# CHLOROPHYLLS: FROM EVANTA BYPRODUCT TO NATURAL PORPHYRIN SOURCE

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## Abstract

Evanta (*Galipea longiflora*) is a medicinal specie used as antiparasitic agent by the Tacana Amazonian community. The total alkaloids of this plant have showed interesting results in clinical studies for the treatment of cutaneous *Leishmaniasis*. During the alkaloids isolation and purification processes, chlorophylls,together with non-useful fractions, are eliminated. Chlorophylls represent an important natural source of porphyrins, which have huge potential applications in many fields (materials, catalysis, nanotechnology, diagnostic and photo- and sono-dynamic therapy). They also possess biological and pharmacological activity (antitumoral, antioxidant and antifungal properties). This work is aimed to isolate, purify and characterize chlorophylls contained in evanta leaves.

L'evanta (*Galipea longiflora*) è una specie medicinale utilizzata come agente antiparassitario dalla comunità amazzonica tacana. Gli alcaloidi di questa pianta hanno mostrato risultati interessanti negli studi clinici per il trattamento della leishmaniosi cutanea. Durante i processi di isolamento e purificazione degli alcaloidi, le clorofille, insieme alle frazioni non utili, vengono eliminate. Le clorofille potrebbero costituire un'importante fonte naturale di porfirine, le quali hanno potenziali applicazioni in molti campi (materiali, catalisi, nanotecnologie, diagnostica e terapia foto- e sono-dinamica). Posseggono inoltre attività biologica e farmacologica(antitumorali, antiossidanti e antifungini). Questo lavoro è volto ad isolare, purificare e caratterizzare le clorofille presenti nelle foglie di evanta.

## Keywords

Chlorophylls, Leishmaniasis, evanta, porphyrins, extraction

## Introduction

Recent studies show that tropical diseases represent 10% of global illnesses; the three most important parasitosis are Leishmaniasis, Malaria and Chagas. Leishmaniasis is caused by protozoan parasites of the genus Leishmania and is spread by small *phlebotomine* sand flies. Leishmaniasis

has diverse clinical manifestations: ulcerative skin lesions [cutaneous leishmaniasis (CL)], destructive mucosal inflammation [muco cutaneous leishmaniasis (MCL)], and inseminated visceral infection (VL) [1,2]. The number of Leishmaniasis cases are increasing in different word areas, e.g. in Brazil cutaneous Leishmaniasis grew from 21800 cases in 1998 to 60000 in 2003, it is the most diffuses and it infects 24 of 26 districts. Only in Bolivia (in 7 of 9 districts), approximately 800000 people are on high risk of infection [3]. It is believed that worldwide 12 million people are affected by this disease [4]. The number of cases and its behaviour along time, are related to economic development and to environmental changes (massive migration from rural to urban areas, unplanned urbanization, deforestation) which increase the infection risk [5]. Nowadays, the most important drugs used against Leishmaniasis are antimonium based ones (Glucantime and Pentostam), which have also several secondary effects such as inflammations and cardiac and kidney toxicity [6]. In patients infected by Leishmania families resistant to those drugs, the treatment involves the use of amphotericin B (Ambisome) in liposomal formulation, a choice which increases the cost of the therapy and limits its use.

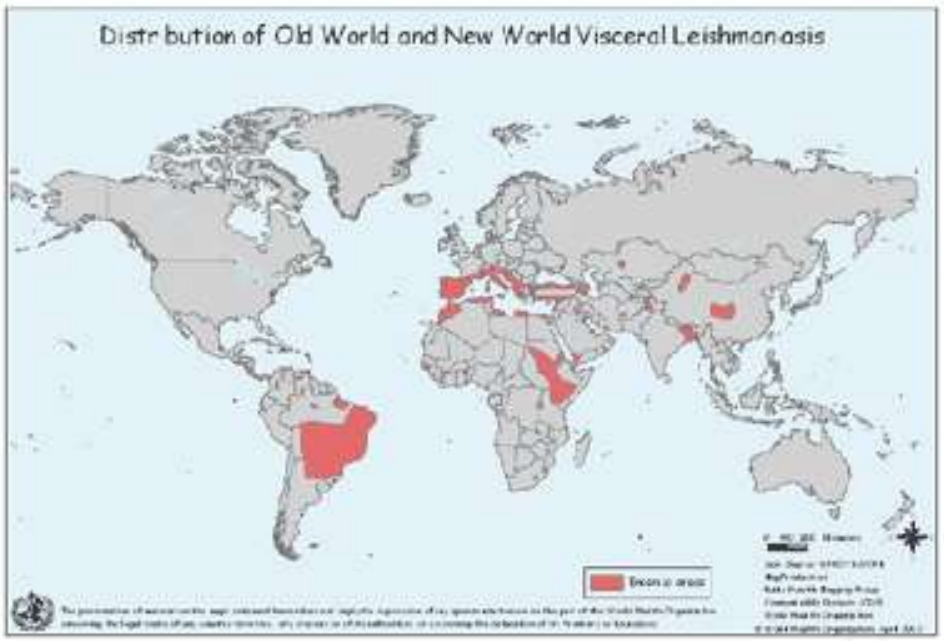


**Figure 1** -Worldwide distribution of visceral Leishmaniasis

To overcome the economic and supply difficulties, the phytotherapeutic approach has been pursued exploring the use of various indigenous plants. Since 1993, the research group of IIFB (Instituto de Investigaciones Fármaco Bioquímicas, Facultad de Ciencias Farmacéuticas y Bioquímicas, Universidad Mayor de San Andrés, La Paz – led by Prof. A. Giménez) is working on biological evaluation of medicinal species included in the traditional pharmacopoeias [7]. They screened more

than 800 plant extracts identifying *Galipea longiflora*, which is present in Boliviain the tropical forest in the last Andean spur of the Beni and La Paz districts, as the medicinal plant with the best antiparasitic profile. The medicinal species *Galipea longiflora*is 12 m long plant with trilobed leaves; it is used as antiparasitic agent (in form of cataplasms and as decoction [8]) by the amazonian ethnic groups of Tacana, Mosetene and Tsimane who known it as Evanta or Yurumahuanaepuna. IIFB's researcher have isolated from Evanta twelve quinolinic alkaloids and have formulated topic and systemic drugs which are in clinical phases I and II (in collaboration with Healthy Ministers of Bolivia and Sweden) [9].

Another relevant part present in each plant is represented by chlorophylls, which are usually considered as unwanted by-products in pharmacological active ingredients extraction. However, chlorophylls are used as food and beverages natural additives and in many other fields, such as: materials [10], nanotechnology [11], fluorescent probes for in-vivo bio-imaging[11]. Considering the common substructure of chlorophylls and porphyrins, they can be employed in new medical applications namely photo- and sonodynamic therapies [12]. In this paper, we describe new extraction and preliminary purification procedures (applicable using Bolivian resources), to obtain a chlorophylls crude fraction (purified from main other natural elements) from *Galipea longiflora* leaves.

**Materials and methods**

Solvents were purchased by Merck and used without further purification. Solvent evaporation was carried out using rotating evaporator (Heidolph Laborota-400) equipped with vacuum pump (Diaphragm Vacuum Puma DC-4). TLC analyses were performed on silica gel aluminium plate (Macherey–Nagel, thick 25µm, F254). NMR spectra were recorded on Bruker Avance 400 operating at 9.4T, samples were dissolved in deuterated chloroform; chemical shift was referenced on the residual peak solvent. UV-Vis spectra were acquired on Cintra 5 spectrophotometer dissolving samples in methanol. HPLC analyses were carried out on Waters instrument made up of 1525EF binary pump, W717 plus auto-sampler and 2996 PDA detector.

Leaves of *Galipea longiflora* (Evanta) were collected by the Giménez's group (Instituto de Investigaciones Fármaco Bioquímicas - IIFB), together with Tacana people, in the region of Sud Yungas in La Paz (Bolivia). The taxonomical identification was possible by comparing with samples at the Herbario Nacional de Bolivia (HLP). Evanta leaves were air driedin the shade for several days, atroom temperature (RT)and protected from humidity and light. Milled material (5 Kg) was extracted with ethanol (25 L) for seven days and then filtered. The filtrate was evaporated

obtaining residue (6g-10g), which was dissolved in ethanol (100 mL). The ethanol solution was extracted with petroleum ether (2 x 100 mL) and the collected organic phases were dried over sodium sulphate and evaporated obtaining 3 g of crude product. The crude was re-dissolved in petroleum ether (80 mL) and washed with methanol (2 x 80 mL). Petroleum ether phase was dried over sodium sulphate and solvent was removed under reduced pressure giving a green sticky solid (2.5 g). Final purification was carried out on Sephadex LH 20 column (26 x 560 mm) eluting with $CHCl_3/CH_3OH$ 1:1. Fractions were collected based on their colour. UV-Vis analysis allows to group fractions in 5 clusters. Each cluster was characterized by HPLC on a Waters XTerra Phenyl column (4.6 x 150 mm, 5 μm), using 0.1% trifluoroacetic acid solution in water (A) and 0.1% trifluoroacetic acid solution in methanol (B) as eluent. Gradient profile was set as follow: (min, %B) 0,65; 15.0,65; 27.4,100; 42.4,100. Wavelength range observed by PDA detector was between 210 and 700 nm.

**Discussion**

The present work was possible thanks to Uni.Coo (UNITO for International Cooperation) project between Italy and Bolivia; more in detail the collaboration involves the Department of Drug Science and Technologies (contact dr. M. Lolli) of the University of Turin (Italy) and the Instituto de Investigaciones Fármaco Bioquímicas (IIFB), Facultad de Ciencias Farmacéuticas y Bioquímicas, Universidad Mayor de San Andrés (La Paz, Bolivia - contact Prof. A. Giménez).

Considering the potential impact of Evanta plant on Bolivian people, we are encouraged to support the IIFB's work by the additional task aimed to recover the main by-products of their extractive process, namely Chlorophylls. Chlorophylls are often unwanted products which contaminate bioactive fractions. However, chlorophylls could be an important natural source of porphyrins which have huge potential of application in many field, including therapeutic purposes. The use of porphyrins in the antitumoral treatment is one of the research field of the Turin's group. So, we develop an extractive method able to recover chlorophylls using solvent and technologies available for Bolivian resources.

Extraction was carried out in ethanol over one week, then the alcoholic solution was concentrated under vacuum. Extraction with petroleum ether allows to isolate a chlorophylls crude portions. By further purification over Sephadex LH20, five chlorophyll containing fractions were collected. UV-Vis spectra, between 200 and 800 nm, gave a preliminary characterization. All fractions show typical absorption bands of Chlorophyll A at 220 and 680 nm and differ only in the high-energy

region. Moving from fraction 1 to fraction 5 the absorption in the range 200-300 nm decrease, indicating a higher purity degree of last fractions (Figure 2).

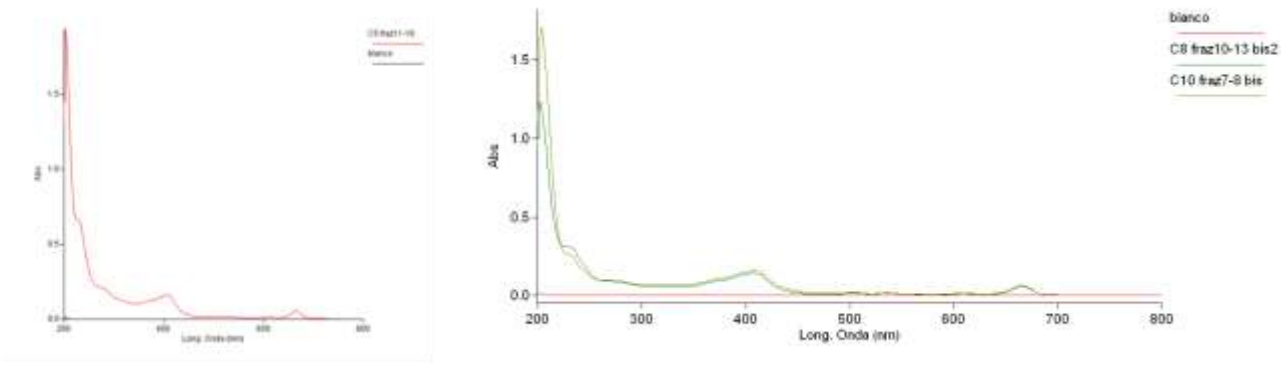


**Figure 2** -UV profiles of fraction 1 (left) and fraction 5 (right). Comparing two profiles it appears evident an absorption decrease in the range 200-300 nm in the right spectrum.

HPLC analyses show similar chromatographic behaviour (405 nm) for all fractions. Chlorophyll portion is eluted at 100% of B and profiles are characterised by three main peaks whit very similar shape (figure 3). These preliminary data could suggest that the composition of the five fractions is very similar in term of chlorophylls content and differ only for "non-chlorophylls" components.

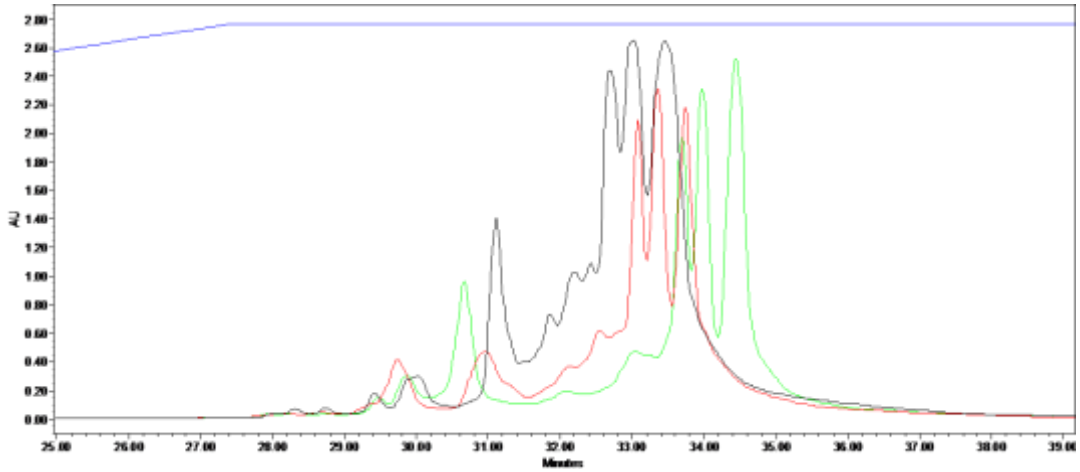

**Figure 3** -HPLC traces at 405 nm of fraction 2 (red), 4 (green) and 5 (black).

$^{1}H$, $^{13}C$ and $^{13}C$-DEPT NMR spectra were acquired in deuterated chloroform to have a fingerprint of these fractions. Aliphatic and olefinic spectroscopic regions, connotative of chlorophylls structures, are very similar in $^{1}H$ spectra of all five fractions. Even ifa deeper spectra interpretation is very difficult, the $^{1}H$-NMR profiles identify the presence of lateral chains bound to the central macro-cyclic core of chlorophylls.

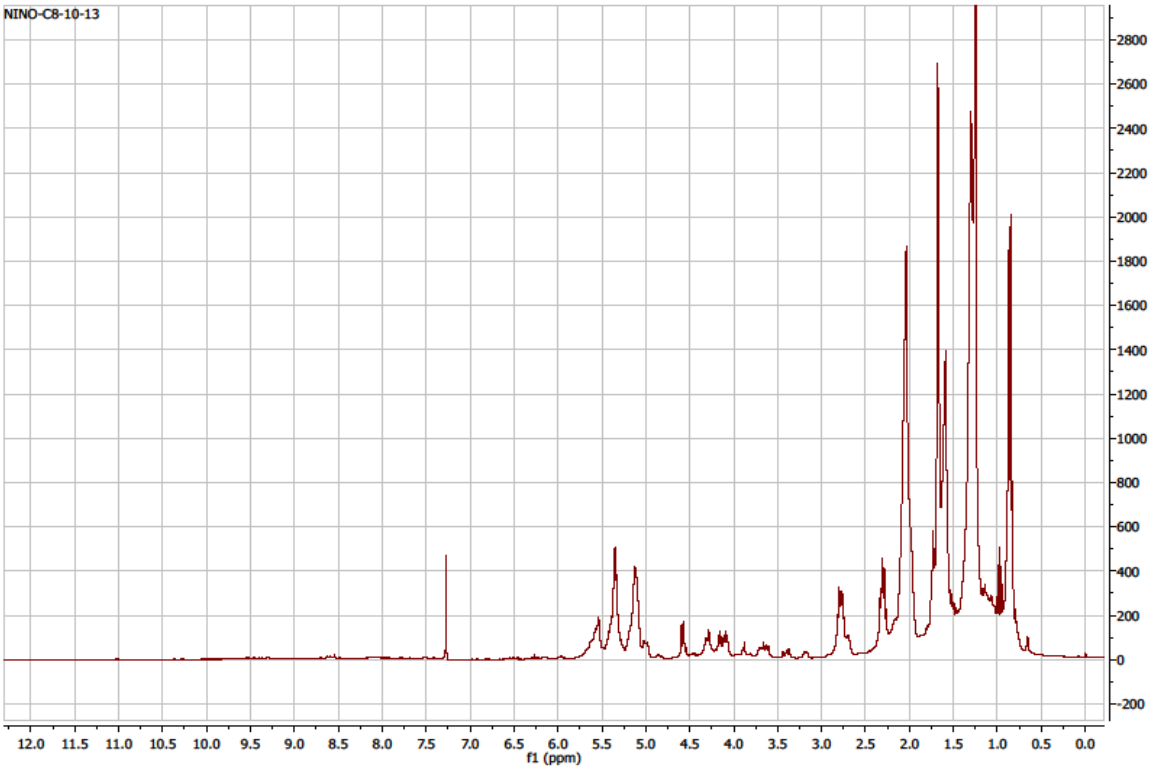


**Figure 4 -** $^{1}$H-NMR spectrum (400 MHz, $CDCl_3$) of fraction 5.

## Conclusions

A new chlorophylls extraction and purification method has been developed starting from Bolivian medicinal plants. By this protocol an enough pure extract has been obtained and its preliminary characterization allows to qualify its composition. This method could represent an alternative strategy to isolate pharmacological active alkaloids and, at the same time, collect chlorophylls for further applications.

To use chlorophylls for biological and pharmacological applications, a further finest purification is required. This task is currently part of our research activities pointed to find natural porphyrins scaffold able to be excited by ultrasound, or light, and generate radicals which induce cells death.

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## Lista degli acronimi

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|---|---|
| CL | Cutaneous Leishmaniasis |
| DEPT | Distortionless enhancement by polarization transfer |
| HLP | Herbario Nacional de Bolivia |
| HPLC | High Performance Liquid Chromatography |
| IIFB | Insituto de Investigaciones Fármaco Bioquímicas |
| MCL | MucocutaneousLeishmaniasis |
| NMR | Nuclear Magnetic Resonance |
| PDA | Photo Diode Array |
| RT | Room temperature |
| TLC | Thin Layer Chromatography |
| UNI.COO | UNITO for International Cooperation |
| VL | Visceral infection |