



Original Article

Antischistosomal activity from Brazilian marine algae


 Erika M. Stein^a, Levi P. Machado^b, Henrique K. Roffato^c, Patricia A. Miyasato^c, Eliana Nakano^c, Pio Colepico^d, Daniel X. Andregueti^{a,*}
^a Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil

^b Núcleo de Pesquisa em Ficologia, Instituto de Botânica, Secretaria do Meio Ambiente do Estado de São Paulo, São Paulo, SP, Brazil

^c Laboratório de Parasitologia, Instituto Butantan, Secretaria de Estado da Saúde, São Paulo, SP, Brazil

^d Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Schistosomiasis may be caused by six different species of the genus *Schistosoma*. Current treatment is based only on two drugs: oxamniquine, which is only effective against the *Schistosoma mansoni* species, and praziquantel, which is ineffective against young parasites. Therefore, research on new drugs and their targets for the treatment of this disease is urgently needed. In the present work, the efficacies of several seaweeds extracts against *S. mansoni* were tested. Worm couples were incubated with different concentration of seaweed extracts for 120 h and monitored after the first 2 h and then every 24 h to evaluate death, mobility reduction and couple detachment. The extracts of 13 different seaweed species were tested in a first trial and the active extracts were further evaluated in lower concentrations. The extracts of *Gracilaria ornata* and species belonging to the genera *Dictyota* and *Laurencia* showed activity at relatively low concentrations. The active extracts were analyzed by LC–MS, and possible candidates are proposed.

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Introduction

Schistosomiasis is the most serious form of parasitism by multicellular organisms and still remains on the list of the neglected diseases prioritized by the World Health Organization (WHO, 2015). The disease, also known as bilharzia or snail fever, is caused by an infection with blood flukes of the genus *Schistosoma*. It predominantly affects the poor population, representing one of the main public health problems in more than 70 developing countries. Among the *Schistosoma* species, *Schistosoma mansoni* is the most widely spread in Africa and Latin America. The infection occurs when the host's skin is penetrated by the cercaria, the infectious form of the parasite life cycle. Once inside the host they transform into schistosomula, mature and form couples in the venous system. The egg is responsible for parasite transmission and is also the main cause of the disease symptoms (Gryssels et al., 2006).

The disease affects approximately 240 million people around the world, resulting in an annual mortality rate of 280 000 people. Disease treatment is based on two therapeutic drugs: praziquantel and oxamniquine (WHO, 2015). Praziquantel is ineffective

against young parasites and larval-stage schistosomula, but is effective against one-month-old worms; while oxamniquine is only active against the *S. mansoni* species, but not against other members of the *Schistosoma* genus. Moreover, both drugs face the problem of resistance development in the parasites and side effects. Therefore, further studies on the targets and new drugs are desired.

Due to the ability to produce a wide range of chemicals with sophisticated structures, marine organisms are considered good candidates to provide new drugs with pharmaceutical activities, including chemicals to parasitic diseases (Torres et al., 2014). It is well established that marine and freshwater synthesize important metabolites with economic impact (Cardozo et al., 2006; Gressler et al., 2011a,b; Stein et al., 2011; Andregueti et al., 2013; Machado et al., 2014; Simas-Rodrigues et al., 2015), such as low-weight hydrocarbons, tannins, fatty acids, acetogenins, saponins, phenolic compounds, lignans, alkaloids and terpenoids (Crews et al., 1978; González et al., 1982; Laus, 2001; Li et al., 2011). Specifically, the last five classes cited have been described for their anthelmintic properties.

In this paper, we show the efficacies of 13 macroalgae extracts against *S. mansoni*. The active extracts from the *Gracilaria*, *Dictyota* and *Laurencia* genera were analyzed by LC–MS, and possible candidates are proposed.

* Corresponding author.

E-mail: andregueti@usp.br (D.X. Andregueti).

Materials and methods

Seaweed material

The species *Chondria littoralis* Harvey – SP 428.154, *Dictyota dichotoma* (Hudson) J.V.Lamouroux SP 469.031, *Dictyota menstrualis* (Hoyt) Schnetter, Hörnig & Weber-Peukert – SP 469.032, *Plocamium brasiliense* (Greville) M.Howe & W.R.Taylor – SP 428.163 and *Spyridia hypnoides* (Bory de Saint-Vincent) Papenfuss – SP 428.171 were collected at Ubu beach, Anchieta – ES while the species of *Laurencia catarinensis* Cordeiro-Marino & Fujii – SP 400.209, *Laurencia dendroidea* J.Agardh – SP 400.198 (designated as (a)) were collected at Castelhanos beach, Guarapari – ES in October 2011. The above seaweed identification was performed by Dr. Mutue T. Fujii from Instituto de Botânica and MSc. Erika M. Stein. The vouchers of each species were placed in Herbário Maria Eneida P. Kauffman Fidalgo at Instituto de Botânica, São Paulo (SP). *Padina gymnospora* (Kützting) Sonder – 18.849, *Gracilaria ornata* Areschoug – 37.629, *L. dendroidea* J.Agardh – 18.852 (designated as (b)), *Ochtodes secundiramea* (Montagne) M.A.Howe – 18.851, *Dictyota mertensii* (Martius) Kützting – SP 469.156 and *Pterocladia capillacea* (S.G.Gmelin) Santelices & Hommersand – 18.853 were collected at Baleia beach, Vitória – ES in July 2013, and the identification was performed by Dr. Levi P. Machado and a voucher of each specie was deposited in the herbarium VIES at the Universidade Federal do Espírito Santo.

Extraction procedure

Extracts were prepared by using different procedures. *C. littoralis*, *D. dichotoma*, *D. menstrualis*, *P. brasiliense* and *S. hypnoides* were freeze-dried, powdered and extracted with supercritical CO₂, with the addition of 4% ethyl alcohol as a modifier solvent. The extractions were performed for 3 h at 45 °C and a pressure of 280 bar with a CO₂ flow of 12 ml/min. The species *L. catarinensis* and *L. dendroidea* (b) were also freeze-dried and powdered, and different extracts were obtained by the crescent order of solvent polarity: first hexane, followed by chloroform and then methanol, three times each for 24 h. The species *P. gymnospora*, *G. ornata*, *L. dendroidea* (a), *O. secundiramea*, *D. mertensii* and *P. capillacea* were freshly extracted with a 2:1 mixture of methylene chloride: methanol, at a proportion of 10 ml/g of seaweed with stirring for one week at a temperature of 20 °C.

Schistosomicidal screening

The life cycle of *S. mansoni* (Samboon, 1907) (Trematoda: Schistosomatidae) (BH strain – Belo Horizonte, MG, Brazil) was maintained in *Biomphalaria glabrata* (Say, 1818) (Gastropoda: Planorbidae) snails and *Mesocricetus auratus* (Waterhouse, 1839) (Mammalia: Cricetidae) hamsters. Female hamsters were infected by subcutaneous injection of 300 cercariae, and six weeks later, *S. mansoni* adult worms were recovered by perfusion of the rodent's portal and mesenteric system. The worms were washed in RPMI 1640 medium (Invitrogen), pH 7.5, supplemented with sodium bicarbonate (2000 µg/ml), penicillin (100 UI/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) and 10% fetal bovine serum (Gibco BRL). Adult worm pairs (male and female) were transferred to each well of a 24-well culture plate containing 1 ml of the medium. The seaweeds extracts were dissolved in DMSO (dimethyl sulfoxide, at 1.5%), diluted in 1 ml of RPMI medium and added to the cultured worms to achieve a final concentration of 500 µg/ml. The parasites were kept for 120 h and monitored after the first 2 h and then every 24 h under a light microscope to evaluate motor activity and the mortality rate. The worms were considered to be dead when no movement was observed. RPMI

1640 with 1.5% DMSO was used in the negative control group, and 4.8 µM (1.5 µg/ml) praziquantel (PZQ) was used in the positive control group. The experiments were carried out in five replicates and repeated at least two times. The active extracts in the assay were re-tested at a lower concentration of 100 µg/ml.

UFLC–MS analysis

The analysis were carried out using an UFLC system (Shimadzu, USA) consisting of a solvent delivery pump (Model LC-20AD) and auto sampler (SIL-20Aht) with a 100-µl loop, degasser (DGU 20A3r) and column oven (CTO-20A) followed by a Bruker ESI-microTOF II mass spectrometer. System operation was performed using otof-Control and HyStar V.3.2, while data collection and analysis were performed using Compass DataAnalysis V.4.1. Gradient elution was performed on a Phenomenex® Luna 3µ C18(2) 100A column (150 mm × 2.0 mm) at 30 °C. Mobile phase A consisted of a aqueous solution of 20 mM NH₄Ac and 0.1% formic acid adjusted to pH 6.4, and solution B consisted of acetonitrile with 0.1% formic acid. Separations were effected by a gradient elution program as follows: solution B was maintained at 0% from 0 to 2 min, followed by a linear change from 0% to 100% from 2 to 75 min, B was keep isocratic at 100% from 75 to 80 min. Solution B was changed to 0% at 80.1 min and was held constant until 90 min. The mobile phase flow rate was 0.2 ml/min, and the injection volume was 10 µl. Mass detection was performed scanning the *m/z* between 50 and 1100 in positive mode with the following parameters: capillary 4500 V; end plate offset –500 V; nebulizer 2.0 Bar; dry heater 180 °C; and dry gas at a flow of 8 l/min.

Results

The active extracts from the *Gracilaria*, *Dictyota* and *Laurencia* genera displayed high activity against *S. mansoni* in comparison with the other extracts. Table 1 summarizes the extraction methods and *in vitro* effects of all of positive tested seaweed extracts, which have been classified into two main groups, red algae (Rhodophyta) and brown algae (Heterokontophyta). As previously mentioned, the extracts displaying activity at 500 µg/ml exposure, were considered to be active and subjected to further testing at a lower concentration (100 µg/ml). The killing time (24, 72 and 120 h) was recorded and Table 1 shows the 100% mortality rate. The supercritical extracts of the red algae *P. brasiliense*, *C. littoralis* and *S. hypnoides* were active only at the higher concentration (500 µg/ml) tested. The macroalgae *P. capillacea*, *O. secundiramea* and *L. dendroidea* extracted with dichloromethane/methanol (2:1), *L. dendroidea* extracted with methanol, and *L. catarinensis* extracted with chloroform did not present anthelmintic activity (data not shown).

The red macroalgae *P. brasiliense*, *S. hypnoides* and *C. littoralis* submitted to supercritical extraction were active only at the higher concentrations (Table 1). Interestingly that *L. dendroidea* and *L. catarinensis* submitted to hexane extraction display distinct results against schistosoma worms. *L. catarinensis* hexanic extracts kill the worms in 24 h and *L. dendroidea*, killed the worms in females in 24 h and males in 72 h. In addition, chloroformic extract of *L. dendroidea* was also effective against male after 120 h incubation. *G. ornata* submitted to the dichlorometane/methanol extraction displays anthelmintic activity (100 µg/ml) only against male worms in after 120 h.

As observed in Table 1, the brown algae, the three *Dictyota* species showed activity at the two concentrations tested. The supercritical extract of *D. menstrualis* kills very effectively both male and female after 24 h exposure. Additionally, *D. dichotoma* submitted to supercritical extraction kills

Table 1

List of seaweeds utilized for screening, extraction methods and antischistosomal activity of the extracts at 500 and 100 µg/ml.

Seaweed	Extraction method	Antischistosomal activity at 500 µg/ml	Antischistosomal activity at 100 µg/ml
<i>Heterokontophyta</i>			
<i>Dictyota dichotoma</i>	Supercritical CO ₂ (4% ethanol)	Active	72 h
<i>Dictyota menstrualis</i>	Supercritical CO ₂ (4% ethanol)	Active	24 h
<i>Dictyota mertensii</i>	Dichloromethane/methanol 2:1	Active	72 h ♂ 120 h ♀
<i>Rhodophyta</i>			
<i>Plocamium brasiliense</i>	Supercritical CO ₂ (4% ethanol)	Active	–
<i>Spyridia hypnoides</i>	Supercritical CO ₂ (4% ethanol)	Active	–
<i>Gracilaria ornata</i>	Dichloromethane/methanol 2:1	Active	120 h ♂
<i>Chondria littoralis</i>	Supercritical CO ₂ (4% ethanol)	Active	–
<i>Laurencia dendroidea</i> (b)	Hexane	Active	72 h ♂ 24 h ♀
	Chloroform	Active	120 h ♂
<i>Laurencia catarinensis</i>	Hexane	Active	24 h

Note: The time shown in the table is related with the couple worm death time, unless the symbols ♂ for male and ♀ for female parasites are indicated.

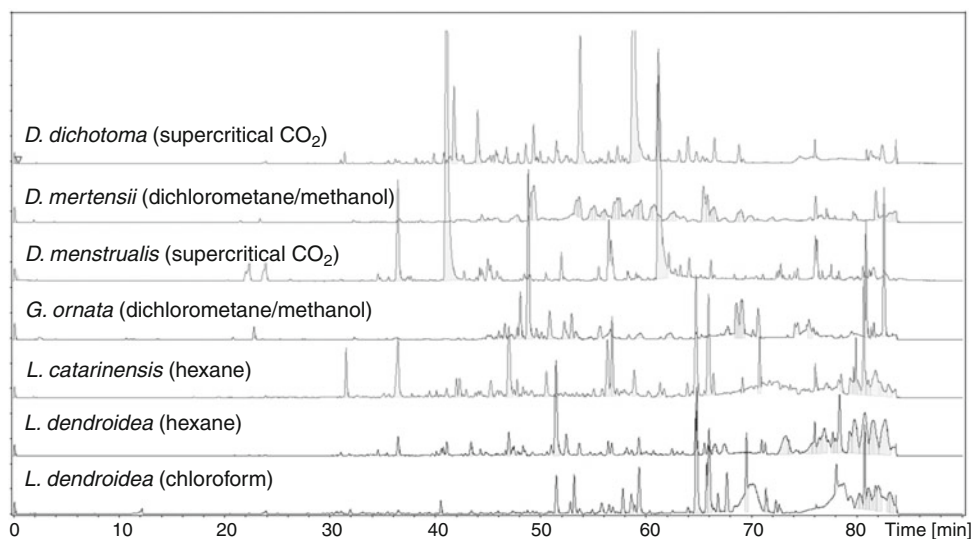


Fig. 1. Chromatograms obtained for the active seaweed extracts against *S. mansoni*. The X-axis represents the retention time for each peak.

the schistosome worms after 72 h. Surprisingly, *D. mertensii* extracted with dichloromethane/methanol kills male after 72 h and female after 120 h incubation. *P. gymnospora* extracted with dichloromethane/methanol did not show anthelmintic activity (data not shown).

In order to compare the chemical composition of the active extracts, samples were submitted to chromatographic analysis applying a UFLC–MS system. Fig. 1 shows extract chromatograms generated from the total ion chromatogram (TIC) mode, where the signal peaks correspond to the sum of each mass detected.

The chromatograms were manually analyzed to identify similar compounds present in different samples. The chromatograms were stacked to standardize the retention times, and the mass spectra

of similar peaks were analyzed individually. It was assumed that the same chemical structure was present in samples when a similar mass spectrum was detected at a similar retention time for both extracts. Table 2 summarizes common *m/z* values obtained in different extract with similar retention times.

Among the *m/z* values detected in more than one extract, we found an $[M+H]^+$ ion at *m/z* 391.283 in five extracts, with a retention time of 76.1 min, indicating a similar secondary metabolite in the extracts of *D. menstrualis*, *D. dichotoma*, *L. catarinensis* and *L. dendroidea* (hexanic and chloroformic). As expected, similar *m/z* values were derived from species belonging to the same genus, *Laurencia*, even among different species or with different extraction methods. However, this pattern did not hold for the *Dictyota* genus, where

Table 2Common $[M+H]^+$ ion at *m/z* values related to each seaweed species analyzed by UFLC–MS in different retention times.

Seaweed extract	Retention time								
	36.1	51.5	64.8	66.1	67.7	72.6	76.1	80.9	81.1
<i>D. dichotoma</i>							391.283	494.564	
<i>D. mertensii</i>									
<i>D. menstrualis</i>	181.123					284.294	391.283		
<i>G. ornata</i>					609.268			494.564	
<i>L. catarinensis</i> EH	181.123		386.328	386.328		284.294	391.283		687.521
<i>L. dendroidea</i> EH	181.123	335.060	386.328				391.283	494.564	
<i>L. dendroidea</i> EC		335.060	386.328	386.328	609.268	284.294	391.283		687.521

EH: hexanic extract; EC: chloroformic extract.

only one metabolite ($[M+H]^+$ ion at m/z 391.283) was common between the species *D. dichotoma* and *D. menstrualis*. Comparing the three genera – *Dictyota*, *Laurencia* and *Gracilaria* – one common metabolite $[M+H]^+$ ion at m/z 494.564 was detected at 80.9 min. The dichloromethane/methanol extract of *D. mertensii* was the only sample that shared no similar metabolites with the other species. A comparison of the extracts from *D. menstrualis* and hexanic extracts from both *L. catarinensis* and *L. dendroidea* – the best three candidates from the antischistosomal screening – identified one common metabolite with a $[M+H]^+$ ion at m/z 181.123 at 36.1 min.

Discussion

Over the past few decades, research on natural sources has identified chemical compounds with important pharmacological roles in medicinal fields. With regard to marine sources, some compounds have shown unique biological properties, in part due to their singular chemical identity, and are currently employed as new therapeutic agents, either unmodified or as prototypes for the design and synthesis of new drugs (Eustáquio et al., 2011; Cherigo et al., 2015). Seaweeds from the phyla of Rhodophyta and Heterokontophyta have led to the discovery of a wide variety of secondary metabolites belonging to different chemical classes, such as terpenoids (monoterpenes, sesquiterpenes, diterpenes, triterpenes, etc.) and their variants (cyclic, acyclic, halogenated) (Paul et al., 1988; La Barre et al., 2010), alkaloids (Güven et al., 2010), steroids (Francavilla et al., 2013), saponins (Jeeva et al., 2012), lignans, and phenolic compounds (Keyrouz et al., 2011).

In recent years, considerable efforts have been dedicated to develop alternative approaches to treat schistosomiasis. In a recent publication, Barbosa de Castro et al. (2013) compiled several therapeutic candidates from natural sources, including alkaloids, flavonoids, saponins, phenolic compounds and terpenoids. Therefore, the presence of those chemical classes in seaweeds justifies the importance of this study.

In antischistosomal assays, the most significant results were obtained when the parasites were exposed to extracts obtained from seaweed that belonged mainly to the three genera, *Dictyota*, *Gracilaria* and *Laurencia*. A commonality among these three genera is the terpenoid compounds (Peres et al., 2012), which may play an important role against *S. mansoni*. Nevertheless, there remains a possibility that the antischistosomal activity can be mediated by another molecule.

The active extracts were analyzed chromatographically in search of a common metabolite among all of the seaweeds. From the three most active extracts, defined for the total mortality reached in a short period of time (24 h) – *D. menstrualis*, *L. dendroidea* and *L. catarinensis* – a common $[M+H]^+$ ion was found at m/z 181.123, which may coincide with the molar weight of a sesquiterpene or even a conjugated monoterpene (Al-Massarani, 2014). Among all of the active extracts, the most common peak separated at 76.1 min and showed a $[M+H]^+$ ion at m/z 391.283, which is compatible with the molar weight of a sesterterpene or a conjugated diterpene (Hirano et al., 2001).

Overall, the most active extracts were obtained by non-polar extraction solvents, such as hexane, chloroform, dichloromethane or supercritical CO₂, and the pattern of the mass spectra (data not showed) obtained for the extracts revealed an abundance of low weight molecules (i.e., 95% of all m/z are under 500 Da for *D. menstrualis*) in all of the active extracts. This leads to our hypothesis that terpenes and/or their variants, such as mono-, di-, tri-, sesqui- or sesterterpenes, may be a promising source of new molecules with activity against *S. mansoni* and represent therapeutic alternatives to treat the disease.

Authors' contribution

DXA contributed to all aspects of this study. EMS and LPM contributed to the seaweed collection, identification, preparation and analysis of the extracts and assays results. HKR and PAM contributed to the biological studies under the supervision of EN, who also contributed to the design of the biological studies. PC supervised all aspects of the study and contributed to design of the study and the critical reading of the manuscript. All of the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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