

Research Article

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Effectiveness of red alga *Asparagopsis taxiformis* extracts against *Leishmania infantum*

DOI 10.1515/biol-2015-0050

Received June 9, 2014; accepted November 20, 2014

Abstract: Leishmaniasis is a disease with a worldwide distribution affecting both humans and animals. There is a need to identify and develop new drugs for the treatment of leishmaniasis. This study showed that crude ethanolic extracts of the red alga *Asparagopsis taxiformis* have a powerful effect against *L. infantum*, the prevalent species of the genus *Leishmania* in the Mediterranean basin. *L. infantum* demonstrated decreased vitality with increasing concentration of the algal extracts. At a concentration of 40 µg/mL, the extracts achieved 100% mortality of the parasite and the LD₅₀ value was 25 µg/mL for promastigotes and 9 µg/mL for amastigotes. Algal extracts caused morphological alterations and apoptosis in *Leishmania* cells. The potential cytotoxic action of crude extracts was investigated by a MTT viability assay on DH82 and Vero cell lines but there was no cytotoxic effect. The potential of red alga *A. taxiformis* metabolites as anti-leishmanial agents merits further pharmacological investigation.

Keywords: Antimicrobials; *Asparagopsis taxiformis*; *Leishmania infantum*; Mediterranean basin; natural drugs

1 Introduction

Leishmaniasis is a parasitic disease caused by an obligate intramacrophage protozoan of the genus *Leishmania*, which is transmitted by female sandflies of the genus *Phlebotomus*. It is a disease with a worldwide distribution, especially in many tropical and sub-tropical countries, affecting both humans and animals. Its incidence is increasing in non-endemic areas due to changing patterns of international travel and population migration. It is classified on the basis of symptomatology as cutaneous, visceral, muco-cutaneous or diffused cutaneous forms. At present, there are 12 million new cases of leishmaniasis in the world per year [1].

Current chemotherapy strategies have several disadvantages, including problems of low efficacy, severe toxic side effects and drug resistance [2]. There are two principal treatments against the several forms of leishmaniasis: the first-choice treatment is pentavalent antimonials, which are potentially toxic and often ineffective, typically with therapeutic failure, undesirable side effects and slow healing of lesions [3,4]; the second-line compounds generally include pentamidine and amphotericin B, which may be very toxic and in some areas are ineffective [5]. Newer therapies, such as the lipid formulations of amphotericin B, are extremely expensive, which is an important factor since, in general, leishmaniasis mainly affects those in the developing world [2]. These pharmaceutical problems point towards the need to develop new antileishmanial drugs.

Interest in marine organisms as a potential and promising source of pharmaceutical agents has increased during the last few years [6-8]. Particularly, seaweeds are being considered as a source of bioactive metabolites characterized by a broad spectrum of biological activities [9]. Compounds with cytostatic, antiviral, anti-helminthic, antifungal and antibacterial activities have been detected in green, brown and red algae [10-22]. Among red algae, *Asparagopsis* species (Bonnemaisoniales, Rhodophyta) are reported to have strong antifungal, antibacterial and

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antiprotozoal properties [23-29]. The present research deals with the evaluation of extracts of the red alga *A. taxiformis* against *L. infantum*.

2 Experimental Procedures

2.1 Asparagopsis taxiformis extracts

Plants of *A. taxiformis* were collected from the Straits of Messina (Sicily, Italy), (38°12'02.10" N; 15°33'34.46" E) in February 2011. Samples were identified by DNA barcoding and had identical COI-5' sequences with an isolate previously deposited in GenBank [ID: JN642177, 29].

Thalli were manually cleaned and stored at -20°C. Crude extracts were obtained with ethanol at room temperature and dried by Rotavapor® at low temperature (35°C) to prevent volatile compounds from evaporation.

2.2 Antileishmanial bioassays

Leishmania infantum promastigotes (IPT1/MON1, received from the Higher Institute of Health - Rome, Italy) were treated in phosphate buffered saline (PBS) and cultured at 25°C and at pH 7.2, in RPMI-PY medium, which consisted of RPMI 1640 (Sigma R0883) supplemented with equal volume of *Pepton-yeast medium* [30], 10% fetal bovine serum (FBS), 1% glutamine, 250 µg/mL gentamicin and 500 µg/mL of 5-fluorocytosine.

Temperature, differentiation time, and acidification of the medium were used as variables for preconditioning of the promastigote cultures of *L. infantum*. The influence of temperature was evaluated by incubating the promastigotes from 25°C at 37°C. The conditioning time for the promastigotes varied from 24 h to 72 h and the pH was lowered with 1 N HCl to 5.4 to obtain amastigote parasites.

Flasks containing 5 mL of culture medium were inoculated with 4×10^6 /mL promastigotes or amastigotes, and respectively treated with serial dilutions of the crude extracts of *A. taxiformis* (5, 10, 20, 30 and 40 µg/mL). After 48 hours treatment at 24°C, percentage of vitality of *L. infantum* promastigotes and amastigotes was observed by counting in a Burker hemocytometer and staining with May-Grünwald-Giemsa. All tests were performed in triplicate and compared with a negative control. The results were expressed as the median lethal dose (LD_{50}), i.e., the drug concentration that decreases the rate of cell vitality by 50%, calculated by regression analysis.

2.3 Cell sensitivity assays: the MTT assay

The potential cytotoxic action of the crude extracts was checked by the MTT viability assay on DH82 and Vero cell lines [31]. The Vero cell line (CCL-81) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The cell line was cultured in Eagle's minimum essential medium (MEM, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 mg/mL). DH82 cells (ATCC CRL- 10389) were propagated in MEM with non-essential amino acids, 2 mM L-glutamine, and 10% FBS. Cells were incubated at 37°C in 5% CO₂ and passaged semi-weekly.

These cells lines were susceptible to intracellular growth of *Leishmania*, proving to be good models for further studies of the necessary conditions for the conversion of promastigotes to amastigotes [32]. The Vero cell line, a non-phagocytic cell, supports the intracellular mechanism of *Leishmania chagasi* [32]. Pessotti et al. [32] demonstrated that *L. chagasi* promastigotes could interact, transform to amastigote forms, and multiply in non-phagocytic cells, proving to be a new model to study the intracellular cycle of this protozoan.

In each experiment, exponentially growing cells were plated in 100 µL aliquots of growth medium into 96-well plates at 10^5 cells per well, respectively, and incubated for 24 h. For loading of the drug, the cells (in 96-well plates) were incubated with algal crude extracts at concentration of 5, 10, 20, 30, 40 µg/mL, and without algal extract as a control. Ten replicates were performed for each concentration. After 48 h incubation, the MTT labeling reagent (final concentration, 5 mg/mL) was added to each including a blank; and after a 4-h incubation at 26°C, 200 µL DMSO was added to dissolve the formazan crystals and obtain a homogeneous blue solution suitable for measurement of the absorbance with an enzyme-linked immunosorbent assay plate reader (wavelength, 570 nm). The absorbance was measured using a microplate reader Spectrostar Nano (BMG LabTech) at 570 nm. The conversion of MTT to the formazan product by the mitochondrial electron transport chain is an indicator of cell viability, and a decrease in the amount of MTT converted indicates toxicity to the cell. The percentage of surviving cells versus the number of surviving control cells was assessed by the formula = $[(\text{absorbance sample} - \text{absorbance blank}) / (\text{absorbance control} - \text{absorbance blank})] \times 100$.

2.4 Statistical analysis

All experiments were performed by two observers in three replicates and repeated with three new batches of parasites. The mean and standard error were determined. The differences between the mean values obtained for experimental groups were evaluated by Student's t test. P-values of 0.05 or less were considered significant. The LD₅₀ values were calculated using the GraphPad Prism(®) 5 (Version 5.01, GraphPad Software, Inc., USA).

3 Results

The viability of *Leishmania infantum* promastigotes treated with serial dilutions of *Asparagopsis taxiformis* extracts is shown in Figure 1A. Cultures of *Leishmania* with a concentration of 5 µg/mL of algal extract showed 95% vitality, and the percentage of viable *Leishmania* decreased proportionally with increasing concentration of the algal extract, reaching 100% mortality of the parasite at 40 µg/mL. From the results observed, the value of LD₅₀ of algal extract obtained is equal to 25 µg/mL. The viability of *L. infantum* amastigotes treated with serial dilutions of *A. taxiformis* extracts is shown in Figure 1B. Also in this case, the vitality of *Leishmania* decreased proportionally with increasing concentration of *A. taxiformis* extracts. From observed results, the value of LD₅₀ of algal extract is equal to 9 µg/mL.

The leishmanicidal effect of *A. taxiformis* was further verified by evaluating the morphological characteristics of promastigotes and amastigotes of *L. infantum* after staining with May-Grünwald-Giemsa, with 48 hours of treatment (Figures 2, 3). At a concentration of 5 µg/mL of algal extracts (Figure 2B), the cultures showed a clear decrease in the number of promastigotes compared with the control (Figure 2A) and, at a concentration of 20 µg/mL (Figure 2D), they were characterized by the presence of abnormal and roundish forms. In cultures with 30 µg/mL of algal extracts (Figure 2E), *L. infantum* cells were aggregates, rounded and without flagella. Finally, in *L. infantum* cultures with 40 µg/mL of algal extracts (Figure 2F) there were no intact forms of protozoa but only apoptotic bodies. At concentrations of 5, 10, 20, 30 µg/mL (Figures 3B, 3C, 3D, 3E), the cultures showed a clear decrease in the number of amastigotes compared with the control (Figure 3A). Finally, in *L. infantum* amastigotes cultures with 40 µg/mL of algal extract (Figure 3F) there were no intact forms of amastigotes but only apoptotic bodies.

The cytotoxicity test (MTT assay) showed a viability of 91.7-94.8% of DH82 cells and of 88.55-93.29% of VERO cells in tested conditions (Table 1). Therefore, algal extracts did not have a cytotoxic effect on Vero and DH82 cells at tested concentrations.

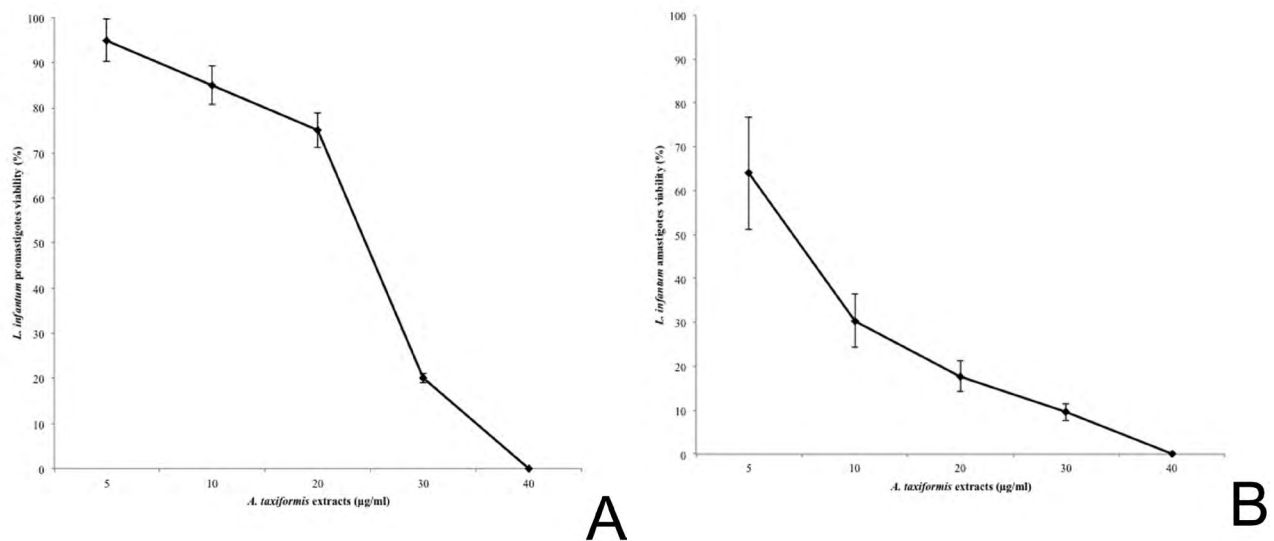


Figure 1. The viability of for *L. infantum* promastigotes (A) and amastigotes (B) treated with serial dilutions of *A. taxiformis* extracts. The LD₅₀ against promastigotes is 25 µg/mL and against amastigotes is 9 µg/mL. The percentage of viable *Leishmania* was calculated by defining the viability of cells without *A. taxiformis* extracts treatment as 100%. Data are the results of three independent experiments and are presented as the mean ± SD and the data obtained are statistically significant at $p < 0.001$.

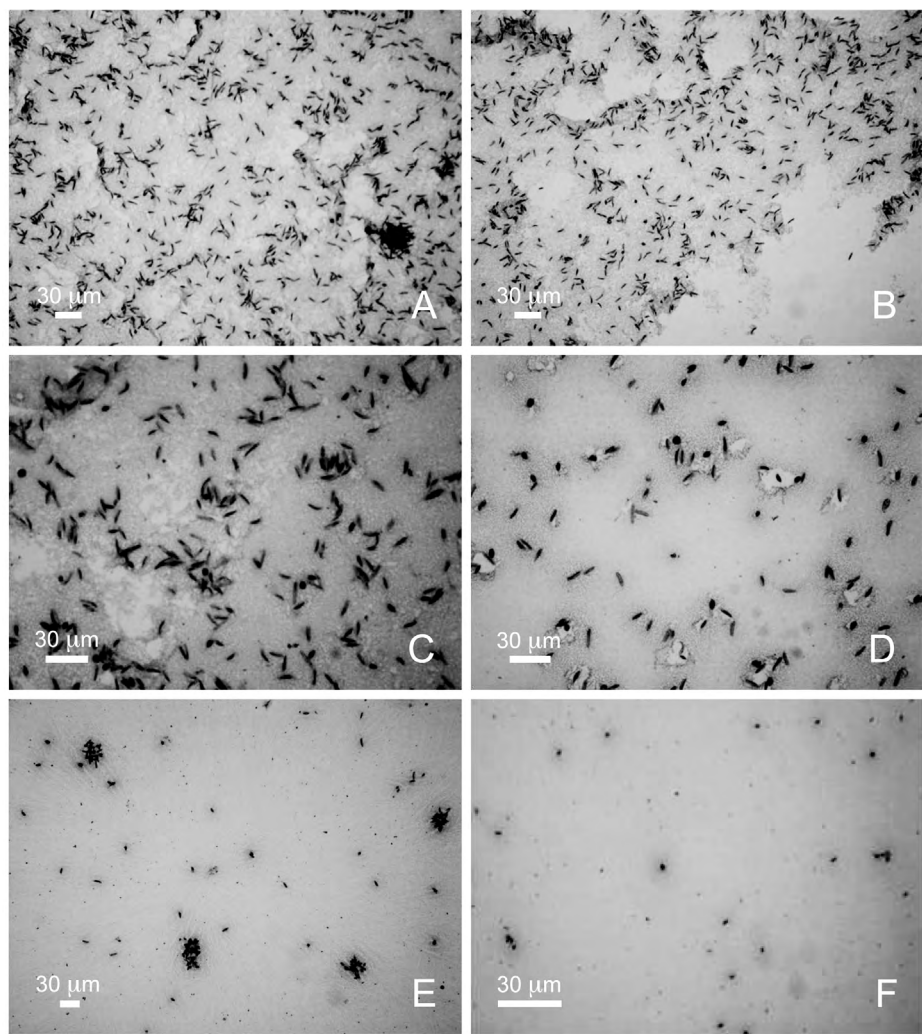


Figure 2. Cultured promastigotes of *L. infantum* stained with May-Grünwald-Giemsa. (A) cultures without algal extracts; (B) cultures with 5 µg/mL of algal extracts; (C) cultures with 10 µg/mL of algal extracts; (D) cultures with 20 µg/mL of algal extracts; (E) cultures with 30 µg/mL of algal extracts; (F) cultures with 40 µg/mL of algal extracts. Scale bar = 30 µm.

Table 1. Viability of VERO cells and DH82 cells incubated with algal extract.

Algal extract concentration (µg/mL)	Cell viability (%)	
	VERO cells	DH82 cells
5	90.76 ± 0.032731	91.7 ± 0.032731
10	90.97 ± 0.027765	92.2 ± 0.027765
20	93.03 ± 0.036041	94.8 ± 0.036041
30	91.41 ± 0.038691	93.7 ± 0.038691
40	93.29 ± 0.035266	93.0 ± 0.035266

4 Discussion

Antileishmanial effects had already been reported for extracts from the red alga *Asparagopsis taxiformis* [25]. The present work extends these findings by showing that ethanolic crude extracts have a powerful action

against *Leishmania infantum*, the prevalent species in the Mediterranean basin. In addition, the algal extracts did not show a cytotoxic effect on immortalized Vero and DH82 cell lines. The treatment of leishmaniasis has been the subject of numerous studies and interest in this disease is very high [33]. Our evaluation of the

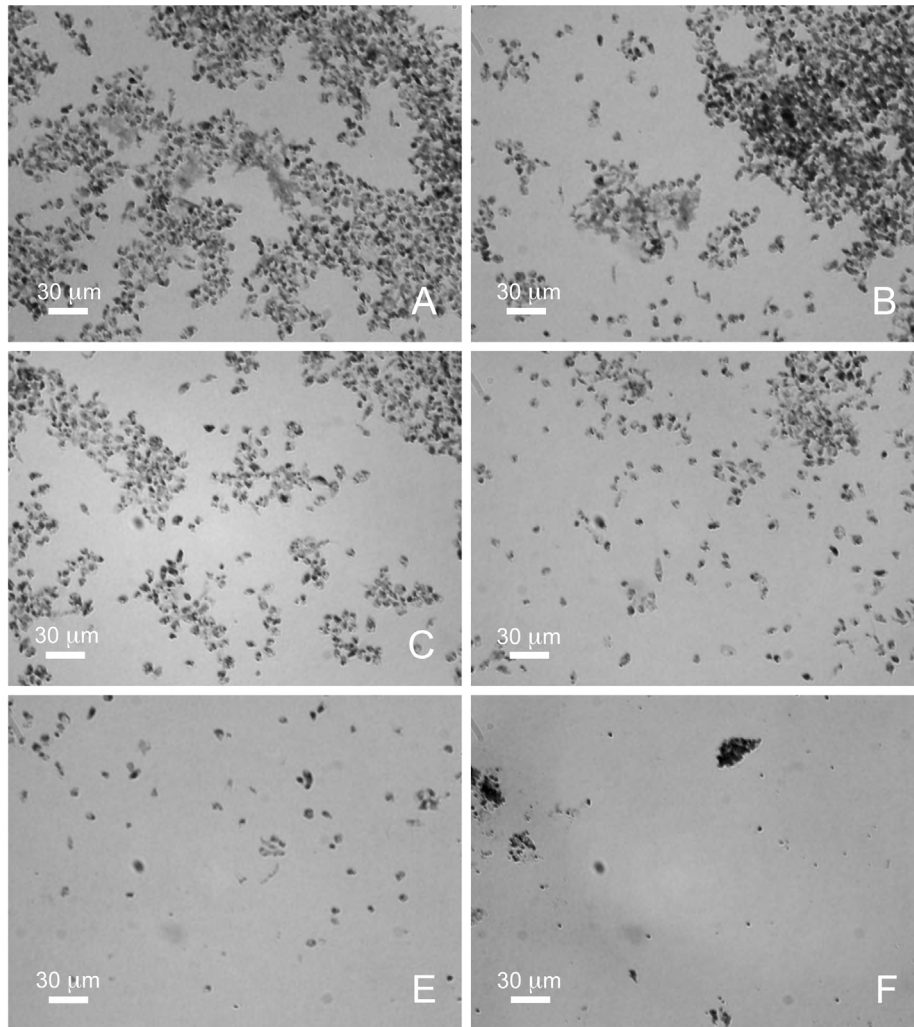


Figure 3. Cultured amastigotes of *L. infantum* stained with May-Grünwald-Giemsa. (A) cultures without algal extracts; (B) cultures with 5 µg/mL of algal extracts; (C) cultures with 10 µg/mL of algal extracts; (D) cultures with 20 µg/mL of algal extracts; (E) cultures with 30 µg/mL of algal extracts; (F) cultures with 40 µg/mL of algal extracts. Scale bar = 30 µm.

antiprotozoal activity of *A. taxiformis*, collected from the Straits of Messina (Sicily), showed that ethanolic extract had antileishmanial activity characterized by a trend dose-effect.

Dogs are the main reservoir for diffusion of the visceral form of leishmaniasis, which for this reason is widespread in the human population [34]. At present, it is an incurable and complex zoonosis, whose true incidence is not known and for ethical reasons drastic measures (e.g. animal culling) are not proposed. Furthermore, there is a large difference between immunocompetent patients, who heal completely after treatment, and patients in which the condition recurs regularly regardless of the drug used. Current drugs decrease the parasitic charge and promote cell-mediated immune response, but fail to eradicate the disease. Therefore, the development of new therapeutic

protocols for the treatment of leishmaniasis and new drugs with leishmanicidal and immunomodulatory activity are needed, in order to achieve total elimination of the parasite. At the same time, it is necessary to develop economical drugs in order to decrease the gap with developing countries, where the impact of leishmaniasis is far greater.

Among marine organisms, seaweeds produce a diverse array of novel secondary metabolites as a chemical defense strategy [35] and these are characterized by a broad spectrum of biological activities [6,10,11,13-15,17,19,20,36].

The study area in Sicily, in the heart of the Mediterranean basin, is an ideal habitat for endemic leishmaniasis infections in both dogs and humans. In this paper, we highlighted the important potentialities of red

alga *A. taxiformis* metabolites as anti-leishmanial agents which merit further pharmacological investigations. In particular, the purification of the active principle could be interesting, although it is not possible to exclude the possibility that several molecules contribute to the antileishmanial activity in a synergic mechanism.

Acknowledgements: This study was supported by grants to F.V. (IZS SI 13/2012 RC) and G.G. (INNOVAQUA PON02_00451_3362185).

Conflict of interest: Authors declare nothing to disclose.

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