

# Anti-leishmanial activity of Brazilian green, brown, and red algae

Marie-Luce Flores Lira · Raphaela Lopes · Alex Portes Gomes ·  
Gabrielle Barcellos · Maurício Verícimo · Kelvin Osako ·  
Fredy Augusto Ortiz-Ramirez · Carlos José Brito Ramos ·  
Diana N. Cavalcanti · Valéria Laneuville Teixeira ·  
Veronica do Amaral

Received: 12 September 2014 / Revised and accepted: 27 January 2015 / Published online: 12 February 2015  
© Springer Science+Business Media Dordrecht 2015

**Abstract** Leishmaniasis is a disease of public health significance, and the available therapy is unsatisfactory. Studies of marine algae as a source of pharmacologically active compounds have increased worldwide. This study evaluated the activity of algal crude extracts and a purified product against *Leishmania amazonensis*. Extracts from *Caulerpa racemosa*, *Osmundaria obtusiloba*, *Styopodium zonale*, *Dictyota ciliolata*, and *Dictyota menstrualis* showed EC<sub>50</sub>/24 h activity with concentrations that ranged from 0.61 to 154.0 µg mL<sup>-1</sup>. The cytotoxicity of these extracts on macrophages showed CC<sub>50</sub>/24 h, with a concentration range of 18.2–240.0 µg mL<sup>-1</sup>. The selectivity index corresponds to values varying from 0.31 to 39.33 for these extracts. A mixture of diterpene isomers isolated from *D. menstrualis* showed interesting activity against *L. amazonensis*. In addition, the anti-leishmanial activity

of the *O. obtusiloba* extract showed potential activity that was better than the effect obtained with pentamidine, a reference drug. The alcoholic extract of *O. obtusiloba* was selected for in vivo tests, and BALB/c mice were infected with *L. amazonensis* and treated with the *O. obtusiloba* extract orally for 4 weeks. The treatment showed the ability to control the dissemination of parasites to the draining lymph nodes as well the evolution of cutaneous lesions, indicating a potential therapeutic use of the alcoholic extract of *O. obtusiloba*. These results provide new perspectives on the development of drugs against leishmaniasis.

**Keywords** Anti-leishmanial activity · *Caulerpa racemosa* · *Osmundaria obtusiloba* · *Styopodium zonale* · *Dictyota ciliolata* · *Dictyota menstrualis*

M.-L. F. Lira · R. Lopes · G. Barcellos · K. Osako · C. J. B. Ramos  
Programa de Pós-Graduação em Ciências e Biotecnologia, Instituto de Biologia, Universidade Federal Fluminense, Centro, 24020-141 Niterói, Rio de Janeiro, Brazil

M.-L. F. Lira  
e-mail: marie.biouff@gmail.com

R. Lopes  
e-mail: raphaelalopes@id.uff.br

G. Barcellos  
e-mail: gabriellebarcellos@id.uff.br

K. Osako  
e-mail: k3kelvin@gmail.com

C. J. B. Ramos  
e-mail: cjbramos@gmail.com

A. P. Gomes · M. Verícimo · V. do Amaral (✉)  
Departamento de Imunobiologia, Instituto de Biologia, Universidade Federal Fluminense, Centro, 24020-141 Niterói, Rio de Janeiro, Brazil  
e-mail: veronicadoamaral@gmail.com

A. P. Gomes  
e-mail: mdvportes@gmail.com

M. Verícimo  
e-mail: vericimo@vm.uff.br

F. A. Ortiz-Ramirez  
Programa de Pós-Graduação em Biologia Marinha, Departamento de Biologia Marinha, Instituto de Biologia, PO Box 100.644, Centro, 24001-970 Niterói, Rio de Janeiro, Brazil  
e-mail: faortizr@gmail.com

D. N. Cavalcanti · V. L. Teixeira (✉)  
Laboratório Algamar, Departamento de Biologia Marinha, Instituto de Biologia, PO Box 100.644, Centro, 24001-970 Niterói, Rio de Janeiro, Brazil  
e-mail: valerialaneuville@gmail.com

D. N. Cavalcanti  
e-mail: dn.cavalcanti@gmail.com

## Introduction

Leishmaniasis is a group of infectious diseases, with clinical significance and epidemiological diversity (Alvar et al. 2012). It is caused by intracellular protozoa of the genus *Leishmania* (Grimaldi et al. 1991). This important disease with epidemiological impacts worldwide especially affects developing countries. Currently, leishmaniasis is present in 98 countries on five continents and affects about 12 million people. Furthermore, it is estimated that 310 million people are at risk of contracting this disease with an annual incidence of 1.3 million new cases (WHO 2014). Despite enormous efforts by scientists researching various aspects of *Leishmania* parasites, the disease remains an important public health problem (Alvar et al. 2012; Bañuls et al. 2007).

The *Leishmania* genus comprises 35 species, with about 20 species known to be pathogenic to humans (Fraga et al. 2013). These parasites are transmitted to vertebrate hosts by phlebotomine vectors (Kato et al. 2010; Lainson and Shaw 2005). Nearly 20,000–40,000 deaths annually are associated with leishmaniasis worldwide (Alvar et al. 2012; WHO 2014). In Brazil, *Leishmania* species are agent of visceral (Leon et al. 1992), cutaneous, and mucosal clinical presentation forms and diffuse leishmaniasis due to *Leishmania amazonensis* (Reithinger et al. 2007; Brito et al. 2012). There is an expansion of the geographic distribution of *L. amazonensis*, and this species was associated with unusual clinical manifestations at new areas of transmission (Azeredo-Coutinho et al. 2007). Furthermore, co-infection between HIV and *Leishmania* has been reported in 35 countries, mainly in Spain, Italy, France, and Portugal (Alvar et al. 2008). Approximately 8.5 % of Brazilian patients with HIV are estimated to be co-infected with *Leishmania* (Lindoso et al. 2014).

Leishmaniasis is a typical example of a re-emerging tropical disease, due to the diversity of animal reservoirs and the difficulty of vector control. This neglected disease needs urgent investment by the pharmaceutical industry and attention by research institutes (Cavalli and Bolognesi 2009; Ameen 2010). The current chemotherapy of leishmaniasis involves parenteral administration of drugs such as pentavalent antimonials, pentamidine, and amphotericin B. However, these drugs have a number of disadvantages, including patient hospitalization and its associated high costs (Singh and Sivakumar 2004). Moreover, these drugs exhibit significant side effects (González et al. 2009). Despite the descriptions of over 25 new substances against *Leishmania* species, few are effectively used in humans (Palumbo 2009) and, in most cases, are used in parenteral form (Singh and Sivakumar 2004). The oral administration of drugs such as miltefosine and ketoconazole has been used with varying effects (González et al. 2009). In addition, most of the available drugs for the treatment of leishmaniasis are expensive, require a long period of use, and become inefficient, requiring the development of new therapies

(Palumbo 2009). Furthermore, the lack of efficient vaccines and the high toxicity of treatment contribute to the spread of the disease (El-On 2009). Considering the inefficiency of current drugs and the fact that some varieties of *Leishmania* are resistant to these treatments, new drugs are being researched in order to find a more selective and effective therapy with fewer side effects. Therefore, our research group conducted studies on new therapeutic agents (Charret et al. 2009, 2013; Marra et al. 2012).

The literature has reported several studies about biological activities of extracts from marine algae (Shalaby 2011). These also have exhibited appreciable anticoagulant, anti-inflammatory, antitumoral, antiparasitic, antibacterial, and antiviral activities (Mayer et al. 2009). In recent years, the interest in the study of marine algae as a source of pharmacologically active compounds has increased worldwide, but there are few investigations that focus on the anti-leishmanial activity of seaweeds (Soares et al. 2012). Thus, the major aim of this work was to evaluate the potential activity against *Leishmania amazonensis* by natural substances isolated from marine algal species such as *Caulerpa racemosa* (Forsskål) J. Agardh (Chlorophyta, Bryopsidales), *Osmundaria obtusiloba* C. Agardh R. E. Norris (Rhodophyta, Ceramiales), *Stypopodium zonale* (J.V. Lamouroux) Papenfuss, *Dictyota ciliolata* Sonder ex Kützing, and *Dictyota menstrualis* (Hoyt) Schnetter, Hörning & Weber-Peukert (Phaeophyceae, Dictyotales), obtained from different geographical regions of Brazil.

## Material and methods

**Preparation of the extracts** Algae were collected along the coastline of Brazil (Table 1) with a license from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, license number 10594-3). Extractions were performed with solvents of low and medium polarity, resulting in 10 extracts identified by abbreviations. The extracts from five marine algae (*C. racemosa*, *O. obtusiloba*, *S. zonale*, *D. ciliolata*, and *D. menstrualis*) were evaluated. The diterpenes pachydictyol A and isopachydictyol A were obtained from the dichloromethanic extract from *D. menstrualis*.

**Isolation of the mixture pachydictyol A and isopachydictyol A** Air-dried *D. menstrualis* (95 g) was extracted in 100 % dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) exhaustively at room temperature, yielding 5 g of crude extract, which was subjected to silica gel (Merck; 0.015–0.045 mm), eluted with CH<sub>2</sub>Cl<sub>2</sub>/EtOAc (4:1), CH<sub>2</sub>Cl<sub>2</sub>/EtOAc (1:1), EtOAc, and 100 % acetone. Fractions 2 and 3 contain a crude mixture of diterpenes isomers pachydictyol A/isopachydictyol A (55 mg). Gel-column chromatography with 100 % hexane furnished 10 fractions. Fractions 3–10 yielded 45 mg of the pure mixture of the diterpenes pachydictyol A/isopachydictyol A (3:1). Pachydictyol A and

**Table 1** Seaweeds species collected, abbreviation code, solvent used, and collection sites at different geographical regions of Brazil

Seaweeds	Code	Solvent	Collection site
<i>Styopodium zonale</i>	Sz-EtOAc-Hex/Mar	EtOAc Hex	Marataízes (Mar)/Espírito Santo
<i>Dictyota menstrualis</i>	DM-EtOAc/SPSP	EtOAc	São Pedro e São Paulo (SPSP)/Pernambuco
	DM-EtOAc/Buz	EtOAc	Búzios (Buz)/Rio de Janeiro
	DM-Hex/Buz	Hex	Búzios (Buz)/Rio de Janeiro
	Pachydietylol A	Hex	Búzios (Buz)/Rio de Janeiro
	-Isopachydietylol A /Buz		
<i>Dictyota ciliolata</i>	DC-EtOAc/Angra	EtOAc	Angra dos Reis/Rio de Janeiro
	DC-Hex/Angra	Hex	Angra dos Reis/Rio de Janeiro
<i>Osmundaria obtusiloba</i>	OO-Et/Buz	EtOH	Búzios (Buz)/Rio de Janeiro
	OO-EtOAc/Buz	EtOAc	Búzios (Buz)/Rio de Janeiro
<i>Caulerpa racemosa</i>	CR-EtOAc/Buz	EtOAc	Búzios (Buz)/Rio de Janeiro
	CR-EtOAc/SPSP	EtOAc	São Pedro e São Paulo (SPSP)/Pernambuco

EtOAc ethyl acetate, Hex hexane, EtOH ethanol

isopachydietylol A were identified by comparison of physical and spectroscopic data with reported values (Durán et al. 1997) and comparison with authentic molecules.

**Animals** Male BALB/c mice, 8 weeks old, were purchased from the animal facility of the Núcleo de Animais de Laboratório (NAL-UFF). The animals were used in therapeutic assay to obtain peritoneal macrophages for cytotoxicity assay. All experiments were conducted according to a protocol approved by the Ethics Committee for Use of Laboratory Animals (Permit Number: 318/13).

**Parasites** *Leishmania amazonensis* strain MHOM/BR/77LTB0016 amastigotes were obtained from mouse popliteal lymph nodes (Bernardino et al. 2006). The amastigotes differentiated into promastigotes after 3 days in culture with Schneider's insect medium (Sigma, USA) at 26 °C, supplemented with 10 % (v/v) inactivated fetal bovine serum (Cultilab, São Paulo, Brazil).

**Anti-leishmania assays** Algal crude extracts and the purified product within a concentration range of 40–320  $\mu\text{g mL}^{-1}$  were tested against *L. amazonensis* promastigotes (in the early stationary phase) starting with a concentration of  $1 \times 10^7$  parasites  $\text{mL}^{-1}$  (Bernardino et al. 2006). Assays were performed in triplicate in 96-well microtiter plates. Dimethylsulfoxide (DMSO, Sigma) and the reference drug pentamidine (Sanofi-Aventis, Brazil) were used as controls. After 24 h, tests were carried out with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT (Sigma, USA), and after 4 h, plates were read using 545-nm filters (Mikus and Steverding 2000).

**Cytotoxicity assays** In vitro evaluation of the cytotoxic activity of natural products on macrophages was performed

according to the technique described by Bernardino et al. (2006). Briefly, peritoneal macrophages from BALB/c mice were obtained after washing with cold DMEM (Gibco, USA) medium. The peritoneal cells were cultured in 96-well plates at a concentration of  $3 \times 10^5$  cells per well and maintained at 37 °C in 5 %  $\text{CO}_2$ . After 24 h, all extracts and purified products (Table 1) in different concentrations (20, 40, 80, and 160  $\mu\text{g mL}^{-1}$ ) were added to the macrophages culture. DMSO, culture medium, and pentamidine were used as controls. Incubation for 24 h was conducted with MTT (5  $\text{mg mL}^{-1}$ ; Sigma) as previously noted. Then, by regression analysis, the  $\text{CC}_{50}/24$  h was determined (Bernardino et al. 2006).

**Evaluation of in vivo activity** Superior values of selectivity index (SI), which corresponds to the ratio between the values of  $\text{CC}_{50}/24$  h and  $\text{EC}_{50}/24$  h were used as criteria for the selection of the extract to be evaluated in vivo. BALB/c mice were inoculated subcutaneously in the left-hind footpad with  $1 \times 10^6$  promastigotes of *L. amazonensis*. Two weeks after infection, BALB/c mice were treated orally daily with an alcoholic algal extract of *O. obtusiloba* (OO-Et/Buz) (5 or 20  $\text{mg kg}^{-1}$ ). Similarly, the positive control group was treated daily with ketoconazole (50  $\text{mg kg}^{-1}$ ), and the negative control group received DMSO. The treatment was performed for 28 consecutive days. Lesion thicknesses were evaluated weekly by measuring the diameters of both rear feet with a direct-reading dial caliper. The size of lesions in millimeters was calculated by subtracting the diameter of the uninfected foot from that of the infected foot.

**Parasite quantification** A determination of the number of parasites in the lymph nodes was estimated by a modified limiting-dilution assay (Buffet et al. 1995). Popliteal lymph nodes of the infected footpads were removed and used to

prepare a cell suspension in phosphate-buffered saline. After centrifugation the pellet was resuspended in Schneider's insect medium, pH 7.2. The suspension was then serially diluted in 12-fold dilutions, incubated at 26 °C for 7 days, and monitored in an inverted microscope for the presence or absence of promastigotes. The parasite-burden per gram in the corresponding organ was calculated according to the method used by Pourrajab et al. (2012). To assay body weight, mice were weighed before, during, and at the end of the experiment to compare the weight of the treated and untreated mice.

**Statistical analysis** All experiments were repeated at least three times. Data obtained were analyzed using Student's *t* test, and  $p \leq 0.05$  were considered significant. For comparison of more than two groups, a one-way ANOVA was performed. If a significant main effect or association was identified ( $p \leq 0.05$ ), the respective group means were compared using Dunnett's multiple comparison test. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., USA).

## Results

This study employed in vitro and in vivo tests to evaluate initial anti-leishmania activity against promastigote forms of *L. amazonensis*, its toxicity against mammalian cells, and after that, the therapeutic activity of Brazilian marine algae extracts. The extract from the brown alga *S. zonale* (Sz-EtOAc-Hex/Mar) inhibited 50 % of the parasitic activity at dilutions ten times less concentrated than that of pentamidine. Similarly, other brown algae extracts from *D. menstrualis* (DM-EtOAc/SPSP, DM-EtOAc/Buz, and DM-Hex/Buz) and *D. ciliolata* (DC-EtOAc/Angra and DC-Hex/Angra) showed high activity against *L. amazonensis* at low concentrations. In addition, the extract from the red alga *O. obtusiloba* (OO-Et/Buz and OO-EtOAc/Buz) and a mixture of isomers pachydietylol A and isopachydietylol A isolated from *D. menstrualis* showed potential anti-leishmania activity. In contrast, the two extracts from the green alga *C. racemosa* showed no significant activity against *L. amazonensis* (Table 2).

In addition, a cytotoxicity assay of the crude extract of *S. zonale* (Sz-EtOAc-Hex/Mar), *D. ciliolata* (DC-EtOAc/Angra, and DC-Hex/Angra), *D. menstrualis* (DM-EtOAc/SPSP, DM-EtOAc/Buz, and DM-Hex/Buz), and *C. racemosa* collected from Buzios (CR-EtOAc/Buz) and the diterpene isomers mixture pachydietylol A and isopachydietylol A showed moderate cytotoxicity on mammalian cells similar to pentamidine. However, extracts of *O. obtusiloba* (OO-EtOAc/Buz and OO-Et/Buz) and *C. racemosa* (CR-EtOAc/SPSP) in the concentration tested were not cytotoxic to murine peritoneal macrophages ( $p \leq 0.05$ ) (Table 2).

**Table 2** Effect of the algae extracts and purified product of *D. menstrualis* on *Leishmania* and macrophages and their respective selectivity indexes (SI)

Extracts/ products	Code	EC <sub>50</sub> /24 h	CC <sub>50</sub> /24 h	SI
		( $\mu\text{g mL}^{-1}$ ) Promastigotes	( $\mu\text{g mL}^{-1}$ ) Macrophages	
<i>Styopodium zonale</i> extract	Sz-EtOAc- Hex/Mar	0.75±0.3	29.5±14.8	39.33
<i>Dictyota menstrualis</i> extract	DM-EtOAc/ SPSP	0.75±0.3	24.7±8.3	32.93
	DM-EtOAc / Buz	0.7±0.2	18.3±6.0	26.14
	DM-Hex/ Buz	0.61±0.2	18.2±6.0	29.83
<i>Dictyota ciliolata</i> extract	Isomers/Buz	23.5±15.4	30.0±7.0	1.27
	DC-EtOAc/ Angra	4.22±2.9	19.0	4.5
	DC-Hex/ Angra	1.15±0.3	23.5±10.6	20.43
<i>Osmundaria obtusiloba</i> extract	OO-Et/Buz	24.5±0.5	240.0±113.1	9.79
	OO-EtOAc/ Buz	22.0±7.0	198.0±172.5	9.0
<i>Caulerpa racemosa</i> extract	CR-EtOAc/ Buz	154.0±6.0	48.0	0.31
	CR-EtOAc/ SPSP	47.5±4.5	115.0	2.42
Pentamidine	Pentamidine	7.6±2.5	19.5±4.9	2.56

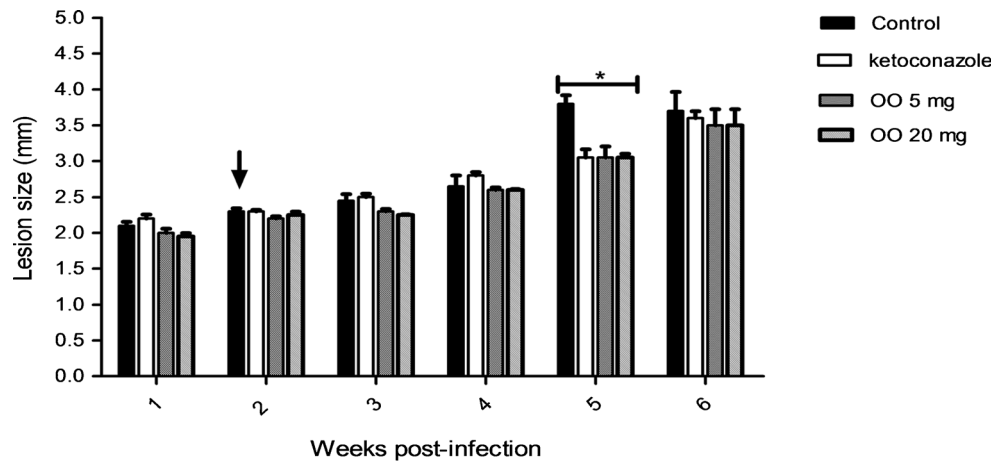
Antileishmanial (EC<sub>50</sub>/24 h) effect against promastigotes forms of *L. amazonensis* and cytotoxicity (CC<sub>50</sub>/24 h) on murine adherent peritoneal cells compared with pentamidine. Mean of EC50 or CC50 ( $\mu\text{g mL}^{-1}$ )±standard deviation of three experiments carried out in triplicate

The selectivity index (SI) was determined to be a secure indicator of the therapeutic use of a new potential compound. Among the marine macroalgae evaluated, a total of eight extracts obtained from *S. zonale*, *D. menstrualis*, *D. ciliolata*, and *O. obtusiloba* had a selectivity index better than pentamidine. The best result was observed in assays with an extract from *S. zonale*, which showed an SI about 15 times higher than pentamidine. This extract was also found to be about 40 times less toxic to murine peritoneal macrophages compared to the promastigote forms of *L. amazonensis* that were affected by the extract. Additionally, extracts of *Dictyota* (DM-EtOAc/SPSP, DM-EtOAc/Buz, DM-Hex/Buz, DC-EtOAc/Angra, and DC-Hex/Angra) and *O. obtusiloba* (OO-Et/Buz and OO-EtOAc/Buz) showed a selectivity index better than pentamidine. However, the mixture of isomers pachydietylol A and isopachydietylol A and *C. racemosa* extracts showed low SI values (Table 2).

The extract of *O. obtusiloba* (OO-Et/Buz) was considered promising and was used for the therapeutic evaluation in mice subcutaneously infected with *L. amazonensis*. At the second week post-infection, BALB/c mice showed small cutaneous lesions initially characterized by mild edema, erythema, and a progressive increase of lesions in all animal groups. However,



**Fig. 1** BALB/c mice infected with *L. amazonensis* after treatment with alcoholic extract of *Osmundaria obtusiloba* (OO 5 mg or OO 20 mg kg<sup>-1</sup>) or ketoconazole (50 mg kg<sup>-1</sup>). Lesion sizes were measured at the indicated times post-infection (mean±standard deviation). The black arrow indicates the start of the oral treatment. \**p*<0.05). These results are representative of one experiment (*n*=6 per group)



at 5 weeks post-infection, a significant increase in the diameters of cutaneous lesions in the untreated animals was observed in relation to animals treated with *O. obtusiloba* extract (Fig. 1). After that, both groups treated with 5 mg kg<sup>-1</sup> of alcoholic extract of *O. obtusiloba* and those treated with 20 mg kg<sup>-1</sup> of the extract had similar skin-lesion progressions compared to the group treated with ketoconazole, a reference drug. Treatment with 5 or 20 mg kg<sup>-1</sup> of the alcoholic extract of *O. obtusiloba* or ketoconazole decreased the parasite load observed in lymph nodes in comparison to the untreated animals (Fig. 2).

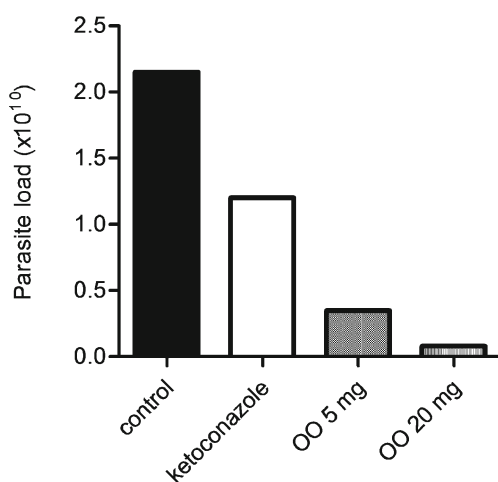
**Discussion**

Leishmaniasis has been considered a neglected disease, despite its high rates of mortality and morbidity (Alvar et al. 2012; WHO 2014). The drugs used in leishmaniasis treatment

have serious side effects (Ameen 2010), and the search for anti-leishmanial activity among natural products such as algae may be useful in the development of new drugs.

Marine organisms constitute a promising source of biologically active compounds. However, few studies have researched marine algae for their potential for the development of leishmanicidal drugs (Soares et al. 2012). Among ten algal extracts examined, this study evaluated *S. zonale* to demonstrate the best selectivity index, approximately 15 times better than pentamidine. Given these considerations, further in vivo studies will be necessary in order to elucidate the role of *S. zonale* extract in *Leishmania* infection. The presence of meroditerpenes, in particular atomaric acid isolated from *S. zonale* that has been selected for antiviral activity (Soares et al. 2007), could also be contributing to anti-leishmanial activity since some terpenes can inhibit target enzymes from *Leishmania* (Gray et al. 2006). Some inhibitors of kinases are cytotoxic to *L. amazonensis* (Becker and Jaffe 1997). We are not aware of any study thus far of the anti-leishmanial action of *S. zonale*, but there are reported studies concerning its antiviral (Mendes et al. 2011), antitumoral, and antioxidant (Penicooke et al. 2013) activities.

The literature also does not report anti-leishmanial activity of *D. menstrualis* and *D. ciliolata*. A comparison of the activity from extracts obtained from *D. menstrualis* (DM-EtOAc/SPSP, DM-EtOAc/Buz, and DM-Hex/Buz) from different sites showed no significant difference in their inhibitory effect on *L. amazonensis* promastigotes. In our study, all *Dictyota* extracts showed better anti-leishmanial activity compared to the reference drug, pentamidine. It is possible that the superior inhibitory effects of the algal extracts could be related to the presence of  $\alpha$ ,  $\beta$ -unsaturated dialdehydes such as xeniane, dichotomane, and cycloxeniane diterpenes isolated from these algae in previous studies (Cavalcanti et al. 2007; De-Paula et al. 2008). In other studies, best values of anti-leishmanial activity were observed with extracts obtained from *Dictyota caribaea* (Freile-Pelegrin et al. 2008). Diterpenes isolated from brown algae *Dictyota pfaeffii* and *D. menstrualis* have



**Fig. 2** Parasite load (number of parasites per mg) in popliteal lymph node of BALB/c mice infected with *L. amazonensis* after treatment with alcoholic extract of *Osmundaria obtusiloba* (OO 5 mg or OO 20 mg kg<sup>-1</sup>) or ketoconazole (50 mg kg<sup>-1</sup>). These results are representative of one experiment (*n*=3 per group)

potentially important biological activities such as antiviral (Abrantes et al. 2010), antimalarial, and antibacterial (Jongaramruong and Kongkam 2007) effects. In addition, diterpenoids of *D. menstrualis* have demonstrated anti-HIV activity (Pereira et al. 2005). More recently, dolabelladienetriol, a diterpene isolated from Brazilian *D. pfaflii*, was shown to be effective against promastigote forms of *L. amazonensis* (Soares et al. 2012). In the present study, the mixture of diterpene isomers pachydictyol A and isopachydictyol A was less active than pentamidine as well as the extracts from *D. menstrualis*. This might be due to the presence of different molecules found in the crude extract that may develop a synergism to anti-leishmanial activities. In addition, the hexanic extract of *D. ciliolata* (DC-Hex/Angra) showed better anti-leishmanial activity than the ethyl acetate extract (DC-EtOAc/Angra). These results can be explained by the greater abundance of diterpenes in hexanic extract, in particular of  $\alpha$ ,  $\beta$ -unsaturated dialdehydes and prenylated guaiane diterpenes (Cavalcanti et al. 2007; De-Paula et al. 2008).

The extract of *C. racemosa* was considerably less active compared to those described in the literature (Süzgeç-Selçuk et al. 2011). Additionally, a sample of the green alga *C. racemosa* (CR-EtOAc/SPSP) collected in Pernambuco State inhibited the growth of parasites about three times more effectively than a sample (CR-EtOAc/Buz) collected in Rio de Janeiro State (Table 2). The difference in  $EC_{50}$  values found was possibly directly related to the fact that these algae have been collected from different regions, as differing environmental conditions influence the production of metabolites. In previous studies, extracts from Brazilian *C. racemosa* resulted in the isolation of caulerpin, a bis-indole alkaloid that has an anti-inflammatory effect (Rocha et al. 2007; de Souza et al. 2009).

Several studies have demonstrated the important antimicrobial activities of *C. racemosa*, *O. obtusiloba*, *S. zonale*, *D. menstrualis*, and *D. ciliolata* (Mayer et al. 2009; Soares et al. 2007; Mendes et al. 2011; Freile-Pelegrin et al. 2008; Abrantes et al. 2010; Süzgeç-Selçuk et al. 2011). Although the activities of many of these seaweeds are notable, the targets of their potential products are unknown. We investigated several alternative therapies for the treatment of leishmaniasis and we have identified several promising natural substances, especially algal species such as *O. obtusiloba*, *S. zonale*, *D. ciliolata*, and *D. menstrualis*, which showed excellent selectivity indices, and which hold promise for in vivo tests.

In the present study, extracts of the red algae *O. obtusiloba* (OO-Et/Buz and OO-EtOAc/Buz) showed interesting anti-leishmanial effects and were not cytotoxic to murine peritoneal macrophages ( $240\text{--}198\ \mu\text{g mL}^{-1}$ , respectively). In addition, SI of extracts showed their security to the host cells and specificity to the parasite. The alcoholic extract (OO-Et/Buz) and the ethyl acetate extract were about ten times less toxic to peritoneal macrophages than

pentamidine was. Studies by de Souza et al. (2012) showed that a lipid extract of *O. obtusiloba* exhibited moderate toxicity toward Vero cells ( $72\ \mu\text{g mL}^{-1}$ ).

The present study was conducted to evaluate the therapeutic activity of an alcohol extract of *O. obtusiloba* in BALB/c mice infected with *L. amazonensis*. A highly significant reduction in the size of cutaneous lesions was observed in animals treated with 5 and 20  $\text{mg kg}^{-1}$  of *O. obtusiloba* alcohol extract compared to the untreated infected group ( $P\leq 0.05$ ), (Fig. 1). We observed an important reduction of parasite load in the popliteal lymph nodes obtained from the infected treated group. The parasite load in the lymph nodes of infected mice treated with 20  $\text{mg kg}^{-1}$  extract ( $0.05\times 10^{10}$  parasites) was approximately 40 times smaller than in the untreated infected group ( $2.13\times 10^{10}$  parasites) and approximately 20 times smaller compared to the group treated with ketoconazole ( $1.12\times 10^{10}$  parasites). In addition, compared to the untreated infected control group, no signs of apparent toxicity were observed with no significant changes in body weight varying from 25 to 34 g between animals receiving ketoconazole or even in those treated with different concentrations of alcoholic extract of *O. obtusiloba*.

Thus, the treatment of the murine model of *L. amazonensis* infection showed the ability to control the dissemination of parasites to the draining lymph nodes as well as the evolution of cutaneous lesion, indicating a potential therapeutic use of the alcoholic extract of *O. obtusiloba*. The probable activity of *O. obtusiloba* extracts could be related to the presence of bromophenols in these extracts with important antimicrobial activity (Liu et al. 2011). Until now, no leishmanicidal effects of *O. obtusiloba* have been described but other biological activities have been reported, such as potent antiviral activity verified in assays with HSV-1 and HSV-2 viruses (de Souza et al. 2012). In a recent review of the natural products isolated from the genus *Osmundaria* (Osako and Teixeira 2013), the authors describe the presence of halogenated substances, such as bromophenols and fimbriolides in various species. These molecules may be responsible for the observed activity.

Upon comparison of these data, *O. obtusiloba* is the algal species with satisfactory activity in both in vitro toxicity and in vivo therapeutic tests against *L. amazonensis*. This fact should encourage further studies of these algae, as new anti-leishmanial products and on the characterization of molecules and mechanism of action in relation to their anti-leishmanial activity.

**Acknowledgments** This work was supported by a CAPES Fellowship to RL, GB, AG, KO, and FOR and grants from CNPq, FAPERJ, and PROPPI/UFF. The authors are grateful to Roberto Villaça for seaweed collections. The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and VLT for Productivity Fellowships. VLT also thank the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) for Cientista do Nosso Estado Fellowship.

## References

- Abrantes JL, Barbosa J, Cavalcanti D, Pereira RC, Fontes CFL, Teixeira VL, Souza TML, Paixão IC (2010) The effects of the diterpenes isolated from the Brazilian brown algae *Dictyota paffii* and *Dictyota menstrualis* against the herpes simplex type-1 replicative cycle. *Planta Med* 76:339–344
- Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet JP, Gradoni L, Ter Horst R, López-Vélez R, Moreno J (2008) The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* 21:334–359
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, WHO Leishmaniasis Control Team (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671. doi:10.1371/journal.pone.0035671
- Ameen M (2010) Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. *Clin Exp Dermatol* 35:699–705
- Azeredo-Coutinho RB, Conceição-Silva F, Schubach A, Cupolillo E, Quintellam LP, Madeira MF, Pacheco RS, Valet-Rosalino CM, Mendonça SC (2007) First report of diffuse cutaneous leishmaniasis and *Leishmania amazonensis* infection in Rio de Janeiro State, Brazil. *Trans R Soc Trop Med Hyg* 101:735–737
- Bañuls AL, Hide M, Prugnolle F (2007) *Leishmania* and the Leishmaniasis: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv Parasitol* 64:1–109
- Becker S, Jaffe CL (1997) Effect of protein kinase inhibitors on the growth, morphology, and infectivity of *Leishmania* promastigotes. *Parasitol Res* 83:273–280
- Bernardino AM, Gomes AO, Charret KS, Freitas AC, Machado GM, Canto-Cavalheiro MM, Leon LL, Amaral VF (2006) Synthesis and leishmanicidal activities of 1-(4-X-phenyl)-N'-[(4-Y-phenyl)methylene]-1H-pyrazole-4-carbohydrazides. *Eur J Med Chem* 41:80–87
- Brito ME, Andrade MS, Dantas-Torres F, Rodrigues EH, Cavalcanti MP, de Almeida AM, Brandão-Filho SP (2012) Cutaneous leishmaniasis in northeastern Brazil: a critical appraisal of studies conducted in State of Pernambuco. *Rev Soc Bras Med Trop* 45:425–429
- Buffet PA, Sulahian A, Garin YJF, Nassar N, Derouin F (1995) Culture microtitration: a sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. *Antimicrob Agents Chemother* 39:2167–2168
- Cavalcanti DN, Rezende CM, Pinto AC, Teixeira VL (2007) Diterpenoid constituents from the brown alga *Dictyota menstrualis*. *Nat Prod Commun* 1:609–611
- Cavalli A, Bolognesi ML (2009) Neglected tropical diseases: multi-target-directed ligands in the search for novel lead candidates against *Trypanosoma* and *Leishmania*. *J Med Chem* 52:7339–7359
- Charret KS, Rodrigues RF, Bernardino AM, Gomes AO, Canto-Cavalheiro MM, Leon LL, Amaral VF (2009) Effect of oral treatment with pyrazole carbohydrazide derivatives against murine infection by *Leishmania amazonensis*. *Am J Trop Med Hyg* 80:568–573
- Charret KS, Lagrota-Cândido J, Carvalho-Pinto CE, Hottz CF, Lira ML, Rodrigues RF, Gomes AO, Bernardino AM, Canto-Cavalheiro MM, Leon LL, Amaral VF (2013) The histopathological and immunological pattern of CBA mice infected with *Leishmania amazonensis* after treatment with pyrazole carbohydrazide derivatives. *Exp Parasitol* 133:201–210
- De-Paula JC, Bueno LB, Cavalcanti DN, Yoneshigue-Valentin Y, Teixeira VL (2008) Diterpenes from the brown alga *Dictyota crenulata*. *Molecules* 13:1253–1262
- Durán R, Zubía E, Ortega MJ, Salvá J (1997) New diterpenoids from the *Dictyota dichotoma*. *Tetrahedron* 53:8675–8688
- El-On J (2009) Current status and perspectives of the immunotherapy of leishmaniasis. *Isr Med Assoc J* 11:623–628
- Fraga J, Montalvo AM, Van der Auwera G, Maes I, Dujardin JC, Requena JM (2013) Evolution and species discrimination according to the *Leishmania* heat-shock protein 20 gene. *Infect Genet Evol* 18: 229–237
- Freile-Pelegrin Y, Robledo D, Chan-Bacab MJ, Ortega-Morales BO (2008) Anti-leishmanial properties of tropical marine algae extracts. *Fitoterapia* 79:374–377
- González U, Pinart M, Rengifo-Pardo M, Macaya A, Alvar J, Tweed JA (2009) Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database Syst Rev* CD004834. doi: 10.1002/14651858.CD004834.pub2
- Gray CA, de Lira SP, Silva M, Pimenta EF, Thiemann OH, Oliva G, Hajdu E, Andersen RJ, Berlinck RGS (2006) Sulfated meroterpenoids from the Brazilian sponge *Callyspongia* sp. are inhibitors of the anti-leishmaniasis target adenosine phosphoribosyl transferase. *J Org Chem* 71:8685–8690
- Grimaldi G Jr, Momen H, Naiff RD, McMahon-Pratt D, Barrett TV (1991) Characterization and classification of leishmanial parasites from humans, wild mammals, and sand flies in the Amazon region of Brazil. *Am J Trop Med Hyg* 44:645–661
- Jongaramruong J, Kongkam N (2007) Novel diterpenes with cytotoxic, anti-malarial and anti-tuberculosis activities from a brown alga *Dictyota* sp. *J Asian Nat Prod Res* 9:743–751
- Kato H, Gomez EA, Cáceres AG, Uezato H, Mimori T, Hashiguchi Y (2010) Molecular epidemiology for vector research on leishmaniasis. *Int J Environ Res Public Health* 7:814–826
- Lainson R, Shaw JJ (2005) New World Leishmaniasis. In: Cox FEG, Wakelin D, Gillespie SH, Despommier DD (eds) Topley & Wilson's microbiology and microbial infections parasitology. Hodder Arnold ASM Press, London, pp 313–349
- Leon LL, Machado GM, Barral A, de Carvalho-Paes LE, Grimaldi Júnior G (1992) Antigenic differences among *Leishmania amazonensis* isolates and their relationship with distinct clinical forms of the disease. *Mem Inst Oswaldo Cruz* 87:229–234
- Lindoso JA, Cota GF, Cruz AM, Goto H, Maia-Elkhoury AN, Romero GA, Sousa-Gomes ML, Santos-Oliveira JR, Rabello A (2014) Visceral leishmaniasis and HIV coinfection in Latin America. *PLoS Negl Trop Dis* 18:e3136. doi:10.1371/journal.pntd.0003136
- Liu M, Hansen PE, Lin X (2011) Bromophenols in marine algae and their bioactivities. *Mar Drugs* 11:1273–1292
- Marra RK, Bernardino AM, Proux TA, Charret KS, Lira ML, Castro HC, Souza AM, Oliveira CD, Borges JC, Rodrigues CR, Canto-Cavalheiro MM, Leon LL, Amaral VF (2012) 4-(1H-Pyrazol-1-yl) benzenesulfonamide derivatives: identifying new active anti-leishmanial structures for use against a neglected disease. *Molecules* 17:12961–12973
- Mayer AMS, Rodríguez AD, Berlinck RGS, Hamann MT (2009) Marine pharmacology in 2005–2006: Marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action. *Biochim Biophys Acta* 1790:283–308
- Mendes G, Soares AR, Sigiliano L, Machado F, Kaiser C, Romeiro N, Gestinari L, Santos N, Romanos MT (2011) In vitro anti-HMPV activity of meroditerpenoids from marine alga *Styopodium zonale* (Dictyotales). *Molecules* 16:8437–8450
- Mikus J, Steverding D (2000) A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. *Parasitol Int* 48:265–269
- Osako K, Teixeira VL (2013) Natural products from marine algae of the genus *Osmundaria* (Rhodophyceae, Ceramiales). *Nat Prod Commun* 8:533–538

- Palumbo E (2009) Current treatment for cutaneous leishmaniasis: a review. *Am J Ther* 16:178–182
- Penicooke N, Walford K, Badal S, Delgoda R, Williams LA, Joseph-Nathan P, Gordillo-Román B, Gallimore W (2013) Antiproliferative activity and absolute configuration of zonaquinone acetate from the Jamaican alga *Styopodium zonale*. *Phytochemistry* 87:96–101
- Pereira HS, Leão-Ferreira LR, Moussatché N, Teixeira VL, Cavalcanti DN, Costa LJ, Diaz R, Frugulhetti IC (2005) Effects of diterpenes isolated from the Brazilian marine alga *Dictyota menstrualis* on HIV-1 reverse transcriptase. *Planta Med* 71:1019–1024
- Pourrajab F, Forouzannia SK, Tabatabaee SA (2012) Novel immunomodulatory function of 1,3,4-thiadiazole derivatives with leishmanicidal activity. *J Antimicrob Chemother* 67:1968–1978
- Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S (2007) Cutaneous leishmaniasis. *Lancet Infect Dis* 7:581–596
- Rocha FD, Soares AR, Houghton PJ, Pereira RC, Kaplan MAC, Teixeira VL (2007) Potential cytotoxic activity of some Brazilian seaweeds on human melanoma cells. *Phytother Res* 21:170–175
- Shalaby EA (2011) Algae as promising organisms for environment and health. *Plant Signal Behav* 6:1338–1350
- Singh S, Sivakumar R (2004) Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother* 10:307–315
- Soares AR, Abrantes JL, Souza TML, Fontes CFL, Pereira RC, Frugulhetti ICDP, Teixeira VL (2007) In vitro antiviral effect of meroditerpenes isolated from the Brazilian seaweed *Styopodium zonale* (Dictyotales). *Planta Med* 73:1221–1224
- Soares DC, Calegari-Silva TC, Lopes UG, Teixeira VL, Paixão ICNP, Cirne-Santos C, Bou-Habib DC, Saraiva EM (2012) Dolabelladienetriol, a compound from *Dictyota pfaffii* algae, inhibits the infection by *Leishmania amazonensis*. *PLoS Negl Trop Dis* 6:e1787. doi:10.1371/journal.pntd.0001787
- Souza ET, Lira DP, de Queiroz AC, Silva DJ, de Aquino AB, Mella EA, Lorenzo VP, de Miranda GE, Araújo-Júnior JX, Chaves MC, Barbosa-Filho JM, de Athayde-Filho PF, Santos BV, Alexandre-Moreira MS (2009) The antinociceptive and anti-inflammatory activities of Caulerpin, a bisindole alkaloid isolated from seaweeds of the genus *Caulerpa*. *Mar Drugs* 7:689–704
- Souza LM, Sasaki GL, Romanos MT, Barreto-Bergter E (2012) Structural characterization and anti-HSV-1 and HSV-2 activity of glycolipids from the marine algae *Osmundaria obtusiloba* isolated from southeastern Brazilian coast. *Mar Drugs* 10:918–931
- Süzgeç-Selçuk S, Meriçli AH, Güven KC, Kaiser M, Casey R, Hingley-Wilson S, Lalvani A, Tasdemir D (2011) Evaluation of Turkish seaweeds for antiprotozoal, antimycobacterial and cytotoxic activities. *Phytother Res* 25:778–783
- WHO (2014) Fact sheet no. 375. Leishmaniasis. World Health Organization, Geneva. <http://www.who.int/mediacentre/factsheets/fs375/en/>. Accessed 28 May 2014