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Chemical Composition and Allelopathic Activity of the Essential Oil from *Callistemon viminalis* (Myrtaceae) Blossoms on Lettuce (*Lactuca sativa* L.) Seedlings

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Abstract

The extraction of essential oils was performed by using the technique of hydrodistillation modified Clevenger apparatus, identification and quantification of the constituents was achieved by Gas Chromatography coupled to Mass Spectrometry, and Gas Chromatography equipped with a flame ionization detector. Assessment of allelopathic activity was evaluated with the use of the method that assesses the direct contact of essential oils on germination and vigor of lettuce seeds. The major constituents that characterize the essential oil from the flowers of *Callistemon viminalis* were 1, 8-cineole, α-pinene and limonene at concentrations of 66.9%, 16.0% and 10.0%, respectively. The essential oil presented allelopathic activity at intensities that varied proportionately to the concentration of the essential oil, with a reduction in the percentage of germination and the germination speed index (GSI) of lettuce seeds and in the dry mass and length of shoots and roots of lettuce seedlings.

Keywords

Biological Activity, Myrtaceae, Allelopathy

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1. Introduction

The chemistry of natural products interacts with different areas of expertise, and its relevance is linked to this interdisciplinarity. Chemical studies of different plant species seek to detect information inherent to secondary metabolites and to contribute to the development of several areas of science. The use of plants by humans dates back thousands of years and relies on chance discoveries that are frequently proven by science. They were used to cure diseases, for food preservation and insect control. Many natural compounds present in plants, herbs and spices have shown biological activity and serve as a source of antimicrobial agents against various pathogens. Among these products, the essential oils receive emphasis. They are also called volatile, ethereal oils or essences because they have an oily appearance at room temperature, are important in phytosanitary control and in the reduction of the negative effects of oxidants, free radicals and microorganisms that cause damage in the food industry [1] [2].

In addition, essential oils possess allelopathic activity and are potent inhibitors of seed germination and the development of different plant species, which accredits these compounds to be used as bioherbicides [3]-[5]. According to Sousa *et al*. (2007), allelopathy has been described as positive or negative interference in the process by which products of secondary metabolism of a particular plant are liberated into the environment, and these substances (allelochemicals) can benefit or harm the receptor plants in natural and agricultural systems. Resistance or tolerance to secondary metabolites is a species specific characteristic; some species, such as *Lactuca sativa* (*lettuce*) and *Lycopersicon esculentun* Mill. (tomato), are more sensitive than others and are considered to be indicators of allelopathic activity [6] [7].

Callistemon viminalis, popularly known as escova-de-garrafa (bottle brush), belongs to the Myrtaceae family. It is a medium-sized tree with persistent foliage. It is found throughout the world, being distributed mainly in the humid tropical regions that include Australia, South America and tropical Asia. Many species of this family are important sources of edible fruits, spices, and ornamental and medicinal plants [8]. Some species of *Callistemon*, especially *Callistemon viminalis*, are widely used as environmental bioindicators, being an important source of chemical compounds with insecticidal, fungicidal and antimicrobial properties [9]. In the present study, the essential oil from *Callistemon viminalis* flowers was studied, and its allelopathic activity against lettuce (*Lactuca sativa* L.) seeds was evaluated.

2. Material and Methods

2.1. Plant Material

The *Callistemon viminalis* (Myrtaceae) blossoms were collected on the campus of the Federal University of Lavras (UFLA) in Lavras, MG, Brazil, in March 2012 in the morning on a day with mild temperatures and no precipitation. Lavras is located in the South of Minas Gerais, Brazil, latitude 21˚14'S, longitude 45˚00'W Gr and at an altitude of 918 m. The species was identified in the Department of Biology at the Federal University of Lavras, and its voucher specimen is found recorded in the ESAL Herbarium of the Federal University of Lavras (record number 26,624).

2.2. Extraction of the Essential Oil

The essential oil from fresh flowers (300 g) was extacted by hydrodistillation over a 2 h period in a modified Clevenger apparatus (Pharmacopoeia, 2010) [10]. After extraction, the hydrolact was centrifuged at 965.36 g for 5 min to obtain a better separation of the essential oil from the water. The essential oil was stored refrigerated in an amber bottle. Together with the extraction, the moisture content was determined according to the method of Pimentel *et al*. [11]. The yield of the essential oil was calculated and expressed in weight of oil per unit weight of plant material on a Moisture-Free Basis (% w/w MFB).

2.3. Chemical Characterization and Quantification of the Constituents of the Essential Oil

Chemical analysis of the essential oil from *Callistemon viminalis* blossoms was performed in the Departamento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, Portugal. Analyses by gas-liquid chromatography (GLC) were performed on a Perkin Elmer 8700 gas chromatograph equipped with two flame ionization detectors (FID), and a data processing system. Two columns of different polarities were installed: a DB-1 fused

silica column with a methyl silicone stationary phase (30 m × 0.25 mm ID, film thickness, 0.25 mm; J & W Scientific Inc.) and a DB-17HT fused silica column (30 m × 0.25 mm, film thickness 0.25 mm; J & W Scientific Inc.). The oven temperature was programmed from 45˚C to 175˚C in increments of 3˚C/min and, subsequently, from 175˚C to 300˚C at 15˚C/min. The temperature was maintained at 300˚C for 10 min. The temperatures of the injector and detector were 290˚C and 280˚C, respectively. Hydrogen, adjusted to a linear velocity of 30 cm/s, was used as the carrier gas. The split ratio was 1:50. The percentage composition of the essential oils was determined by integration of peak areas without the use of correction factors. The values given represent the average of two injections.

In the gas-liquid chromatography/mass spectrometric (GLC/MS) analysis, an Autosystem XL chromatograph equipped with a fused silica column DB-1 (30 m × 0.25 mm ID, film thickness 0.25 mM one; J & W Scientific Inc.) and connected to a Perkin-Elmer Turbo Mass (program version 4.1) spectrometer was used. The oven temperature was programmed from 45˚C to 175˚C at a rate of 3˚C/min and, subsequently, at 15˚C/min to 300˚C, where the temperature was maintained for 10 min. The temperature of the transfer line was 280˚C; the ionization chamber temperature was 220˚C; the carrier gas was helium, adjusted to a linear velocity of 30 cm/s. The split ratio was 1: 40; the ionization energy, 70 eV; ionization current, 60 μA; mass range, 40 - 300 U; and the scan time, 1 s. The identity of the compounds was determined by comparison of their retention indices with respect to the C9-C16 n-alkanes. The mass spectra were compared with those of standard commercial and reference compounds present in essential oils existing the laboratory and with a mass spectral library developed in the laboratory [12].

2.4. Allelopathic Activity

The germination and growth bioassays were conducted in the UFLA Seed Testing Laboratory. For the preparation of the stock solution, 0.250 mL of the essential oil emulsified in Tween 80 was used in a 1:1 (v/v) ratio and dissolved in distilled water to give a 1% stock solution. The remaining concentrations (0.1 and 0.01 v/v) were prepared by dilution, and a 1.0% v/v solution of Tween 80 in water was used as a control. The test solutions were added only at the start of the bioassays, and, when necessary, only distilled water was added [5]-[13].

Four replicates containing 50 seeds of each species, totaling 200 seeds per treatment, were used for the germination test. The seeds were placed in gerbox-type germination boxes containing two sheets of sterilized blotting paper, which were soaked with solutions containing different concentrations of the essential oil, whose volumes were equivalent to 2.5 times the mass of the dry paper. The germination boxes were maintained in a BOD at 20˚C using a 12 h photoperiod [14]. Seed germination was monitored for seven days, with daily counts of lettuce seedlings until the seventh day of sowing and assessment of normal seedlings [14].

The verification of the effect of essential oils from the flowers of *Callistemon viminalis* was performed by the following tests using four concentrations:

First germination count: count of normal seedlings germinated (that presented the perfect vegetative structures) on the fourth day after sowing [14].

Germination Speed Index (GSI): determined according to the formula given by Maguire (1962), 16 GVI = N1/N2 + 1/2 + ⋯ + Nn/n where Nn = number of germinated seeds observed on the first, second and nth days after planting the seeds, respectively. Thus, the GSI may range from zero, if no seed germinated, to a hundred, if all the seeds germinated on the first day.

Average length of root and hypocotyl: at the end of the germination test, normal seedlings had their aereal parts and root parts measured using a millimetric ruler and the average results were expressed in centimeters per seedling [14].

Determination of dry matter: after performing the measurement of the seedlings, they were packaged in paper bags and dried in an oven at 60˚C to constant weight. The means were expressed in grams [15].

2.5. Statistical Analysis

A completely randomized design (CRD) with four replications for the variables—first germination, germination speed index (GSI), length of root, shoot length and dry weight—was employed. The results were subjected to analysis of variance, and quantitative variables that were significant according to the F test were subjected to regression analysis. Data were analyzed using the Analysis of Variance for Balanced Data System (Sisvar) statistical program, according to Ferreira (2011) [16].

3. Results and Discussion

3.1. Chemical Characterization and Quantification of the Chemical Constituents of the Essential Oil

The average essential oil content found in the flowers of *C. viminalis* was 0.24% [w/w, Moisture-Free Basis (MFB)]. In agreement with the data observed in this study, Pires *et al*. (2013) [17] in studies with leaves and flowers of *C. viminalis* observed that these showed yields of essential oils from 1.42% to 0.30% for leaves and flowers, a value close to that obtained in this study compared with the flowers.

The chemical constituents present in the essential oil extracted from the flowers of *C. viminalis*, followed by their retention indices and their concentrations, are presented in **Table 1**. Compounds belonging to the monoterpene class have been identified of in various concentrations. The principal compounds were 1, 8-cineole

Table 1. Composition of the essential oil from the flowers of *C. viminalis* identified by CG/MS and quantified by GC-FID.

Compounds	RI*	Composition** (%)
α-Thujene	924	0.10
α-Pinene	930	16.00
Camphene	938	t
Sabinene	958	t
β-Pinene	963	0.58
6-Methyl-5-hepten-2-one	960	t
β-Myrcene	975	0.09
α-Phelandrene	995	0.15
Isoamyl isobutyrate	995	t
α-Terpinene	1002	t
p-Cymene	1003	t
1,8-Cineole	1005	66.93
Limonene	1009	10.04
γ-Terpinene	1035	0.42
Terpinolene	1064	t
Linalool	1074	0.09
endo-Fenchol	1085	0.15
α-Campholenal	1098	t
trans-Pinocarveol	1106	t
Pinocarvone	1121	t
Borneol	1134	66.93
Terpinen-4-ol	1148	10.04
α-Terpineol	1159	0.42
trans-Carveol	1189	t
2-Phenyl ethyl acetate	1228	2.19
Geraniol	1236	t
Eugenol	1327	t
Geranyl acetate	1370	t
3,3,5,5,8,8-Hexamethyl-7-oxabicyclo[4, 3, 0]non-1(6)-ene-2,4-dione	1488	t
3-*cis*-Hexenyl benzoate	1533	t
Spathulenol	1551	0.13
Globulol	1566	t
Viridiflorol	1569	t

*RI = Calculated retention index relative to C_9-C_{16} *n*-alkanes on the DB-1 column, **t = trace (<0.05%).

(66.93%), α-pinene (16.00%) and limonene (10 04%). Silva *et al*. (2010) evaluated the chemical composition of the essential oils from the leaves of *C. viminalis* collected in Brazil and found 1, 8-cineole (65.0%), α-pinene (12.0%) and α-terpineol (13.0%) to be the principal compounds. They found α-terpineol in much smaller amounts (0.22%) in the essential oil from the flowers, a result that disagrees with the findings of the present study. According Gobbo-Neto and Lopes (2007) [18], climatic factors, the age and development of the plant, and the different plant organs can influence not only the total amount of metabolites produced, but also the relative proportions of the constituents in the mixture.

3.2. Allelopathic Activity

There was a dose-dependent effect **Figure 1** for the variables—dry mass, GSI, shoot length and length of the

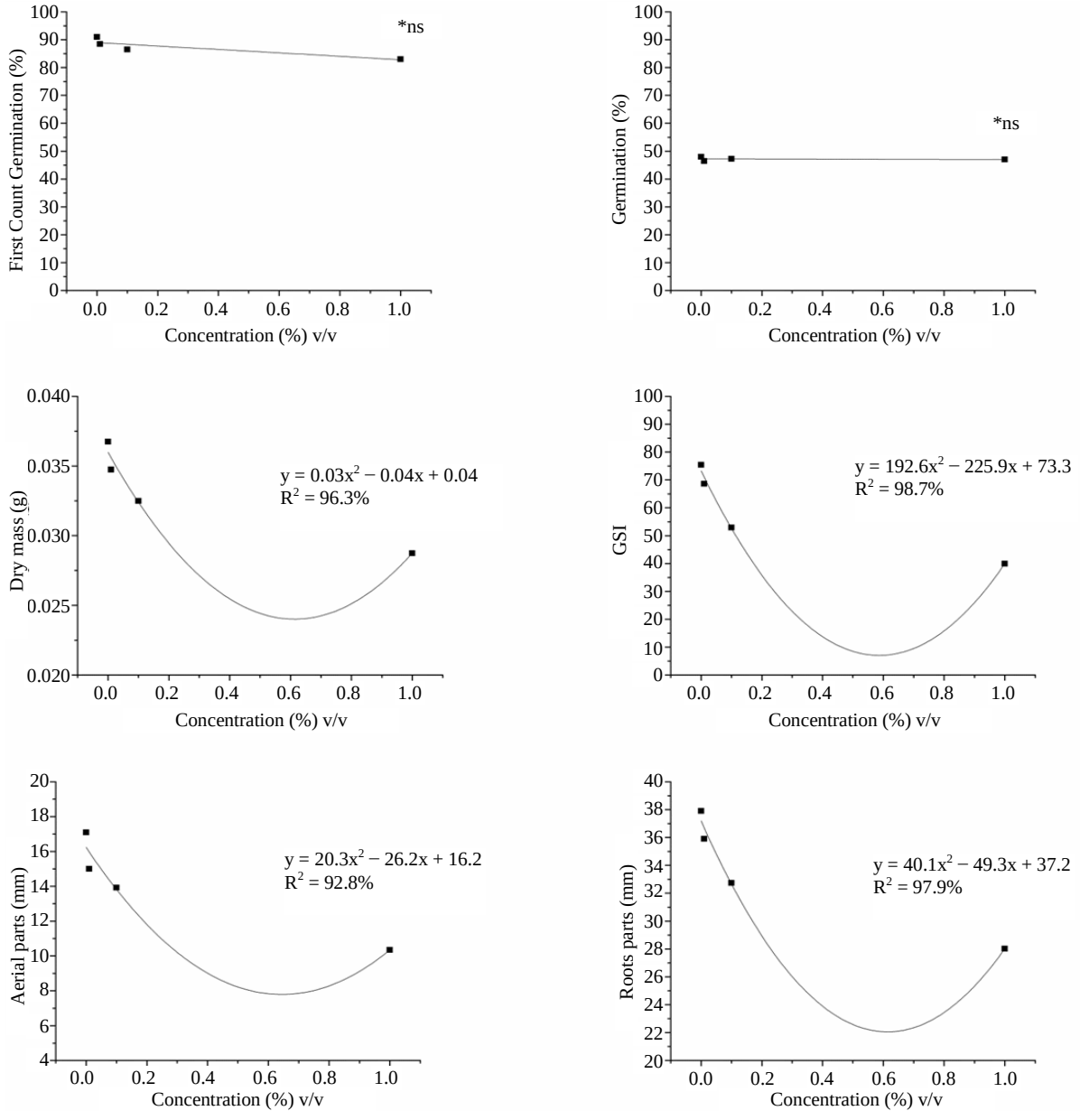

Figure 1. The effect of the concentrations of the essential oil obtained from the *Callistemon viminalis* flowers on the percent of germination and germination speed index of lettuce seeds and the dry weight and the lengths of the shoots and roots of lettuce seedlings.

roots—that is, their values were inversely proportional to the concentration of the essential oil. The different concentrations of the essential oil did not affect the first count and the germination of the lettuce seeds.

Singh *et al*. (2005) [19] detected a high toxicity of the essential oil from eucalyptus species (*Eucalyptus citriodora*), which belong to the same family as *C. viminalis* (Myrtaceae), against the weed Parthenium hysterophorus. The authors observed that the seedling length decreased with increasing concentration of the eucalyptus oils (0.2 - 5.0 $\mu L \cdot mL^{-1}$). Their result corroborated that observed in the present study. The germination was completely inhibited at 5.0 $\mu L \cdot mL^{-1}$. This result substantiates the bioherbicidal effect of the essential oil on the germination and initial growth of weeds and differs from the results obtained in this study. Similarly, Yamaguchi *et al*. (2011) [20] studied aqueous extracts of eucalyptus (*Eucalyptus globulus Labill.*), which belongs to the Myrtaceae family, and found that the percentage of germination within seven days of all cultivated species decreased with increasing concentrations of the extract. A greater decrease (24%, 12% and 7% germination of lettuce seeds) was observed with the application of higher concentrations of the extract (70%, 90% and 100%, respectively).

All the concentrations of the essential oil from the flowers caused a significant decrease in the GSI. A reduction in germination rate occurred because of the delay in seed germination during the first three days of the experiment **Figure 1**. The essential oil also affected the growth of lettuce seedlings and caused a reduction in the length of shoots and the root system, this decrease being proportional to the concentration of the essential oil **Figure 1**. Similar results were found by Verdeguer *et al* (2009) [21], who observed a decrease in the rate of of germination and growth of *Amaranthus hybridus* and *Portulaca oleracea* with increasing concentrations of 0.125.; 0.5 and 1 $\mu L \cdot mL^{-1}$ when essential oils from Eucalyptus (*Eucalyptus camaldulensis*) were used.

The decrease in the dry weight of the plants was proportional to the concentration of the essential oil applied **Figure 1**. This observation could be confirmed by the results for the length of shoots and root parts because they demonstrate the manner in which the plant grows and the amount of dry mass is directly linked to these parameters.

According to Rodrigues, Rodrigues and Reis (1999) [22], allelopathic compounds inhibit germination and growth by interfering with cell division, membrane permeability and the activation of enzymes. This allelopathic effect presented by the essential oil from *C. viminalis* may be due to the presence of the principal constituents 1, 8 cineole, α-pinene and limonene or to chemical synergism between the compounds in the essential oil.

4. Conclusion

The principal constituents that characterized the essential oil from the flowers were 1, 8-cineole, α-pinene and limonene at concentrations of 66.9%, 16.0% and 10.0%, respectively. The essential oils from the flowers presented allelopathic activity at intensities that were proportional to the concentration of the essential oil and caused a reduction in the germination speed index (GSI) of lettuce seeds and in the dry mass and length of shoots and roots of lettuce seedlings.

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