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

Rym Mansour*, Najoua Haouas, Amel Ben Kahla-Nakbi, Saoussen Hammami, Zine Mighri, Farouk Mhenni and Hamouda Babba

*Unité of Recherche 12-04, Chimie Appliquée et Environnement, Faculté of Sciences of Monastir, Monastir 5000, Tunisie. *Laboratoire of Parasitologie-Mycologie (99UR/08-05), Faculté de Pharmacie, Département de Biologie, Clinique B, 1 Rue Avicenne, Monastir 5000, Tunisie. *Laboratoire of Biodiversité and Biotechnologie Marine, Institut National des Sciences and Technologies of la Mer, Annexe Monastir, BP 59, Monastir 5000, Tunisie

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 *Leishmania infantum* 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 *Leishmania infantum* 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 us 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
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 canker 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 fecalis*, *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 decomplemented 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 × 10⁶ live promastigotes/mL. The viability of
**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 microplates 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 μg/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 (IC₅₀) 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).

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

The concentration of anthocyanins was calculated using this equation:

\[ C = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times \text{cuvette width} \times \text{conversion factor}} \]  

MW (molecular weight) = 449.2 g / mol for cyanidin-3-glucoside (Cyd-3-glu) (Figure 1); DF = dilution factor 
\[ \varepsilon = 26900 \text{ molar coefficient of extinction} \]  
\[ 10^3 = \text{conversion factor from g to mg} \]

**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...
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 ethanol extract IC$_{50}$ = 108.85 µg/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 ethanol extract on *Leishmania* were different from those caused by Glucanthime. Indeed, *Leishmania* incubated in the presence of the ethanol extract of *V. vinifera* 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 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).
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 anticarcinogenic 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 IC\textsubscript{50} 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 IC\textsubscript{50} = 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.

**Acknowledgments**

This work was supported by the finances from the Tunisian Ministry of High Education and Scientific Research.

**Conflict of interest:** This work has no conflict of interest

**References**

(1) Aoun K, Jeddi F, Amri F, Ghrab J and Bouarabine A. Actualités épidémiologiques de la leishmaniose viscérale en Tunisie. Méd. Mal. Infect. (2009) 39: 775-779.

(2) Faraut-Gambarelli F, Pierratx F, Giussiano B, Marty P, Michel G, Faugère B and Dumon H. in- vitro and in-vivo resistance of Leishmania infantum to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. Antimicrob Agents Chemother (1997) 41: 27-830

(3) Carrio J, Riera C, Ga´ llego M and Portu´ s M. In-vitro activity of pentavalent antimony derivatives on promastigotes and intracellular amastigotes of Leishmania infantum strains from humans and dogs in Spain. Acta. Trop. (2001) 79: 179-180.

(4) Belhadj S, Pratlong F, Hammami M, Kallel K, Dedet J and Chaker E. Human cutaneous leishmaniasis
due to *Leishmania infantum* in the Sidi Bourouis focus (Northern Tunisia): epidemiological study and isoenzymatic characterization of the parasites. *Acta. Trop.* (2003) 85: 83-86.

(5) Kalled K, Pratlong F, Belhadj M, Cherif F, Hammami M, Dedet J and Chaker E. La leishmaniose cutanee due a *Leishmania infantum* MON-24 en Tunisie: extension du foyer vers le centre du pays. *Ann Trop. Med. Parasitol.* (2005) 99: 11-19.

(6) Gradonil L, Gramiccia M, Pettoello M, Di Martino L and Nocerino A. A new *Leishmania infantum* isoenzymatic variant, agent of an urban visceral case unresponsive to drugs. *Trans. R. Soc. Trop. Med. Hyg.* (1987) 81: 927-8.

(7) Gradonil L and Byrceason A. The increase of risk factors for leishmaniasis worldwide. *Trans. R. Soc. Trop. Med. Hyg.* (2001) 95: 239-43.

(8) Lwn S, Aarmstrong M, Chilton D and Whitty C. Electrocardiographic and biochemical adverse effects of sodium stibogluconate during treatment of cutaneous and mucocutaneous leishmaniasis among returned travellers. *Trans. R. Soc. Trop. Med. Hyg.* (2006) 100: 264-269.

(9) Domingo P, Ferrer S and Kolle L. Acute pancreatitis associated with sodium stibogluconate treatment in a patient with Human Immunodeficiency Virus. *Arch. Intern. Med.* (1996) 156: 1029-1032.

(10) Sundar S. Drug resistance in Indian visceral leishmaniasis. *Trop. Med. Int. Health.* (2001) 6: 849-854.

(11) Das V and Ranjan A. Magnitude of unresponsiveness to sodium stibogluconate in the treatment of visceral leishmaniasis in Bihar. *Nat. Med. J. India.* (2005) 18: 131-133.

(12) Haouas N, Gorcii M, Chaguie N, Aoun K, Bourratbine A, Messaadi A, Masmoudi M, Zili J, Ben Said M, Pratlong F, Dedet J, Mezhoud H, Azaiez R and Babba H. Leishmaniasis in central and southern Tunisia: Current geographical distribution of zymodesmes. *Parasite* (2007) 14: 239-246

(13) Bombardelli E, Morazzonni P and Vitisvininfera L. *Fitteroterapia* (1995) 66: 291-317.

(14) Baytop T, Bikiler ‘Ile Tedavi (Gec.mis.teveBugün). *Nobel. Tip. Kitabevleri.* Istanbul, (1999) 357-358.

(15) Kalled K, Haouas N, Pratlong F, Kaouech E, Belhadj S, Anane S, Dedet J, Babba H and Chaker E. La leishmaniose cutanee due a *Leishmania infantum* MON-24 en Tunisie: extension du foyer vers le centre du pays. *Bull. Soc. Pathol. Exot.* (2008) 101: 30-31.

(16) Kapoor L. *Handbook of Ayurvedic Medicinal Plants*. CRC Press, Boca Raton, USA, (1990) 45-56.

(17) Felicio J, Santos R and Goin E. Chemical Constituents from *Vitis vinifera* (Vitaceae). *Sao Paulo.* (2001) 68: 47-50.

(18) Griewe A. *Am Bernd Herba!*, Dover Books New York Company, White River Junction, USA (1971) 2-10.

(19) Onstad D. Whole Foods Companion: A Guide for Adventurous Cooks, Curious Shoppers & Lovers of Natural Foods. Chelsea Green Publishing (1996).

(20) Bown D. *The Herb Society of America.* New encyclopedia of herbs and their uses. 3rd ed. Dorling Kindersley Ltd., London (2001) 407-411.

(21) VanWyke B and Wink M. *Medicinal Plants of the World*. 3rd ed. Timber Press, Portland, USA (2004) 23-30.

(22) Kang J, Lee W, Lee C, Yoon W, Kim N, Lee H, Park H, Han S and Yun J. Improvement of high-fat diet-induced obesity by a mixture of red grape extract, soy isoflavone and 1-carnitine: Implications in cardiovascular and non-alcoholic fatty liver diseases. *Food Chem. Toxicol.* (2011) 49: 2453-2458.

(23) Orhan N, Aslan M, Orhan D, Ergun F and Erdem Y. *In-vivo* assessment of anti-diabetic and antioxidant activities of grapevine leaves (*Vitis vinifera*) in diabetic rats. *J Ethnopharmacol.* (2006) 108: 280–286.

(24) Hebash K, Fadel H and Soliman M. Volatile components of grape leaves. *J. Islam. Acad. Sc.* (1991) 4: 26-28.

(25) Nilüfer Orhan, Mustafa Aslan, Didem Deliorman Orhan, Fatma Ergun and ErdemYesilada. *In-vivo* assessment of anti-diabetic and antioxidant activities of grape vine leaves (*Vitis vinifera L.*) in diabetic rats. *J. Ethnopharmacol.* (2006) 108: 280–286.

(26) Mansour R, Ayed L, Hammami S, Mighri Z, Bakhrouf A and Mhenfi F. Proprietés tinctoriales et Activités antibactériennes d’extraits de feuilles de *Vitis vinifera* L.de TUNISIE. *Tunisian J. Med. Plants. Nat. Prod.* (2011) 6: 126-132.

(27) Jing-Ming K, Lian-Sai C, Ngoh-Khang G, Tet-Fatt C and Bourillard R. Analysis and biological activities of anthocyanins, *Phyto. Chimistry* (2003) 64: 923–933

(28) Ezzi B., Habib J, Damé G and Chemli R. Influence of the date of pre-lavage sur les teneurs en anthocyanes et en éléments minéraux des feuilles du cépage Alicant Bouschet cultivé à El Khengue. *Bull OIV.* (1997) 70: 795-796.

(29) Penchev PI. *PhD thesis*: Étude des procédés d’extractions et de purification de produits bioactifs à partir de plantes par couplage de techniques séparatives à basses et hautes pressions. *Toulouse, Toulouse University.* (2010) 50-53.

(30) Hmamouchi M, Es-Safi N and Essassi E. Oligomeric and polymeric proanthocyanidins from *Morocccangrapewine* (*Vitis vinifera*) leaves. *Fitteroterapia* (1997) 68: 332-337.

(31) Monagas M, Gómez-Cordovés C and Bartolomé B. Evolution of the phenolic content of red wines from *Vitis vinifera* L. during ageing in bottle. *Food Chemistry* (2012) 95: 405-412

(32) Murphy Cowan M. Plant Products as Antimicrobial Agents. *Clinical Microbiol Rev* (1999) 12: 564–582.

(33) Konczaki and Zhang W. Anthocyanins-More Than Nature's Colours. *J. Biomed. Biotech.* (2004) 5: 239-240.

(34) Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. NY. Acad. Sci.* (2002) 957: 146-61.

(35) Kolodziej H and Kiderlen A. Antileishmanial activity and immune modulatory effects of tannins and related
compounds on *Leishmania* parasitised RAW 264.7 cells. *Phyto. Chemistry* (2005) 66: 2056-2071.

(36) Lewin G, Cojean S, Gupta A, Verma S, Puri K and Loiseau P. *In-vitro antileishmanial properties of new flavonoids against Leishmania donovani*. *Biomedicine and Preventive Nutrition* (2011) 1: 168-171.

(37) Nour A, Khalid S, Kaiser M, Brun R, Abdalla W and Schmidt T. The antiprotozoal activity of methylated flavonoids from Ageratum conyzoides L. *J. Ethno. Pharmacol.* (2010) 129: 127-130.

(38) Valadares G, Duarte C, Oliveira S, Chávez-Fumagalli A, Martins T, Costa E, João V, Santoro M, Régis C, Tavares A and Coelho A. Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species. *Parasitol. Internat.* (2011) 60: 335-544.

(39) Ghazanfari T, Hassan M and Khamesipour A. Enhancement of peritoneal macrophage phagocytic activity against L. major by garlic (*Allium Sativum*) treatment. *J. Ethno. Pharmacol.* (2006) 103: 333-337.

This article is available online at http://www.ijpr.ir
۳۰ درصد تخفیف نوروزی ویژه کارگاه‌ها و فیلم‌های آموزشی

اصول تنظیم قراردادها

پروپوزال نویسی

آموزش مهارت های کاربردی در تدوین و چاپ مقاله