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Antileishmanial activity of extracts from a brown seaweed *Bifurcaria bifurcata* the atlantic coast of Casablanca (Morocco)

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ABSTRACT

In this work we performed antileishmanial activity for hexane, ethereal, chloroform and methanolic extracts from seaweed *Bifurcaria bifurcata*, the extracts obtained by Soxhlet extraction system yields excerpts represent about 1 % to 8 % by weight of the raw alga. The antileshmanien activity was carried out in vitro by MTT colorimetric method (3 - (4,5- dimethylthiazol -2- yl) -2,5- diphenyltetrazolium Bromide) on *Leishmania infantum* parasites. Meanwhile, a test was performed cytotoxicity Brine Shrimp larvae of *Artemia salina* to measure the power of toxicity of the extracts studied. The overall results show that the products extracted from seaweed *Bifurcaria bifurcata* antileishamnien show remarkable activity, where the IC_{50} concentrations of 50% inhibition, respectively, of various extracts of 46.83 µg/mL at 63.83 µg/mL, and the median lethal dose of cytotoxicity test of less than 40.46 µg/mL. These results may be subject to the application of the products obtained from seaweed *Bifurcaria bifurcata* in treating leishmaniasis.

KEYWORDS

Brown alga;
Bifurcaria bifurcate;
Extraction;
Antileishamnien activity;
Cytotoxicity test.

INTRODUCTION

The marine environment and the organisms that live there are infinite sources of active molecules to original chemical structure. These compounds are synthesized by methods different from those observed in terrestrial pathways. Among the marine organisms, algae, which are most often fixed to a substrate, have developed chemical defenses to prevent colonization by other species, including microorganisms[1].

The use of seaweed for therapeutic purposes is far from a new phenomenon. If the active seaweed extracts used in pharmacy principles are few, the current scientific works are significant. Thousands of compounds have been identified. These are polysaccharides, lipids, or other phenolic metabolites[2], terpene[3-4]. The operations described are primarily various antibacterial activities[5], antifungal[6], antiviral[7] and for other activities.

On the other hand, leishmaniasis is a very common disease in the world. It affects many species of mammals, including humans. In some embodiments the spontaneous evolution without treatment is fatal in a few months. In Morocco, as in most Mediterranean coun-

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tries, leishmaniasis is an important public health problem[8]. Whether or zoonotic anthroponotic, cutaneous or visceral, these conditions are well represented, from the mountains of the Rif to the palm perarides foothills of the Anti-Atlas[8-9]. where the objective of this work is to develop extracts of seaweed Bifurcaria bifurcata the Atlantic coast of Morocco in the anti-leishmanien activity by MTT assay in vitro vis-respect to Leishmania infantum. Thus, the cytotoxicity test was performed Brine Shrimp larvae of Artemia salina to measure the power of the extracts studied toxicity.

MATERIALS AND METHODS

Harvest seaweed

The brown alga Bifurcaria bifurcata was harvested at the beach Florida - Dar Bouaaza (south of Casablanca MOROCCO) in the period of low tide (June 2013). After harvest, the seaweed are washed and are dried for one day at room temperature and arbitrary light, then they are dried in an oven at 60 ° C for 72 h.

Preparation of extracts

The extract used in this work by a Soxhlet extraction system. It allows for continuous extraction of the solid - liquid by spraying cycles - condensation of the solvent. This method has the advantage of being easy to use the extracts obtained from solvents of increasing polarity, such as: Hexan, ether, chloroform and methanol.

Antileishmanial activity

The MTT method used is that described by A. Dutta (2005)[10], this is a colorimetric technique, which allows determining the antileishmanial activity of promastigote forms of Leishmania. The principle of the test is based on the conversion of a tetrazolium salt MTT (3 - (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) to formazan by enzymatic reduction. In short, it contains the tetrazole ring is reduced by mitochondrial succinate dehydrogenase active formazan in living cells. The product is a colored formazan (dark purple), insoluble in water and the intensity of this color is proportional to the number of living cells present in the test well as their metabolic activity.

The antileishmanial evaluations were performed on cultures of Leishmania infantum promastigotes. Promastigotes were maintained at 25 ° C by subculturing every five days using the RPMI 1640 phenol red-free, buffered with 25 mM $NaHCO_3$ (pH = 7.2) and supplemented with 20% fetal bovine serum (FBS). The parasites were incubated in culture flasks with a starting concentration of 5 x 10^5 parasites per mL[11]. The parasites were counted on Thoma cell, then divided into a 96-well ELISA plate at a concentration of $2x10^5$ parasites / well in 90 µL of RPMI medium. The test products are dissolved in DMSO 1% (to final concentrations of 20, 30, 40 and 50 µg/mL) and the solvent control (100 µL of parasitic cultivation + l0 µL DMSO) are then added in triplicate in wells for an additional 10 µL volume. After 72 hours incubation at 25 ° C was added a volume 10 µL of MTT (5 mg / mL) and the plates are returned to the incubator. After 1 hour incubation at room temperature and 3 hours at 37 ° C the developed purple color, the plates were centrifuged at 2000 rpm for 5 min and was added to a volume of 200 µL DMSO. The absorbance of each well was measured at 492 nm on an ELISA spectrophotometer[11].

The determination of the concentration value of 50% inhibition IC_{50} is determined by linear interpolation of the curves showing percent viability based on the logarithm of concentration tested.

It is noted that the percentage of viability is given by the relationship:

$$\% \text{ Viability} = \frac{ABS_{Control} - ABS_{Sample}}{ABS_{Control}} \times 100$$

Cytotoxicity test

The cytotoxicity test Brine Shrimp used in this work is described by P.Vanhaecke in 1981[12]. Briefly, this test determines the toxic activity of the products tested their effects on the larvae of brine shrimp: Artemia salina. It also allows for determining the median lethal dose LD50 which can determine the power of toxicity compared to other products of references. Test samples are dissolved in 2% DMSO, with concentrations of 20, 40, 60 and 100 µg / ml and, immediately determined volumes of the prepared solution are added to tubes containing the larvae of Artemia salina. The tubes are placed in a chamber at room temperature and the results were read after 24 hours is by counting under a

dissecting microscope. If the lamp contains dead larvae, the percentage mortality is corrected using the following formula:

$$\%\,M = \frac{NLP}{NLT} \times 100$$

With; % M : Percent mortality; NLP : Number of dead larvae in the Presence of the Product; NLT : Number of dead larvae in the Presence of Control (solvent).

RESULTS AND DISCUSSION

Extraction

After harvesting and drying seaweed Bifurcaria bifurcata four products extracted H, E, C and M are obtained from successive extractions 500g biomass soxhlet with solvents of increasing polarity: hexane, the ether, chloroform and methanol. Once the products were obtained, it was determined their colors and returns relative to the initial amount of dry seaweed. Data for the products obtained are given in the TABLE 1.

TABLE 1 : The different extracts obtained *Bifurcaria bifurcata*

Sample	Color	weight (g)	Yield (%)
H	dark green	20.23	4.04
E	Green - yellow	4.6	0.92
C	green	7.16	1.43
M	brown	43.28	8.66
Marc (*)	brown	422	84.4

(*) (After evaporation)

Antileishmanial activity

Extracts H, E, C and M obtained from the brown alga Bifurcaria bifurcata were tested at concentrations 20, 30, 40 and 50 µg/mL. Thus, a positive control value of percentage viability of Leishmania in the concentration function are given in TABLE 2. From this table, it is seen that the percentage viability with increasing concentration. This is well illustrated by the curves of Figure 1. Note that the extract M has no antileishmanial activity. The values of the concentrations of 50% inhibition IC_{50} obtained from the linear function expressions of viability of logarithm of the concentration tested are shown in TABLE 3, all the results obtained show that the extracts H, E and C have remarkable activity,

TABLE 2 : Results of the antileishmanial activity

Extract	Concentration ($\mu g.mL^{-1}$)	ABS_{Sample}	$ABS_{Control}$	Viability (%)
H	20	0.536	0.579	7.309
	30	0.436	0.579	24.604
	40	0.420	0.579	27.453
	50	0.206	0.579	64.431
E	20	0.534	0.579	7.654
	30	0.493	0.579	14.762
	40	0.430	0.579	25.669
	50	0.232	0.579	59.942
C	20	0.559	0.579	3.338
	30	0.540	0.579	6.676
	40	0.431	0.579	25.553
	50	0.316	0.579	45.381
M	20	n.d	0.579	-
	30	n.d	0.579	-
	40	n.d	0.579	-
	50	n.d	0.579	-

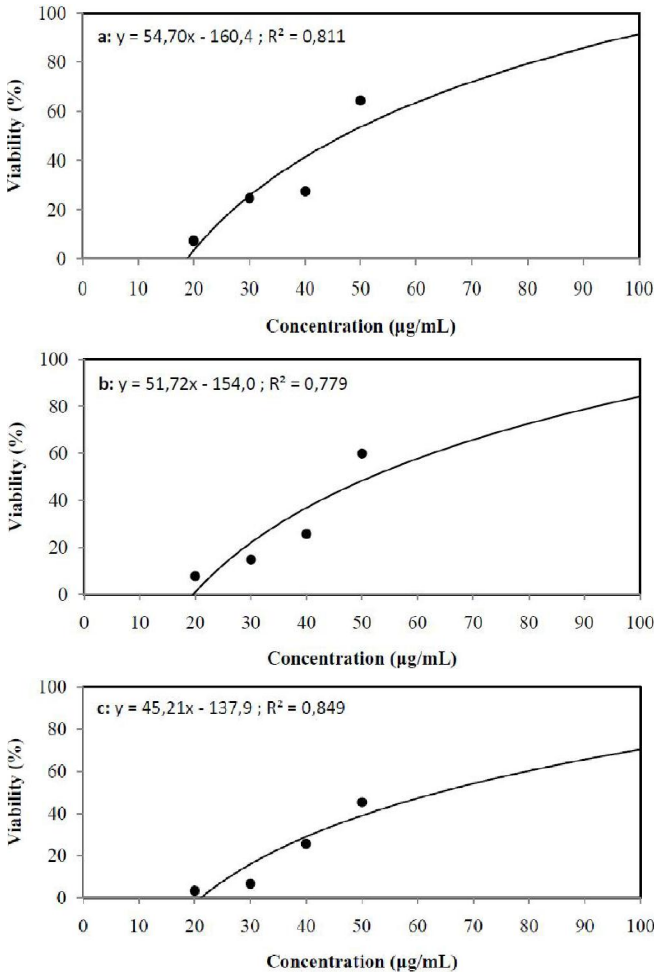

Figure 1 : Viability curves depending on the concentration of antileishmanial activity (a: extract H, b: extract E, c: extract C)

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where the values of concentrations of 50% inhibition IC_{50} extracts H, E and C are of the order: 46.83 µg/mL, 51.64 µg/mL and 63.83 µg/mL.

The values obtained compared to other organic extracts of marine algae (TABLE 4) show that extracts *Bifurcaria bifurcata* had interesting activities compared to extracts *perindusiata Padina* and *Sargassum fluitans*, they are similar to extract *Lobophora variegata* (IC_{50} = 49.9 µg/mL) and lower extracts *Bostrychia tenella* activities (4.3 <IC_{50}< µg/mL), *Dictyota caribaea* (IC_{50} = 24.4 µg/mL) and *Turbinaria turbinata* (IC_{50} = 10.9 µg/mL).

TABLE 3 : IC_{50} extracts *Bifurcaria bifurcate*

Extract	IC_{50} (µg/mL)
H	46.83
E	51.64
C	63.83
M	n.d
Amphothéricine B[12]	0.24

n.d.: not detected

TABLE 4 : IC_{50} values of the antileishmanial activity of the organic extracts of some algae

Algae	*Leshmania species*	IC_{50} (µg/mL)	Reference
Bostrychia tenella	*L. amazonensis*	4.3 – 22.2	[13]
Dictyota caribaea	*L. mexicana*	24.4	[14]
Lobophora variegata	*L. mexicana*	49.9	[14]
Padina perindusiata	*L. mexicana*	> 100	[14]
Sargassum fluitans	*L. mexicana*	> 100	[14]
Turbinaria turbinata	*L. mexicana*	10.9	[14]

TABLE 5 : Results of the cytotoxicity test Brine Shrimp

Extract	Concentration (µg.mL^{-1})	NLT	NLP	% M
H	20	10	5	50
	40	10	6	60
	60	10	8	80
	100	10	9	90
E	20	10	4	40
	40	10	5	50
	60	10	5	50
	100	10	7	70
C	20	10	2	20
	40	10	6	60
	60	10	8	80
	100	10	9	90
M	20	10	0	0
	40	10	0	0
	60	10	0	0
	100	10	0	0

Cytotoxicity test

The extracts were tested at 20, 40, 60 and 100 µg/mL. The values of the percentages of mortality of larvae in the concentration function are given in TABLE 5.

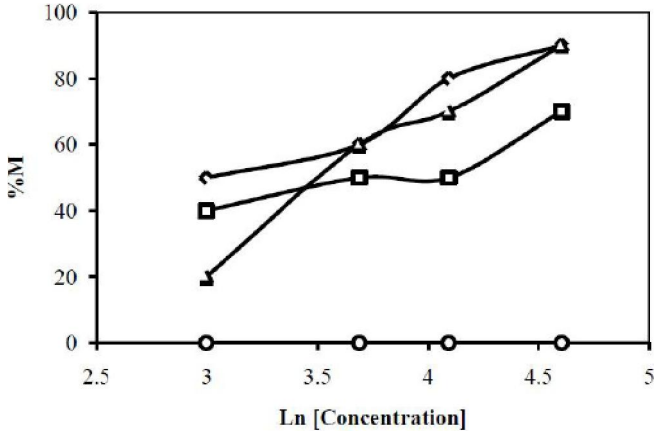

Figure 2 : Curve representing the percentage of mortality as a function of concentration (◇: extract H, □: extract E, △: extract C, ○: Extract M)

From this table it follows that the percentage of larval mortality increases as a function of the concentration. This is well illustrated by the curves shown in Figure 2 which show the percentages of mortality according to logarithm of the concentrations. The values of LD_{50} lethal doses 50 different extracts obtained by different linear expressions are shown in TABLE 6. Toxicity extracts H, E and C of Bifurcaria bifurcata compared to the other products with respect to larvae of Artemia salina shows that all the samples are less than podophyllotoxin (2.4 µg/mL) and greater than digitalis (77.2 µg/mL) and strychnine sulfate (151 µg/mL). Against by the methanol extract of Bifurcaria bifurcata showed no cytotoxic activity.

TABLE 6 : LD_{50} values of extracts *Bifurcaria bifurcate*

Extract / Product	LD_{50} (µg/mL)
H	21.8
E	40.46
C	37.05
M	n.d
Podophyllotoxin	2.4[15]
Digitalin	77.2[15]
Strychnine sulphate	151[15]

n.d.: not detected

CONCLUSION

The overall results obtained in this work show that the H, E and C have significant values for antileishmanial activity and low toxicity compared to the cytotoxicity test products so they have a positive outlook, which proves the use of this algae in several pharmaceutical and biological applications and this by the study of different organic fractions of the algae which will be shown capable of providing biologically active molecules.

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