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Essential oils from *Piper aduncum* inflorescences and leaves: chemical composition and antifungal activity against *Sclerotinia sclerotiorum*

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ABSTRACT

Even though essential oils from *Piper aduncum* (Piperaceae) have different biological activities, little is known about their application to agricultural areas. White mold is a plant disease caused by the phytopathogen *Sclerotinia sclerotiorum*, which needs to be controlled by alternative measures. This study aimed at evaluating the effect of essential oils from *P. aduncum* leaves (PL-EO) and inflorescences (PI-EO) on the mycelial growth of *S. sclerotiorum*. Essential oils from *P. aduncum* were obtained by hydrodistillation by a Clevenger-type apparatus while their chemical composition was analyzed by GC-MS and GC-FID. Piperitone (23.4 %), myristicin (12.4 %), terpinen-4-ol (12.3 %), β-caryophyllene (7.2 %), α-humulene (6.9 %), germacrene-D (6.9 %) and dillapiol (6.3 %) were the main constituents found in oils from *P. aduncum*. The *in vitro* antifungal activity showed that PI-EO dose above 30 µL inhibited mycelial growth in 100 %, whereas PL-EO at 50 µL inhibited it in 98.74 %. This is the first report of the chemical composition of PI-EO and results suggest that the essential oils under evaluation have high potential to control the phytopathogenic fungus *S. sclerotiorum*.

Key words: *Piper aduncum*, essential oils, piperitone, antifungal activity, *Sclerotinia sclerotiorum*.

INTRODUCTION

Control of plant diseases has been often carried out by synthetic fungicides. However, the use of fungicides is prohibited in organic production processes by certification agencies and they should be replaced by alternative products (Fonseca et al. 2015). Indiscriminate use of agrochemicals to control plagues and diseases may cause both severe risks to human health and environmental contamination, besides possible problems with resistant pathogens (Fonseca et al. 2015).

Sclerotinia sclerotiorum is a phytopathogenic fungus which occurs mostly in the soil and has damaged many plants of economic interest. It has

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been known as white mold due to its resulting symptoms, such as wet root rot, i.e., roots are covered by white mycelium on the soil surface and/or on the host tissue and may yield resistant structures called sclerotia (Dildey et al. 2014).

Researches which aim at an alternative control of *S. sclerotiorum* by means of essential oils extracted from different species of plants have increased and revealed promising potential application over recent years (Soylu et al. 2007, Chutia et al. 2009, Pansera et al. 2013). Fonseca et al. (2015) reinforce the importance of studies of essential oils since many have fungicidal and fungistatic potential. They also mention that these natural resources may be used by agricultural producers in the future.

Piper aduncum, popularly known as *falso-jaborandi*, *jaborandi do mato* and *pimenta de macaco*, is a widely distributed species in tropical regions. Some species of the genus *Piper*, such as *P. aduncum*, *P. brachystachyum*, *P. falconeri*, *P. guineese* and *P. hispidum*, which belong to the family Piperaceae, are rich in essential oils with important biological activities (Oliveira et al. 2013a).

Studies carried out by this research group have aimed at analysing the chemical composition and biological activities of essential oils (Oliveira et al. 2016, 2017, Lemes et al. 2017, Estevam et al. 2018), and this one, specifically, addresses the chemical composition and the *in vitro* antifungal activity of *P. aduncum* leaves and inflorescences against *S. sclerotiorum.*

MATERIALS AND METHODS

PLANT MATERIAL

Piper aduncum leaves and inflorescences were collected on August 2016 at 8 am, in Rio Verde, Goiás, Brazil, on the campus of the Universidade de Rio Verde (UniRV) (17°47'22.776"S and 50°57'56.894"W). The plant was identified by the botanist Luzia Francisca de Souza and a sample was deposited at the Herbarium Jataiense Professor Germano Guarim Neto at exsiccate number HJ 7872.

EXTRACTION OF ESSENTIAL OILS

Samples of *P. aduncum* leaves and inflorescences were subjected to hydrodistillation for 2 hours by a Clevenger-type apparatus (Carneiro et al. 2017). In order to carry out the analysis, 300 g plant material was divided into three 100-g samples, and 500 mL distilled water was added to each sample. After manual collection of the essential oil (EO) samples, traces of remaining water in the oils were removed with anhydrous sodium sulfate, and then followed by filtration. The extraction procedure was done in triplicate. Isolated oils were stored under refrigeration up to the analysis and test. Yields (w/w) were calculated from fresh leaf and inflorescences weight and expressed as the average of triplicate analyses.

IDENTIFICATION OF THE CHEMICAL COMPOSITION OF ESSENTIAL OILS

Gas chromatography (GC) analyses were performed by a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with FID and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30-m x 0.25-mm i.d.; 0.25-µm film thickness) was employed. Operation conditions were as follows: column temperature programmed to rise from 60 to 240 °C at 3 °C/min and, then, to hold at 240 °C for 5 min; carrier gas = He (99.999 %), at 1.0 mL/min; injection mode; injection volume, 0.1 µL (split ratio of 1:10); and injector and detector temperatures = 240 and 280 °C, respectively. Relative concentrations of components were obtained by peak area normalization (%). Relative areas were the average of triplicate GC-FID analyses.

GC-MS analyses were carried out by a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column was a RTX-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). Electron ionization mode occurred at 70 eV. Helium (99.999 %) was employed as the carrier gas at a constant flow of 1.0 mL/min. Injection volume was 0.1 µL (split ratio of 1:10). Injector and ion-source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC. Mass spectra were taken at a scan interval of 0.5 s, in the mass range from 40 to 600 Da.

Identification of volatile components of *P. aduncum* leaves and inflorescences (Table I) was based on their retention indices on an Rtx-5MS capillary column under the same operating conditions as the ones in the case of GC relative to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectra libraries and their fragmentation patterns were compared with literature data (Adams 2007).

ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM *P. aduncum* AGAINST THE PHYTOPATHOGEN *S. sclerotiorum*

The isolate of *Sclerotinia sclerotiorum* Ss12 (BRM 29673) was provided by the Embrapa Arroz e Feijão, whose headquarters is in Santo Antônio de Goiás, GO, Brazil. Assays were carried out in the agricultural microbiology lab at IF Goiano – Campus Rio Verde and the antifungal activity of essential oils from *P. aduncum* leaves and inflorescences was evaluated in agreement with the disc-diffusion method described by Xavier et al. (2016), whose doses of essential oils were 10 – 50 µL for PI-EO and PL-EO. Negative controls were dishes with no addition of essential oil (witness) whereas the positive control was the fungicide Frowncide 500 SC, at 10 µg/mL of the active ingredient. Petri dishes were sterilized and prepared with PDA culture medium. After medium solidification, essential oils, at previously mentioned doses, were added and smeared on the surface of the dish with the help of a Drigalski spatula. Afterwards, 5 mm diameter PDA medium discs with 10-day-old mycelium were placed in the center of the dishes. Then, they were incubated at 28 ± 2°C and mycelial growth was measured daily, up to the full growth of the fungus on the control dishes. The treatment was carried out in quadruplicate and the experimental design was thoroughly randomized. Data were submitted to the analysis of variance (ANOVA) and the means of the treatments were evaluated by the Scott-Knott test at 5% significance level by the ASSISTAT software.

The percentage of inhibition of mycelial growth (IMG) was calculated by the following formula:

$$IMG(\%) = \frac{(control\ growth - treatment\ growth)}{control\ growth} x100$$

RESULTS AND DISCUSSION

Both GC-MS and GC-FID analyses identified forty five compounds for the essential oil from *P. aduncum* inflorescences and forty one compounds for the essential oil from *P. aduncum* leaves, which corresponds to 99.4 % and 99.1 % of the total of compounds, respectively (Table I).

Yields of essential oils from *P. aduncum* inflorescences (PI-EO) and leaves (PL-EO) after extraction were 0.42 % and 0.34 %, respectively. In general, the yield was much lower than the one reported in the literature for essential oils from *P. aduncum*, i. e., from 2.5 to 4.0% (Silva et al. 2013).

The essential oil from *P. aduncum* inflorescences consisted mainly of oxygenated monoterpenes (43.9 %), followed by sesquiterpene hydrocarbons (30.5 %) and phenylpropanoids (10.0 %). The essential oil from *P. aduncum* leaves

TABLE I
Chemical composition of essential oils from *P. aduncum* inflorescences (PI-EO) and leaves (PL-EO) collected in Rio Verde, Goiás, Brazil.

RT (min)	*Compounds*	RI_{exp}	RI_{lit}	*RA %*	
				PI-EO	*PL-EO*
11.54	Myrcene	993	991	-	0.2
12.17	α-Phellandrene	1007	1005	-	0.3
12.73	α-Terpinene	1019	1018	0.1	0.3
13.12	p-Cymene	1028	1026	0.3	1.0
13.32	β-Phellandrene	1032	1031	0.7	1.2
13.74	*cis*-β-Ocimene	1041	1040	0.1	1.9
14.27	*trans*-β-Ocimene	1053	1050	0.5	4.1
14.75	γ-Terpinene	1063	1059	1.5	1.9
15.17	*trans*-Sabinene hydrate	1070	1068	2.0	0.4
16.16	α-Terpinolene	1084	1084	1.0	0.9
16.67	*cis*-Sabinene hydrate	1095	1097	2.8	0.2
17.79	*cis*-*p*-Menth-2-en-1-ol	1127	1129	3.2	0.9
18.12	Alloocimene	1138	1140	-	0.2
18.67	*trans*-*p*-Menth-2-en-1-ol	1145	1142	2.5	0.9
19.98	α-Borneol	1162	1165	0.4	0.1
20.57	**Terpinen-4-ol**	1183	1179	**12.3**	**6.3**
21.14	α-Terpineol	1192	1189	1.1	0.3
21.95	*p*-Menth-1-en-3-ol	1205	1203	1.0	0.8
24.31	**Piperitone**	1252	1254	**23.4**	**11.8**
25.67	Safrole	1282	1285	0.9	1.8
27.74	Bicycloelemene	1349	1345	0.6	2.5
28.27	α-Cubebene	1351	1351	0.3	-
29.08	Cyclosativene	1369	1367	0.8	-
29.25	α-Ylangene	1373	1373	0.1	-
29.44	α-Copaene	1378	1376	3.8	1.7
30.05	β-Cubebene	1392	1389	1.8	-
30.13	β-Elemene	1394	1391	-	1.4
30.90	α-Gurjunene	1412	1409	0.2	-
31.37	**β-Caryophyllene**	1420	1418	**7.2**	5.4
31.71	Unknown	1432	1432	0.5	0.8
31.87	γ-Elemene	1435	1433	0.4	-
32.13	Aromadendrene	1440	1439	0.5	-
32.77	**α-Humulene**	1458	1456	**6.9**	3.9
32.97	β-Santalene	1463	1461	0.8	-
33.70	Unknown	1481	1481	0.3	-
33.96	**Germacrene-D**	1482	1480	3.3	**6.9**
34.11	β-Selinene	1491	1495	1.4	0.8

TABLE I (continuation)

RT (min)	*Compounds*	RI_{exp}	RI_{lit}	*RA %*	
				PI-EO	*PL-EO*
34.57	Bicyclogermacrene	1502	1504	0.3	3.1
34.89	(*E.E*)- α-Farnesene	1510	1508	1.0	1.0
35.20	γ-Cadinene	1514	1512	0.6	-
35.37	Sesquisabinene hydrate	1528	1530	-	2.4
35.79	**Myristicin**	1534	1534	**6.5**	**12.4**
35.92	Cadina-1.4-diene	1535	1531	0.5	-
36.90	Elemicin	1559	1556	0.4	1.8
37.64	Germacrene D-4-ol	1572	1574	-	1.1
37.79	Spathulenol	1576	1576	-	1.5
37.99	Caryophyllene oxide	1580	1581	1.0	2.1
38.37	Viridiflorol	1600	1590	1.0	4.4
38.98	1.2-Epoxide-humulene	1608	1607	1.4	2.1
39.62	Dillapiol	1644	1644	2.2	**6.3**
40.32	τ-Cadinol	1648	1645	0.9	0.7
40.63	α-Cadinol	1653	1653	0.9	1.3
	Monoterpene hydrocarbons			9.0	12.6
	Oxygenated monoterpenes			43.9	21.1
	Sesquiterpene hydrocarbons			30.5	29.1
	Oxygenated sesquiterpenes			5.2	13.2
	Phenylpropanoids			10.0	22.3
	Not identified			0.8	0.8
	Total			**99.4**	**99.1**

RT: Retention time; RI_{exp}***:*** Retention index determined relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; RI_{lit}***:*** Retention index from literature (Adams, 2007); ***RA%:*** relative area (peak area relative to the total peak area in the GC-FID chromatogram), average of three replicates.

had mostly sesquiterpene hydrocarbons (29.1 %), followed by phenylpropanoids (22.3 %) and oxygenated monoterpenes (21.1 %) (Table I). The major constituents found in the essential oil from *P. aduncum* inflorescences were piperitone (23.4 %, **1**), terpinen-4-ol (12.3 %, **2**), β-caryophyllene (7.2 %, **3**), α-humulene (6.9 %, **4**) and myristicin (6.5 %, **5**) (Figure 1). The major ones identified in the essential oil from *P. aduncum* leaves were myristicin (12.4 %), piperitone (11.8 %), germacrene-D (6.9 %, **6**), terpinen-4-ol (6.3 %) and dillapiol (6.3 %, **7**) (Figure 1). The chemical composition of essential oil from *P. aduncum* inflorescences has been described by this study for the first time.

Specifically, the chemical composition of the essential oil from leaves (PL-EO) was found to be similar to other chemical compositions that had already been reported in the literature regarding other species of *P. aduncum* from other Brazilian regions (Oliveira et al. 2006, 2013b).

The major constituents had been previously identified in the essential oil from four populations

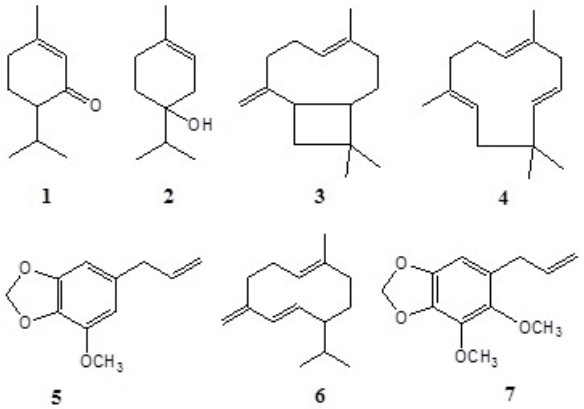

Figure 1 - Chemical structures of major constituents identified in the essential oils from *P. aduncum* inflorescences and leaves: piperitone (**1**), terpinen-4-ol (**2**), β-caryophyllene (**3**), α-humulene (**4**), myristicin (**5**), germacrene-D (**6**) and dillapiol (**7**).

of *P. aduncum* leaves found in the Federal District, Brazil (Potzernheim et al. 2012). Similarity was also found when the chemical composition of essential oils from *P. aduncum* were compared with the ones of other species that belong to the family Piperaceae, such as *P. manausense*, *P. demeraranum*, *P. malacophyllum* and *P. tuberculatum* (Andrade et al. 2005, 2006, Santos et al. 2012, Sales et al. 2017).

The comparison between chemical compositions of PL-EO and the essential oil of *P. aduncum* leaves found in Bocaiúva, a city located in Minas Gerais state, Brazil (Oliveira et al. 2013a) showed that they were very different. It may be evidence of the existence of a new chemotype of the species *P. aduncum* in the southeast of Goiás state, Brazil. In addition, a recently published study describes nine different chemotypes of the species *P. aduncum* and a new one that was found in Cuba (Monzote et al. 2017). In the essential oil of aerial parts of *P. aduncum* in Cuba, the following major constituents were identified: piperitone (23.7%), camphor (17.1%) and viridiflorol (14.5%) (Monzote et al. 2017). Only piperitone was identified as a major constituent of PI-EO and PL-EO, with 23.4% and 11.8%, respectively (Table I).

In vitro antifungal activity of essential oils from *P. aduncum* inflorescences and leaves was evaluated against the phytopathogenic fungus *Sclerotinia sclerotiorum*. Percentages of inhibition of mycelial growth (IMG) of essential oils from *P. aduncum* inflorescences and leaves are shown in Figure 2 and Figure 3, respectively.

Results of the analysis of inhibition of mycelial growth showed the high antifungal potential of essential oils extracted from *P. aduncum* inflorescences and leaves. The study of the means found by the Scott-Knott test revealed that doses above 20 µL of the essential oil from inflorescences did not differ statistically from the commercial fungicide Frowncide 500 SC, which was used as positive control (Figure 2). Concerning the essential oil from *P. aduncum* leaves, doses above 10 µL had similar results; its inhibition values were above 95% (Figure 3). Besides, the antifungal activity of the essential oil from *P. aduncum* inflorescences should be highlighted, since above 30 µL oil, the inhibition potential of the mycelial growth of *S. sclerotiorum* was 100%, equal to the one of the commercial fungicide Frowncide 500 SC.

The literature describes that the aqueous extract from *P. aduncum* fruit inhibited mycelial growth of *S. sclerotiorum* in 42.86% and that the essential oil of this Piperaceae makes the oil show fungicide activity because it has dillapiol in its chemical composition (Garcia et al. 2012). In addition, major chemical constituents piperitone, terpinen-4-ol, β-caryophyllene, α-humulene, germacrene-D and myristicin, which were found in the essential oils from *P. aduncum* in Goiás state, along with dillapiol, may explain the promising anti-*Sclerotinia sclerotiorum* activity observed by this study, since these compounds have already had their antifungal activities well described in the literature (Costa et al. 2000, Mondello et al. 2006, Francescato et al. 2007, Maxia et al. 2012, Benmansour et al. 2016).

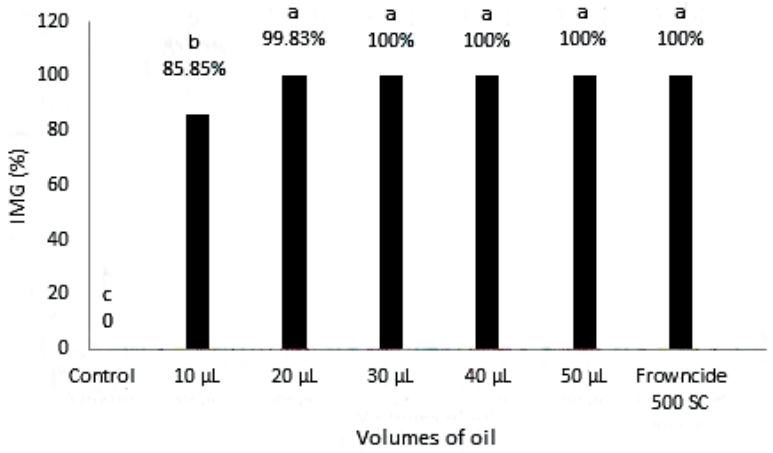

Figure 2 - Percentages of inhibition of mycelial growth of *Sclerotinia sclerotiorum* at different volumes of essential oils from *P. aduncum* inflorescences. Means followed by the same letter do not differ from each other by the Scott-Knott test.

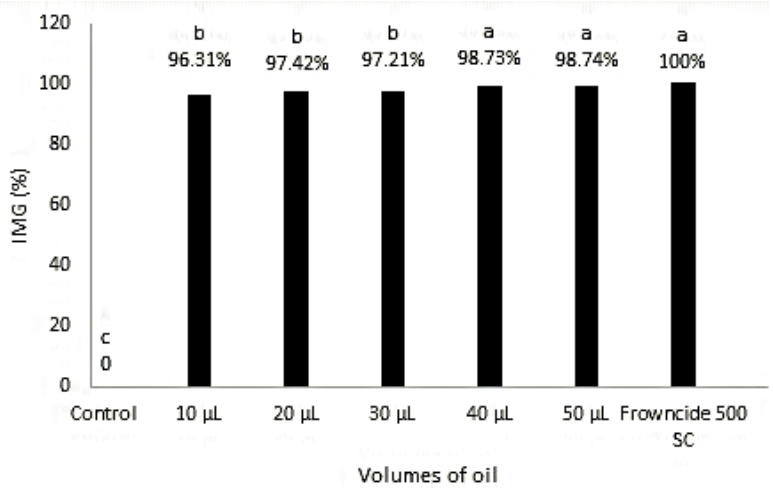

Figure 3 - Percentages of inhibition of mycelial growth of *Sclerotinia sclerotiorum* at different volumes of essential oils from *P. aduncum* leaves. Means followed by the same letter do not differ from each other by the Scott-Knott test.

Likewise, Silva and Bastos (2007) described significant inhibitory activity of essential oils from leaves of other *Piper* species in the mycelial growth of other fungi, such as *Crinipellis perniciosa*, *Phytophthora palmivora* and *Phytophthora capsici*. The essential oil from *P. aduncum* dry leaves also had promissing activity when it was tested against the fungus *Colletotricum musae*; at concentrations above 100 µg/mL, the oil inhibited mycelial growth and conidial germination in 100% (Bastos and Albuquerque 2004). In short, essential oils from *P. aduncum* inflorescences and leaves were more active than the essential oil from *Cardiopetalum calophyllum,* another species found in the Cerrado in Goiás, i. e., 300 µL of the latter inhibited mycelial growth of *S. sclerotiorum* in 87.63% (Xavier et al. 2016). This is the first report of the evaluation of antifungal activity of essential oils from *P. aduncum* inflorescences and leaves against *S. sclerotiorum*.

In sum, results of this study show that essential oils from *P. aduncum* inflorescences and leaves have strong antifungal activity against *S. sclerotiorum*, a fungal pathogen that causes damage to many plants of economic interest. Previous studies of the chemical composition and major chemical constituents identified in essential oils from *P. aduncum* also corroborate the potential observed in this *in vitro* investigation. Therefore, results of this study show that there is good prospect of using these essential oils experimentally to control phytopathogens in both greenhouse and field conditions.

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