

The Effect of *Vitis vinifera* L. Leaves Extract on *Leishmania infantum*

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Abstract

Vitis vinifera L. is a traditional Asian herb widely used for different health problems. In the present research, the ethanolic and the aqueous extracts of *Vitis vinifera* L. leaves collected from shrub, grown in Tunisia, were prepared and evaluated for the antileishmanial activity against *Leishmani ainfantum* promastigotes. The inhibitory concentration 50 (IC₅₀) was determined and the results showed that the ethanolic extract is more active than the aqueous one (IC₅₀ = 0.108 mg/mL). Microscopic observations showed that the ethanolic extract promoted the destruction of cytoplasmic and nuclear membranes of *Leishmani ainfantum* promastigotes and altered the overall shape of the cell. In order to explain the difference of antileishmanial activity between ethanolic and aqueous extracts, anthocyanins amount was determined by spectrophotometry. It was found that the ethanolic extract is richer in anthocyanins than the aqueous one which can explain the higher antileishmanial activity of the ethanolic extract.

Keywords: *Vitis vinifera* L.; Extraction; Leishmaniasis; Antileishmanial activity; Anthocyanins

Introduction

Leishmaniasis is a real public health problem encountered in several countries over the world including Tunisia. In Tunisia, incidence was assessed to be more than 4000 and 150 case per year for the cutaneous and the visceral leishmaniasis form respectively, (1, 2). Visceral leishmaniasis form is caused by *Leishmania (L.) infantum* species and is endemic in the North and the Center of Tunisia (3, 1). The same species may also be isolated in several cases of

cutaneous leishmaniasis encountered in the same foci (4, 5).

In absence of vaccination against the parasite *Leishmania (L)*, the use of drugs remains the only way for treatment. In fact, pentavalent antimony such as Glucanthime[®] (meglumine antimony) and Pentostam[®] (sodium stibogluconate) were used for the treatment of both cutaneous and visceral leishmaniasis (6). In case of resistance, these drugs were substituted by amphotericin B and miltefosine[®] (7). However, many signs of stibio-intoxication such as cardiac conduction disorders, hepatic cytolysis, dysfunction of renal tubular and pancreatitis have been attributed to these drugs (8, 9). In addition, resistance

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to pentavalent antimony has been reported in Bihar, India in 60% of treated patients (10, 11). Treatment failure was also observed in the Mediterranean region (12, 2). Accordingly, an urgent need to find new antileishmanial agents, more efficient and less harmful for patients is highly recommended.

Vitis (V.) vinifera (Vitales, Vitaceae), is an Asian native perennial woody vine. From different parts of this plant essentially fruits, several preparations used in folk medicine have been derived (13). In Ethnopharmacology, the infusion of the leaves of red varieties has been used as haemostatic and for diarrhea treatment. Fresh leaves have been used externally to heal wounds and to lance abscesses (14). Grape leaf-based medicines are traditionally used for diarrhea, hepatitis and stomachaches (15, 16, 13, 17). Grapes, seeds, and leaves have been used for preventing heart and blood vessels diseases, varicose veins, hemorrhoids, "hardening of the arteries" (atherosclerosis), high blood pressure, swelling after injury or surgery, heart attack and stroke.

Moreover, grape leaf has been used for attention deficit-hyperactivity disorder (ADHD), chronic fatigue syndrome (CFS), diarrhea, heavy menstrual bleeding, uterine bleeding, and cancer sores. It has been also used as a mild laxative for constipation (16-24).

Few studies have been conducted on the biological effects of leaves. Nilüfer *et al* have shown that the aqueous extract from leaves of *Vitis vinifera* L. possess antidiabetic and antioxidant activities (25). It was also mentioned that the aqueous extract of *Vitis vinifera* L. leaves shows antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Vibrio alginolyticus* (26). The aim of this study was to evaluate the antileishmanial activity of the aqueous and ethanolic extract of *Vitis vinifera* L. leaves and to quantify their anthocyanins amount as anthocyanins family is well known by the richness of its biological activities (27).

Experimental

Plant material

The *Vitis vinifera* L. Black Grenache leaves are collected from "Bir Bou Ragueba", a suburb in the city of Nabeul, Tunisia. Bir Bou Ragueba's

latitude is 10°25' and its longitude is 36° 37' W. Leaves were collected on December 8th 2010. They were dried and reduced to fine powder (28).

Preparation of extracts

One gram of dried leaves of *Vitis vinifera* L. was extracted with 20 mL of distilled water during 1 hour at 95°C. The mixture was passed through a filter paper in order to remove plant debris. The aqueous extract was then sterilized by filtration through a 0.22 µm membrane filter. It was used freshly for the preparation of different concentrations in order to evaluate the antileishmanial activity. As for the ethanol extract, it was obtained by Soxhlet with acidified ethanol (ethanol: HCl 99: 1 v / v: = 0.1 N HCl) till the reflux of this mixture. (29)

Preparation of stock solution

The aqueous extract was directly used. However, the ethanolic extract of *Vitis vinifera* L. leaves was initially dissolved in dimethyl sulfoxide (DMSO) at 1%. 10 mg of the ethanolic extract were dissolved in 100 µL of pure DMSO and then added to 900 µL of culture medium suitable for a final concentration of 10 mg/mL. This solution was sterilized by passage through a filter of 0.22 µm in a laminar flow hood. It was then diluted to different concentrations for the antileishmanial activity that the highest concentration tested is 1 mg/mL. In this way the final concentration of DMSO never exceeded 1% in the medium tested. This concentration had no effect on the growth of *Leishmania*.

Maintenance and counting of parasite

The *L. infantum* strain (MHOM/TN/2010/44M) was isolated from a visceral leishmaniasis human case and typed by isoenzyme and molecular methods in the laboratory of Parasitology, Faculty of Pharmacy of Monastir, Tunisia. Promastigote Culture was maintained at 25 °C in RPMI 1640 medium supplemented with 10% of decomplexed fetal calf serum (Gibco Invitrogen Corporation, New York, NY) pH 7.0, in a cell culture dishes with weekly subcultures. The promastigotes were counted using Neubauer chamber and re-suspended in fresh medium at a final concentration of 1.0×10^6 live promastigotes/ mL. The viability of

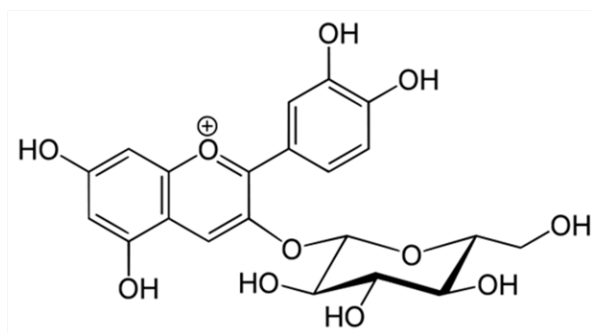


Figure 1. Chemical structure of cyanidin-3-glucoside, Anti-leishmanial activity of *Vitis vinifera* L. (*Vitales, Vitaceae*) leaves extracts, Rym Mansour.

Leishmania was assessed by mobility and lack of color in the presence of trypan blue.

Anti-leishmanial activity

The trials were conducted in a liquid medium in microplaques of 96 round bottom wells. Promastigotes of the logarithmic phase were re-suspended to a concentration of 10^6 *Leishmania*/mL and were treated with ethanolic and aqueous extracts of *V. vinifera* L. leaves. For the ethanolic extract, the tested concentrations were 1, 10, 100, and 1000 $\mu\text{g/mL}$. While for the aqueous extract concentrations were 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. Two wells containing the negative control cultures without extract supplemented or not with 1% DMSO were used. A positive control well containing culture with Glucantime[®], was also tested. The plates were incubated at 27 °C for 72 h to assess the anti-proliferative effect of the extracts. The number of mobile and viable promastigotes was quantified by counting the parasite using the Neubauer chamber. Inhibitory concentration 50 (IC_{50}) was determined by the method of logarithmic regression analysis of data obtained.

Leishmania cell morphology was also evaluated by depositing 20 μL of culture treated with ethanolic *V. vinifera* L. leaves extract on microscopic slides. After spreading and drying in ambient air, slides are fixed with absolute methanol, stained with 10% Giemsa, and examined under an oil immersion objective of the light microscope.

Anthocyanins quantification

The amount of anthocyanins in the extracts

was determined according to the method of Giusti and Wrolstad. By changing the pH different values of absorbance were obtained. Extracts were diluted 10 times in two buffers. The aqueous solution consisted of potassium chloride (0.025 M) with pH 1 and a second aqueous solution consisting of sodium acetate (0.045 M) after 15 min incubation at room temperature, the absorbance of both extracts were measured at 520 nm and 700 nm. The total anthocyanins content was given in mg of cyanidin-3-glucoside (cy-3-glu) / liter (2), and the equipment used was an UV-visible CE-202 spectrophotometer (27).

$A = (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ pH } 1.0 - (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ pH } 4.5$ (1)

The concentration of anthocyanins was calculated using this equation:

$$C = \frac{A \times MW \times DF \times 10^3}{l \times \epsilon} \quad (2)$$

MW (molecular weight) = 449.2 g / mol for cyanidin-3-glucoside (Cyd-3-glu) (Figure 1);

DF = dilution factor

$l = 1 \text{ cm}$ (cuvette width)

$\epsilon = 26 \text{ 900 molar coefficient of extinction in } \text{L}^{-1} \times \text{cm} \times \text{mol}^{-1}$, for Cyd-3-glu, and $10^3 =$ conversion factor from g to mg.

Statistical Analysis

In order to assure the reproducibility of results, all experiments were repeated thrice. The means and standard deviation were determined. The data were then analyzed by SPSS version 17.0. The Student t-test was applied and

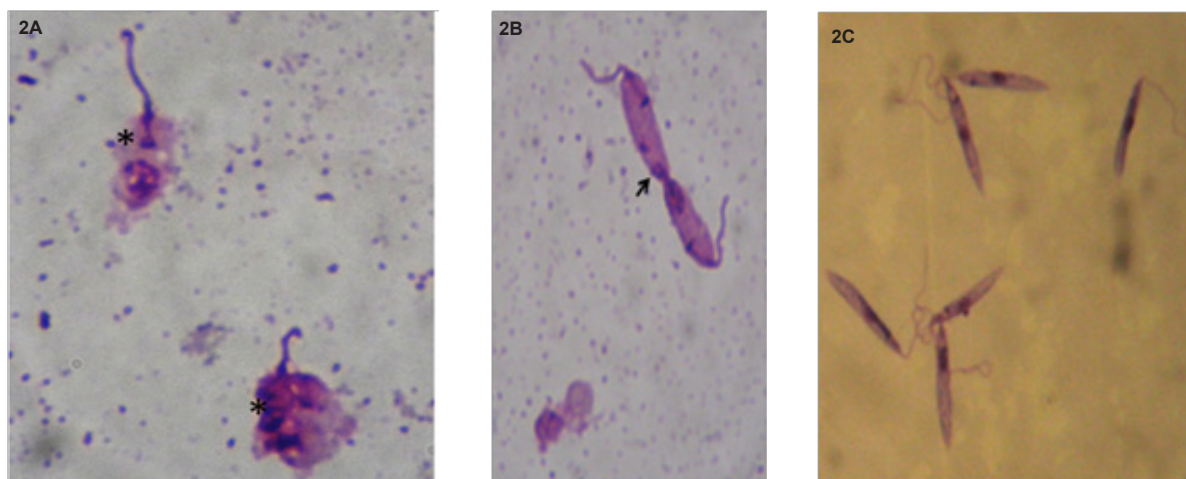


Figure 2. a: membrane destruction, Anti-leishmanial activity of *Vitis vinifera* L. (*Vitales, Vitaceae*) leaf extracts, Rym Mansour. b: Unequal division, Anti-leishmanial activity of *Vitis vinifera* L. (*Vitales, Vitaceae*) leaf extracts, Rym Mansour. c: Untreated *Leishmania infantum* culture., Rym Mansour.

the p-value less than 0.05 was considered significant.

Results

Vine leaves extracts were tested to evaluate their activity against promastigotes of *L. infantum*. For aqueous extract, $IC_{50} = 12.55$ mg/mL and for ethanolic extract $IC_{50} = 108.85$ μ g/mL. In addition, the ethanol extract showed an IC_{50} lower than glucanthime ($IC_{50} = 8.504$ mg/ml). (Table 1)

In order to assess the viability of promastigotes of *L. infantum* in the presence of the ethanol extract, treated cultures were stained with May-Grunwald Giemsa and examined under an optical microscope. The effects of ethanolic extract on *Leishmania* were different from those caused by Glucanthime. Indeed, *Leishmania* incubated in the presence of the ethanolic extract of *V. vinifera* L. leaves showed the following result: destruction of cytoplasmic and nuclear membranes and, thus, altered the overall shape of the cell (Figure 2a, 2b). An abnormality at the cell division: an imbalance in the nuclear division (Figure 2b) and the division of the nucleus and the flagellum however, without duplication of the kinetoplast (Figure 2c).

The quantity of anthocyanins present in the ethanolic and aqueous extracts was calculated using the spectrophotometric method according

to the protocol of Giusti and Wrolstad. Thus, we found that the aqueous extract and ethanolic extract contained respectively 3.89 mg/g and 5.01 mg/g (mg anthocyanins/g of dry material). The ethanol extract was richer in anthocyanins than the aqueous extract.

Discussion

This study examined the antileishmanial activity of aqueous and ethanolic *V. vinifera* L. leaves extracts. The ethanolic extract showed a prominent activity against *L. infantum* promastigotes.

Vitis vinifera leaves are rich in tannins, flavonoids, procyanidins and also contain organic acids, lipids, enzymes and vitamins (13, 30, 31, 17). Furthermore, the quantitative analysis of compounds found in leaves has also been evaluated by Monagas *et al.* (2006). They found that *V. vinifera* is rich in anthocyanins and other flavonoids (32).

We found that the difference between the IC_{50} could be the result of the difference in the anthocyanin content. The ethanol extract, having a higher content of anthocyanins than the aqueous extract, seems to be more effective against *L. infantum* promastigotes. This could be due to the number of hydroxyl groups of anthocyanins which is supposed to be the cause of the relative toxicity to microorganisms (33).

Table 1. IC₅₀ extracts of *Vitisvinifera*L. leaves against *L.infantum*.

	Aqueous extract				Ethanollic extract				Glucanthime			
Concentrations (mg/L)	50	25	12.5	6.25	1	0.1	0.010	0.001	200	100	50	25
Percentage of Viability	0	3.	99.9	100	31.65	46.23	71.69	99.91	10.10	42.29	52.17	81.23
IC50 (mg/L)	12.53				0.108				8.504			

Anthocyanins are the anthocyanidins in their glycoside form (linked to a sugar moiety). The anthocyanidins are composed of an aromatic ring attached to a heterocycle that contains oxygen, which is also linked by a carbon-carbon to a third aromatic ring (34). The anthocyanidins are flavonoids which are, in their turn, a subdivision from the family of polyphenols.

Anthocyanins display a wide range of biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities. In addition, they display a variety of effects on blood vessels, platelets and lipoproteins able to reduce the risk of coronary heart diseases (34).

Kolodziej *et al.* (2001), have reported that proanthocyanidins possess antileishmanial activity against the species *L. donovani* with IC₅₀ between 0.8 and 10.6 nM (35).

Lewin *et al.*, (2011) have also studied the antileishmanial activity of the flavonoids against *L. donovani* (36). Moreover, in 2010 Nour *et al.*, found that *Ageratum conyzoides* methylated flavonoids have an antileishmanial activity against *L. donovani* with IC₅₀ = 3.4 µg/mL (37).

Flavonoids from *Agaricus blazei* Murill had an antileishmanial activity against *L. amazonensis*, *L. chagasi* and *L. major* (38). Several studies have shown that many natural compounds such as naphthoquinones, alkaloids, lignans and triterpenes possess antileishmanial activity against many *Leishmania* species (39). Nevertheless, to our knowledge, our study is the first to show the correlation between anthocyanins content and IC₅₀ of aqueous and ethanolic extracts of *V. vinifera* L. leaves against *L. infantum* promastigotes.

Conclusion

For 50 years, pentavalent antimony was the drug most used for the treatment of leishmaniasis.

These molecules have been used to treat all forms of leishmaniasis. However, recently liposomal amphotericin B has replaced the pentavalent antimony as a treatment of choice for visceral leishmaniasis. Nevertheless, these drugs have dangerous side effects. In this research, we have tried to evaluate the anti-leishmanial activity of aqueous and ethanolic extracts of *V. vinifera* L. leaves. Ethanolic extract showed interesting activity against *L. infantum* promastigotes. This extract is currently undergoing detailed investigations with the objective of isolating biologically active molecule(s). Based on the results of this study, we plan to develop bandages with *V. vinifera* extract inhibiting the dermatological proliferation of leishmaniasis, by nano-microencapsulation.

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Conflict of interest: This work has no conflict of interest

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