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Influence of Plant Age on Chemical Composition, Antimicrobial Activity and Cytotoxicity of *Varronia curassavica* Jacq. Essential Oil Produced on an Industrial Scale

Jéssica Pires Farias [1], Ana Luisa Alves Nogueira Barros [2,3], Alyne Rodrigues de Araújo-Nobre [1], Enoque Pereira Costa Sobrinho-Júnior [4], Michel Muálem de Moraes Alves [4], Fernando Aécio de Amorim Carvalho [4], Klinger Antonio da Franca Rodrigues [1], Ivanilza Moreira de Andrade [1], Francisco Artur e Silva-Filho [5], Daniel Carneiro Moreira [2], David Fernandes Lima [6], Massimo Lucarini [7], Alessandra Durazzo [7,*], Daniel Dias Rufino Arcanjo [4,8] and José Roberto de Souza de Almeida Leite [1,2,3,*]

1 Núcleo de Pesquisa em Biodiversidade e Biotecnologia, Biotec, Campus Ministro Reis Velloso, Universidade Federal do Delta do Parnaíba, Parnaíba 64202-020, Brazil
2 Núcleo de Pesquisa em Morfologia e Imunologia Aplicada, Faculdade de Medicina, Campus DarcyRibeiro, Universidade de Brasília, Brasília 70910-900, Brazil
3 Programa de Pós-graduação em Medicina Tropical, Núcleo de Medicina Tropical, Faculdade de Medicina, Campus Darcy Ribeiro, Universidade de Brasília, Brasília 70910-900, Brazil
4 Laboratório de Atividade Antileishmania, Núcleo de Pesquisas em Plantas Medicinais, Campus Ministro Petrônio Portella, Universidade Federal do Piauí, Ininga, Teresina 64049-550, Brazil
5 Programa de Pós-graduação em Química, Universidade Estadual do Piauí, Parnaíba 64002-150, Brazil
6 Faculdade de Medicina, Universidade Federal do Vale do São Francisco, Paulo Afonso 48605-560, Brazil
7 CREA-Research Centre for Food and Nutrition, Via Ardeatina 546, 00178 Rome, Italy
8 Laboratório de Estudos Funcionais e Moleculares em Fisiofarmacologia, LAFMOL, Departamento de Biofísica e Fisiologia, Universidade Federal do Piauí, Teresina 64049-550, Brazil
* Correspondence: alessandra.durazzo@crea.gov.it (A.D.); jrsaleite@gmail.com (J.R.d.S.d.A.L.)

Abstract: Considering the therapeutic potential of *Varronia curassavica* Jacq. essential oil and the great value in the pharmaceutical market, this study aims to evaluate the influence of plant age on the chemical composition and biological activities of *V. curassavica* Jacq. essential oil. The plant age is a parameter that can influence the chemical composition of the essential oil, as well as its pharmacological potential. For this purpose, essential oils from aerial parts of *V. curassavica* produced at different ages (4, 10, 14 and 18 months-age) were used. According to chromatograms obtained by GC-MS, the essential oils were mainly composed of α-pinene, *trans*-caryophyllene, α-santalene, alloaromadendrene and α-humulene. The chemical composition of *V. curassavica* essential oils varied qualitatively and quantitatively with the aging of the plants, and the essential oils from plants at 18 month-age appeared to be the most distinct from the others. The tested essential oil samples showed inhibitory activity against *Candida albicans* (MIC = 1000 µg/mL) but did not show antibacterial activity against the tested bacteria. The cytotoxic activity levels against the murine macrophages varied among the oils extracted from the plants at different ages; the IC_{50} values of the essential oils increased with age (171.90 µg/mL at 18 month-age). More studies should be carried out to assess whether age also affects the therapeutic effects of essential oils, resulting in the manufacture of plant-derived formulations that balance production costs, toxicity and therapeutic effects.

Keywords: *Cordia verbenacea*; essential oil; medicinal plant; GC/MS; antifungal; cytotoxicity

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1. Introduction

Varronia curassavica Jacq., also known as *Cordia verbenacea* DC., is an aromatic plant known as "*erva-baleeira*" that belongs to the Boraginaceae family [1]. This plant is native to the Brazilian Atlantic Forest, of the restinga vegetation class, with distribution ranges from the east of the Amazon region to the south of the state of Rio Grande do Sul [2]. The

shrubs of this species have leaves covered with glandular trichomes (sites of production and storage of essential oil (EO)), white flowers (ranging between four to six petals) in the axis of inflorescence and red fruit, and can reach up to 2 m in height [3–5].

The aerial parts of this species are widely used in traditional medicine in many communities, with anti-inflammatory, analgesic and wound-healing properties [6–9]. Other biological and pharmacological activities have also been attributed to *V. curassavica*, such as antibacterial [10], antifungal, antiprotozoal [11,12], antitumor [13], antioxidant [14] and antiulcerogenic [15] properties. Recently, Andrade et al. [16] demonstrated the potential of *V. curassavica* EO for natural pest control, and Oliveira et al. [17] used *V. curassavica* EO to study bioassays of fumigation toxicity, aiming to control a common species of ant (*Dorymyrmex thoracicus*), showing that the oil was able to repel and decrease the motility of the ants.

Many of the previously described therapeutic effects of *V. curassavica* are associated with plant secondary metabolites, whose preferred source of industrial production is the essential oil extracted from the leaves [18]. Acheflan®, an anti-inflammatory phytomedicine produced in Brazil by the Aché Laboratory, approved in 2004 by ANVISA (the Brazilian National Sanitary Surveillance Agency (NSSA)) and used for treatment of trauma, tendinopathy and muscular pain, is manufactured from essential oil from *V. curassavica* aerial parts (containing 5 mg of EO and approximately 0.130 mg of α-humulene). Currently, Acheflan®is the most prescribed drug in this market segment, with sales of more than $8 million USD in the pharmaceutical market, being exported to countries in the Americas and Japan [3,19,20].

Considering the commercial value of essential oils (up to $5800.00 USD per liter), the best technique for obtaining the essential oil is to improve the quantity and quality of the *V. curassavica* EO process, including an exhaustion time of 1.5 h for extracting the oil, with faster extraction of monoterpenes compared to sesquiterpenes, using the steam distillation method, which has been demonstrated to be the best method for extraction [20,21]. The contents of the specific plant secondary metabolites and the chemical composition of the essential oils are influenced by both genetic and environmental factors; generally, the main constituents of *V. curassavica* EO are *trans*-caryophyllene, β-santalene, α-pinene and α-humulene [16,22–24]. The anti-inflammatory activity of α-humulene and *trans*-caryophyllene was proven in previous studies, and these two compounds in the EO extracted from *V. curassavica* leaves for Acheflan®production are used for control by ANVISA here in Brazil [3,4].

Considering the expected effect of age on the composition of plant essential oils and the derived industrial products, this study aimed to assess the influence of the age of *V. curassavica* plants on the chemical composition of the essential oil extracted at the industrial scale. Moreover, we also analyzed the effect of age on the cytotoxicity against murine cells (BALB/c peritoneal macrophages) and on the activity against bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*) and yeast (*Candida albicans*).

2. Material and Methods

2.1. Plant Material and Essential Oil Production

The plants were cultivated in an agricultural production area by the Centroflora Group (Parnaíba, Piauí, Brazil). The *V. curassavica* plantation is located in a central pivot area covering 59.8 hectares, divided into sub-areas that are numbered and differentiated from each other according to their planting date. The exsiccate of the botanical material used was deposited in the Herbarium Delta do Parnaíba (HDELTA, Parnaíba, Piauí, Brazil), under voucher specimen no. 3930. The area selected for the development of the project corresponded to sub-area 13, at a latitude of 3°1.720′ S and longitude of 41°45.021′ W, with dimensions of 3.34 hectares, with the genotype characterized as ID 13 by Lima et al. [25], with an average height of 70.66 cm and an average diameter equal to 14.01 mm after one year of planting (Figure 1).

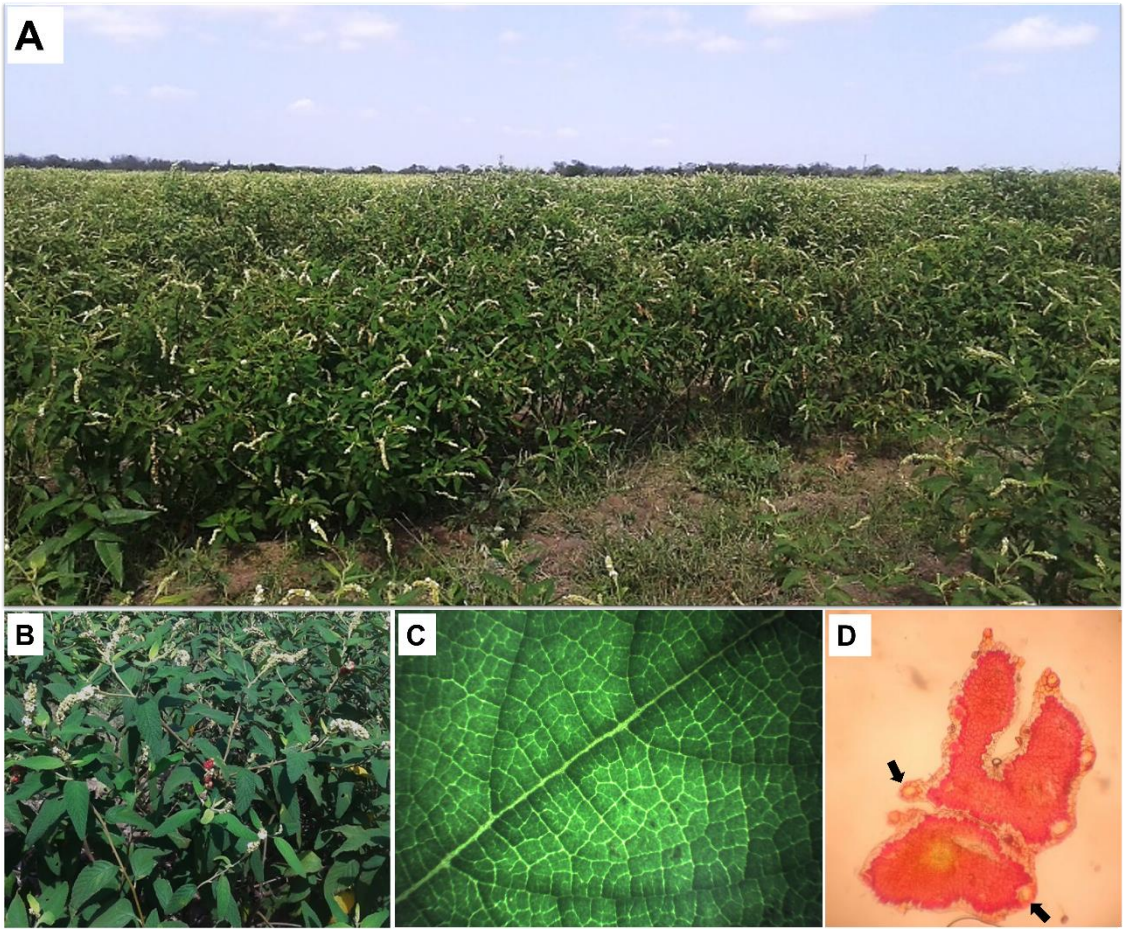

Figure 1. Images of *V. curassavica*. (**A**) at the place of cultivation. (**B**) The leaves, flowers and fruit of the plant. (**C**) The leaf details. (**D**) A leaf under microscopy showing the epidermis and the essential oil storage location (black arrows).

The aerial parts were harvested at the same time (05:00) at different ages (4, 10, 14 and 18 months-age) in (i) September 2016 and (ii) March, (iii) July and (iv) November 2017. The parts were collected mechanically with the aid of a forage harvester for essential oil production by the Centroflora Group. The leaves were taken to a laboratory for microscopic study. Briefly, the slides were made with freehand paradermal and transversal cuts of the stem and the midrib of the leaf. The samples were clarified with 2% sodium hypochlorite and later stained with 0.5% safranin for viewing under a stereoscopic microscope, through which it was possible to observe the globular trichomes, where the plant stores and secretes essential oil (Figure 1D) [26,27].

The essential oil (EO) was extracted by steam distillation on an industrial scale, at 09:00 on the same day the material was harvested. Briefly, the aerial parts were uniformly distributed and compressed in a stainless-steel vat. Steam distillation was carried out for two hours (Figure 2).

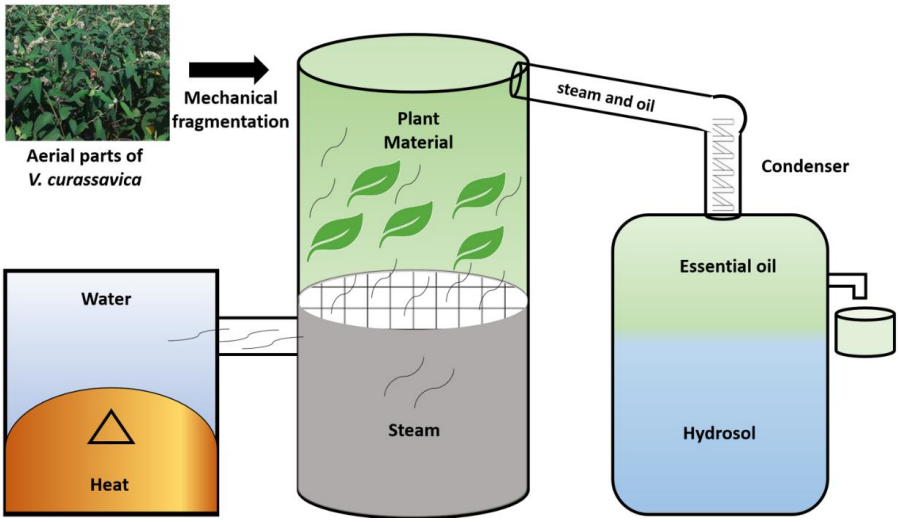

Figure 2. Representative diagram of essential oil extraction process of *V. curassavica* on an industrial scale.

2.2. Phytochemical Characterization of V. curassavica Essential Oil

Samples of essential oil extracted from plants at different ages (4, 10, 14 and 18 months-age) were characterized using a gas chromatography system coupled to a mass spectrometer with electron impact ionization (GC-MS-QP210 SE, Shimadzu, Kyoto, Japan). Separation was achieved using the Equity®-1 Capillary GC Column (L × I.D. 60 m × 0.25 mm, df 1.00 µm; Supelco, Bellefonte, PA, USA), following the temperature programming from 100 to 240 °C (5 °C/min), where it remained for 5 min, followed by heating from 240 °C to 280 °C (10 °C/min) for the same duration. Helium was used as the inert gas at 1 mL/min, with a split ratio of 1:10 and an injected volume of 1 µL. The injector was kept at 250 °C and the detector at 280 °C. The following conditions were used for the mass spectrometer: ion source temperature: 200 °C; interface temperature: 290 °C; 5 min delay time; analysis mode: scan (35–350 m/z). Dibutyl phthalate was used as the internal standard, following the method validated by the Centroflora Group. Compounds were identified by comparing the mass spectra of samples with those in the NIST 14 library; the retention times of samples with those of the analytical standards of α-pinene, *trans*-caryophyllene and α-humulene; and the calculated Kovats index values of sample constituents with those available in the literature [28].

2.3. Cytotoxicity Assay

Murine peritoneal macrophages were isolated from BALB/c mice and treated with essential oils or α-pinene, and their viability was assessed using an MTT (3-(4, 5-dimethylthiazol-2-118 yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) [29]. Briefly, macrophages were obtained from female BALB/c mice (4–5 weeks old) obtained from the Animal Facility of Universidade Federal do Piauí (UFPI, Teresina, Piauí, Brazil) and kept in a controlled environment with a constant temperature of 24 ± 1 °C and a 12 h light/dark cycle. Then, 3.0% thioglycolate (1.5 mL) was intraperitoneally administered, and after three days the sterile phosphate-buffered saline (PBS, pH 7.4) was injected into the abdominal cavity to the remove the cells. This procedure was approved by the Ethics Committee for the Use of Animals from Universidade Federal do Piauí (CEUA-UFPI, Teresina, Piauí, Brazil) under approval n°457/18.

Then, the macrophages were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10% FBS), penicillin (10,000 IU/mL), and streptomycin (10 mg/mL) (Sigma-Aldrich, St Louis, MO, United States) at a concentration of 1.0×10^5 cells per well, where they were exposed to treatments with *V. curassavica* EO samples and α-pinene at 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL in triplicate. Afterwards, 10 µL of MTT

(5 mg/mL in PBS (*w/v*) was applied to each well and incubated for 4 h throughout the course of 48 h. The supernatant was then discarded, and 100 µL of DMSO was immediately added to each well. To completely dissolve the formazan salt, the plate was stirred for 30 min. The absorbances were measured at 540 nm using the ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA). A concentration–response curve was plotted to determine the mean inhibitory concentration (IC_{50}), using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, United States).

2.4. Antimicrobial Assays

The minimum inhibitory concentration (MIC) values, i.e., the lowest concentrations inhibiting visible microbial growth, of the essential oils were determined following Clinical and Laboratory Standards Institute procedures [30,31] against the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus epidermidis* (ATCC 12228), in addition to yeast *Candida albicans*. Briefly, the bacteria were inoculated in Mueller–Hinton broth (Difco™) at a concentration of 5×10^5 CFU/mL in 96-well microplates, where they were exposed to serial dilutions of *V. curassavica* EO samples (7.8–1000 µg/mL), then were incubated for 24 h at 37 °C in triplicate. For yeast, the essential oils were tested at the same concentrations against 5.0×10^2–2.5×10^3 CFU/mL of *C. albicans* in 96-well plates with a U-shaped base. After the incubation period (24 h/37 °C), the result was analyzed via visual readings of microbial growth.

2.5. Statistical Analysis

The relative contribution of each compound identified in the essential oil was used for a principal component analysis of extractions at different plant ages using Minitab software v.19 (Minitab, State College, PA, USA). The levels of α-pinene, *trans*-caryophyllene and α-humulene were compared using a one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism software v.6.1 (GraphPad, San Diego, CA, USA). The results of the cytotoxicity assay were compared to the respective control untreated group using a one-way ANOVA followed by Dunnett's multiple comparisons test. At all instances, p values below 0.05 were considered to indicate statistical significance.

3. Results and Discussion

The chemical characterization via GC/MS identified 19 molecules in *V. curassavica* essential oils at 4, 10, 14 and 18 months-age old, as reported in Table 1. The respective yields of those essential oils were 0.093%, 0.096%, 0.083% and 0.112% (*w*/*w*) (Table 2). Regardless of age, the most abundant compounds in the essential oils were α-pinene, *trans*-caryophyllene, α-santalene, alloaromadendrene and α-humulene (Table 1). The compositions varied according to the plant age, mainly for less abundant molecules, which were detected in some samples and in others could not be accurately identified. α-Pinene was the most abundant compound of *V. curassavica* EOs obtained from different regions of Brazil (São Paulo, Rio de Janeiro, Minas Gerais, Bahia, Sergipe and Ceará) [3,27,32,33]. On the other hand, plants of the same species cultivated in Minas Gerais and in the southern region of Bahia (Brazil) showed β-pinene, β-caryophyllene and α-humulene as the major chemical compounds, while α-pinene and caryophyllene were also found [33–35].

Table 1. Chemical composition of the essential oils from *V. curassavica* at different ages extracted by steam distillation on an industrial scale. Data were obtained from three independent GC-MS chromatograms and are presented as (%) percentages of area of each substance relative to the total chromatogram.

Compound	R_T (min)	KI^{lit}	KI^{c}	Sample A (%)	Sample B (%)	Sample C (%)	Sample D (%)
α-tujene	10.74	930	941.98	-	-	1.35	1.56
Sabinene	10.78	975	943.24	1.20	1.49	1.05	0.95
α-pinene	**11.17**	**961**	**955.50**	**33.08**	**34.60**	**33.45**	**37.55**
Myrcene	12.08	990	984.12	1.1	1.22	-	-
β-pinene	12.33	979	991.98	1.34	1.30	1.13	1.42
D-limonene	13.60	1040	1031.93	-	-	0.45	-
1, 8-Cineole	13.72	1031	1035.70	1.79	1.74	1.56	1.65
Bornyl acetate	21.25	1288	1272.52	0.78	0.56	-	-
δ-elemene	23.25	1338	1335.42	3.35	4.13	3.66	4.03
Copaene	24.55	1376	1376.30	0.73	0.56	-	0.58
β-elemene	24.84	1390	1385.42	4.15	5.66	5.26	4.62
Trans-α-bergamotene	25.24	1434	1398.00	1.09	1.10	1.03	0.96
α-santalene	25.65	1417	1410.90	8.29	8.67	7.74	7.34
***Trans*-caryophyllene**	**25.95**	**1419**	**1420.33**	**13.05**	**16.25**	**18.25**	**12.52**
β- cedrene	26.10	1420	1425.05	1.74	-	1.54	1.42
β-funebrene	26.22	1429	1428.82	-	1.75	-	-
β-santalene	26.58	1447	1440.15	0.77	-	-	-
α-humulene	**26.98**	**1454**	**1452.73**	**2.69**	**3.32**	**4.17**	**2.62**
Alloaromadendreno	27.13	1452	1457.44	9.51	6.85	7.04	7.37
9-Epi-E-cariofilene	27.28	1466	1462.16	2.91	2.14	2.27	2.16
β-bisabolene	27.59	1528	1471.91	-	0.59	0.56	0.53
β-sesquifilandrene	28.05	1522	1486.38	0.81	-	0.69	0.66
δ-cadinene	28.24	1523	1492.35	-	-	-	1.99
Amorpha-4, 7 (11)-diene	28.33	1481	1495.18	2.38	1.98	1.99	-
Bergamotol	32.59	1690	1629.16	-	-	0.86	-
α-santalona	32.78	1577	1635.14	0.96	0.96	-	1.23
Santalol (Z)-α	33.17	1675	1647.40	1.20	1.24	1.06	1.46
Total				93	96.37	95.19	92.75
Unknown				7	3.63	4.81	7.25

Legend: Sample A = 4 month-age; sample B = 10 month-age; sample C = 14 month-age; sample D = 18 month-age.

Table 2. Vegetal raw material, resulting mass and yield of essential oils produced from *V. curassavica* at different ages (area of 3.34 ha).

Plant Age (Month-Age)	Vegetal Raw Material (kg)	Essential Oil (kg)	Yield (%)
4	1252.00	1.160	0.093%
10	1300.00	1.250	0.096%
14	1200.00	0.996	0.083%
18	889.43	1.000	0.112%

The data on the chemical characterization of essential oils (Table 1) were further analyzed using a principal component analysis and plotted as a score plot (Figure 3). As expected, the three independent essential oil extractions from plants at the same age clustered together (Figure 3); this indicated that variability in the concentrations of the 19 identified compounds is sufficient to separate essential oil samples according to plant age. The essential oil from plants at 18 month-age appeared as the most distinct from the others; the three samples clustered in the negative areas in principal component analyses 1 (*x*-axis) and 2 (*y*-axis). The first component (PC1) separated plants harvested at 4 and

18 month-age from those harvested at 10 and 14 month-age. The second component (PC2) isolated the essential oil from plants at 18 month-age from all other groups, except for one sample from plants at 10 month-age. The compounds had a rather balanced contribution for PC1; the highest coefficient was 0.239 for eucalyptol and β-elemene and the lowest was 0.201 (i.e., a narrow range of 0.201–0.239). In the case of PC2, this component was mainly a combination of β-myrcene (−0.529), β-gurjunene (0.336), α-thujene (−0.289), α-pinene (−0.276) and α-humulene (0.271).

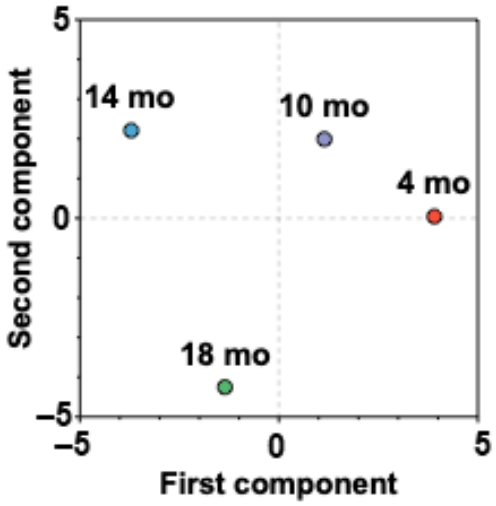

Figure 3. The principal component analysis (PCA) of three independent essential oil samples extracted from *V. curassavica* at different ages (4, 10, 14 and 18 months-age) via steam distillation on an industrial scale. The relative concentrations of the 19 identified compounds (detailed in Table 1) were used to perform the analysis.

α-Pinene and *trans*-caryophyllene were described as the major constituents of *V. curassavica* EO from another geographic area [10]. A similar profile was observed in the *V. curassavica* EO cultivated in Minas Gerais (Brazil), whose main constituents were α-pinene, α-santalene and (E)-caryophyllene [2]. On the other hand, the most abundant compounds in the essential oil of this species vary according to the cultivation site due to the different environmental conditions, directly influencing the chemical composition. For instance, α-pinene and (E)-caryophyllene were the major oil components in plants from Minas Gerais (Brazil) [35], and tricyclene, α-zingiberene, turmerone, camphene and (E)-caryophyllene were the major oil components in plants from Sergipe (Brazil) [12].

Several factors, including climatic, altitude, soil, area of cultivation, processing, and genetic variations, explain such differences [2]. Moreover, it should be noted how these variations in content are especially relevant for industrial purposes since these main identified compounds exert biological activity. The assessment of interactions of biologically active compounds represents a first step in the evaluation of the biological activity of a plant [36–38]. α-Pinene and *trans*-caryophyllene present anti-inflammatory properties through reductions in PGE2, COX-2 and inducible NOS levels [39]. Alloaromadendrene presents cytotoxic and antiproliferative activities against cancer cells [40]. α-Santalene has been used in the perfume and flavoring industries, and its analogues present some pharmacological properties such antifungal, antibacterial and antitumor activities [41].

Acheflan®contains α-humulene obtained from *V. curassavica* essential oil, and the α-humulene and trans-caryophyllene contents are controlled by the Brazilian regulatory agency, ANVISA [3,18]. Therefore, we used specific analytical standards to quantify the contents of three essential oil components, namely α-pinene, *trans*-caryophyllene and α-humulene. In Figure 4, the concentrations of the selected compounds (α-pinene, α-humulene and *trans*-caryophyllene) in the essential oil extracted from *V. curassavica* at different ages (4, 10, 14 and 18 months-age) via steam distillation on an industrial scale are

reported. The α-pinene content at 10 month-age increased by 128% compared to the content at 4 month-age. At 14 month-age, the content of α-pinene decreased by 27% compared with that in the essential oil of 10-month-old plants, and at 18 month-age it returned to levels similar to those at 4 month-age (Figure 4A). The α-humulene levels tended to increase as the plants aged, reaching their maximum value at 14 month-age and then decreasing at 18 month-age (Figure 4B).

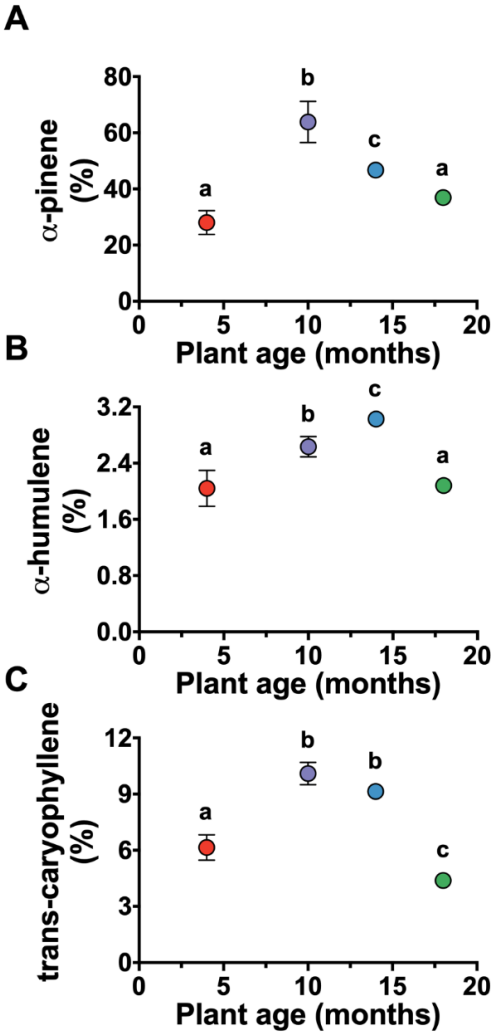

Figure 4. Concentrations of selected compounds in the essential oils extracted from *V. curassavica* at different ages (4, 10, 14 and 18 months-age) via steam distillation on an industrial scale: (**A**) α-pinene; (**B**) α-humulene; (**C**) *trans*-caryophyllene. Different letters (i.e., groups not sharing any letter) denote statistically significant differences (one-way ANOVA followed by Tukey's multiple comparisons test). Data are presented as means ± SDs.

The essential oil from plants at 14 month-age contained 48%, 15% and 45% more α-humulene than that from plants at 4, 10 and 18 months-age, respectively; the trans-caryophyllene levels followed an inverted-U pattern, reaching their maximum values at 10 month-age (Figure 4C). According to Facanali et al. [42], the average yield of an EO is affected by the season, with higher production rates in spring and lower rates in winter. In addition, the α-humulene content can also be affected by the genotype and season, being produced in greater quantities in spring and summer. At 18 month-age, the trans-caryophyllene content decreased, reaching values that were 29% lower than in plants at 4 month-age.

Ontogenetic effects on the compositions of essential oils have also been observed for other plant species [16,43,44]. As plants age, reductions in the activity of the metabolic pathways can be observed, which can lead to reductions in the synthesis of essential oil compounds [44,45]. On the other hand, studies have also shown increases in oil production with increasing age, demonstrating variable behavior between species, such as for *Melissa officinalis* L. oil, which has a richer chemical composition at two years of age when compared to oil extracted from younger plants [46,47]. For *V. curassavica*, the relationship between the plant age and chemical composition was demonstrated for the first time in our study.

When studying the effect of the Artemisia annua harvest age on essential oil production, Damtew et al. [48] demonstrated that there was no change in the essential oil content, but the content of artemisinin, an antimalarial compound, was significantly altered. The same result was found by Rocha et al. [49] when studying the influence of the age of *Cymbopogon citratus* (lemon grass) on the chemical composition of the essential oil of this species. The authors reported that there was no change in the extracted oil content, only in the chemical composition.

Our cytotoxicity assay results showed significant differences in relation to the control group in all essential oil concentrations tested at 10, 14 and 18 month-age. In contrast, the EO at 4 month-age at a concentration of 6.25 µg/mL did not differ from the control (Figure 5). A very similar pattern was observed when the cells were treated with pure α-pinene (Figure 5E). The essential oil at 18 month-age had the lowest toxicity among the essential oils, with an IC_{50} of 171.90 µg/mL (Figure 5F). The cell viability tended to increase as the essential oil aged. Another study identified an IC_{50} of 120 µg/mL for J774 macrophages exposed to essential oil of the same species [11].

Pereira et al. [27] also reported on the cytotoxicity of *V. curassavica* EO, which is rich in α-pinene, in mammalian cells (L929 fibroblasts) at concentrations below 200 µg/mL (LC_{50} of approximately 140 µg/mL). An analysis of the cytotoxic effects of crude and supercritical extracts of *V. curassavica* against tumor cells resulted in IC_{50} values of 133.96 and 198.40 µg/mL, respectively [13].

The second most abundant compound, trans-caryophyllene, was present regardless of the plant age, and at 100, 200 and 400 µM the survival of L-929 (murine fibrosarcoma) cells significantly decreased by approximately 54, 100 and 100%, respectively [50]. In addition, the high cytotoxicity of the other essential oils could be due to the capacity of this compound to enhance the penetration of other bioactive substances, increasing the permeability of the plasma membrane, thereby increasing the overall cytotoxicity of the oil [51]. These observations suggest that the content of trans-caryophyllene could have a significant influence on the cytotoxicity of the essential oils extracted from young plants.

The *V. curassavica* EO exerted little antimicrobial activity, regardless of the plant age, in any of the strains of bacteria and yeast of *Candida albicans* that were tested (Table 3). Studies have previously described the antimicrobial activity against *S. epidermidis* (500 µg/mL), *S. aureus* (MIC ranging from 64 to 250 µg/mL), *E. coli* (MIC ranging from 64 to 2000 µg/mL) and *P. aeruginosa* ($\leq$1024 µg/mL) and the fungistatic activity against *Candida albicans* (MIC = 512 µg/mL) [52,53]. Antimicrobial activity is associated with the presence of specific compounds, which are usually tested at a much higher concentration in their pure form than that in the essential oil and with the strain susceptibility used in the different studies [54]. Thus, the discrepancies described above might be attributed to variations in the levels of essential oil components.

Table 3. Minimum inhibitory concentration (MIC, µg/mL) values of essential oil samples produced from *V. curassavica* at different ages against bacteria and yeast.

Microorganism	ATCC®ID	Plant Age (Month-Age)			
		4	10	14	18
Bacteria					
Escherichia coli	25922™	>1000	>1000	>1000	>1000

Table 3. *Cont.*

Microorganism	ATCC®ID	Plant Age (Month-Age)			
		4	10	14	18
Pseudomonas aeruginosa	27853™	>1000	>1000	>1000	>1000
Staphylococcus aureus	29213™	>1000	>1000	>1000	>1000
Staphylococcus aureus (MRSA)	43300™	>1000	>1000	>1000	>1000
Staphylococcus epidermidis	12228™	>1000	>1000	>1000	>1000
Yeast					
Candida albicans		1000	1000	1000	1000

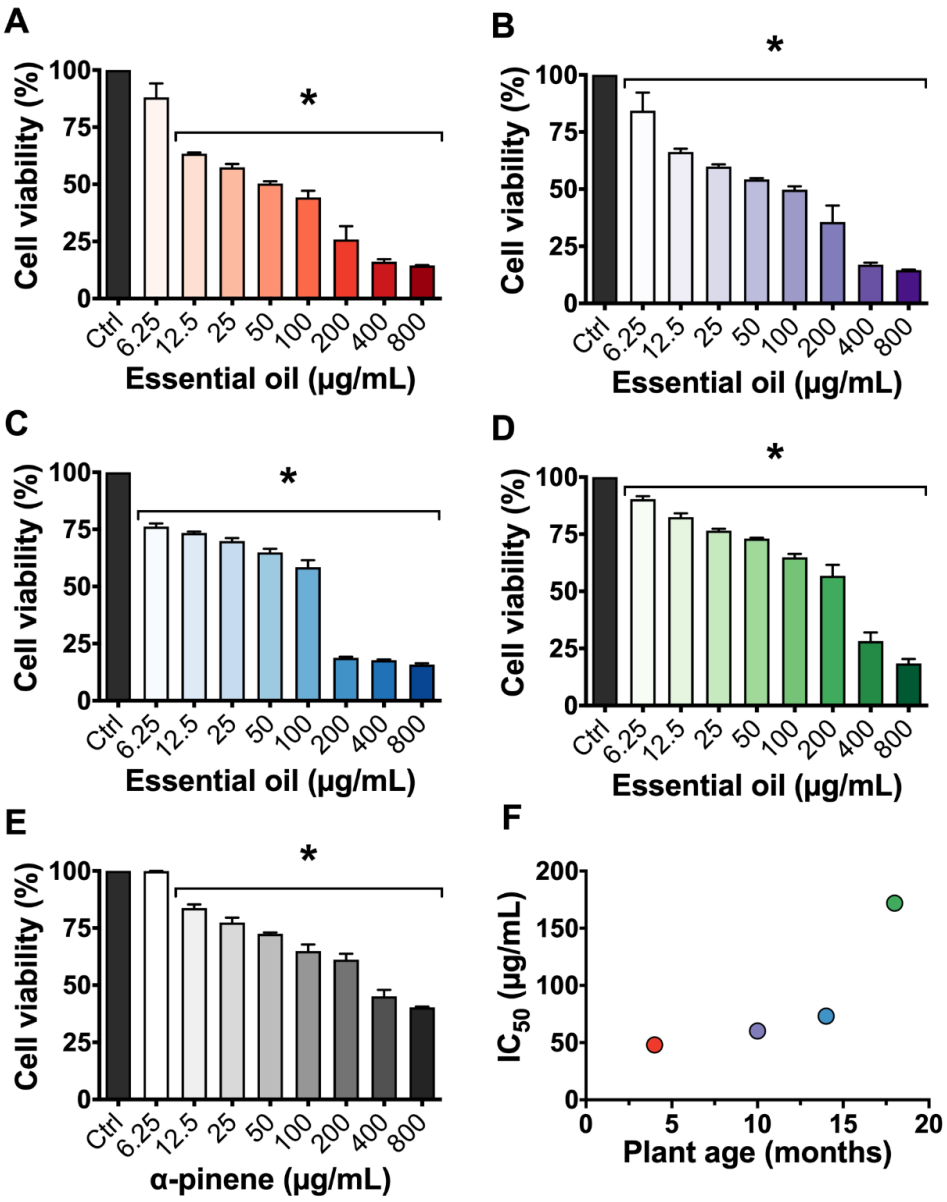

Figure 5. Effects of essential oils extracted from *V. curassavica* on cell viability as assessed using an MTT assay. Murine peritoneal macrophages were treated with 6.25–800 µg/mL essential oil from plants at 4 (**A**), 10 (**B**), 14 (**C**) and 18 (**D**) month-age. Cells were also treated with 6.25–800 µg/mL *α*-pinene (**E**) for comparison. The data in panels (**A**–**D**) were used to calculate the IC_{50} values plotted in panel (**F**). The asterisks denote statistically significant differences in comparison to the untreated control cells (one-way ANOVA followed by Dunnett's multiple comparisons test).

4. Conclusions

Understanding the factors that can affect the essential oil contents of species that have commercial value is very important. We observed that the chemical compositions of *V. curassavica* EOs produced on an industrial scale varied with plant age. Additionally, the cytotoxicity decreased as the plants aged. Our results indicate that the plant age significantly affects the chemical composition of the essential oils, with significant changes in their cell viability. Further studies should aim to unveil whether age also affects the therapeutic effects (e.g., anti-inflammatory) of plant essential oils. This will allow the manufacture of plant-derived formulations that balance production costs, toxicity and therapeutic effects.

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Data Availability Statement: The raw data and detailed results of the PCA analysis are available under request to corresponding authors.

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