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ANTIFUNGAL ACTIVITY OF *Piper diospyrifolium* KUNTH (*PIPERACEAE*) ESSENTIAL OIL

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ABSTRACT

In vitro activity of the essential oil from *Piper diospyrifolium* leaves was tested using disk diffusion techniques. The antifungal assay showed significant potencial antifungal activity: the oil was effective against several clinical fungal strains. The majority compounds in the essential oil were identified as sesquiterpenoids by GC-MS and GC-FID techniques.

Key words: *Piper diospyrifolium*; essential oil; antifungal activity

The family *Piperaceae* is pantropical in distribution. In Brazil, it is represented by five genera (*Piper*, *Peperomia*, *Potomorphe*, *Ottonia,* and *Sarcorhachis*) and about 500 species, which are quite common in forests, particularly in the Atlantic Tropical Forest (24, 26). known for the aromatic properties of many members, the family's best-know representatives belong to the genus *Piper*, including species that are currently used as producers of essential oils, such as *Piper arboreum*, *P. hispidum*, *P. crassinervium*, *P. lanceaefolium*, and *P. auritum* (4, 13, 14, 16, 17, 19, 25). Studies by Fazolin *et al*. (5) gave promising results for the use of essencial oils from *P. hispidinervum* and *P. aduncum* as insecticides, at concentrations above 3.0% (v/v) and 2.5% (v/v), respectively. Species of Piperaceae are widely used in folk medicine, and their essential oils have high economic and commercial importance (2). The aim of this study was to determine the potential antifungal activity, *in vitro*, of the essential oil obtained from leaves of *P. diospyrifolium*. The composition of the oil was determined by GC-MS and GC-FID techniques.

Fresh leaves of native *P. diospyrifolium* were collected at Maringá, state of PR, southeastern Brazil, in March 2009, from Horto Florestal Dr. Luis Teixeira Mendes (51º30´54W and 22º30´30S), urban zone of Maringá, 556m altitude and 37ha of land. A voucher specimen, HUEM 9392, was deposited in the Herbarium of State University of Maringá.

The essential oil was obtained in a Clevenger apparatus by steam distillation. After 4 h of distillation, 300 g of fresh leaves was extracted with 2.5 l water. The oil was separated from the water and stored in a freezer at -20ºC.

The essential oil was diluted in acetone 1:10 (v/v) and 1 µL of the sample was analyzed by GC-MS, performed using an Agilent GC (6890 Series) – quadrupole MS system (5973), with a fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film, coated with DB-5), operating at 70 eV. The injection port and detector temperatures were set at 250ºC

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(1:20 division ratio). The oven temperature program was 40°C for 1 min and 40-240°C at 3°C/min, and helium was employed as the carrier gas (1 mL min^{-1}), with a total analysis time of 78 min.

The compounds were identified based on comparison of the calculated retention index (Kovats Index (KI)), and the mass spectra with literature data (1), or by comparison with mass spectra recorded in a database (Wiley 275).

The chromatographic conditions from GC-FID analyses were identical to those used for GC-MS.

Yeast strain patterns of *Candida albicans* ATCC-90028, *C. parapsilosis* ATCC-22019 and *C. tropicalis* ATCC-28707 were used. From *C. Albicans* yeast, isolates from urine, catheter and blood culture were used; from *C. parapsilosis*, isolates from catheter and blood culture and, from *C. tropicalis*, clinical samples isolates from urine, blood culture and oro-tracheal tube. Samples were donated by the Santa Casa de Maringá-PR or by the Hospital Universitário de Maringá-PR, and grown at 37°C in Sabouraud broth.

In vitro antifungal activity of the *P. diospyrifolium* essential oil was determined by the agar disk diffusion method according to Rubio *et al.* (21). Yeasts suspensions with turbidity equivalent of the McFarland 0.5 standard were poured on Petri plates containing solid Sabouraud agar. Sterile filter-paper discs (Whatman No. 1, 6.0 mm diameter) were impregnated with 20 µl of the oil and placed on the inoculated plates. Nystatin (Sensifungidisc, Cecon) (100 U.I.) was used as a positive control. These plates were allowed to dry at room temperature for 2 h, and were incubated at 37ºC for 24 to 48 h. After the incubation, the presence or absence of inhibition zones was checked. The diameters of the inhibition zones were measured in millimeters, and their means were calculated (12). All the tests were performed in triplicate.

The essential oil from *P. diospyrifolium* leaves afforded 0.1%, similar to other *Piper* species (*P. arboreum*, *P. crassinervium*, *P. dilatatum* and *P. tuberculatum*) tested by Cysne et al. (4).

The main compounds were sesquiterpenoid hydrocarbons (68.21%) and monoterpene hydrocarbons (19.41%). The majority compounds were eudesmane-type compounds including (E)-eudesma-6,11-diene (21.12%) and γ-muurolene (10.57%) and caryophyllene-type (E)-caryophyllene (16.76%) (Table 1).

Table 1. Chemical constituents of essential oil from leaves of *Piper diospyrifolium*

Constituents	% FID	IK[a]
Monoterpenoids hydrocarbons		
α-Tujene	1.46	929
Sabinene	2.76	971
Limonene	8.47	1024
(E)-*β*-ocimene	5.76	1048
α-humulene	1.04	1449
Oxygenated monoterpenoids		
Bornyl angelate	1.12	1567
Sesquiterpenoids hydrocarbons		
α-copaene	0.96	1374
β-elemene	4.29	1392
(E)-caryophyllene	16.76	1418
(11)Drima – 7,9– diene	0.90	1472
γ-muurolene	10.57	1476
γ-himachalene	2.20	1479
(E)-eudesma -6,11-diene	21.12	1490
α- cuprenene	1.92	1504
γ-cadinene	3.29	1515
Germacrene B	6.20	1559
Oxygenated sesquiterpenoids		
Spathulenol	3.85	1574
Carotol	0.86	1592
α–acorenol	2.23	1634
Epi-*α*-cadinol	1.01	1638
Cubenol	3.23	1646

Percentages are the mean of three runs and were obtained from electronic integrations measurements using flame ionization detection (FID).

[a]The retention index was calculated for all volatile constituents using a homologous series of n-alkanes C_{5-6}, C_{10-19}, C_{21-23} e C_{25-26}.

Mesquita *et al*. (11) found a similar predominance of sesquiterpenoids in other species (*P. aduncum, P. amalago, P. arboreum, P. cernuum, P. hispidum*, *P. regnelii, P. submarginalum, P. vicosanum* and *Pothomorphe umbellate*). Three species of *Piper* from gallery forests of the Cerrado (*P. arboreum subsp arboreum, P. dilatatum* and, *P. hispidum*) also showed a predominance of sesquiterpenoids (18).

Yeats are emerging as an important cause of diseases, not only because of their commensal nature but especially due to their implication as important agents of infections. Candidiases primarily infect immunocompromised patients or hospitalized patients with serious underlying diseases (3, 10, 15). Many antifungal agents are available for the treatment of candidal infections, such as polyenes (amphotericin B and nystatin) and azoles (itraconazole and fluconazole). However, due to the occurrence of undesirable outcomes, such as the unpredictable resistance to recently developed antifungal agents, mainly in immunodepressed individuals, as well as their toxic effects, further studies are needed to develop new, more effective antifungal medicines with fewer disadvantages (23, 6, 7, 8).

The essential oil was effective against several clinical isolates and for standard samples of *C. albicans* (Table 2). The essential oil was more effective against *C. parapsilosis* (ATCC 22019), with an inhibition zone of 15.0 ± 1.0 mm.

Table 2. Investigation of antifungal activity (inhibition zone in mm) from essential oil of *P. diospyrifolium* and standard antifungal used as positive control.

Microorganisms	Origin*	*P. diospyrifolium* oil	Nystatin
C. albicans	ATCC 90028	12.3 ± 0.5	26.0 ± 1,5
C. albicans	Urine	10.7 ± 0.5	23.0 ± 0,4
C. albicans	Catheter	11.7 ± 0.5	27.0 ± 0,6
C. albicans	Blood culture	11.3 ± 1.1	26.0 ± 0,8
C. parapsilosis	ATCC 22019	15.0 ± 1.0	32.0 ± 1,9
C. parapsilosis	Blood culture	9.7 ± 1.5	36.0 ± 1,0
C. parapsilosis	Catheter	9.3 ± 1.5	38.0 ± 0,7
C. tropicalis	ATCC 28707	9.3 ± 0.5	27.0 ± 2,2
C. tropicalis	Urine	10.3 ± 1.1	26.0 ± 0,4
C. tropicalis	Oro-tracheal tube	8.3 ± 0.5	26.0 ± 0,6
C. tropicalis	Blood culture	11.3 ± 1.1	28.0 ± 0,9

*Except to ATCC microorganisms all of others are human clinical isolates. Mean of inhibition zone by oil of *P. diospyrifolium*: Effective (diameters > 10mm) and resistant (diameters ≤ 10mm)(22). The values represent the average of three determinations ± standard deviations

Rodrigues-Silva *et al*. (20) reported that essential oil extracted from *P. ovatum* leaves showed an effect against *C. tropicalis* ATCC 28707 and *C. tropicalis* from urine clinical isolates (22.6±3.1 and 18.7 ±2.1 mm respectively. The main constituents found in the oil were *δ*-amorphene (16.5%), *cis*-muurola-4(14%),5-diene (14.29%) and γ-muurolene (13.26%).

Eudesmane-type sesquiterpenoids from Asteraceae exhibit a wide range of biological activities, and include compounds that are plant-growth regulators, insect antifeedants, antifungals, anti-tumor compounds and antibacterials (27, 9). The derivative from (E)-caryophyllene like caryophyllene oxide, used as preservative in food, drugs, and cosmetics, has been tested in vitro as an antifungal against dermatophytes. Its antifungal activity has been compared to ciclopirox olamine and sulconazole, wich are commonly used to treat onychomycosis (28).

In conclusion, the essential oil of *P. diospyrifolium*, under the test conditions, showed interesting antifungal properties. This activity merits further study of this drug as a possible new phytotherapeutic or natural fungicide.

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