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Chemical composition and antifungal potential of essential oils from different aerial parts of Protium ovatum Engl.

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Abstract

Protium ovatum is a Brazilian endemic species widely distributed between the Cerrado and Amazon biomes. Here, we evaluate the chemical composition of the essential oils (EO) of different shoot organs from *P. ovatum* including stems, petioles, leaves, flowers, ripe and unripe fruits, and investigate their antifungal potential against *Sclerotinea sclerotiorum*. The EO were obtained by hydrodistillation in a Clevenger-type apparatus and analysed by GC-MS, followed by PCA analysis. The antifungal activity was performed by agar diffusion. Fruits had the highest essential oil contents among the shoot parts. The constituents were found varyingin the different organs: α-Pinene (0.80-18.3%), β-Pinene (0.58-5.17%), Myrcene (0.52-27.3%), Limonene (3.15-59.7%), Caryophyllene E (3.67-16.4%), Germacrene D (6.34-27.4%), and δ-Cadinene (2.29-7.63%). The essential oil from ripe fruit showed the strongest antifungal activity, with the highest Inhibition of Mycelial Growth (IMG) (50.11%) at the lowest concentration assayed (18.75µg.mL^{-1}). This is the first report on the chemical composition of the essential oils from stems, petioles, flowers, and ripe fruits of *P. ovatum* and their antifungal activity against *S. sclerotiorum*, making it a potential source of antimicrobial agents.

Keywords: *Protium ovatum*; essential oils; stems; petioles; leaves; flowers; fruits; *Sclerotinea sclerotiorum*.
Abbreviations: EOs_essential oils; URF_unripe fruit; RF_ripe fruit; UniRV_University of Rio Verde; Na_2SO_4_anhydrous sodium sulfate; GC_MS/MS_gas chromatography_mass spectrometry; EI_electron ionization; IK_Kovats index; PCA_principal components analysis; HCA_hierarchical cluster analysis; PDA_Potato_Dextrose_Agar; IMG_Percentage Inhibition of Mycelial Growth.

Introduction

The Burceraceae family comprises 17 genera and approximately 750 species distributed throughout the pantropical region. The highest occurrence of the Protieae tribe is recorded in the Neotropical regions around the world (Costa-Lima 2018; Rosalem et al., 2017). This family shares tree or shrub habits and is found on rocky soils such as *campos rupestres* (open mountaintop vegetation), or more rarely over other species (Costa-Lima 2018). In Brazil, seven genera and 104 species have been reported throughout the country, except in the state of Rio Grande do Sul (BFG, 2015).

The genus Protium is the most widespread in Brazil, and approximately 74 species have already been described. *Protium ovatum*. Engl. is one of the fourteen endemic species in the country. It is commonly known as "vick-do-cerrado" and widely distributed between the Cerrado and the Amazon (Castelo et al., 2010; Daly, 2015; Marques et al., 2010; Rosalem et al., 2017). *P. ovatum* is a shrub 0.4 to 4.0 m tall, branches glabrous, with few lenticels. Leaves are ovate, glabrous, with petioles 3-5 cm long. Leaflets are subcoriaceous, shiny, and ovate, with petioles 0.3-1.1 cm long. Petioles of terminal leaves are larger with possible presence of dark trichomes. Inflorescences are 2.5 cm long, flowers 6 mm long, 4-merous,yellow-greenish or greenish color (Lima et al., 2005). This genus is known for its high oleic potential, rich in essential oils and aromatic resins often used in folk medicine (Costa-Lima, 2018).

Studies on the essential oils (EOs) from leaves and unripe fruits of *P. ovatum* have already been reported in the literature by Castelo et al., (2010). Estevam et al. (2017, 2018), also reported the biological potential of EOs from unripe fruits and leaves against *Trypanosoma cruzi* and *Leishmaniasis amazonensis*, and cell toxicity in pathogens. Essential oil biosynthesis is sensitive to biotic, abiotic, genetic, physiological, and morphological factors, and may present chemical variation among the plant parts, e.g., stems, petioles, leaves, flowers, fruits, and rhizomes (Barbosa et al., 2017; Tian et al., 2014; Ud-Daula et al., 2016; Zhang et al., 2015).

Essential oils are often used in several industrial processes, including pharmaceuticals, agricultural, food, sanitary, and cosmetics, according to their antibacterial, antifungal, antimycotic, antihelmintic, antiseptic, antispasmodic, antioxidant properties , and their flavour and fragrance features (Mobin et al., 2016; Ud-Daula et al., 2016). EOs have been used as natural antifungal agents in the control of pathogenic fungi such as white mold in vegetables, which is caused by *Sclerotinea sclerotiorum* (Moraes et al., 2018;

Valadares et al., 2018). Therefore, the objective of this study was to evaluate the variation in content and chemical composition of *Protium ovatum* essential oils extracted from different shoot parts (e.g., stems, petioles, leaves, flowers, ripe and unripe fruits), as well as to evaluate its antifungal activity against *Sclerotinea sclerotiorum*.

Results and Discussion

Constituents of EO from P. ovatum stems, petioles, leaves, flowers and fruits (ripe and unripe)

The essential oil yield of the different shoot parts of *P. ovatum* is shown in Figure 01. The stems presented the lowest yield (% v/w) of essential oil (0.09%), below those of petioles (0.18%), leaves (0.24%), and flowers (0.23%). The highest yields were recorded for unripe fruits (0.45%) and ripe fruits (0.48%), which were significantly different from the other parts.

Similar contents to those of the leaves and fruits of *P. ovatum* obtained in this study were reported by Estevam et al., 2017, 2018,who also collected in the region of Rio Verde-GO. They obtained yields close to 0.30% for leaves and 0.50% for unripe fruits. This difference in EO yield between different shoot parts is usually due to biotic and abiotic factors that cause changes in the density of oil glands, secretory cells, modified parenchymal cells, epithelial cells, and lysigenous cavities, or glandular trichomes on plant tissue (Barbosa et al., 2017; Figueiredo et al., 2008; Miguel, 2010; Moghaddam et al., 2015; Morshedloo et al., 2018).

The results showed that the lowest percentage of EOs occurred in the stems of *P. ovatum*. Our findings were similar to those of *Origanum vulgare*, *Etlingera sphaerocephala*, *Anvilleagarcinii*, *Seseliannum*, and *Protium heptaphyllum* with yields ranging from 0.1 to 1.3% (m/m) (Khan et al., 2015; Kovacevic et al., 2016; Morshedloo et al., 2018. The low oil yield of *P. ovatum* stems is associated with a lower density of oil trichomes present in the stem tissue (Morshedloo et al., 2018; War et al., 2012).

The chemical composition of the essential oils from the different shoot parts of *P. ovatum* is presented in Table 01. In total, 51 compounds were identified by mass spectrometry, representing between 99.80 and 99.99% of the total compounds of the essential oils, consisting mainly of hydrocarbon monoterpenes (10.4-100%), oxygenated monoterpenes (0-4.84%), hydrocarbon sesquiterpenes (0-81.0%), and oxygenated sesquiterpenes (0-12.3%). A great variation in the chemical profiling of the essential oils from *P. ovatum* was found among the different shoot parts.

The essential oils extracted from the petioles and leaves of *P. ovatum* consisted predominantly of sesquiterpenes, which accounted for 81.4% and 81.0% of their total chemical composition, respectively. On the other hand, the essential oils of unripe and ripe fruits showed a chemical profile of monoterpenes, with more than 99.8% of the total compounds identified in this class. The essential oils of stems and flowers contain mainly monoterpenes and sesquiterpenes, which constitute, more than 82% of the total compounds identified.

Compounds were identified in the essential oils of stems (30), petioles (27), leaves (20), flowers (23), unripe fruits (8), and ripe fruits (6). Among the constituents common to all shoot parts, α-pinene (0.80-18.3%), β-pinene (0.58-5.17%), myrcene (0.52-27.3%), and limonene (3.15-59.7%) were most prevalent.

Previous studies on essential oils of *P. ovatum* leaves and fruits confirm the presence of monoterpenic compounds in concentrations similar to those obtained in our study: a low concentration of monoterpenes in the leaves (17.1%), and a higher concentration in the fruits (91.4%) (Estevam et al., 2017, 2018). Similar results have been described for other species of *Protium* spp. and for different shoot parts, which include resin, leaves, and fruits (Carvalho et al., 2013; Mobin et al., 2016; Mobin et al., 2017; Moraes et al., 2013; Pinto et al., 2010; Pontes et al., 2010, 2007; Siani et al;, 2004; Silva et al., 2016; Silva et al., 2013; Souza et al., 2016; Zoghbi et al., 2005).

Cluster analysis of chemical variation of essential oil

Results of EO chemical composition for the different organs of *P. ovatum* were classified by the hierarchical cluster analysis (HCA) (Figure 02). A dendrogram was generated from the HCA clustering and the results were separated into four different groups (Figure 02). The first group with the highest similarity comprises the essential oils from unripe and ripe fruits (URF, RF), with similar chemical composition and rich in α-pinene (15.4% for URF and 18.3% for RF), myrcene (27.3% for URF and 18.4% for RF), and limonene (41.1% for URF and 59.7% for RF). The second group is formed by essential oils from leaves and flowers and has a greater abundance of germacrene D (21.0% for URF and 17.5% for RF) and germacrene A (28.6% for URF and 21.4% for RF). The main constituents that separated the third group formed by the essential oils from stems were mainly myrcene (10.9%), limonene (11.8%), and caryophyllene E (16.4%). The fourth group formed by essential oils from petioles showed the largest difference between the various shoot parts and is rich in germacrene D (27.4%) and α-muurolene (22.8%).

The results of this study show considerable differences in the chemical composition of essential oils from different shoot parts of *P. ovatum*. This chemical variation in the essential oils may be due to the non-continuous and non-homogeneous production of secondary metabolites in the different organs and development stages of the plant (Gobbo-Neto et al., 2007; Tian et al., 2014). Chemical variation similar to that observed between different shoot parts of *P. ovatum* has already been reported in other species (Kovacevic et al., 2016; Masoudi et al., 2017; Ud-Daula et al., 2016).

Antifungal activity of EO of P. ovatum leaves, flowers and ripe fruits

The evaluation of the antifungal activity of the essential oils from leaves, flowers, and ripe fruits of *P. ovatum* is shown in Figure 3. All EOs inhibited *S. sclerotiorum* mycelial growth in a dose-dependent manner. The results indicated significant differences in mycelial growth inhibition with the increase in the concentrations of the essential oils.

The stronghest antifungal activity was found for ripe fruit EOs, which had the highest IMG (50.11%) at the lowest concentration assayed (18.75 $\mu g.mL^{-1}$). On the other hand, the leaf EOs had the weakest antifungal activity compared with the other treatments and IMG of 42.23% at the highest concentration assayed (600 $\mu g.mL^{-1}$).

In vitro antifungal activity of plant extracts and essential oils of different plant species against *S. sclerotiorum* were

Table 1. Chemical composition of the essential oil from different shoot organs of *Protium ovatum*.

Compounds	RT (min)	RIexp	RIlit	RA (%)					
				STE	PET	LEA	FLO	URF	RF
α-thujene	5.257	917	924	0.22	-	-	-	0.44	-
α-pinene	5.433	924	932	2.59	0.8	3.28	3.96	15.44	18.28
Sabinene	6.466	963	969	5.96	-	-	-	0.48	0.27
β-pinene	6.583	967	974	0.63	0.58	1.14	0.90	5.17	2.96
Trans-isolimone	6.911	979	980	-	-	0.92	-	-	-
Myrcene	6.920	980	988	10.91	0.52	1.63	2.98	27.27	18.36
δ-3-carene	7.566	1003	1008	2.26	2.83	3.12	24.76	8.60	-
p-cymene	8.009	1015	1020	2.06	-	-	-	-	-
o-cymene	8.014	1016	1022	-	-	-	-	-	0.39
Limonene	8.144	1019	1024	11.82	4.74	3.15	4.39	41.12	59.73
β-ocimene Z	8.412	1027	1032	1.32	0.97		1.83	1.36	-
β-ocimene E	8.779	1037	1044	-	-	0.66	0.91	-	-
Terpinolene	10.265	1079	1086	0.88	-	-	1.59	-	-
Terpinen-4-ol	13.801	1169	1174	4.84	-	-	-	-	-
α-copaene	22.062	1366	1374	2.4	2.73	4.22	1.32	-	-
β-Panasinsene	22.450	1375	1381	0.28	-	-	-	-	-
β-cubebene	22.663	1380	1387	0.2	0.36	-	-	-	-
β-bourbonene	22.995	1388	1387	-	-	0.43	0.72	-	-
Sibirene	23.214	1393	1400	-	-	0.54	-	-	-
Caryophyllene E	23.865	1409	1417	16.39	-	13.34	3.68	-	-
β-4.8-epoxy- caryophyllene	24.422	1423	1423	-	7.78	-	-	-	-
γ-elemene	24.651	1428	1434	0.68	-	-	-	-	-
β-gurjunene	24.835	1433	1431	0.42	-	-	-	-	-
Epi-β-santalene	25.091	1439	1445	0.52	-	-	-	-	-
α-humulene	25.242	1443	1452	1.16	1.32	1.76	0.92	-	-
Cis-3.5-diene- muurola	25.380	1446	1448	-	1.07	-	0.23	-	-
Allo-aromadendrene	25.554	1451	1458	0.24	-	-	-	-	-
γ-muurolene	26.356	1471	1478	7.19	-	-	-	-	-
β-acoradiene	26.639	1478	1469	-	0,58	-	-	-	-
Germacrene D	26.997	1486	1484	6.34	27.38	21.04	17.51	-	-
α-muurolene	27.554	1500	1500	-	22.84	-	-	-	-
Germacrene A	27.563	1500	1508	-	-	28.59	21.43	-	-
Cubebol	27.686	1504	1514	0.18	1.48	-	-	-	-
γ-cadinene	27.695	1504	1513	-	0,7	-	-	-	-
α-bulnesene	27.706	1504	1509	-	-	-	0.86	-	-
δ-amorphene	27.977	1511	1511	-	1.55	-	-	-	-
δ-cadinene	28.049	1.513	1522	7.14	6.64	7.63	2.29	-	-
γ-bisabolene E	28.804	1533	1529	1.23	0.81	-	-	-	-
cis-sesquisabinenehydrate (IPP vs. OH)	29.332	1546	1542	-	1.02	-	-	-	-
Trans-dauca-4(11).7-diene	29.909	1561	1556	-	5.34	-	-	-	-
Germacrene B	29.922	1562	1559	-	-	3.42	1.62	-	-
Spathulenol	30.147	1567	1577	5.29	1.82	3.09	4.17	-	-
Caryophyllene Oxide	30.371	1573	1582	3.21	1.53	1.12	-	-	-
Viridiflorol	30.954	1588	1592	-	-	-	1.87	-	-
Globulol	31.142	1593	1590	0.67	-	-	1.12	-	-
α-epi-cadinol	32.515	1630	1638	1.89	-	-	0,19	-	-
α-epia-muurolol	33.076	1645	1640	-	2,14	-	-	-	-
α-muurolol (=Torreyol)	33.300	1651	1644	-	0,2	-	-	-	-
α-cadinol	33.594	1659	1652	-	0,83	0,37	0,97	-	-
α-epi-bisabolol	34.111	1673	1683	1.08	-	0,55	-	-	-
Nerolidylisobutyrate Z	34.629	1787	1784	-	1,24	-	-	-	-
Hydrocarbon monoterpenes				38.65	10.44	13.9	41.32	99,88	99,99
Oxygenated monoterpenes				4.84	-	-	-	-	-
Hydrocarbon sesquiterpenes				44.18	81.36	80.97	50.47	-	-
Oxygenated sesquiterpenes				12.32	8	5,12	8,12	-	-
Total identified				99.99	99.8	99,99	99,91	99,88	99,99

RT: Retention time; **RI_{exp}**: Experimental retention index using a series of n-alkanes (C_8-C_{31}); **RI_{lit}**: Retention index reported in scientific literature (Adams, 2007); **RA**%: Relative area (relative area of the peak in relation to the total area of the peak in the GC-MS chromatogram) of essential oils from different shoot parts of *Protium ovatum*. **STE**: Stems; **PET**: Petioles; **LEA**: Leaves; **FLO**: Flowers; **URF**: Unripe Fruits; **RF**: Ripe Fruits.

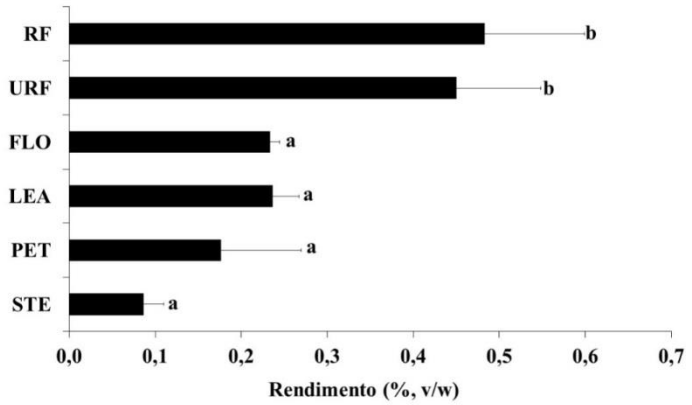

Fig 1. Essential oil yield (%, v/m) of different shoot parts of Protium ovatum. STE: Stems; PET: Petioles; LEA: Leaves; FLO: Flowers; URF: Unripe Fruits; RF: Ripe Fruits. Bars followed by the same letter are not significantly different.

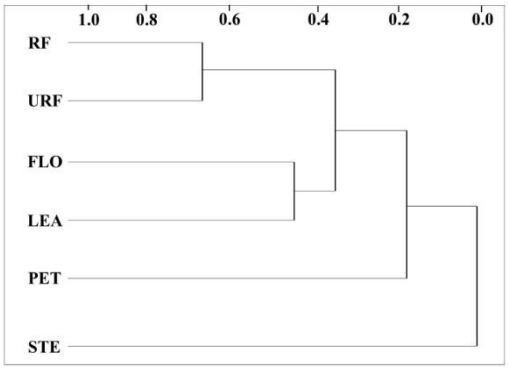

Fig 2. Dendrogram of the hierarchical cluster analysis (HCA) of the chemical similarity between the essential oils from different shoot parts of *P. ovatum* collected in July 2017. **RF**: Ripe Fruits; **URF**: Unripe Fruits; **FLO**: Flowers; **LEA**: Leaves; **STE**: Stems; **PET**: Petioles.

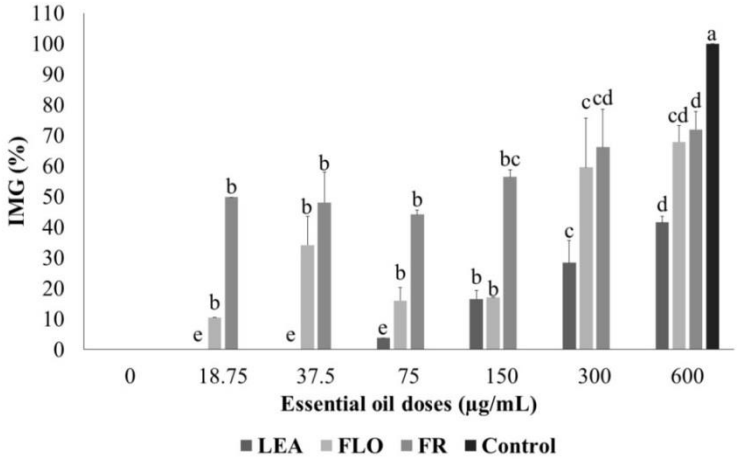

Fig 3. Percentage inhibition of mycelial growth (IMG), by the essential oils from leaves, flowers, and fruits of *Protium ovatum* against the fungus *Sclerotinia sclerotiorum*. **LEA**: Leaves; **FLO**: Flowers; **RF**: Ripe fruits. Bars with different letters represent significant difference for the concentration ($p < 0.05$) between treatments and controls.

already reported (Moraes et al., 2018; Silva et al., 2018; Valadares et al., 2018).

Ours results indicated considerable antifungal activity of EOs from leaves, flowers, and ripe fruits of *P. ovatum*. The antifungal activity of *P. ovatum* EOs against pathogenic plant fungi is reported in the literature. In this study, the EOs of ripe fruits showed the highest inhibitory activity against *S. sclerotiorum* compared with the other oils studied. According to Burt (2004) and Negi (2012), several factors, including chemical structure, bioactive compound, essential oil concentration, and type of microorganism play important roles in essential oil efficiency. Table 01 shows the different chemical profiles of the EOs. Limonene represents 59.38% of the EO compounds in ripe fruits, 4.39%in flowers, and 3.15% in leaves, with a very strong antifungal property effective against fungi that cause food spoilage (Cheng et al., 2006). Limonene also has an effective antibacterial property against pathogenic bacteria (Alonso-Gutierrez et al., 2013). The highest EO activity of the ripe fruit may be related to its higher limonene concentration compared with the other oils studied.

Materials and Methods

Plant material

The plant material was collected from as ingle population of *P. ovatum* in an area of Cerrado belonging to the University of Rio Verde (UniRV), Rio Verde, Goiás, Brazil (17°47'15.0"S 50°57'59.9"W, 839 m altitude). The shoot system parts (stems, flowers, petioles, and leaves) were collected at the plant flowering stage in June 2017. Fruits were collected between August (unripe fruits) and September (ripe fruits), in 2017. Voucher specimens of the botanical material were deposited in the Herbarium of the Federal Institute of Education, Science and Technology - Campus Rio Verde, and assigned the collection number 628.

Extraction of EO

Essential oils of different parts of *P. ovatum* were extracted from fresh matter collected between 7 a.m. and 10 a.m. After collection, the plant was separated between different organs: stems from petioles, petioles from leaf, and ripe from unripe fruits. Then, each plant part was weighed (100 g), soaked in 500 mL of ultrapure water, and subjected to hydrodistillation extraction using a Clevenger-type apparatus, at 70 °C for 3 h. The EO obtained was dried using 3.0 g of anhydrous sodium sulfate (Na_2SO_4), filtered, and stored in a glass vial protected from light, sealed, and kept at an average temperature of 4 °C for further analysis. EO extraction was performed for each *P. ovatum* organ in triplicate (n = 3).

The average EO yield (%) was calculated based on fresh plant matter according to Zhang et al. (2015).

Analysis of EO

The analysis of the chemical profile of *P. ovatum* essential oils was performed on a gas chromatograph coupled to a sequential mass spectrometer (CG-MS/MS) equipped with a Combi PAL AOC-5000 automatic sample injector, a Restek RTx-5MS fused silica capillary column (30 m × 0.250 mm × 0.250 µm), and a Sequential Mass Spectrometer (MSTQ-8040 Shimadzu). Mass spectra data were recorded in electron ionization mode (EI) at 70 eV. The initial column temperature was programmed at 60 °C for 3 minand riseto 200 °C at 3 °Cmin^{-1}, riseto 280 °C at 15 °C.$min^{-1}$and hold for 1 min. The injector temperature was 230 °Cand the detector temperature was 300 °C. Helium was used as carrier gas with injection pressure of 57.4 KPa, in the splitless mode: 150, mass detection rangefrom 43 to 550 Da, and flow rate of 3 $mL.min^{-1}$. Identification of oil components was based on the linear retention index (Kovats index - IK) related to retention times of a homologous series of *n*-alkanes (C_7 to C_{40}) and the observed fragmentation pattern in mass spectra, comparing with the literature (ADAMS, 2007) and the Nist®11 mass spectral library.

PCA analysis of the chemical variation in essential oil composition

Principal component analysis (PCA) was applied to evaluate the interrelationships between the chemical constituents of the essential oils from different *P. ovatum* parts using the software Pirouette®4.0 (Infometrix Inc., Bothell, WA, USA). Hierarchical cluster analysis (HCA) was used to evaluate the similarity between the samples according to component distribution and hierarchical grouping was performed according to the Ward's minimum variance criterion (Ward, 1963).

Anti-sclerotinia assay

The biological assay was performed at the Plant Microbiology Laboratory of the Goiás Federal Institute - Rio Verde Campus. The phytopathogenic fungus *Sclerotinia sclerotiorum* Ss12 (BRM 29673) was kindly provided by Embrapa Rice and Beans Research Center, Santo Antônio de Goiás, GO. Initially, essential oils of leaves and fruits of *P. ovatum* were prepared in emulsion [water and 0.05% Tween®80 (m/v)] in different concentrations (18.75, 37.5, 75, 150, 300, and 600 $\mu g.mL^{-1}$). Aliquotes of 100 µL of each oil concentration was spread over all the surface of plates containing sterilized and solidified Potato-Dextrose-Agar (PDA) culture medium, using a sterilized Drigalski loop. After seven days of fungal culture, a *S. sclerotiorum* mycelial disc was placed in the center of the plate (BALBI-PEÑA et al., 2006; SILVA et al., 2009). Mycelial radial growth of colonies was measured daily, starting from 24 h after the inoculation until the complete growth of colonies in the control treatment.

The antifungal activity was measured by both the mean of mycelial growth inhibition of fungus treated with the essential oils and the controls. Sterile distilled water was used as negative control and the fungicide fluazinam as Frowncide 500 SC (10 $\mu g.mL^{-1}$active ingredient) was used as a positive control. Tween®80 [0.05% (w/v)] was used as adjuvant for homogenization of the essential oils in water. The antifungal activity was calculated by the Percentage Inhibition of Mycelial Growth (IMG), according to the equation:

$$\text{IMG}\ (\%) = \frac{(\text{Control growth} - \text{Treatment growth})}{\text{Control growth}} \times 100$$

as described by Venturoso et al. (2011) and Andrade et al. (2018).

The results of this study were analysed by analysis of variance and the means were compared by the Tukey's test at 5% of probability, using the statistical software BioEstat version 5.0.

Conclusion

The results of this work show that the content and chemical composition of *Protium ovatum* essential oils varied according to the different shoot organs. This study has also confirmed the antifungal potential of these essential oils against *Sclerotinea sclerotiorum*, and showed that the essential oils of ripe fruits have the highest cytotoxic activity, making it a natural source of antimicrobial compounds forutilization by the food and agricultural industry.

Disclosure statement

The authors declare no conflict of interest.

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