	Fresh flowers (IC50–mg mL^{-1})	Dried leaves (IC50–mg mL^{-1})
Achol inhibition	79.56 ± 0.69	45.39 ± 2.37	59.70 ± 2.46	0.32 ± 0.02	>1

Achol: acetilcolinesterase; **IC_{50}:** Inhibitory concentration 50%.

It is observed that the most active essential oil was the one from the fresh flowers. In this oil, the hydrocarbon monoterpene fraction accounts for 79,29% of its composition, and 57.26% of this fraction is related to the constituent α-pinene. Expressive activity was also observed in the essential oil obtained from dried leaves, containing 57,75% of hydrocarbon monoterpenes, where 47.61% is associated with α-pinene. However, the fresh leaves which presents higher concentration in monoterpenes than dried leaves, was less effective, with an inhibitory activity below 50%.

4. Discussion

Terpenes can be subdivided into four groups with respect to the value of the arithmetic indices (AI), thus there are: monoterpene hydrocarbons - up to 1100; oxygenated monoterpenes - 1100 to 1300; sesquiterpene hydrocarbons - 1300 to 1540 and oxygenated sesquiterpenes - above 1540 [8].

It is possible to observe the presence of monoterpene and sesquiterpene hydrocarbons, and oxygenated monoterpenes and sesquiterpenes in the essential oils of *C. cruciata*, as presented in Table 2. Monoterpene hydrocarbons is the main sub-class of secondary metabolites in all the essential oils analysed, with α-pinene as the major constituent. There is a higher concentration of oxygenated sesquiterpenes in the oil from the fresh flowers and leaves compared to the oil from the dried leaves. In the oil from the fresh and dried leaves there is a higher concentration of sesquiterpene hydrocarbons compared to the oil from the flowers, especially in the dried leaves. This could be linked to the drying process of the leaves, which may lead to the loss of more volatile compounds when using more vigorous conditions.

Considering the study on chemical composition of the essential oils of the species *Campovassouria cruciata* from Lebon Régis - SC, compared to the same species collected and studied in the state of Rio Grande do Sul [15], it is noted some similarities in the chemical composition, but in different concentrations. It is also noted that in the species collected in Rio Grande do Sul there was a greater number of constituents present in the essential oils.

The result of biological activities regarding the antimycoplasmatic assays may be associated to the rich unsaturated monoterpene composition, with a maximum value of 79.29% found in the oil from fresh flowers. The olefinic bond is an important structural feature contributing to the antimicrobial activity [16].

The flower is the reproductive structure of angiosperm plants. The function of a flower is to reproduce seeds through sexual reproduction [17-18]. This justifies the high concentration of the monoterpene class in its composition, since monoterpenes have low molecular mass and are apolar, they turn to be volatile compounds and responsible for attracting pollinators.

However, all the samples were moderately active against *Mycoplasma pneumoniae* strain, MIC ranging from 125-250 μg mL^{-1}. The results observed against *M. capricolum* were less significant. In this assay MIC ranged from 500-1000 μg mL^{-1} which means an

activity from moderate to weak. Synergism between the monoterpenes might be the cause of such activity [18-19].

As can be seen, DMSO exerts some toxic effect on the growth of mollicutes, contrary to what occurs with typical bacterial species, *Pseudomonas sp, E. Coli, and Staphylococcus sp*. This fact is probably due to the characteristic of mollicutes that they do not have a cell wall, making them more sensitive to osmotic changes and other aggressions to the cell membrane [20].

The difficulties in cultivating these microorganisms explain, in part, the scarcity of publications related to the evaluation of the antimicrobial activity of natural products against mollicutes [21].

The genus *Eupatorium* (Asteraceae) which is a synonym of the target species has provided extracts and essential oils against strains of bacteria for the development of new drugs. Among the species with the greatest microbial potentials are *E. adenophorum, E. odoratum and E. triplinerve* [16]. In particular, the literature does not bring antimicrobial studies for the genus *Campovassouria*, nor for the species *C. cruciata*.

Analyzing the results obtained, it is noted that the essential oil of the flowers has a stronger activity against *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus* and *Escherichia coli*, which showed the greatest resistance to the samples tested. The moderate activity observed for most of the samples should be related to the high concentration of hydrocarbon monoterpenes in the samples, especially due to the presence of α-pinene.

This high resistance of Gram-negative bacteria may indicate the complexity of the cell wall of this group of microorganisms, which have an external membrane that favors the surface of the bacteria with strong hydrophilicity and limits the diffusion of hydrophobic compounds through its lipopolysaccharide layer [22-23].

Essential oils from *Baccharis uncinella* and *B. semiserrata* (Asteraceae) were tested against 5 bacteria strains [24]. The general activity was less significant than the present results. The best MIC, 500 μg mL^{-1}, was obtained from twig of *B. semiserrata* against the Gram-(+) *S. aureus*. The essential oils from these species were rich in hydrocarbon and oxygenated sesquiterpenes, reinforcing the importance of monoterpenes for the observed antimicrobial activity.

Some other research developed with aqueous extract of dried leaves of *Eupatorium maximiliani* showed efficacy against Gram-positive bacteria *Staphylococcus aureus* [25]. Extracts and in essential oil of the species *E. intermedium* had the efficacy tested against Gram-positive bacteria *S. aureus* and *L. monocytogenes*, with special attention to the extract obtained with supercritical CO_2 at 80 °C and 250 bar. The tested Gram-negative bacteria, *S. typhimurium* and *E. coli*, were completely resistant to the tested extracts and essential oil [26].

In other countries studies of this nature have also been conducted [27-28-29-30-31]. These works tested essential oils from inflorescences, leaves, roots, and stems of the species *Eupatorium adenophorum* Spreng. against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* and *P. aeruginosa* and obtained strong activity. Also, in 2008 research tested essential oils from the aerial parts of *Eupatorium altissimum* L. and achieved strong activity against *S. aureus* and *E. coli* [32].

Samples capable of inhibiting 50% of the enzyme activity are considered promising [13]. As for the results found in the studies, the essential oil from fresh flowers is the most effective inhibitor for acetylcholinesterase. Due to the low content of sesquiterpenes in the sample, it is possible to associate these properties to the major monoterpenic compounds, since α-pinene has been proven to present inhibitory activity on the acetylcholinesterase enzyme [14]. Although the lowest concentration of α-pinene is present in dried leaves which was effective inhibitor of acetylcholinesterase, 59,70%, its IC_{50} was very ineffective, higher de 1 mg mL^{-1}.

A study shows that the assay of anticholinesterase activity performed with the essential oil of *E. bupleurifolium* and other species of *Eupatorium* against the enzyme acetylcholinesterase of bovine erythrocytes, showed similarity in qualitative chemical composition. All analyzed samples exerted moderate inhibitory action on the acetylcholinesterase enzyme [15].

The inhibitory activity of several terpenes on the acetylcholinesterase enzyme has already been observed [33-34-35], such as the monoterpenes limonene and p-cymene, and the oxygenated sesquiterpene caryophyllene oxide, identified in the volatile oil of species of the genus *Eupatorium*. A high inhibitory activity against acetylcholinesterase was also observed for α-pinene in *E. polystachyum* [36].

The final activity exerted on the acetylcholinesterase enzyme may not be associated only with the summed effect of the individual activities of the various essential oil constituents, but the sum of synergistic and antagonistic effects of them. Associations, for example, between 1,8-cineole and α-pinene, or 1,8-cineole and caryophyllene oxide exert a synergistic effect, thus increasing enzyme inhibition [35].

Thus, even though the compound or compounds responsible for the enzyme inhibition were not identified, it is possible that the effects observed are related to the synergistic action mainly by the various hydrocarbon monoterpenes, but also in less extend due to the sesquiterpene components present in the essential oil.

5. Conclusions

The chemical composition of the essential oil *of Campovassouria cruciata* (Vell.) R. M. King & H. Rob from Santa Catarina was described for the first time. Terpenes are the main class identified in the samples from fresh flowers, fresh leaves, and dried leaves of this species. In the chemical characterization by GC-MS and GC-FID it was possible to observe a greater predominance of the monoterpene class. Preliminary infrared analysis confirmed the presence of functional groups referring to saturated and unsaturated hydrocarbons. In the antimycoplasmatic assays a higher activity against *M. pneumoniae* was verified. The antibacterial evaluation showed stronger activity of the essential oils of *C. cruciata* against *Pseudomonas aeruginosa*. The essential oil from fresh flowers was also effective against *Staphylococcus aureus.* All the cited activities are classified as moderate. *Escherichia coli* was the less susceptible bacterium. For acetylcholinesterase inhibition, the activity was significant for the essential oil from fresh flowers.

In future studies, it is suggested an investigation of the influence of seasonality on chemical composition of the essential oils from aerial parts of *C. cruciate* and the fractionation of the oils to be tested in the inhibitory assays of acetylcholinesterase.

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