IN VITRO ANTI-PROTOZOAL ASSESSMENT OF
Vernonanthura patents EXTRACTS

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Abstract

In this work, it was performed a chemical-biological study of *Vernonanthura patens* (Kunth) H. Rob.'s aerial parts, which grows on the Ecuador's coast. This work begins with the obtaining of extracts, the isolation and purification of leaves and stems's fractions and compounds of the species by extraction with ethanol by maceration, chromatography column with increasing polarity's solvents and TLC. Structural identification was performed by Gaseous chromatography - mass spectrometry and NMR. Anti-protozoal activity was evaluated against Leishmania amazonensis MHOM/77BR/LTB0016's and Plasmodium berghei ANKA. The majority chemical compounds identified by GC-MS with more than 50% relative abundance were: hexadecanoic acid (palmitic), tetracosanoic acid (lignoceric), tetradecanoic acid (myristic), aristolona, α-Amyrin acetate, lupeol acetate, lupeol, Lupeol palmitate, stigmasta-5,22-dien-3-ol. Leave's ethanol extract showed antileishmanial´s activity and selectivity with IC50 of 24, 3 µg/mL and IS 12, higher than Pentamidine´s positive control (IS 9) such as reported for species that grows in other countries. Investigation´s results are reported for the first time for the ecuatorian species.

Key words: Vernonanthura patens, Anti-protozoal activity, *Leishmania amazonensis*, *Plasmodium berghei.*

Introduction

The use of medicinal plants in Ecuador is part of the everyday practices of its inhabitants, mainly due to the vast ancient medical knowledge. It reported 3,118 species belonging to 206 families of plants used for medicinal purposes, among which, the families that have a greater number of species are Asteraceae, Fabaceae, Rubiaceae, Solanaceae and Aracaceae (Batista et al., 2009).

The species *Vernonanthura patens* (Kunth) H. Rob, (Asteraceae), is a wild shrub native to South America that grows in the provinces of Loja, El Oro, Guayas, Manabi and Los Rios. The residents of Loja, in the southwest of Ecuador, used this plant to clean wounds, given its healing properties, as well as against headache pain, according to their analgesic action. In the Marcabeli Canton, Oro Province, is widely used as anti-inflammatory, to soothe coughs and fight some cancers. Also it has been employed in the treatment of leishmaniasis, and its leaves as a poultice, to combat athlete's foot (Blair and Madrigal, 2005). For Ecuadorian species have been realized several chemical studies wich their major chemical components are pentacyclic triterpenes and other compounds such as fatty acids (Manzano et al., 2012a and 2012b; Manzano et al., 2013a, 2013b and 2013c). In this paper, we make in vitro anti-protozoal assessment of *V. patents* extracts, in order to validate their use in traditional medicine.

Methods

Extracts from *V. patents*

The plant material of adult leaves and stems of *Vernonanthura patens* (laritaco) were used from plants at the florewing state which were growing in the citadels "July 25", "Imbabura" and belonged to the Canton Marcabelí, province El Oro, Ecuador. Leaves and stems were collected at early morning on the month of august in 2011.

Botanical identification was performed and voucher specimens of the herbs were prepared and deposited at the National Herbarium of Ecuador (QCNE) and a duplicated sample (CIBE037) was kept as herbal witness in the laboratory of the CIBE-ESPOL Bioproducts, Ecuador.

The extraction was carried out by maceration with ethanol p.a. in a closed container, with absence of light and stirring in a shaker (New Brunswick Scientific model C-10, USA). The extraction time was eight days until complete exhaustion of the vegetable material. The extracts were mixed and filtered using filter paper, then were concentrated until dryness on a rotary evaporator (Heidolph Brand model 4001 Laborota, Germany) under reduced pressure at 50°C, each dry extract was used for the biological evaluation.

Fractionations were carried with whole extracts from leaves and stems, by column chromatography using increasing polarity solvents. The extracts and fractions were analyzed for structural identification by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph with an Agilent 5975 detector (Avondale, PA.USA) equipped with a column HP-5MS of 5m long (0.25 mm in diameter and 0.25 cm inside diameter, USA). Helium was used as the carrier gas; the analytical conditions were: initial temperature: 100°C (increasing 8°C per minute to a final temperature of 250°C); inlet temperature and mass detector: 250 oC and 300 °C respectively. The mass detector was used in scan mode ("scan") with a range of 100 to 400 amu.

Biological study
Reference drug
Choloroquine phosphate (Sigma, USA) for *Plasmodium berghei*. Pentamidine (Richet, Buenos Aires, Argentina) for *Leishmania amazonensis* was used. Both compounds were diluted in sterile distilled water.

Parasites
After thawing of *Plasmodium berghei* ANKA stocks (gently donated by the Laboratory of Microbiology, Parasitology and Hygiene of University of Antwerp, Belgium), the parasite population was maintained in vivo by serial transfer of parasitized erythrocytes from infected to naive mice.
Leishmania amazonensis (MHOM/77BR/LTB0016) was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil.

Parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26°C in Schneider's medium (Sigma) containing 10% heat-inactivated fetal bovine serum (HFBS) (Sigma), 100 mg of streptomycin/ml, and 100 U penicillin/mL. The parasites were not used after the tenth passage.

Antiplasmodial activity
Short-term in vitro cultures of *P. berghei* ANKA blood stages were performed as described before (Janse & Waters, 1995). Briefly, erythrocytes infected with parasites of *P. berghei* ring forms/young trophozoites were prepared at 1% parasitemia and 1% final erythrocytes concentration in complete culture medium (RPMI 1640 supplemented with 27.5 mM NaHCO3, 25mM Hepes, 50 µg/ml neomycin and 20 % HFBS, (Sigma) containing serial dilutions of samples from 200 µg/ml to 12.5 µg/ml, each in duplicate wells of 96-well culture plates. Control wells were prepared with 0.5% of DMSO. These plates were incubated overnight (16 – 20 h) at 37 °C, under a candle jar gas mixture, until more than 40 % maturation to schizonts for control wells. Schizont maturation for each test well was determined by light microscopy of post-culture blood slides after Giemsa staining and was evaluated by the WHO Mark III method (WHO, 2001) slightly modified for the threshold of schizont growth which has been adapted to the appearance of red cells with well-defined merozoites. The antimalarial activity was expressed as inhibitory median concentration (IC50), defined as the concentration of extract that induces 50% reduction of production of schyzonts compared to control wells, which was calculated by interpolation (Huber and Koella, 1993). IC50 was expressed as mean ± SD of tests performed in three experiments. The selection of active extracts was performed based on criteria reviewed by Batista et al. (2009).

Antileishmanial activity
The activity of the extracts against intracellular amastigotes was evaluated as described previously Caio et al. ,1999. The peritoneal macrophages were harvested and plated at 106/mL in 24-Well Lab-Tek (Costarâ, USA) and incubated at 37°C under an atmosphere of 5 % CO2 for 2 h.

Non-adherent cells were removed by washing with pre-warmed phosphate-buffered saline (PBS). Stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio, and the cultures were incubated for further 4 h. The cell monolayers were washed three times with pre-warmed PBS to remove free parasites. Then, 999 mL of RPMI completed medium and 1 mL of the different products dissolved in DMSO were added in duplicate for further 48 h. The cultures were then fixed in absolute methanol, stained with Giemsa, and examined under a light microscope. The number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages per each sample. In addition, the percentage of infected macrophages was calculated. The results were expressed as percent of reduction of the infection rate in comparison to that of the controls, where the infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages (Delorenzi 2001). The IC50 value was determined from the linear regression of concentration-response curves.

Cytotoxicity assay
The IC50 of the extracts for the viability of mouse peritoneal macrophages was determined. Macrophages were collected from peritoneal cavities of normal BALB/c mice in ice-cold RPMI 1640 medium (Sigma) supplemented with antibiotics, and seeded at 30000 cells/well. The cells were incubated for 2 h at 37°C in 5 % CO2. Non-adherent cells were removed by washing with PBS, and then, 1 µL of product solutions were added to 200 µL medium containing 10 % HFBS and antibiotics. Macrophages treated with 1 µL DMSO were included as controls. The cytotoxicity was determined using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma). MTT solutions were prepared at 5 mg/mL in saline solution, filtered and sterilized at the moment of use, and 15 µL was added to each well. After incubation for an additional 3 h the formazan crystals were dissolved by addition of 100 µL DMSO. The optical density was determined using an EMS Reader MF Version 2.4-0, at a test wavelength of 560 nm and a reference wavelength of 630 nm (Sladowski y col., 1993).

The IC50 was obtained by fitting a sigmoid Emax model to dose-response curves. Selectivity indexes were calculated by dividing the IC50 for peritoneal macrophage of BALB/c mice by the IC50 for *L. amazonensis* amastigotes (Shioji 2005).

Results

A total of 10 leaves' fractions and 7 stems' fractions obtained by column chromatographic were analyzed by CG-SM, using the library computer and taking into consideration those compounds that exceeded 90% of confidence and relative abundance greater than 50% in any of the organs studied extracts. Were identified seven compounds at leaves and three compounds at species' stems. Main chemical found groups were: free fatty acids and triterpenoids pentaciclic and free fatty acids, triterpenoids pentaciclic and sesquiterpenoid cetone for the leaves and stems respectively. Compounds mostly identified that presented more than 50 % of relative abundance are shown at table 1.

Extracts from leave and stems from *V. patents* were profiled in vitro for their anti-protozoal activities. The extracts showed no activity against *P. berghei* (Table 2); while more interesting results were obtained against Leishmania parasite. A highest selectivity was found in extracts from leaves while the extract of stems showed cytotoxicity.

Discussion

The results that are shown in this chapter have not been previously registered for the species *V. patens*. As ethnomedical knowledge, the use of *V. patents* to treat malaria and leishmaniasis has been reported by population from Peru (Valadeau et al., 2009) and Ecuador (Gachet et al., 2010) use to treat malaria and leishmaniasis.

Qualitative and quantitative differences were observed in chemical composition between leaves and stem extracts. Among studied fractions, lupeol and hexadecanoic acid were found the main compounds, respectively. Similar results were previously reported in methanol extract from leaves of *V. patents* (Manzano et al., 2013c).

Promissory results were found to leaves ethanolic extract against *L. amazonensis in vitro*, granted to evaluated activity more superior than yielded by Valadeau et al., 2009 (CI_{50} >100 mg/ml) from Peruvian species (Valadeau et al., 2009). In addition, antileishmanial activity and selectivity antileishmanial observed for *V. patens* leaves differ from Jakupovic´s reported and published by Fournet and Barrios, 1994. They reported that there is not activity in the leaves and stems extracts and fractions from the Bolivian species. This contradictory result could be to previous knowledge related to natural products, due to herbal medicines may vary by part of plant used, time of harvest, active constituents levels and type of extract (Gagnier et al., 2006) .Compared with pentamidine, a drug clinically used, a better selectivity was obtained to leaves extract from *V. patents*. Surveying the literature on antileishmanial pharmacological reports of Ecuadorian plants, we found that extract from *Minquartia guianensis, Bocconia integrifolia, Cupania cinerea* and *Scoparia dulcis* also have been displayed potentialities against *L. donovani* (Gachet et al ., 2010).

In addition, extracts of leaves from *V. patens* collected in Yanesha, Peru, was active *in vitro* against *P. falciparum* (IC_{50}=38.7 mg/ml) (Valadeau et al ., 2009) ; while lupeol showed also inhibitory activity against *P. falciparum (Al Musayeib et al., 2013 and* Kumar et al., 2008). However, in our study, no inhibitory activity on P. berghei was observed in any of extracts studied. The differences could be addressed due to genetic differences in *Plasmodium* species (Orjuela-Sánchez et al., 2012) used herein.

Previous studies reported potentialities of lupeol against *Leishmania infantum (Al Musayeib et al., 2013), Leishmania amazonensis* (Schinor et al., 1992; Barrios, 1994; and Siddique and Salem, 2011) and *Plasmodium falciparum* and *Plasmodium berghei* (Gallo and Sarachine, 2009), suggest that the majority presence of this compound in the ethanolic extract of the leaves of *V. patens* could be the responsible of antileishmanial activity observed in this extract.

In conclusion, considering that leishmaniasis constitutes a serious public health problem and that high morbidity have been reported in Ecuador (Calvopina et al., 2004), the search for new drugs

with high activity and reduced adverse effects deserves more efforts. We think that the use of crude extracts and the pharmacological activity observed justify the continuation of the studies with *V. patents*, as a promissory source of potential antileishmanial drugs, particularly pure compounds identified that have not been studied against *Leishmania* parasites. Additionally, the biological properties of other plants growing in Ecuador could be explored.

Acknowledgments

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No	Compounds concentration major of 50% of relative abundance	L	S
	Hexadecanoic acid (palmitic)	X	X
	Tetracosanoic acid (lignoceric)	X	-
	Tetradecanoic acid (myristic)	X	-
	Aristolone	-	X
	α-Amyrin acetate	X	-
	Lupeol acetate	X	-
	Lupeol	X	-
	Stigmasta-5,22-dien-3-ol	X	X

Table 1. Compounds majoritarian with more than 50% relative abundance identified by CG-EM

Tested Products	IC50[a] ± SD[b] (µg/ml)			SI[c]
	P. berghei	L. amazonensis	Macrophage	
Leaves	>200	24.3 ± 7.4	280.5 ± 1.8	12
Stems	>200	ND[d]	36.9 ± 4.3	-
Chloroquine	(8.12 - 40.67 / ng/ml)d	-	-	-
Pentamidine	-	1.3 ± 0.1	11.7 ± 1.7	9

Table 2. Activity of *Vernonanthura patents* against protozoa parasites

a: IC50: Concentration of drug that caused 50 % of mortality
b: SD: Standard deviation
c: SI: Selectivity index: IC50 macrophage / IC50 Leishmania
d: ND: Not Detected. The extract caused a high toxicity in the lower concentration tested (100 µg/ml)