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Chemical composition, antimicrobial and cytotoxic activities of *Piper hispidum* Sw. essential oil collected in Venezuela

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ABSTRACT

This article provides an evaluation of the chemical composition, antimicrobial and cytotoxic activities of the essential oil of *Piper hispidum* Sw collected in the Venezuelan Andes. The chemical composition was examined by GC/MS analysis. Thirty four compounds were identified representing 95.2 % of the total oil. The major components were α-pinene (15.3 %), β-pinene (14.8 %), β-elemene (8.1 %), caryophyllene oxide (7.8 %) and δ-3-carene (6.9 %). Antimicrobial activity was observed against Gram positive bacterial strains *S. aureus* ATCC 6538, *S. epidermides* CECT232, *S. saprophyticus* CECT 235, *B. cereus* CECT496, *B. subtilis* CECT39 and E. *faecalis* CECT735 showing MIC values between 6.25 to 15 µg/mL and MBC values of 12.5 to 15 µg/mL however a low activity was observed against Gram negative bacterial strains *E. coli* CECT99, *P. mirabilis* CECT 170 and *P.aeruginosa* AK 958 performing MIC and MBC values of > 200 µg/mL. On the other hand, Candida albicans yeast presented a moderate activity with MIC 100-200 µg/mL and MBC > 200 µg/mL.Cytotoxic activity was also determined against HeLa (cervix carcinoma), A-459 (lung carcinoma), MCF-7 (breast adenocarcinoma) human cancer cell lines and against normal Vero cells (African green monkey kidney), exhibiting potent antiproliferative effects with IC_{50} values ranging from 18.6 to 37.7 µg/mL.

INTRODUCTION

Piper is the most economically and ecologically important genus of the Piperaceae family. It contains around 700 species widely distributed in the tropical and subtropical regions of the world (Tebbs 1993). Particularly, in Venezuela four *Piper* species has been reported, *Piper dilatatum, P. hispidum, P. tuberculatum* and *P. aduncum* growing wild especially in the mountain areas (Steyermark 1984).

Traditionally, *Piper* species has been used in the Indian and Chinese herbal medicinal for the treatment of numerous diseases such as bronchitis, fever asthma, abdominal pain, arthritis, rheumatism, gastrointestinal and venereal diseases (Kirtikar *et al.*, 1933; Matsui *et al.*, 1975). In Latin America, many of these species have also been used in folk medicine to alleviate different diseases. *Piper amalago* is used in Brazil and Mexico as anti-pain and anti-inflammatory agent (Dominguez *et al.*, 1985).

In Jamaica, *P. aduncum* is listed as remedy for stomach aches (Asprey *et al.*, 1954). Hydrogenated components isolated from *Piper arboreum* and *P. tuberculatum* from Brazil are proved to be active against *Cladosporium sphaerospermum* and *C. cladosporioides* fungus (Vasquez *et al.*, 2002), whereas the leaves of *Piper carpunya* are widely used as anti-diarrheal, anti-parasitical and as ailment for skin irritations (Quileza *et al.*, 2010). *Piper obliquum* from Ecuador has been analyzed for their antimicrobial activity against *Gram* positive, *Gram* negative bacteria, dermatophyte and phytopathogenic fungi (Guerrinia *et al.*, 2009). Additionally, insecticide activity has also been proved from extracts of *Piper* spp (Scott *et al.*, 2008).

A number of investigations carried out with different *Piper* species have reported a variety of chemical compounds such as alkaloids, amides, neolignans, steroids, kawapyrones, chalcones, dihydrochalcones, piperolides, flavones and flavanones (Parmar *et al.*, 1997; Jagbeer *et al.*, 2011). Different biological activities evaluated for these components revealed antifungal activity forchalcones and flavonones (Vieira *et al.*, 1980),

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flavonoids (Plazas *et al.,* 2008), amides (Navickiene *et al.,* 2000; Alécio *et al.,* 1998) and antiplasmodial activity for benzoic acid derivatives (Avella *et al.,* 1994; Friedrich 2005).

In our continuing interest for the evaluation of biological proprieties and chemical composition of the Venezuelan Andes aromatic plants, in this paper the chemical composition, antimicrobial and cytotoxic activities of the essential oil of *Piper hispidum*, that grows spontaneously in the mountainous areas of Mérida, is being studied. To the best of our knowledge, this is the first report on antimicrobial and cytotoxic activities for the essential oil of this species.

MATERIAL AND METHODS

Plant Material

Piper hispidum Sw. leaves were collected in Chiguará, Mérida State at 840 m above sea level, Venezuela, in February 2006.

The botanical sample was identified by Ing. Juan Carmona Arzola and a voucher specimen (code 1054) was deposited in the Dr. Luis E Ruiz Terán Herbarium, Faculty of Pharmacy and Bioanalysis, University of Los Andes, Venezuela.

Essential oil isolation

3000 g of fresh leaves were subjected to hydrodistillation for 4 h using a Clevenger- type apparatus. The oil (7.5 mL) was dried over anhydrous sodium sulfate and stored in sealed vials at + 4 °C in the dark until was analyzed and tested. The yield (0.25 %) was calculated based on the dry weight of the plant material.

Gas chromatography

GC analysis was carried out with an HP 5890 Series II gas chromatograph (FID), using a 30 m x 0.35 mm x 0.25 µm HP-5 fused silica capillary column. The temperature program was from 60 °C to 210 °C at 3 $°Cmin^{-1}$, and from 210 °C to 250 °C (2-min hold) at 5 $°Cmin^{-1}$. Detector and injector temperature was 250 °C and the carrier gas was N_2, with split sample introduction.

Gas chromatography-mass spectrometry

was performed with a FINNIGAN GCQ ion trap bench-top mass spectrometer. All conditions were as above except that carrier gas was He at a linear velocity of 31.9 cm/sec^{-1} and DB-5MS (30 m x 0.25 mm x 0.25 µm) capillary columns were used. The positive ion electron ionization mode was used, with a mass range of 40-400 amu.

Identification of the compounds was based on comparisons with published MS data (Adams 1995) and a computer library search (the database was delivered together with the instrument) and also by comparison of their Kovats indices with those of authentic references and with literature values (Adams 1995). The identification was confirmed with the aid of authentic samples. Kovats indices were calculated mainly from the GC-MS analysis results (Kovats, 1965). Solvents and other chemicals used were of high purity (analytical grade).

Antimicrobial Activity

Antimicrobial activity was determined against Gram-positive (*Bacillus subtilis* CECT39, *B. cereus* CECT496, *Staphylococcus aureus* ATCC 6538, *S. epidermidis* CECT232, *S. saprophyticus* CECT 235, *Enterococcus faecalis* CECT735), Gram-negative (*Escherichia coli* CECT99, *Proteus mirabilis* CECT 170, *Pseudomonas aeruginosa* AK 958) bacteria and *Candida albicans* CECT 1039 (yeast).

The bacteria cultures were developed in nutrient broth (NB) or brain heart infusion broth (for *E. faecalis* containing 0.06 % Tween 80), and the yeast was cultured in Sabouraud liquid medium at 37 °C. All media were purchased from Oxoid. The minimal inhibitory concentration (MIC) was determined for each sample by triplicate, using the broth microdilution method (De Leo´n *et al.,* 2005).

All samples were dissolved in DMSO, several wells were also filled with the same proportions of DMSO as controls and never exceeded 1% (v/v). The starting microorganism concentration was approximately (1-5) × 10^5 CFU/mL, growth was monitored by measuring the optical density increasing at 550 nm (OD_{550}) using a microplate reader (Multiskan Plus II). The MIC was defined as the lowest concentration of the essential oil where growth inhibition was observed after 24 h of incubation in a rotatory shaker at 37 °C.

All well with no visible growth were sub-cultured by transferring 100 µL to nutrient, brain-heart infusion or sabouraud agar plates. After overnight incubation, colony counts were performed and the MBC was defined as the lowest concentration of the essential oil that produced ≥ 99.9% killing of the initial inoculum.

Cytotoxic Activity

HeLa (human carcinoma of the cervix), A-549 (human lung carcinoma), MCF-7(human breast adenocarcinoma), and Vero (African green monkey kidney) cell lines were grown as a monolayer in Dulbecco's modified Eagle's medium, DMEM (Sigma), supplemented with 5 % fetal calf serum (Gibco) and 1 % of penicillin-streptomycin mixture (10.000 µL). Cells were maintained at 37 °C in 5% CO_2 and 98 % humidity. Cytotoxicity was assessed using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay (Mosmann1983).

Cell suspensions (0.1 mL of 2 x 10^4 cells/well) in lag and log growth phase were incubated in a microtiter well plate (96-well Iwaki) along with the essential oil, pre-dissolved in DMSO,at different concentrations. After 48 h, the optical density was measured using a micro ELISA reader (Multiskan Plus II) at 550nm after dissolving the MTT formazan with DMSO (150µL). The viability percentage was plotted against the sample concentration, and 50% cell viability (IC_{50}) was calculated from the curve. Cytotoxic assays were carried out by triplicate, variations were measured, calculated and the average value was estimated less than 10%.

RESULTS AND DISCUSSION

Essential oil analysis

A yellow coloured essential oil was analyzed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). All components (34, representing 95.2 % of the total oil) were characterized by comparison of each MS with the Wiley GC/MS library data and also from its retention index (RI).α-pinene (15.3 %), β-pinene (14.8 %), β-elemene (8.2 %), caryophyllene oxide (7.5 %) and δ-3-carene (6.4 %) were the major compounds observed in the present investigation. A list of the identified components, along with their percentages of the total oil, is given in Table 1.

Table. 1: Chemical composition of *Piper hispidum* Sw. essential oil.

Compounds[a]	%	RI[b]
α-pinene	15.3	934
camphene	0.4	971
verbenene	0.5	974
β-pinene	14.8	978
myrcene	0.9	992
δ-3-carene	6.9	1012
p-cymene	2.3	1025
limonene	2.3	1029
β-phellandrene	0.3	1032
unknown	0.8	1101
1,3,8-p-menthatriene	0.2	1109
E-pinocarveol	0.5	1140
myrtenol	0.6	1198
unknown	2.4	1220
E-ocimenone	0.6	1254
unknown	0.8	1313
α-copaene	1.8	1377
β-bourbonene	0.5	1386
β-elemene	8.1	1394
β-caryophyllene	6.2	1421
β-gurjunene	0.8	1431
α-humulene	0.6	1455
γ-gurjunene	0.4	1478
valencene	0.9	1487
viridiflorene	1.0	1496
germacrene A	0.9	1501
γ-cadinene	0.8	1507
Z-calamenene	0.6	1517
germacrene B	5.2	1566
spathulenol	5.0	1580
caryophyllene oxide	7.8	1585
β-eudesmol	2.6	1652
unknown	1.5	1656
unknown	1.1	1719

[a]Compounds are listed in sequence from a DB-5 MS column elution.
[b]Kovats retention indices (RI) were calculated against C_9 to C_{24} *n*-alkanes Series on a DB-5 MS column

The oil was characterized by a high percentage of sesquiterpenes, 55.2 % (44. 8 % none oxygenated and 13.79 % oxygenated), while monoterpenes were represented by 44.8 % of the total oil being 34.5 % of them none oxygenated whereas only 6.9 % were oxygenated. The chemical profile of *Piper hispidum* essential oil in this study turned to be different from those of previous reports. *P.hispidum* from Brazil showed γ-cadinene (25.1 %), camphene (15.6 %), α-guaiene (11.5 %) and γ-elemene (10.9 %) as major components (Machado *et al.*, 1994), whereas for the essential oil of same species collected from Cuba, β-eudesmol (17.5%) was observed in high concentrations (Pino *et al.*, 2004); another sample studied in Colombia showed *trans*-nerolidol (23.6%) as main component (Pino *et al.*, 2009). An additional study in Colombia by (Delgado *et al.*, 2007); reported β-pinene (14.5 %) and α-pinene (13.5%) as major compounds, being those results very closed to the ones obtained in the present investigation. Ferreira *et al.*, also reported α-copaene (28.7%; 36.2%), α-pinene (13.9%; 7.1%), β-pinene(13.3%; 7.5%), and *trans*-nerolidol (2.9%; 7.0%) as main components of the essential oil of ripe and unripe fruits of the same species collected from Brazil (Ferreira *et al.*, 2011), while Facundo *et al.*,also in Brazil, studied the essential oil of the roots finding a very different composition with dillapiole (55.5 %), elemicine (24.5 %) andapiole (10.2 %) as major compounds (Facundo *et al.*, 2008).

Antimicrobial activity

Antimicrobial activity of *Piper hispidum* essential oil was evaluated against *Gram* positive, *Gram* negative bacterial strains and *Candida albicans* yeast. The essential oil showed activity against *S. aureus*, *S. epidermides*, *S. saprophyticus*, *B. cereus*, *B. subtilis*, *E. faecalis* with MIC values ranging between 6.25-12.5 µg/mL; MBC values were observed between 12.5-15 µg/mL. For *P. mirabilis*, *E.coli* and *P. aeruginosa* a higher concentration, 200 µg/mL, was necessary to cause grow inhibition, thus, *P. hispidum*is consider none active against those bacteria. Additionally, *C. albicans* showed a moderate activity with values ranging 100-200 µg/mL. Table 2 summarizes these results.

Table. 2: Antibacterial activityof *Piper hispidum* Sw. essential oil.

Microorganism	Essential oil (µg/mL)		Antibiotic (µg/mL)	
	P. hispidum		Cefotaxime[a]	
	MIC	MBC	MIC	MBC
*S. aureus*ATCC 6538	12.5-6.25	12.5	2.5-1.25	NT
S. epidermides CECT232	12.5-6.25	12.5	2.5	>20
S. saprophyticus CECT 235	12.5-6.25	12.5	0.625-0.313	NT
*B. cereus*CECT496	12.5-6.25	12.5	10	>20
*B. subtilis*CECT39	12.5-6.25	12.5	8	NT
*E. faecalis*CECT735	15.0-12.5	15.0	NT	NT
P. mirabilis CECT 170	>200	>200	NT	NT
E.coli CECT99	>200	>200	NT	NT
P. aeruginosa AK 958	>200	>200	NT	NT
C. albicans CECT 1039	200-100	>200	NT	NT

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration, [a]Cefotaxime (positive control).
ATCC: American type culture collection; CECT: Colecciónespañola de cultivostipo (spanish type culture collection)

Cytotoxic activity

The essential oil was subjected to screening for possible cytotoxic activity using a representative panel of cancer cell lines, HeLa (cervix carcinoma), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma), alone with Vero cells (African green monkey kidney), using 6-mercaptopurine as a positive control.As shown in Table 3, *P. hispidum* essential oil turned to be active against the tested tumor cell lines, especially on HeLa cells (IC_{50} 18.6 µg/mL) after 48 h of exposure. In addition, when comparing the activities against cancer cells with none tumorigenic (Vero) cell, some degree of selective cytotoxicity was observed, especially when these were added in lag phase.

Thus, a selectivity index (SI) of 2.01 for this cell combination was exhibited. Low selectivity was also found against A-549 and MCF-7 (SI ≥ 1), of 1.35 and 1.13 respectively, as was demonstrated by a higher IC_{50} values against the non-tumor mammalian Vero cells. Many essential oils and their individual aroma components have shown cancer suppressive activity on some human tumor cell lines such as glioma, gastric cancer, colon cancer, breast cancer, pulmonary tumors and others (De Angelis 2001). Anticancer activity *in vitro* against a series of human malignant cells lines (A549, MCF-7, K562, HL60) has also been reported (De Souza *et al.*, 2004). Monoterpenes, in particular, have shown chemopreventive as well as chemotherapeutic activities in some tumor models (Elson 1995; Wattenberg 1992; Morse *et al.*, 1993). In the present investigation, *P. hispidum* essential oil was constituted by 44.8 % of monoterpenes. Limonene (2.3 %) and myrcene (0.9 %), present as components in the essential oil, have been reported as candidates for the chemoprevention of some type of cancer (Bodake 2002; Ozbek *et al.*, 2003; Stratton *et al.*, 2000). On the other hand, sesquiterpenes represented 55.2 % of the total oil with the presence of elemenein 8.1 %, this sesquiterpene has shown cytotoxic activity in previous studies (Tan *et al.*, 2000). To the best of our knowledge, this is the first report on the antibacterial and cytotoxic activity of *Piper hispidum* Sw. essential oil.

Tabla. 3: Cytotoxic activity of *Piper hispidum* Sw. essential oil.

Compounds	HeLa		MCF-7		A-549		Vero	
	a	b	a	b	a	b	a	b
Essential oil	18.6	36.6	32.9	37.5	27.7	34.2	37.5	37.7
Control[c]	0.5	0.7	0.24	1.0	8.0	8.4	11.5	>20.0

a: Lag phase, *b*: Log phase, [c]6-mercaptopurine (positive control). All assays were repeated at least three times and IC_{50} values are represented in µg/mL

CONCLUSSION

In the present investigation, several differences were observed in the composition of *P. hispidum* essential oil comparing to previous reports, this might be attributed to geographical environment, seasonality, physiological age of the plant, harvesting time, among other conditions. Regarding the cytotoxic and antimicrobial activities we strongly recommend that further investigations must be carried out using other tumor cell lines and microorganisms, since this species might represent a new alternative in the prevention/therapy of infection or tumors diseases.

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