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Allelopathic Activity and Chemical Analysis of the Essential Oil of *Croton limae* A. P. S. Gomes, M. F. Sales & P. E. Berry (Euphorbiaceae)

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Abstract

The allelopathic activity and chemical composition of the essential oil of *Croton limae* A. P. S. Gomes, M. F. Sales & P. E. Berry (marmeleiro-prateado) have been evaluated in this study. The essential oil was extracted by hydro-distillation. A completely randomized design was used to test the influence of the essential oil from fresh leaves of *C. limae* in concentrations of 0.10%, 0.25%, 0.50%, 0.75%, 1%, 1.25%, 2.50%, 3.75%, and 5% on the germination and growth of *Lycopersicum esculentum* Mill. (tomato) seeds by indirect contact, with a control using water and dimethyl sulfoxide (DMSO). Each treatment consisted of five replicates using twenty seeds in each one. The chemical analysis of the essential oil was carried out using gas chromatography coupled with mass spectrometry. The analysis showed the occurrence of 14 chemical compounds, the majority of which were cedrol (28.4%), eucalyptol (17.4%) and α-pinene (13.8). The *C. limae* essential oil inhibited germination of the tomato seeds at concentrations of 2.50% and upwards and it affected the caulicles and radicles of tomatoes at all concentrations tested. The tests proved that *C. limae* presents phytotoxic activities.

Keywords: germination, growth, inhibition, allelochemicals, marmeleiro-prateado

1. Introduction

According to the National Health Surveillance Agency (ANVISA), Brazil has been the biggest consumer of pesticides worldwide since 2008, and this is due to intensive agricultural growth. In 2010, the Brazilian consumption of pesticides represented US $7.3 billion, which corresponded to 14.25% of the world total of US $51.2 billion (Molina, 2012). However, the large-scale use of pesticides has caused diverse environmental problems and human health hazards (Peres & Moreira, 2007). With this in mind, it is important to develop alternatives weed control techniques through research of nontoxic pesticides which, unlike synthetic pesticides, do not cause damage to the environment. Thus the number of studies on allelopathic activity of flora elements has increased in recent decades.

Allelopathy encompasses various processes involving secondary metabolites production in plants, algae, bacteria and viruses, leading to direct influence on the growth and development of biological and agricultural systems (Gniazdowska & Bogatek, 2005).

The plants of the Cerrado produce diverse secondary metabolites acting as defense mechanisms in response to the constant metabolic stress (water and nutritional shortages) that they are exposed to. The Cerrado biome has an important role in the maintenance and conservation of biodiversity, even though it occurs in relatively restricted areas, and is being threatened by the intense anthropogenic use of its natural resources. Therefore many efforts are currently being made to maintain and conserve these areas (Almeida, Proença, Sano, & Ribeiro, 1998; Scariot, Silva, & Felfili, 2005; Silva, Martim, Silva, Young, & Ladeira, 2006).

The genus of *Croton* are rich in chemical compounds which have important biological activities (Matos, 2000). Some of these species have had their chemical and biological activities analyzed and verified, notably *Croton argyrophylloides* Müll. Arg., *Croton cajucara* Benth., *Croton urucurana* Baill., *Croton sonderianus* Müll. Arg., *Croton zehntineri* Pax. & K. Hoffm. and *Croton nepetifolius* Baill. (Morais et al., 2006a; Oliveira-Junior, Silva, Araújo, Santos Júnior, & Arnaud, 2008; Carneiro et al., 2011).

Croton limae A. P. Gomes, F. Sales & P. E. Berry was found in areas of Cerrado located in the Chapada do Araripe, in the south of the state of Ceará. This species was identified in 2009; it is still little known and no research on its allelopathic activity and chemical composition has been performed until now. Thus this study aims to analyze the allelopathic activity and chemical composition of the essential oil of *C. limae*.

2. Materials and Methods

Both vegetative phase material (leaves) for the essential oil extraction and reproductive phase material for the posterior botanical taxonomy of *C. limae* were collected between 8am and 10am in an area of Cerrado located in the Chapada do Araripe. The sample area is known as Barreiro Novo (07º17′77″S, 39º32′62″W, altitude 923 m). The collected material was kept in plastic bags and subsequently partly herborized and partly refrigerated. The taxonomic identification was carried out by Dr. Margareth Ferreira de Sales, a taxonomist from the Herbarium Sérgio Tavares (HST) located at the Federal Rural University of Pernambuco (UFPRE) in Recife – PE. The voucher specimens have been housed at the Herbarium Caririense Dárdano de Andrade-Lima (HCDAL), number 6285, located in the Cariri Regional University – URCA.

The essential oil of *C. limae* was extracted from the leaves by hydro-distillation. The leaves were weighed, crushed and kept in a volumetric flask coupled to a hydro-distillation unit containing distilled water in sufficient quantity to cover the leaves. The essential oil was removed two hours after the water began to boil and sodium sulfate was added to remove the remaining water. The oil was cooled for 24 hours. After this period the essential oil was removed and kept cooled for later use.

2.1 Analysis of the Chemical Composition of the Essential Oil

The chemical analysis of the essential oil was performed by gas chromatography coupled with mass spectrum (GC/MS) in a SHIMADZU instrument with mass selective detector QP5050A, operating on 70 eV of ionization energy. A Capillary column Agilent DB-5HT (30 m × 0,25 mm of internal diameter × film thickness 0,1 μm) was used, with the following specifications: gun temperature of 270 ºC gun and detector temperature of 290 ºC, using helium gas as carrier gas (1.0 mL/min), linear speed of 47.3 cm/sec; total flux of 24 mL/min; carrier flux of 24 mL/min; pressure of 107.8 kPa; the oven temperature of the column was set to 60º C (2 min) -180 ºC (1 min) at 4 ºC/min and 180 – 260 ºC at 10 ºC/min (10 minutes).

The identification of the compounds was carried out by comparisons between the respective mass spectrum and patterns registered in the Wiley 229 database and between the retention index calculations and specialized literature values (Adams, 2001).

2.2 Allelopathic Activity Assays

The influence of the essential oil from leaves of the donor species *C. limae* collected in the Chapada do Araripe was tested on seed germination and seedling growth of the receptor species *Lycopersicum esculentum* Mill. by indirect contact. The assays were performed in the Laboratory of Applied Botany – LBA, of the Departament of Biological Sciences at the Cariri Regional University – URCA. *L. esculentum* was chosen as the receptor species due to presenting fast and uniform germination.

The allelopathic test was carried out by emulsifying the essential oil with dimethyl sulfoxide (DMSO) in the ratio 1: 1 and then diluting in distilled water to give 15 mL solutions at concentrations of 0.10, 0.25, 0.50, 0.75, 1; 1.25, 2.50, 3.75 and 5%; for the control group, a solution of DMSO and distilled water was used at a concentration of 1% (Table 1).

Table 1. Quantity of essential oil, dimethylsulfoxide and water to prepare solutions of the respective concentrations

Concentrations %	Essential oil µL	Dimethyl sulfoxide (DMSO) µL	Water µL
Control	-	150	14 850
0.10	15	15	14 970
0.25	37.5	37.5	14 925
0.50	75	75	14 850
0.75	112.5	112.5	14 775
1	150	150	14 700
1.25	187.5	187.5	14 625
2.50	375	375	14 250
3.75	562.5	562.5	13 825
5	750	750	13 500

The treatments consisted of five repetitions each, using 20 seeds of the receptor species for each repetition, totaling 100 seeds per treatment. The seeds were sown in 9 cm diameter sterilized Petri dishes containing three filter papers moistened with 3 Ml of distilled water. At the top of the Petri dishes, two filter papers were placed and 3 mL of solution at the appropriate concentration was added, following indirect contact methodology.

The assays were performed in a BOD type germination chamber set to a constant temperature of 25 ºC and photoperiod of 12 h/12 h of light and dark during seven days. After this time, germination percentage and radicle and caulicle lengths were analyzed. The biometric analysis was conducted using a five seedling sample per repetition for each treatment.

A completely randomized design was used and the data was submitted to Analysis of Variance and Analysis of Regression, with the model best fitted to the data obtained being used (Figure 1).

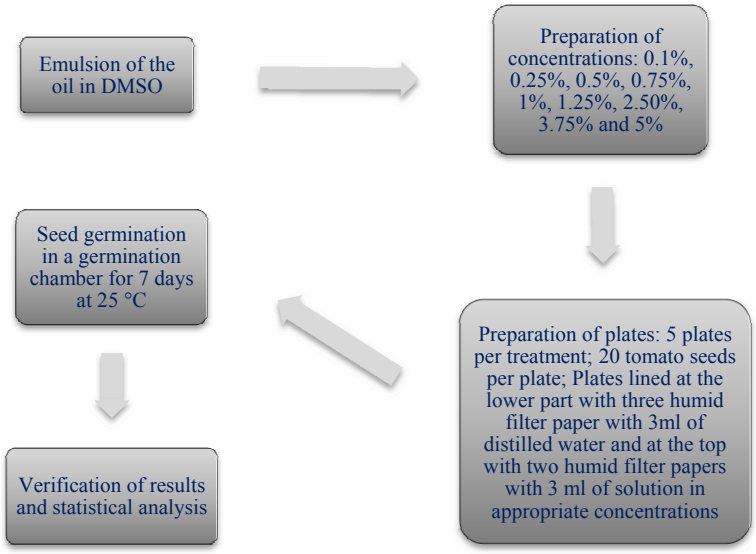

Figure 1. Diagram showing the methodology used in allelopathic test

3. Results

3.1 Chemical Analysis of Essential Oil

The yield of essential oil from the fresh leaves of *C. limae*, obtained from the leaf mass, was 0.36%. In the chemical analysis of the oil, 14 compounds were identified and quantified, representing 84.7% of the total chemical composition. The main compounds were cedrol (28.4%), eucalyptol (17.4%) and α-pinene (13.8%),

representing 59.6% of the total oil composition. The chemical compounds identified and their respective quantities, retention time and Kovats index are summarized in Table 2.

Table 2. Chemical compounds of the essential oil from fresh leaves of *C. limae*

Compounds	**RT[a](min)**	**RI[b]**	**(%)**
α-pinene	5.2	939	13.8
ß-pinene	6.2	980	3.0
ß-myrcene	6.8	991	1.5
p-cymene	7.7	1026	4.2
eucalyptol	7.8	1033	17.4
linalool	10.1	1098	2.5
cryptone	12.8	1186	1.3
p-cymen-8-ol	12.8	1189	1.3
ß-caryophyllene	20.1	1418	3.8
α-humulene	21.1	1452	2.3
alloaromadendrene	21.2	1458	1.2
Spathulenol	22.9	1576	2.8
cariofilene oxide	23.7	1581	1.2
cedrol	24.9	1589	28.4
Total identified			**84.7**

Note. [a] Retention time, [b] Kovats index (Adams, 2001).

3.2 Effects of Allelopathic Activities of Essential Oil

The essential oil of fresh leaves of *C. limae* exhibited meaningful inhibitory action on the germination of *L. esculentum* only at concentrations above 2.5% (Figure 2a, Table 3). The caulicle length suffered meaningful reduction at all concentrations tested (Figure 2b, Table 3).

The result of the statistical analysis of the radicle biometric showed a meaningful reduction of radicular development in all treatments (Figure 2c, Table 3), verifying that increasing concentration caused a greater inhibition of the radical elongation, mainly at concentrations of 2.50% and 3.75%.

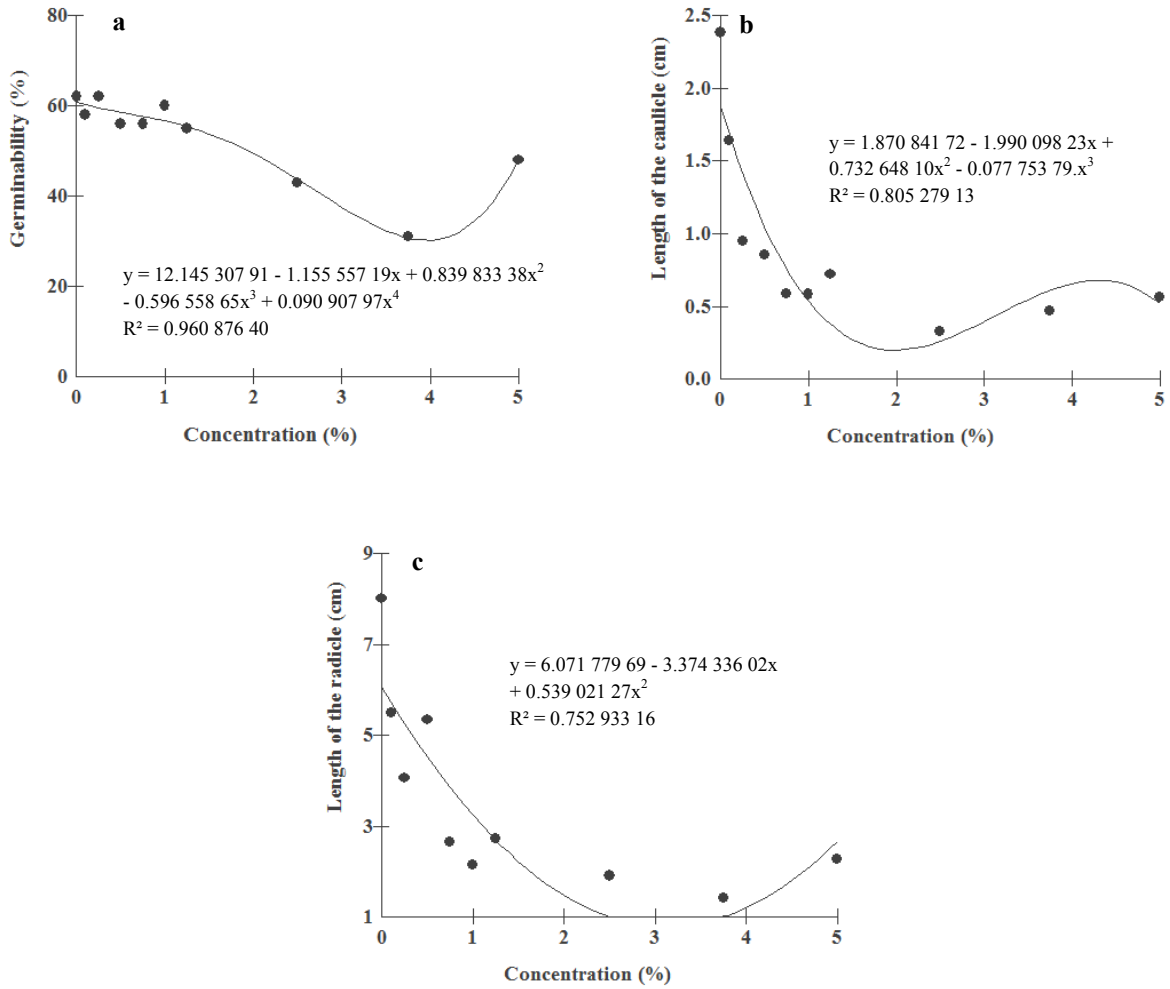

Figure 2. Effect of essential oil of fresh leaves of *C. limae* at concentrations of 0.10; 0.25; 0.50; 0.75; 1.00; 1.25; 2.50; 3.75 and 5.00 % on average values of germination index (A), caulicle length (B) and radicular length of seedlings of *L. esculentum* (C)

Table 3. Analysis of variance of seeds germination and caulicle and radicle length of seedlings of *L. esculentum*. Subject to effects of essential oil from fresh leaves of *C. limae*

Sources of Variation	DF	MS		
		G	Length of the caulicle	Length of the radicle
Treatments	9	19.264 44*	2.005 57 **	21.887 50 **
Residue	40	5.310 00	0.090 08	1.553 00
CV (%)		21.70	33.08	34.57

Note. ** significance at 1%, * significance at 5% by Ftest ; CV = coefficient of variation; MS = mean squares; DF = degrees of freedom; G = germination.

4. Discussion

4.1 Chemical Analysis of Essential Oil

Other species of *Croton* have presented a chemical composition partly similar to the one found in our research; as in the case of Compagnone et al. (2010), who found α-pinene, β-pinene, β-myrcene, linalool, β-caryophyllene, alloaromadendrene, spathulenol and cedrol in the chemical composition of *Croton micans* Muell. Arg. and *Croton matourensis* Aubl.. However, there is a divergence regarding the majority compounds present; for the

leaf oil of *C. micans*, the majority ones were fenchyl acetate (25.3%). β-caryophyllene (20.7%), α-selinene (12.8%) and for the flower oil of *C. micans*, fenchyl acetate (41.6%), β-caryophyllene (12.6%) were shown to predominate.. For *C. matourensis* leaf oil, the majority compounds were fenchyl acetate (19.5%), metyleugenol (14.2%) and isoelimicine (11.3%). Still others present a chemical composition totally divergent from that found in this study, as is the case of *Croton zehntneri* Pax & K. Hoffm. (estragol variety), which presented 1,8-cineole, eugenol, mircene, biciclogermacrene, β-ocimene and sabinene in the composition of its leaf essential oil (Costa el al., 2008).

Various studies have shown that phenylpropanoids, such as anetol and compounds derived from eugenol which are present in fennel, clove and basil oils, are among the main components of the essential oils of the species of *Croton* found in many places of the world, such as *Croton malambo* H. Karst. and *Croton cuneatus* Klotzsch in Venezuela (Suárez, Vásquez, Manzano, & Compagnone, 2005), *Croton pseudoniveus* Lundel and *Croton suberosus* Kunth in Mexico (Perez-Amador, Monroy, & Bustamante, 2003), *C. zehntneri* and *C. nepetifolius* in Brazil (Morais et al., 2006b).

A study carried out on the essential oil extracted from different parts of *Croton blanchetianus* Baill. (leaves, flowers, roots and trunk bark) collected in different regions of the state of Ceará at different times of the day enabled the investigation and identification of 32 compounds. Among these, the majority compounds were β-phellandrene (20.4%) in the leaves, bicyclogermacrene (29.1%) in the flowers and also in the leaves (17.7%), β-elemene (17.8%) in the flowers and also (22.0%) in the trunk bark, cyperene (14.2%) in the roots and *D*-germacrene (12.8%) in the trunk bark (Dourado & Silveira, 2005).

The diversity of chemical composition found in the distinct species of *Croton* could be due to the richness of this genus in terms of the number of species.

Souza et al. (2006) analyzed the chemical composition of the fixed oil of *C. cajucara* bark from a regional market ("Ver o peso" market, located in Belém, state of Pará, Brazil) and noted the occurrence of cycloisosativene, α-copaene, ciperene, α-bergamotene, 2,6-dimethyl-6-(4-methyl-3pentenyl), bicyclo [3.1.1] hep-2-eno, *cis*-cariofileno, alloaromadendrene, α- longipinene, δ-guaiene, α-muurolene, δ-cadinene, nerolidol, spathulenol, viridiflorol. Only the compounds alloaromadendreno, linalool and spathulenol found by Souza et al. (2006) matched with the compounds detected in this study.

According to a bibliographical survey carried out by Torres (2008) the compounds most commonly found in *Croton* species studied in Brazil were diterpenes, which form the class of terpenoids and alcaloids.

4.2 Effects of Allelopathic Activity of Essential Oil

According to Sisodia and Siddiqui (2010) the aqueous extracts of roots, trunks and parts of the leaves of *Croton bonplandianus* Baill at concentrations of 2 and 4% significantly inhibited the growth of seedlings of *Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., *Brassica rapa* L., *Melilotus albus* Medik., *Vicia sativa* L., and *Medicago hispida* Gaertn. The inhibitory effect was greatest on seedlings of *M. albus*.

The fact that many chemical compounds present in the essential oil of *C. limae* present allelopathic activity has been substantiated by many authors. β-pineno inhibited the germination and development of *Salvia leucophylla* Greene, reducing the mitotic rate of the apical region of the roots (Nishida, Tamotsu, Nagata, Saito, & Sakai, 2005). According to Zunino and Zygadlo (2004), eucalyptol inhibited the *Zea mays* L. (corn) root growth through induction of oxidative stress by the production of malondialdeidos, conjugated diene and peroxids. Cedrol inhibited the growth of *Echinochloa crus-galli* (L.) P. Beauv. roots (He et al., 2006).

Singh, Batish, Kaur, Arora and Kohli (2006), analyzing the allelopathic effect of α-pinene in the germination and establishment of seeds of *Triticum aestivum* L. "HD-2329" (wheat), *Cassia occidentalis* L. (coffee), *Amaranthus viridis* L. (green amaranth), *Cicer arietinum* L. "GL-470" (chickpea) and *Pisum sativum* L. "AP-1" (pea), noted inhibition of germination of the first three species and inhibition of radicle growth of all of the species tested. The exposure to α-pineno caused a rupture of the membrane resulting in an excessive liberation of ions. This occurred due to an oxidative stress caused by increased production of reactive oxygen species (ROS). It also promoted increased levels of malondialdehyde or MDA (the main thiobarbituric acid-reactive species, or TBARS) in C. occidentalis roots. According to Almeida, Zucoloto, Zetun, Coelho, and Sobreir (2008), most of the allelochemicals produce ROS which trigger oxidative stress. The ROS can act directly or indirectly as signaling in cellular degradation processes, disabling germination, initial development and vital physiological process for the plants.

Various types of stress, including environmental and pollutant, biotic and abiotic, promote the production of ROS causing oxidative stress production. Plant cells possess specific enzymes for the production of ROS

(Desikan, Hancock, Neill, & Bogatek, 2005). The ROS, ($^1O_2$, O_2^-, OH^- e H_2O_2), are highly reactive molecules that can affect the permeability of the membrane, causing damage to DNA, proteins, lipids and photosynthetic pigments (Smirnoff, 2005; Weir, Park, & Vivanco, 2004; Almeida, Zucoloto, Zetun, Coelho, & Sobreir, 2008). The increase in lipid peroxidation and the leaking of electrolytes resulting in the loss of membrane integrity are among the factors which determine cell damage. The generation of ROS and the related oxidative stress has recently been proposed as one of the forms of activity of plant growth inhibition by allelochemicals (Weir et al., 2004; Blokhina, Virolainen, & Fagerstedt, 2003).

According to Souza-Filho, Vasconcelos, Zoghbi, and Cunha (2009), the biological activity presented by a certain allelochemical substance is determined by its concentration and by the limit of the response from the affected species. In the case of inhibition, the limit is not constant; it depends on the sensibility of the receptor species, the metabolic and physiological processes of the plant and the environmental conditions.

Considering that the cytotoxic action of α-pinene, β-pinene, eucalyptol and cedrol has been proved, it can be concluded that these compounds are among those responsible for the cytotoxic activity cited of *C. limae* and that they probably act in synergy. These compounds possibly act to promote the increase of ROS production, thus enabling oxidative stress. It can be possible that the consequences of this oxidative stress cause the cellular membrane rupture, but also damage DNA, proteins, photosynthetic pigments or lipids. Essential oil fractionation studies are fundamental to determine which compound or compounds are responsible for the phytotoxicity noted for *C. limae.*

The essential oil of *C. limae* is composed of 14 compounds, with cedrol, eucalyptol and α-pinene being the main ones. The compounds cited, together or isolated, are able to inhibit the germination of tomato seeds at concentrations of 2.50% and upwards and also inhibit the caulicle and radicle development of tomato seedlings at all the concentrations tested.

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