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Full Length Research Paper

Composition and screening of antifungal activity against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* of essential oils of leaves and fruits of *Piper* species

Andreia de A. Morandim[1,2*], Aline R. Pin[1], Natalia A. S. Pietro[1], Alberto C. Alecio[2,5], Massuo J. Kato[3], Claudia M. Young[4], José E. de Oliveira[2] and Maysa Furlan[2]

[1]Departamento de Engenharia Química, Centro Universitário da FEI, Av. Humberto de Alencar Castelo Branco, 3972, 09850-901, São Bernardo do Campo-SP, Brazil.
[2]Instituto de Química, Universidade Estadual Paulista, CP 355, 14800-900, Araraquara-SP, Brazil.
[3]Instituto de Química, Universidade de São Paulo, CP 26077, 05599-970, São Paulo-SP, Brazil.
[4]Instituto de Botânica, Instituto de Botânica, CP 4005, 10051, São Paulo-SP, Brazil.
[5]Universidade Paulista, Av. Alberto Benassi, 200, 14804-300, Araraquara-SP, Brazil.

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This study investigated the composition and antifungal activity against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* of essential oils of leaves of *Piper cernuum*, *Piper diospyrifolium*, *Piper crassinervium*, *Piper solmsianum* and *Piper umbelata* and fruits of *P. cernuum* and *P. diospyrifolium*. The essentials oils were analyzed by GC-MS and submitted of the antifungal activity tests. The essential oils of fruits from *P. cernuum* and leaves of *P. crassinervium* and *P. solmsianum* showed potential antifungal activity against *C. sphaerospermum* and *C. cladosporioides*. In addition, this is the first report of the composition of essential oils of fruits of *P. cernuum* and *P. diospyrifolium.*

Key words: Piperaceae, *Piper*; essential oil composition, antifungal activity, GC-MS.

INTRODUCTION

Actually, plants have been an interesting alternative to explore, specifically as a source of natural fungicides to control phytopathogens that affect crops once plants produce numerous and varied organic compounds including monoterpenes and sesquiterpenes compounds present in essential oils, of which the majority does not directly participate in the plant's growth and development and are generally called secondary metabolites. For the plants, the synthesis and formation of these compounds allow them to defend themselves from herbivores, pathogens (bacteria, fungi and viruses) and from other plants, in addition, providing protection from adverse physical effects, such as damaging radiation, water loss and low temperatures (Monsálvez et al., 2010).

The sesquiterpenes make up a group of over 5000 compounds that have a wide spectrum of biological effects and appear to play an important part among the plants' defense mechanisms. Among the sesquiterpenes, the polygodial is one of the most known and stands out for its insecticide effect, although, it has also shown to have an antifungal effect against *Saccharomyces cerevisiae* and *Candida utilis*. For example, several aromatic and officinal plants and ornamental plants synthesize various active substances (phenols, terpenes, terpenoids and glucosides) which may exhibit antifungal properties (Corato et al., 2010; Monsálvez et al., 2010).

In this context, *Piperaceae* species are mostly pioneer shrubs with economic and medicinal importance (treat many diseases including gynecological maladies, vaginitis, intestinal disorders, psychotropic, antimicrobial, antioxidant and cytotoxic effects) and are widely spread in tropical regions and show the accumulation of several classes of physiologically active natural products such as alkaloids,

*Corresponding author. E-mail: andreia.morandim@gmail.com. Tel: +55-011-43532900. Fax: +55-01141095994.

amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids, lignans, neolignans, chromenes and terpenes (Batista et al., 2008; Felipe et al., 2007; Navickiene et al., 2006; Lago et al., 2004; Silva et al., 2002; Benevides et al., 1999; Moreira, 1998; Maia et al., 1998; Parmar et al., 1997).

This family includes the *Piper* and *Peperomia* genus. The *Piper* genus is largely distributed in tropical and subtropical regions of the world and had been studied and it has identificated amides, flavonoids, lignans, aristolactams, long and short chains esters, terpenes, steroids, propenylphenols, chromenes and alkaloids and the genus *Peperomia* had been used for the treatment of malignant tumors but there are a few studies when comparing with *Piper* genus (Felippe, 2008; Tanaka et al., 1998).

The volatile components from aerial parts of *Piperaceae* species, have been subjected to a number of investigations and are variable mixtures with predominance of monoterpenes (C_{10}) and sesquiterpenes (C_{15}) although diterpenes (C_{20}) and phenylpropanoids have also been identified and the essential oils exhibited antimicrobial, antioxidant, antiinflammatory, antispasmodic, relaxing properties and affect hemostasis have been described both in animals and humans (Tognolini et al., 2006; Dorman and Deans, 2000).

The essential oil from *Ottonia anisum*, *Piper amplum, P. arboretum, P. aduncum, P. tuberculatum, P. dilalatum, P. goesii, P. hispidum, P. hoffmanseffianum, P. nigrum, Piper gaudichaudianum*, *P. guineense, P. molliconum*, *Peperomia blanda*, *P. gaudichaudianum*, *P. regnellii* and *P. cernuum* showed biological activity including strong molluscicidal activity against *Biomphalaria glabrata*, cytotoxic, fungistatic, insecticide and antibacterial activities (Navickiene et al., 2006; Santos et al 2001; Costantin et al., 2001; Moreira, 1998; Maia et al., 1987).

As soon, in this paper, we wish to describe the composition of essential oil in fruits of *P. cernuum* and *P. diospyrifolium* and leaves of *P. cernuum*, *P. diospyrifolium, P. crassinervium*, *P. solmsianum* and *P. umbelata*. Additionally, their antifungal activity as evaluated against the fungi *C. sphaerospermum* and *C. cladosporioides* by direct bioautography. Furthermore, this is the first analysis of essential oil of fruits of *P. umbelata*.

MATERIALS AND METHODS

Plant material

P. umbelata (L.) MIQ. was collected at Institute of Chemistry (UNESP) in Araraquara-SP, Brazil. The voucher specimen has been deposited at Herbarium of Botânica, São Paulo, Brazil.

P. cernuum Vell. and *P. crassinervium* Kunth were collected at the Campus of São Paulo University in São Paulo city, Brazil and identified by Dr. Guillermo E. Delgado (Universidad Nacional Pedro Ruiz Gallo, Peru). Voucher specimens (Kato-0137 and Kato-0084, respectively,) were deposited at Herbarium of Instituto de Botânica, São Paulo, Brazil.

P. solmsianum (Miq.) Yunck and *P. diospyrifolium* Kunth were collected at the campus of Universidade de São Paulo (Brazil) and were identified by Dr Elsie Franklin Guimarães (Jardim Botânico do Rio de Janeiro, Brazil). As voucher specimens (Kato-0027 and Kato-0431, respectively,) were deposited at the Herbarium of Jardim Botânico, Rio de Janeiro, Brazil.

Extraction of essential oil

Plant material (100 g of each *Piper* species) was subjected to hydrodistillation in a Clevenger-type apparatus for 2 h. The oil layers obtained were dried over anhydrous Na_2SO_4. The average yields were obtained over three experiments and calculated on the basis of dried weight material.

Essential oil analyses

GC-MS analyses:

Analyses were conducted on Shimadzu GC-MS automated chromatograph model GC-17A/QP-5050A coupled to a model AOC20i auto-sampler and its manipulation was accomplished by the software GCMS Solutions v. 1.02 workstation (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a fused-silica capillary nonpolar column DB–5-MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness; J and W Scientific, Folsom, CA, USA) with phenyl arylene polymer virtually equivalent to a (5%-Phenyl)-methylpolysiloxane as the stationary phase. All analyses were obtained using EI/MS (in positive mode) with scan acquisition mode (40 to 500 m/z). The oven temperature was initially at 60°C and then increased at the rate of 3°C min^{-1} to 240°C, which was kept for 10 min, performing 70 min of total time of analysis (Adams, 1995). The carrier gas was helium at a constant flow rate of 1 mL min^{-1} and sample aliquots (1 µL) were injected in the split mode (1: 20) without pre-treatment solvent delay. Moreover, the injector and detector temperatures were maintained at 220 and 240°C, respectively.

The relative amounts of individual components were determined on the basis of their GC peak areas, without corrections for FID response factors. The retention index was calculated for all constituents using a homologous series of *n*-alkanes and the chemical components was based on the comparison of their mass spectral with the National Institute for Standard Technology – NIST62 library, comparison of calculated retention indexes with literature values and co-chromatography of some constituents with authentic components on the DB-5 capillary column (Adams, 1995)

Antifungal assay:

The microorganisms used in the antifungal assays *C. sphaerospermum* (Penzig) SPC 491 and *C. cladosporioides* (Fresen) de Vries SPC 140 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. For the antifungal assay – 10.0 µL of solutions corresponding to 400, 200, 100, 50, 10 and 1 µg of essential oils were applied to pre-coated TLC plates. TLC plates were developed with $CHCl_3$ for all of the essential oils and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of *C. sphaerospermum* or *C. cladosporioides* in glucose and salt solution and incubated for 72 h in darkness in a moistened chamber at 25°C. Clear inhibition zone appeared against a dark background indicating the minimal amount of the essential oils required for it. Nystatin was used as positive control (Rahalison et al., 1994; Homans, 1970).

RESULTS AND DISCUSSION

The hydrodistillation of fruits of *P. cernuum*, *P. diospyrifolium* and leaves from *P. cernuum*, *P. diospyrifolium*, *P.*

Table 1. Composition of the essential oils of different *Piper* species.

Compound	RI	A	B	C	D	E	F	G
Yield (%)		96.74	70.09	97.12	85.30	78.07	78.18	95.31
Tricyclene	914	-	-	1.40	-	-	-	-
α-Pinene	939	2.10	-	1.06	-	0.13	-	-
β-Pinene	980	-	-	2.10	-	0.21	-	-
P-Cymene	1026	-	3.69	-	-	-	-	-
Limonene	1031	-	-	3.20	-	-	-	-
α-Copaene	1376	2.73	6.49	1.31	1.06	47.73	0.29	-
β-Elemene	1391	7.15	4.90	0.78	-	-	0.62	-
β-Caryophylene	1418	22.23	9.81	8.11	-	12.27	2.88	3.00
Aromadendrene	1439	0.45	0.18	1.65	4.85	2.02	2.16	2.26
Geranyl Acetone	1453	-	-	-	4.40	-	-	-
α-Humulene	1454	2.51	1.86	3.03	1.24	5.68	0.35	1.08
Allo-Aromadendrene	1461	-	0.42	-	-	-	1.41	-
γ-Muurolene	1477	-	-	3.25	0.43	0.68	-	8.94
Germacrene D	1480	9.30	14.27	14.04	-	0.28	1.68	34.19
β-Selinene	1485	1.49	-	-	-	0.24	0.93	-
Biclyclogermacrene	1493	25.10	6.48	9.17	-	2.40	1.27	8.95
α-Muurolene	1499	-	-	-	-	0.77	-	1.33
Z-α-Bisabolene	1504	5.69	-	0.76	-	-	-	-
Germacrene A	1505	-	3.02	-	-	-	0.35	-
Butylated hydroxytoluene	1499	-	-	-	-	0.97	-	3.76
γ-Cadinene	1513	-	2.68	1.78	0.53	0.31	-	5.89
Cubebol	1514	-	-	0.60	0.73	0.45	0.75	1.45
epi-α-Selinene	1516	-	-	5.04	-	-	0.50	-
cis-Calamenene	1521	-	-	1.02	-	0.31	-	-
δ-Cadinene	1524	3.00	-	-	-	-	-	15.02
α-Cadinene	1538	-	-	0.43	-	-	-	1.51
α-Calacorene	1542	-	-	1.27	-	-	-	-
Elemol	1549	-	-	3.17	-	-	-	-
E-Nerolidol	1564	1.46	1.65	8.23	18.18	3.07	-	4.41
Spathulenol	1576	7.24	9.68	9.82	25.37	0.44	5.17	2.30
Caryophylene oxide	1581	-	-	-	7.67	-	1.78	-
Globulol	1583	1.16	-	-	6.64	-	-	-
Viridiflorol	1590	-	-	-	1.82	-	-	-
Guaiol	1595	-	-	5.80	-	-	-	-
humulene epoxide II	1606	-	-	-	6.88	-	-	-
1-*epi*-Cubebol	1627	0.81	0.43	-	-	-	-	-
epi-α-Cadinol	1640	-	2.37	-	-	0.11	-	-
epi-α-Muurolol	1644	2.14	-	-	-	-	4.59	-
α-Muurolol	1645	-	-	-	-	-	-	-
β-Eudesmol	1649	-	-	10.10	-	-	-	-
α-Cadinol	1653	2.18	4.16	-	-	-	-	-
7-*epi*-α-Eudesmol	1658	-	-	-	5.50	-	-	1.22
E-Isoelemicin	1660	-	-	-	-	-	53.45	-

* Leves of *Piper cernuum* (A), Fruits of *P. cernuum* (B), *P. crassinervium* (C), Leaves of *P. diospyrifolium* (D), fruits of *P. diospyrifolium* (E). *P. solmsianum* (F) and *P. umbelata* (G)

crassinervium, *P. solmsianum* and *P. umbelata* gave higher yields, calculated on basis of a dry weight, 1.60, 1.40, 0.23, 1.46, 0.94, 2.57 and 0.11%, respectively. The identification of monoterpenes and sesquiterpenes was carried out by automated interpretation of mass spectra of constituents each oil and also by retention index. A total of 8 monoterpenes and 47 sesquiterpenes identified are showed in order of elution on a DB-5 column (Table 1).

Table 2. Antifungal activity of essential oil of leaves and fruits of *Piper* species against *C. cladosporioides* and *C. sphaerospermum.*

Specie	*C. cladosporioides*	*C. sphaerospermum*
P. cernuum (leaves)	-	-
P. cernuum (fruits)	++	++
P. diospyrifolium (leaves)	-	-
P. diospyrifolium (fruits)	-	-
P. crassinervium (leaves)	+	+
P. solmsianum (leaves)	++	++
P. umbelata (leaves)	-	-
Nistatin (Positive control)	+++	+++

+++ = High activity, ++ = moderate activity, + = few activity.

In spite of similarity between the different species, only four sesquiterpenes (β-caryophyllene, aromadendrene and spathulenol) occur in significant level in the seven oil analyzed and comparing the composition of oils of fruits of *P. cernuum* and *P. diospyrifolium* showed accumulation of germacrene D (14.27 %) and α-copaene (47.73 %), respectively.

Ever, the essential oil from *P. solmsianum* showed accumulation of *E*-isoelemicin (isolated earlier in inflorescence of this species) and *P. cernuum* the compound majority was, in both, the bicyclogermacrene (25.10%). Germacrene D was the majorly compound in *P. crassinervium* and *P. umbelata* (14.04 and 34.19%, respectively,) ever *P. diospyrifolium* showed spathulenol as the most representative compound (25.37%). Concluding, *P. solmsianum* showed the major presence of *E*-isoelemicin (53.45%), compound isolated of this species (Rahalison et al., 1994).

The sesquiterpenes were very abundant and structurally diversified in the oils from leaves and fruits of the seven species. The most general representative was spathulenol accounting up to 7.24, 9.68, 9.82, 25.37, 0.44, 5.17 and 2.30 of leaves and fruits from *P. cernuum*, leaves from *P. crassinervium* and *P. diospyrifolium*, fruits from *P. diospyrifolium* and leaves from *P. solmsianum* and *P. umbelata*, respectively. The antifungal activity of the essential oils was evaluated by means of direct bioautography on TLC plate. The detection limits of samples (Table 2) were obtained according to methodology described. The essential oils from fruits of *P. cernuum* showed high activity against *C. cladosporioides* and *C. sphaerospermum*, respectively, when compared with the standard nystatin and the essential oil of leaves of *P. solmsianum* too. Ever, the essential oil of leaves of *P. crassinervium* showed a few activated against this fungi.

These results also confirm the effectiveness of the essential oils of *Piper* species as botanical-antifungal against *C. sphaerospermum* and *C. cladosporioides* observed in other work (Navickiene et al., 2006). It is also important to note the non-phytotoxic effect of the essential oils has been reported in the literature. The literature data reports a narrow or wide range of activity of the essential oils, because different substances could own a strong antimicrobial activity against certain fungi, but may be ineffective against other pathogens (Corato et al., 2010). Further investigations looking at determinations of bioactive constituents should be carried out.

Conclusions

In this study, a total of 8 monoterpenes and 45 sesquiterpenes were identified and four sesquiterpenes (β-caryophyllene, aromadendrene and spathulenol) occur in significant level in the seven oils analyzed. The antifungal activity of the essential oils of *P. cernuum, P. solmsianum* and *P. crassinervium* species showed antifungal activity. *C. cladosporioides* and *C. sphaerospermum* were evaluated by means of direct bioautography on TLC plate.

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