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CHARACTERISATION OF ISOELEUTHERINE IN AQUEOUS EXTRACT OF *ELEUTHERINE PLICATA* HERB, IRIDACEAE, ACTIVE AGAINST *ENTAMOEBA HYSTOLITICA*/ *ENTAMOEBA DISPAR* IN-VITRO

M. S. Nascimento^{1,4}, J. M. S. Vieira^{3,4}, L. C. S. Malheiros^{1,4}, J. O. C. Silva JÚNIOR⁴, L. C. S. Rodrigues² and W. L. R. Barbosa^{*1,4}

Laboratory of Phytochemistry of the Faculty of Pharmacy¹, Institute of Health Sciences, Federal University of Para, Belem, Para, Brazil

Laboratory Clinical Parasitology², Faculty of Pharmacy, Institute of Health Sciences, Federal University of Para, Belem, Para, Brazil

Laboratory of Microbiology of the Faculty of Pharmacy³, Institute of Health Sciences, Federal University of Para, Belem, Para, Brazil

Program Post-graduate of Pharmaceutical Sciences⁴, Institute of Health Sciences, Federal University of Para, Belem, Para, Brazil

ABSTRACT

Eleutherine plicata Herb., Iridaceae, is an herbaceous Iridaceae, vernacular marupazinho, largely used as tea, in the Amazonian popular phytotherapy, to treat diarrhoea alleged to amoeba infestation. To investigate the form of use of this medicinal plant and its alleged activity, and to perform a phytochemical analysis and an anti-amoebae assay. Bulbs of the plant were dried, grinded and extracted in boiling water. After concentration the extract (AEEp) was analysed by LC-DAD and tested against trophozoitic forms of three strain of *Entamoeba histolytica*/*Entamoeba dispar*. A significant peak in the LC-DAD chromatogram of AEEp can be observed and attributed to isoeleutherine, a naphthoquinone also an anti-amoebian activity was detected for this extract. Isoeleutherine would be one of the active substances responsible for the anti-amoebae activity of *Eleutherine plicata* Herb., since naphthoquinone show anti-protozoal activity and the substance shows pro-oxidative properties. Noteworthy is the fact that the decoction shows 3.5mg dry residue/mL, 40% higher than the concentration of the extract used in the bioassay (2.5mg/mL). The detected anti-amoebian activity for AEEp aligns to the popular attribution of its use and the presence of Isoleutherine can partially explain this observation.

Keywords:

Anti-amoebae activity,
Naphthoquinone,
Marupazinho,
Amoebiasis,
LC-DAD

Correspondence to Author:

Wagner Luiz Ramos Barbosa

Laboratory of Phytochemistry of the
Faculty of Pharmacy, Federal
University of Para, Rua Augusto
Correa, no. 01, CEP 66075-110,
Guama, Belem, Para, Brazil

E-mail: barbosa@ufpa.br

INTRODUCTION: *Eleutherine plicata* Herb., Iridaceae, vernacular marupazinho, marupari, marupá-piranga, palmeirinha, is an herbaceous, bulbous and rhizomatous plant, native in tropical America¹. In the Amazon region, this plant is an important therapeutic option, used in the primary health care. Jardim *et al.* (2007)² demonstrated the use of a tea from the bulbs of this plant in the popular phytotherapy, to treat

diarrhoea. An ethnopharmaceutical survey performed by Pinto and Barbosa (2009)³ discloses a convergence of popular use⁴ of *E. plicata* of about 80% for diarrhoea, which rises to about 100% when others symptoms of amoebiasis are associated (WLR Barbosa, UFPA, personal communication). These surveys were carried out in different cities in Para State, Amazonia, Brazil.

Entamoeba dispar is accepted as a protozoa species genetically distinct but morphologically similar to *E. histolytica* and can be associated to a non-invasive Amoebiasis⁵, but the disease invasive type is defined as a human infection caused by *Entamoeba histolytica*. The species show high prevalence in tropical regions, where both the education and sanitary conditions are considered deficient⁶.

Epidemiological data from World Health Organization⁶ indicate that *Entamoeba histolytica* cause circa 100.000 deasee per year, infecting 500 million people all over the world.

Several epidemiological surveys have been conducted to estimate the incidence and prevalence of amoebiasis in Brazil. These studies are concentrated in Brazil North and Northeast regions and use different methods of analysis and detection⁷⁻¹³.

Epidemiological studies conducted in the North show the incidence of 12.9% in Amazon villages of Tome-Acu⁸, 21% in Manaus¹² and up to 29% in Belém¹¹.

The use of synthetic medicines to treat amoebiasis can lead to unsatisfactory results, mainly due to their toxicity, associated to the long term therapeutic schedule, which results in low percentage of cure¹⁴ or development of resistance¹⁵.

To overcome this situation integrative and complementary treatments can be adopted using medicinal plants as therapeutic resource themselves or as source for the development of safer and more efficient anti-amoebaeen agents.

In this context, the popular knowledge, which alleges to *E. plicata* utility in the treatment of diarrhoea associated to amoebiasis, provides basis to the here reported work which aims to contribute to the standardization of the aqueous extract of the plant and the validation of the alleged use.

MATERIALS AND METHODS

Sample preparation: Bulbs of *Eleutherine plicata* Herb., acquired in July 2010 at a herb market next to Belém, Pará State, Brazil (collected around 1°22' S and 48°27' W), were selected, washed, sliced, dried at 40°C and grinded in a knives mill. The plant material was

characterised by comparison with a voucher deposited at Museu Paraense Emilio Goeldi, Belem-PA, Brazil, under nr. 10543.

The organoleptic and physicochemical characteristics of the drug were evaluated, as well, the humidity and the total and in acid insoluble ashes.

The aqueous extract (AEEp), the form used in the popular phytotherapy in Para State (Amazonia-Brazil), was prepared by decoction of 50g of dried and grinded bulbs, in 250mL purified water, 20% (w/v). After the extraction, the decoction was frozen and then lyophilised. The anti-amoebaeen activity of *E. plicata* Herb. was assayed using this aqueous extract.

Characterization of isoeleutherine in the decoction by LC-DAD:

The chromatographic profile of AEEp was determined by LC-DAD using a Merck Hitachi[®] LaChrom7000 system equipped with diode array detector (DAD) and Agilent LiChrospher 100 column (250mm x 4.6mm), kept by 26°C (±1°C), using a method based on Paramapojn et al. (2008)¹⁶. Detection at 250nm was used to investigate the presence of Isoeleutherine in the sample.

The mobile phase contains water (A) and acetonitrile (B), in gradient, as follow: t= 0min, 85%A; t= 10min, 70%A; t= 20min, 50%A; t= 30min, 20%A; t= 35min, 20%A; t= 40min, 85%A; t= 45min, 85%A; at 1mL/min.

The method specificity was verified by comparison of the retention time of a peak in the chromatogram of the sample, with that one of the same isolated substance, here, Isoeleutherine. The presence of Isoeleutherine in AEEp, could be characterised by reversed and direct search in equipment library, comparing ultraviolet spectra of the isolated substance, with that associated to the peak Rt= 19,15min (Figures 2 and 3).

Assayed Strains: Three polixenic strains of *E. histolytica/E. dispar* obtained from different regions of the Amazon region were kept under polixenic culture condition according to Pavlova (1938)¹⁷ method, modified by Silva (1972)¹⁸. The characteristics of assayed strains are summarized in **table 1**.

TABLE 1: CHARACTERISTICS OF THE STRAINS USED IN THE EVALUATION OF THE ANTI-AMOEBAEAN ACTIVITY OF *E. plicata* HERB.

Strain	Origin	Clinical form	Serology		Virulence		IP	Z
			IIFR	ELISA	Hamster	Rat		
17BB	Belém – Pará	S - NDC	-	-	+	+	I	NP
LC	Ananindeua – Pará	[[As]]	-	-	-	-	I	NP
10096	Macapá – Amapá	S - DC	+	+	+	+	II	P

IIFR- Indirect ImunoFluorescence Reaction; **ELISA**- Imunoenzimatic Test; **IP**- Isoenzimatic Profile; **Z**- Zimodem; **S** - Symptomatic; **As**- Asymptomatic; **NDC**- Not dysenteric Colitis; **DC** - Dysenteric Colitis; **NP**- Not pathogenic Profile; **P**- Pathogenic Profile.

The *in vitro* anti-amoebaean activity of *E. plicata* Herb. was assayed using 5mL of culture of each strain, after 48 hours incubation at 37°C¹⁷, to which 0.5mL of a solution containing 2.5mg of dried AEEp/mL of buffered distilled water (pH 7.2). After 24 hours at 37°C, the viability of the trophozoit was evaluated analysing aliquots of each sample using optical microscopy by 40x. Metronidazole (2µg/mL) was used as positive control¹⁹.

RESULTS: The drug, dried and grinded bulbs of *E. plicata*, shows the following pharmacognostic characteristics: red-wine colour, characteristic odour and bitter taste; humidity by 1.59%, total ashes 12.7% and acid insoluble ashes 1.38%. The content of humidity in the herbal drug lies below the interval established by Brazilian Pharmacopoeia IV, which it is of 8 and 14%²⁰. On the other hand, the content in acid insoluble ashes is in accordance with the literature that recommends values of about 1%²¹.

The decoction prepared with this drug left, as dry residue, 3.534mg/mL and shows the presence of saponines, phenols and tannins, reducing sugars, polysaccharide and azulene.

Isoeleutherine (**Figure 1**), one of the important constituents of genus *Eleutherine* already described in the literature²²⁻²⁴, could be detected in the analysed decoction with a peak purity of 99.14%.

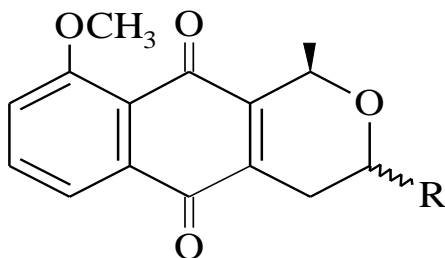


FIG. 1: ISOELEUTHERINE, ISOLATED FROM A TINCTURE OF *Eleutherine plicata* HERB.

Characterisation of Isoeleutherine in the Decoction by LC-DAD: **Figure 2** shows a chromatogram of AEEp, which presents a significant peak at Rt= 19, 15min, and another chromatogram describing the peak of Isoeleutherine at 18, 13min, both registered at 250nm and under the same analytical conditions.

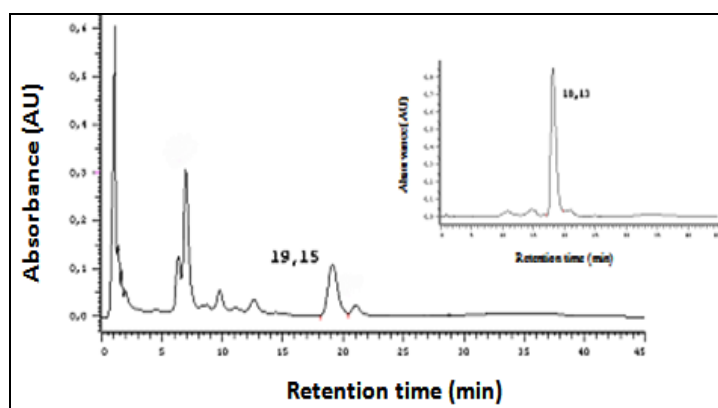


FIG. 2: HPLC-CHROMATOGRAM FROM DECOCTION OF *E. plicata* HERB. (250nm) AND ISOELEUTHERINE (DETAIL)

The ultraviolet spectra corresponding to the above depicted peaks (**Figure 3**) show coincident absorption maxima and the correlation between both the spectra has a factor of 0.9994, inferring the presence of this substance in the decoction.

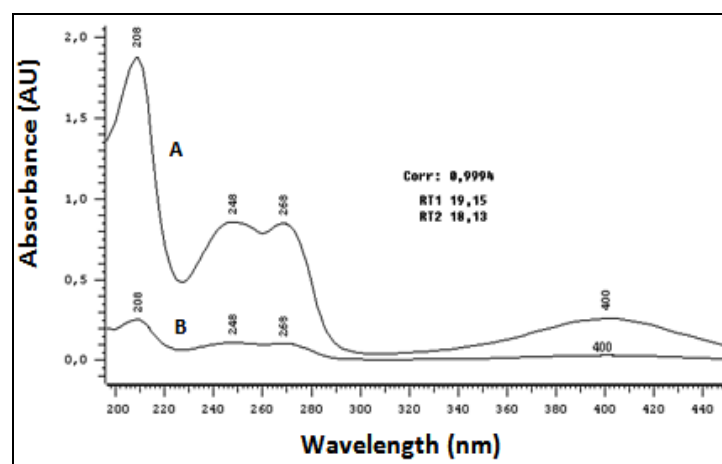


FIG. 3: CORRELATION BETWEEN THE UV-SPECTRA OF ISOELEUTHERINE (A) AND THE PEAK AT RT= 19.15min (B), REGISTERED BY THE HPLC ANALYSIS OF DECOCTION

Evaluation of the anti-amoebae activity of the species: The aqueous extract of *Eleutherine plicata* bulbs, in the assayed concentration, shows, *in vitro*, a complete growth inhibition of *E. histolytica* trophozoite in 24 hours, as shown the **table 2**.

TABLE 2: RESULTS OF EVALUATION IN VITRO SENSITIVITY OF POLYXENIC STRAINS OF *E. histolytica*/*E. dispar* FRONT OF THE AQUEOUS EXTRACT OF *E. plicata* HERB.

Strain	Reading in 24h	Control in 24h
17BB	No growth	No growth
LC	No growth	No growth
10096	No growth	No growth

DISCUSSION: The humidity of the drug is very low and this result can indicate a high chemical and microbiological stability of the material, since the water promotes reactions and propitiates the microbial growth. The content in ashes is also low and this data also indicates that the quality of the drug is high, in terms of contamination or falsification with inorganic elements.

The detected anti-amoebae activity can be associated to different classes of secondary metabolites found in the phytochemical approach of the sample, but mainly to the key substance Isoeleutherine, a naphthoquinone found in the decoction and characterised by LC-DAD, applying the specificity criterion of the analytical method according to the Brazilian regulation²⁵. The difference in the retention time (less than 10%) registered for Isoeleutherine in both chromatograms (Figure 2) can be due to the presence of other substances in the extract, which also interact with the stationary phase delaying the elution of Isoeleutherine, even though both analyses were performed under the same analytical conditions. The comparison of both spectra corresponding to the marked peaks (Figure 2) reinforce the specificity of the method because they show a very high correlation factor (>0,99) calculated by the equipment.

Naphthoquinones show important activities like antimicrobial, antitumor and inhibition of cellular repairing systems, they also promote oxidative stress by inducing the endogenous formation of Reactive Oxygen Species (ROS), which can damage some important cellular components²⁶. Isoeleutherine, a naphthoquinone, presents an important activity against

Cladosporium sphaerospermum, phytopathogenic fungi²⁴ and inhibits virus HIV²³.

A buffered aqueous solution containing 2.5mg of dried AE/mL showed anti-amoebae activity against all the tested strains, after 24 hours. It is to note that the tested sample presents lower concentration than that determined for the decoction (3.534mg/mL), which was prepared according to popular knowledge but adequate according to the Brazilian Pharmacopoeia IV²⁰, being indeed sufficient to promote the total annihilation of the trophozoite tested.

The characterization of the anti-amoebae activity of decoction prepared with bulbs of *E. plicata*, vernacular marupazinho, contributes to validate its alleged popular use. The detection of Isoeleutherine in the analysed decoction can explain partially the here reported anti-amoebae activity, which can be attributed to the pro-oxidant activity of the substance. These results can contribute to the standardization of derivatives of this plant species.

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