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Essential oils of Amazon *Piper* species and their cytotoxic, antifungal, antioxidant and anti-cholinesterase activities

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ABSTRACT

The hydrodistilled oils of *Piper hispidum*, *Piper aleyreanum*, and *Piper anonifolium*, collected in the Carajás National Forest, Pará state, Brazil, were analyzed by GC and GC–MS and then, evaluated their antioxidant, antifungal, anticholinesterase and cytotoxic activities. In total, 87 constituents were identified in the *Piper* oils. The sesquiterpenes were the most highly represented classes and the main compounds found in the *Piper* oils were selin-11-en-4-α-ol, β-elemene, β-selinene, α-selinene, bicyclogermacrene, β-caryophyllene, α-humulene, and δ-elemene. All analyzed oils showed powerful antifungal activity, with the detection limit (DL) from 0.1 to 1.0 μg against the *Cladosporium cladosporioides* and *Cladosporium sphareospermum* fungi, as well they have no lytic effect against the mice erythrocytes. In the anticholinesterase evaluation, the oils of *P. anonifolium* (DL = 0.01 ng) and *P. hispidum* (DL = 0.01 ng) were hundred times more potent than the standard physostigmine (DL = 1.0 ng). Moreover, the oil of *P. aleyreanum* showed high in vitro cytotoxic activity against the human melanoma cell line SKMEL-19 (IC_{50} = 7.4 μg/mL) and significant antioxidant activity (DPPH = 412.2 mg TE/mL). The higher cell growth inhibition induced by the oil of *P. aleyreanum* is probably due to elemene (β-, δ-, and γ-elemene), as well as other structurally related compounds, which were previously reported in the proliferation inhibition, stimulation of apoptosis and induction of cell cycle arrest in malignant cells.

1. Introduction

Piperaceae comprises about five genera and more than 2000 species (Jaramillo and Manos, 2001). The genus *Piper* is mainly distributed in the tropical and subtropical region of the world and has been extensively investigated as the source of new natural products with potential antifungal, antitumoral, antioxidant, antiplasmodial, and tripanocidal properties (Lago et al., 2009). Moreover, *Piper* species are used to treat diseases, including fever, jaundice, rheumatism, and neuralgia in the folk medicine of various countries (Christophe, 2006).

The species *Piper hispidum* Sw., *Piper anonifolium* Kunth, and *Piper aleyreanum* C. DC., are commonly found in the Brazilian Amazon. *P. hispidum* is known by the vernacular names of "matico" and "aperta-ruão", and its leaf infusion is used in the folk medicine as diuretic and anti-hemorrhagic (Andrade et al., 2009). In addition, it has been reported for their antifungal (Navickiene et al., 2000), insecticidal (Santos et al., 2010), and antimicrobial and cytotoxic (Morales et al., 2013) activities. *P. aleyreanum* is known by the indigenous name "paninixpu", and its essential oil is mentioned as immunomodulator, analgesic, and antidepressant in local folk medicine. Recently, the oil of *P. aleyreanum* showed antinociceptive and anti-inflammatory activities, as well as a gastro-protective activity (Lima et al., 2012). *P. anonifolium* is known as "pimenta longa", and there are only few chemical studies reporting mono and sesquiterpenes from their essential oil (Andrade et al., 2005, 2009).

Chemical studies have shown that *Piper* has many classes of compounds, such as unsaturated amides, flavonoids, lignans,

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aristolactams, long and short chain esters, steroids, and alkaloids (Navickiene et al., 2000). Moreover, the oils of *Piper* in the Amazon have showed terpenoid and phenylpropanoid compounds as major constituents, always with the predominance of one over the other (Silva et al., 2011; Andrade et al., 2011).

The aim of this study was to identify the composition of the oils of *P. hispidum*, *P. anonifolium* and *P. aleyreanum* and evaluate their cytotoxic, antifungal, antioxidant and anticholinesterase properties.

2. Materials and methods

2.1. Plant material

The aerial parts of *P. aleyreanum* C. DC. (MG 189271), *P. anonifolium* Kunth (MG 170246) and *P. hispidum* Sw. (MG 189262) were collected in the Carajás National Forest, Municipality of Parauapebas, Pará state, Brazil, during the rainy season (February 2008). The plants were identified by Dr Elsie Guimarães from Jardim Botânico do Rio de Janeiro, Rio de Janeiro, Brazil, and they were deposited in the herbarium of Emilio Goeldi Museum, Belém, Pará state, Brazil.

2.2. Plant processing and extraction of the essential oils

The aerial parts of the plants (leaves and thin stems) were air-dried, ground and submitted to hydrodistillation using a Clevenger-type apparatus (100 g, 3 h). The oils were dried over anhydrous sodium sulfate, and their percentage contents were calculated on the basis of the plants dry weight. The moisture contents of the samples were calculated after phase separation using a Dean–Stark trap (5 g, 60 min) and toluene as the solvent phase.

2.3. Oil-composition analysis

The analysis of the oils were carried on a GC–MS Thermo Focus DSQ II, under the following conditions: DB-5ms (30 m × 0.25 mm; 0.25 mm film thickness) fused-silica capillary column; programmed temperature, 60–240 °C (3 °C/min); injector temperature, 250 °C; carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); injection type, splitless (2 mL of a 1:1000 hexane solution); split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS, electron energy, 70 eV; temperature of the ion source and connection parts, 200 °C. The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a FOCUS GC/FID operated under similar conditions for the GC–MS, except the carrier gas which was nitrogen. The retention index was calculated for all the volatiles constituents using a homologous series of *n*-alkanes (C_8–C_{32}, Sigma–Aldrich), according Van den Dool and Kratz (1963).

2.4. Antioxidant assay

The antioxidant activity of the oils of *Piper* was determined by the DPPH radical-scavenging assay. DPPH is a stable dark violet free radical with a maximum absorption at 517 nm, which is reduced in the presence of antioxidants. Each sample (10 μL) was mixed with 900 μL of 100 mM Tris–HCl buffer (pH 7.4), 40 μL of ethanol, and 50 μL of 0.5% (w/w) of Tween 20 solution and then added to 1 mL of 0.5 mM DPPH in ethanol. The standard curve was prepared to using Trolox, an antioxidant derived from water-soluble vitamin E, expressed as milligrams of Trolox equivalent per milliliter of sample (Choi et al., 2000).

2.5. Antifungal assay

The bioautographic method was applied to the microorganisms that may grow directly on TLC plates (Rahalison et al., 1994). Ten μL of the oil solution (corresponding to 0.1, 0.5, 1.0, 10.0, 50.0, and 100.0 μg) were applied to pre-coated TLC plates, developed on *n*-hexane/ethyl acetate (8:2) solution and dried for complete removal of the solvent. The plates were sprayed with spore suspension of *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* in glucose and salt solution and, then, incubated for 48 h in a dark and moistened chamber, at 22 °C. A clear inhibition zone appeared against a dark background indicating the minimal amount of the used oils. Miconazole was used as a positive control (Silva et al., 2011).

2.6. Acetylcholinesterase assay

The enzyme acetylcholinesterase (500 U) was dissolved in tris–hydrochloric acid buffer (pH 7.8) and stabilized by the addition of bovine serum albumin – fraction V (0.1%). TLC plates were spotted with the essentials oils in ranging from 0.01 to 1000 ng/spot. The alkaloid physostigmine (eserine) was used as the positive control. The plates were sprayed with the enzyme solution (3.33 U/mL) and then dried and incubated at 37 °C for 20 min (moist atmosphere). Enzyme activity was detected by spraying with a solution of 0.25% of 1-naphtyl acetate in EtOH (5 mL) plus 0.25% aqueous solution of Fast Blue B salt (20 mL). Potential acetylcholinesterase inhibitors have appeared as clear zones on a purple colored background (Marston et al., 2002).

2.7. Cytotoxicity assay (against cancer cell lines)

The MTT colorimetric assay was used to measure the activity of cellular enzymes, by indicating the cell viability (Mosmann, 1983). The oils (0.2–25 μg/mL) were tested for cytotoxic activity against three cancer cell lines: HCT-116 (colon), SKMEL19 (melanoma), ACP-03 (gastric). All cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C with 5% CO_2. Each oil was dissolved to obtain a concentration of 10 mg/mL with DMSO. The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). Essential oils (25 μg) were incubated with the cells for 72 h. The negative control received the same amount of DMSO (0.001% in the highest concentration). The cell viability was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product. The IC_{50}'s were calculated by nonlinear regression using GraphPad program (Intuitive Software for Science, San Diego, CA).

2.8. Cell membrane disruption

The potential of the cell membrane lyses is evaluated by the release of the erythrocytes in the medium. The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl solution, containing $CaCl_2$ (10 mM). The oils, diluted as mentioned above, were tested at 200 μg/mL. After incubation at room temperature for 1 h, followed by centrifugation, the supernatant was removed, and the liberated hemoglobin was measured spectrophotometrically at 540 nm. DMSO was used as the negative control and Triton X-100 (1%) as the positive control (Oliveira et al., 2011).