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Separation and evaluation of natural antileishmanial potential against *Leishmania major* and *infantum* isolated from the Tunisia strains

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Abstract

Besides its nutritional value as a dietary supplement, *Tribulus terrestris* is used as a remedy for fertility disorder in Ayurveda and Chinese medicine as well as by modern herbalists. The aim of this study was to explore the biological potential (antileishmanial effect) of an extract rich in saponins from Tunisian tribulus. The chloroform extract of the various parts of *T. terrestris* was subjected to partial purification by solvent partitioning with ethanol and *n*-butanol. All prepared extracts were tested for their anti-leishmanial activity. The result showed that *n*-butanolic extract (saponin fraction, when isolated from leaves part) exhibited the best antileishmanial effect against both pathogenic parasites *Leishmania L. major* (GIC94) and *L. infantum* (LV50) evaluated *in vitro* assessment through MTT assay. *n*-Butanolic extract had been detected, quantified and purified using the RP-HPLC finger print (Hypersil ODS coupled to UV-vis). High peak area (5116.82 at 3.03 min) was detected at 205 nm.

Introduction

Leishmaniasis, one of the parasitic diseases caused by trypanosomated protozoan *Leishmania L.* (transferred by the female phlebotomus sand fly), is one of the important health problems of Mediterranean, tropical and subtropical regions. Human taint is caused by about 21 of 30 species of the genus that infects mammals. Nearly 12 million new cases of the most common shape, cutaneous (causes skin sores) and visceral (effects internal organs of the body such as spleen, liver and bone marrow). The World Health Organization classified leishmaniasis as a major public health problem, the control of leishmaniasis left over a

serious problem.

An effective vaccine versus leishmaniasis is not available, and chemotherapy is the only efficient way to treat all the forms of diseases. However, usual therapy is expensive, toxic and the resistance has emerged as a serious problem, which has pushed the researches to find new antileishmanial agents (Monozote, 2009).

The search for new natural drugs is an important field, including new less toxic and cost-effective drugs from medicinal plants, disposable to people infected with *L. parasites*. Among these medicinal plants we choose *Tribulus terrestris L.* *T. terrestris* growing in Tunisia flora as plant samples to investigate its antileishmanial



activity related to its chemical composition.

The genus *Tribulus*, belonging to family Zygophyllaceae, comprises about 20 species in the world, of which three species, viz *Tribulus cistoides*, *T. cistoide*, *T. terrestris*, and *Tribulus alatus* *T. alatus*, are of common occurrence in India (Samuelsson et al., 1993). *T. terrestris* is a well-patronized medicinal herb by Ayurveda (Tomova et al., 1981) and Peruvian Amazonia for its anti-inflammatory medicinal effect (Bonnier, 1911; Ross et al., 2010), as well as by modern herbalists (Combarieu et al., 2003). The plant is used individually as a single therapeutic agent or as a prime or subordinate component of many compound formulations and food supplements. *T. terrestris* (Zygophyllaceae) is an annual creeping herbaceous with a worldwide distribution shrub found in Mediterranean, subtropical, and desert climate regions in: India, China, southern USA, Mexico, Spain, and Bulgari. Known as puncture vine (Ross et al., 2010), since ancient times it was regarded as an aphrodisiac in Ayurveda therapy, thus to its beneficial claims on various ailments such as inflammations, oedema, urinary infections, and ascites (Gauthaman et al., 2008). Recently, some of the main biological molecule of Bulgarian and Chinese *T. terrestris* are identified and purified, including steroidal furostanol and spirostanol saponins protodioscin, terrestrinins A and B, gitogenin, hecogenin, diosgenin and others. Biological value of the herbaceous was determined by the amount of the active compounds, which is varying considerably depending on the area where the plant is growing and depending on the part of plant used (Kostova et al., 2005).

Saponins are secondary metabolites which are widely distributed in the plant kingdom. They act as a chemical barrier or shield in the plant defense system to counter pathogens and herbivores (Augustin et al., 2011). Therefore, they are found in plant tissues which are able to fungal or bacterial attack and insect predation (Wina et al., 2005). Saponins divided into two major classes: Triterpenoid and steroid glycosides with a structure depending on the numbers of sugar units attached at different positions (Hostettmann and Marston, 1995). The classification and occurrence of saponins in the plant kingdom are reviewed in detail by Vincken (Vincken et al., 2007). The discovery of biological activities of saponins was not only limited to traditional uses, but more recently, in pharmaceutical and food industry applications (Güçlü-Üntündağ et al., 2007). Saponins appeared to have hemolytic, molluscicidal, anti-inflammatory, antifungal, antibacterial, antiparasitic, antiviral (Sparg et al., 2004) and mostly antitumor. They are capable to interact with targets involved in the signaling pathways leading to the apoptosis of cancer cells lines. They're used as a starting point for the semi-synthesis of steroidal drugs in pharmaceutical sectors (Sheng and Sun, 2011). The

clinical significance of triterpene saponins in prevention and treatment of metabolic and vascular disease has been demonstrated. Due to the large variety of their structures and applications, biological effect of saponins needs to be more investigated in details by the medicinal and natural biochemists in the future (Man et al., 2010).

In general, the study of secondary metabolites derived from plants is an important field research, is requiring sophisticated tools and current approaches to precisely quantify and identify bioactive molecules with high biological activities. In our last publication we have shown a strong inhibitory activity of Tunisian *T. terrestris* on ovary cancer cell lines (Bouabdallah et al., 2015), so in the present study we propose to investigate the antileishmanial effect on *L. major* and *L. infantum* of *T. terrestris* according to, solvent extraction and the part of the plant used.

Materials and Methods

Reagents

Analytical grade ethanol, chloroform, acetonitril, hexane and *n*-butanol, obtained from Merck (Nottingham, UK), all reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France). The murine macrophage cell line RAW 264.7 (ATCC, TIB-71) was used in this work. For the chemicals, thiazolyl blue tetrazolium bromide [3-(4, 5- dimethyl thiazol-2-yl)2, 5-diphenyltetrazolium bromide] (MTT), isopropanol, sodium dodecyl sulfate (SDS) and all reagents used for cell culture were purchased from Sigma (St. Louis, MO USA), Fluka Chemie (Buchs, Switzerland) and Merck (Nottingham, UK). (PNA) (Sigma, Saint-Quentin Fallavier, France).

Biological material

T. terrestris was collected from samples grown spontaneously in the region of "Ain-Halloufa, Boukrim Elhawaria" (north east of northern Tunisia). The sampling was conducted during (September-October 2014). The identification of the the plant was previously described as mentioned in our last publication (Bouabdallah et al., 2012; 2015).

Saponin fraction extraction

The dried and powdered plant material (powder: 30 g) was extracted in a succession by chloroform at room temperature (3 × 270 mL × 1 hour) and 70% v/v ethanol (reflux at 80°C, 3 × 450 mL × 2 hours). The combined ethanol solutions were concentrated under vacuum at 70°C to a small volume-150 mL and extracted in the separator funnel with *n*-butanol (3 times 60, 45, 45 mL). The butanol layers were concentrated to dryness giving the crude fraction.

Parasite culture

L. major (MHOM/TN/95/GLC94) and *L. infantum* strains isolated from Tunisian patients were used. Amastigotes were obtained after passage in BALB/c mice footpad and harvested from skin lesions. Promastigote were cultured in solid medium at 26°C, and then progressively adapted to an RPMI 1640 medium containing 10% of fetal calf serum (FCS) and supplemented with 100 U penicillin mol/ μ L, 100 μ g streptomycin mol/ μ L and 2 mM L-glutamine. With a starting concentration of 3×10^6 parasites/mL. When the stationary phase was reached, the metacyclic promastigotes were then purified as reported by (Sghaier et al., 2016) and a negative selection with peanut agglutinin (PNA). PNA- and PNA+ fractions were separated by density gradient centrifugation. The PNA- metacyclic promastigotes were then washed and diluted at the desired density.

Cell and parasite treatment

RAW 264.7 cells were dispatched at 3×10^4 cells/well in 96 well tissue culture plates, and kept for one night before treatment. Promastigotes parasites were washed twice with PBS, counted and dispatched at 10^7 parasites/well. Cells and parasites were then incubated for 24 hours in the presence of increasing concentrations of various extracts (ranging from 12.5 to 400 μ g/mL serially diluted). Negative controls correspond to cells or parasites cultured in the absence of plant extracts.

Parasites viability assay

The effects of extracts on *L. promastigotes* were evaluated by the MTT assay as described by Dutta (Dutta et al., 2005). After treatment with plant extracts, microtitre plates were centrifuged at 2500 g for 10 min and supernatants were removed and replaced with the same volume of 2 mg/mL of MTT freshly dissolved in PBS. Plates were incubated over night at room temperature and centrifuged at 2,500 \times g. Formazan salt formed inside the parasite mitochondria was solubilized by discarding supernatants and adding SDS 20% for 2 hours and 30 min at 37°C in the dark. Absorbance was measured at 540 nm using an ELISA plate reader. Optical density of each treated sample was compared to those grown without extracts. Each assay was performed in duplicate and independent experiments were realized at least twice.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis were carried out on a gas chromatograph; an HP7890 series (II) coupled to an HP 5972 mass spectrometer (Agilent Technologies, USA) with electron impact ionization (70 eV). Separation was carried out using phenylmethyl silox as capillary column (30 m \times 0.25 mm, 0.25 μ m film thickness Agilent Technologies,

Hewlett-Packard, USA). Column temperature was programmed as follows 40°C for 1 min, 8°C/min to 100°C for 5 min, 10°C/min to 200°C for 3 min, 12°C/min to 300°C for 20 min. The carrier gas was helium with a flow rate of 0.9 mL/min and a split ratio of 100:1. Scan time and mass range were 1 sec and 50-550 m/z, respectively. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system and other published mass spectra. Determination of the components percentages was based on peak area normalization.

Identification of biological molecule

Further identification was made by matching their recorded mass spectra with those stored in the Wiley 9/NIST11 mass spectral library of the GC/MS data system and other published mass spectra. Determination of the component percentages were based on peak area normalization.

RP-HPLC analysis

The bioactive compound analysis were carried out using an Agilent Technologies 1100 series liquid chromatography (RP-HPLC) coupled with an ultraviolet/visible spectrum multi wave length detector. The separation was performed on a 250 \times 4.6 mm, 4 μ m Hypersil ODS C18 (Thermo Fisher Scientific Inc., USA) at ambient temperature. The mobile phase consisted of acetonitrile (Solvent A) and water with 0.2% formic acid (Solvent B). The flow rate was maintained at 0.7 mL/min. The gradient program was as follows: 15% A 85% B for 0-12 min; 40% A 60% B for 12-14 min; 60% A 40% B for 14-18 min; 80% A 20% B for 18-20 min; 90% A 10% B for 20-24 min, and 100% A 24-28 min. Peaks were monitored at 205 and 305 nm.

Statistical analysis

Log transformed IC₈₀ and IC₅₀ values for cytotoxic and antileishmanial was calculated using regression analysis and expressed as mean of 80% of cell viability, 50% inhibitory activity, respectively. Statistical significance of the differences between the treated and the control sample means were evaluated by Student's t-test and ANOVA test, for pairwise and multiple comparisons respectively. A p value <0.05 was considered to imply significance. All computations were performed using Excel software (Microsoft Corporation, 2003).

Results

Antileishmanial activity

Four various extract (chloroform, ethanol, *n*-butanol and aqueous) from different parts (leaf, seed and root) of *T. terrestris* at six concentrations (12.5-400 μ g/mL)

Table II

***In vitro* leishmanicidal and cytotoxic activities of various extracts from TT**

Plant	<i>L. infantum</i> IC ₅₀ (µg/mL)	<i>L. major</i> IC ₅₀ (µg/mL)	Raw264.7 IC ₈₀ (µg/mL)	selectivity index of <i>L. infantum</i>	selectivity index of <i>L. major</i>
Chloroform extract of leaf	314.5 ± 0.7	367 ± 0.7	591.4	1.9	1.6
<i>n</i> -Butanol extract of root	183.8 ± 0.4	176.5 ± 0.3	< IC ₅₀	Not available	Not available
<i>n</i> -Butanol extract of leaf	169.8 ± 0.2	227 ± 0.1	< IC ₅₀	Not available	Not available

Leishmanicidal activity and cell viability were expressed as 50% inhibitory concentration (IC₅₀) and 80% inhibitory concentration (IC₈₀) respectively; The selectivity index (SI) is calculated as the ratio of the IC₈₀ values for macrophages to the IC₅₀ for *L. major* or *L. infantum*.

was screened for their antileishmanial activity (Table I). The screening was carried out on two *Leishmania* species: *L. major* (GLC94) (Kebaier et al., 2001) and *L. infantum* (LV50) (Aoun et al., 2001). The cytotoxicity was evaluated by the conventional thiazolyl blue tetrazolium bromide (yellow), [3-(4, 5- diméthyl thiazol-2-yl)2, 5-diphényltetrazolium bromide] MTT assay, according to the methods described by Mosman, so MTT will be transferred to formazan (bleu) via dehydrogenase activity (synthesized by the mitochondria of parasites).

At observation, leaf *n*-butanolic extract (saponin fraction) exhibited the best antileishmanial effect (*L. infantum*: 169.8 ± 0.2 µg/mL; *L. major*: 227 ± 0.1 µg/mL) in terms of inhibitory concentration of 50% (Table II). Given that *Leishmania* is an obligate intracellular parasite that affect essentially macrophages, so all extracts were also investigated for their potential cytotoxic effect towards Raw murine macrophagic 264.7 (ATCC, TIB-71) cells (as model of non-infected cell).

Characterization of selectivity index

The best extract is the one that inhibits *Leishmania* and presents low toxicity against raw macrophages (264.7), so three (03) extracts shown sensitive response to the drug under a concentration of 400 µg/mL after 72 hours of treatment against *L. major* and *L. infantum*, only chloroformed extract has small cytotoxic effect on macrophage prompted us to define its selectivity index (SI): Against *L. major* and *L. infantum* as follows 1.9; 1.6 (Table II).

Identification of volatile compound by GC-MS

GC-MS separation was achieved using Phenyl Methyl Silox as capillary column. The interpretation on mass spectrum GC-MS high resolution was accomplished and the spectra of the unknown component were compared to the spectrum of the known constituents stored in the WELY and NIST library. A total of 43 bioactive compounds (low proportions) were characterized and identified from *n*-butanolic extract and retention times for all mineurs peaks varied

between 3.1 and 39.8 min.

Analysis of bioactive compound by RP-HPLC

The crude extracts of *T. terrestris* were analysed by RP-HPLC, eluting mixture of formic acid/water-acetonitrile that was tested at various compositions in an attempt to resolve the polar compounds. The result indicates that the crude sample contains nine high polar molecular peak at 205 nm and two peaks detected at 305 nm. Retention times for both peaks corresponded respectively to 3.2 and 3.6 min. Samples (peaks) were eluted with a mobile phase mixture of formic acid/water-acetonitrile (35% B; 65% C). The most abundant compound at 205 nm is one of peak 3 (RT: 3.7 min; Teneur: 5578.2 Mau) (Figure 1).

Discussion

During the second half of the 20th century, it was generally accepted that anti-infectious agents from medicinal plants have less side effects and thus are advantageous for therapeutic purposes than synthetic. EOs exhibited wide spectrum of biological effects such as antibacterial (Burt, 2004), antimycotic (Pisseri et al., 2008), antiviral (Lee et al., 2009) and anti-parasitic properties (Anthony et al., 2005) and even recently, they have been used as adjuvant in current anti-leishmanial therapy (Sanchez-Suarez et al., 2013). Moreover, researchers are encouraged to investigate the pharmacological activity of natural products as the traditional use agents.

T. terrestris is used in folk medicines as an aphrodisiac, it is a vital compound of Gokshuradi Guggul, tonic, palliative, diuretic, astringent, antihypertensive, stomachic, lithotriptic, and urinary disinfectant.

For the first time, we discovered the inhibitory effect of the *Tribulus* on the *L. major* and *L. infantum*. Twelve extracts were prepared by sequential extraction using different solvent such as, chloroform, ethanol, *n*-butanol and aqueous one.

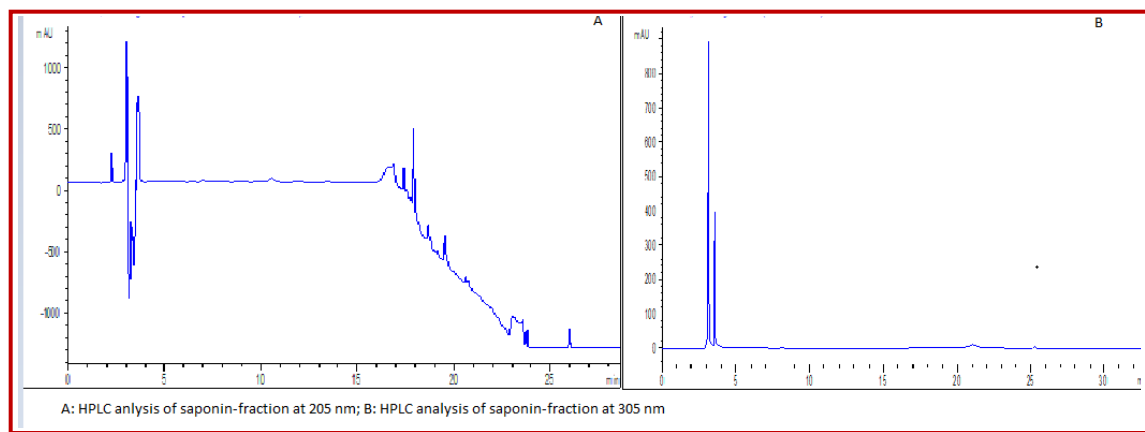


Figure 1: Analysis of bioactive compound by RP-HPLC

Chloroform extract was used to recuperate apolar-molecular like fatty-acid, chlorophyll, and caretonoids. Ethanolic fraction was prepared to recuperate polar-molecular such as flavonoids, phenolic-compounds, *n*-butanolic extract was used to concentrate saponin fraction. Our principal goal is evaluating the antileishmanial potential of *n*-butanolic fraction (saponin fraction) and comparing it with the inhibitory effect of others extracts (chloroformed, ethanolic, aqueuse). Our result showed that *n*-butanolic fraction when isolated from leaves part exhibited the best antileishmanial effect on *L. major* and *L. infantum*.

Analysis of chemical composition of the bioactive extracts is an important stage in order to determine the properties of the major compound responsible for the biological activity. From the first interpretation we can conclude that the biological effect studied before is due to the total effect of the extract including both of the volatile and the non-volatile fraction, but this conclusion is so general and need more information. That's why we have to precise which fraction is responsible for the best activity. So, we intend to analyze the active extracts by sophisticated analytical methods adapted to each fraction isolated.

Firstly, we're going to start by studying the first hypothesis: The biological effect of the plant extract could be due to the volatile fraction. We analyzed the *n*-butanolic extract by GC-MS (gas chromatography coupled to mass spectrometry is considered as an excellent method for detection and quantification of volatile compounds) to determine the major volatile compound. Unfortunately GC-MS chromatogram showed a very poor profile of all the compounds with small proportions.

Secondly, we're going to treat the second hypothesis: The biological effect of the plant extract could be due to the non-volatile fraction. We analyzed the same extract (*n*-butanolic) by RP-HPLC (reversed phase liquid-

chromatography used in our study is known as a sophisticated tool to detect, quantify, chemical saponin class). (Lee et al., 2009; Choon et al., 2014) in order to characterize the properties of the non-volatile compounds (Tenon et al., 2017). Fortunately the liquid chromatography profile showed an important prominent peaks detected and quantified at 205 nm (the wavelength that proves the existence of the saponin compound).

Based on those practical results, we can conclude that the first hypothesis was rejected and the second one was holded back.

So, we can attribute the antileishmanial activity of *n*-butanolic extract to the synergetic effect of saponin molecules highly detected by HPLC finger print at 205 nm.

As described in the introduction section, recently saponins have become a research hotspot due to their multiple and strong bioactivities, especially their cytotoxic activities on a series of tumor cell lines related to their chemical structure (Bouabdallah et al., 2015; Nath et al., 2016; Zhang et al., 2016; Zhou et al., 2017). However our result also confirmed that the *n*-butanolic extract (saponin fraction) exhibited the best inhibitory activity on both of the leishmania parasites tested (Table II).

Now how to explain the death of parasites generated by the effect of the saponin fraction evaluated by *in vitro* assay?

To explain this event, we propose to evoke two mechanisms:

The first one is the mechanism of action of synthetic molecule prescribed to the patients affected by leishmaniasis.

The second mechanism is concerning the potential targets of the saponin that kills the parasites.

1. Amphotericin B is an antifungal polyene effective against leishmaniasis. This molecule has a mechanism of action different from other antileishmanians. It's based on a combination with the parasite ergosterol membrane which increases permeability and leads to the parasite death (Vertut-Croquin et al., 1983; Fournier et al., 1998; Gagos et al., 2001; Herec et al., 2005; Umegawa et al., 2008; Grudzinski et al., 2016).

Amphotericin B liposomale is composed of distaroyl phosphatidyl-glycerol and cholesterol. The interaction of distearoyl phosphatidylglycerol and cholesterol with a charge transfer complex present on the parasite membrane (presence of membrane sterols). Liposomal amphotericin B intercalates and stabilizes in the parasitic membrane. The liposome ruptures and releases amphotericin B then amphotericin B damages the parasitic membrane (Seibel et al., 2016).

2. Saponins are complex heterosides belonging to the cyclic terpenes or to the steroid (Cheok et al., 2014). They are divided into steroidal saponin and triterpenic saponin, the steroidal saponin are characterized by structures similar to those of the cholesterol, so they are capable to interact with sterol of cell membrane by inducing membrane pores which will modify the cellular integrity and disrupt the exchanges with the external media (Shilu et al., 2011). In the majority of cases this disorder will lead to cell death.

Conclusion

The present study reports for the first time the anti-leishmanial effect of the medicinal herbaceous (*Tribulus*) extracts and saponin fraction evaluated by *in vitro* assessment through MTT assay. This investigation revealed that the saponin fraction when isolated from leaves part exhibited the best inhibitory effect on *L. major* and *L. infantum*. This anti-leishmanial activity was probably due to the synergetic effect of saponin molecules highly detected by HPLC finger print.

Conflict of Interest

Authors declare no conflict of interest

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