SUPPLEMENTARY MATERIAL

Antimicrobial and antiparasitic activities of three algae from the northwest coast of Algeria

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Abstract: The objective of this study was to investigate the biological activities of Algerian algae, *Sargassum vulgare*, *Cladostephus hirsutus* and *Rissoella verruculosa*. Antimicrobial activity of the crude extracts and their fractions was assessed using the disc diffusion assay, the minimum inhibitory concentration and the minimum bactericidal concentration. Antiparasitic activity was studied *in vitro* against the blood stream forms of *Trypanosoma brucei brucei* and the intraerythrocytic stages of *Plasmodium falciparum*. Ethyl acetate (EA) fractions of the three tested algae showed more potent antimicrobial activity against *S. aureus* (7-14.5 mm) and *B. cereus* (7-10.75 mm), MIC values ranged from 0.9375 to 7.5 mg mL^{-1} and MBC values >15 mg mL^{-1}. Concerning the antiparasitic activity, EA factions of *S. vulgare* (IC_{50} = 9.3 µg mL^{-1}) and *R. verruculosa* (IC_{50} = 11.0 µg mL^{-1}) were found to be more effective against *T. b. brucei*, whereas the three EA fractions were little active against *P. falciparum*.

Keywords: *Sargassum vulgare*; *Cladostephus hirsutus*; *Rissoella verruculosa*; antimicrobial activity; antiparasitic activity.

Experimental

Algal materials

Algae used in this study were *S. vulgare*, *C. hirsutus* and *R. verruculosa*. They were collected by handpicking from Ain Timouchent region situated in the Northwest Coast of Algeria (Latitude 35°18′ N, Longitude 1°28′ O) during June 2013. Samples collected were initially washed thoroughly with sea water to remove epiphytes and other dirt particles and transported to the laboratory immediately in plastic bags containing water to prevent evaporation, then rinsed with tap water followed by distilled water to remove salts, and shade dried at room temperature. Dried algae were ground in an electric mixer and stored at 4 °C for further experiments. Algal species were identified by Dr. BENGUEDDA Wacila from the University of Tlemcen (Algeria) according to Fischer et al. (1987) , and voucher specimens are deposited in Natural Products Laboratory Herbarium of Tlemcen University (Algeria).

Preparation of algal crude extracts and fractionation

Powdered samples (100 g) were extracted by using a Soxhlet apparatus with 500 mL methanol:chloroform (2:1 V/V) for 6 h, according to Duan et al. (2006). Extraction was repeated twice and the combined organic solution was evaporated under vacuum to give a crude extract, which was dissolved in 100 mL of 90% aqueous methanol. First fractionation was carried out with 3 × 100 mL petroleum ether. The aqueous methanol solution was evaporated under reduced pressure to give a semisolid, which was further dissolved in 200 mL distilled water and then extracted successively with 3 × 100 mL of ethyl acetate, 3 × 100 mL of dichloromethane and 3 × 100 mL of n-butanol, respectively. Resulting fractions were evaporated to dryness, to yield the petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), butanol (BOH) fractions, and aqueous residue (AR).

Antimicrobial activity

Microbial strains

The *in vitro* antimicrobial activity of algal crude extracts and their fractions was assessed against three representative Gram-positive strains *Bacillus cereus* (ATCC 10876), *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 9341), three Gram-negative strains *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853)

and *Klebsiella pneumoniae* (ATCC 700603) and one yeast strain *Candida albicans* (ATCC 10231).

Preparation of inoculums

Test organisms were grown in Brain Heart Broth (Fluka Analytical) and incubated for 24 h at 37 °C. Cell suspensions were diluted to OD_{625} of 0.08 - 0.1 to obtain initial cell count of about 10^8 CFU mL^{-1} for bacteria and OD_{530} of 0.12 - 0.15 to obtain about 1 - 5 x 10^6 CFU mL^{-1} for yeast.

Antimicrobial assay

Antibacterial activity was carried out using the disc diffusion assays described by Boulekbache-Makhlouf et al. (2013), with minor modifications. Petri plates were prepared with 20 mL of sterile Mueller Hinton agar for bacteria and 20 mL of Sabouraud Dextrose Agar for fungi. Test cultures were uniformly inoculated with a sterile swab on the surface of appropriate solid media and allowed to dry for 10 min at room temperature. Three different concentrations (50, 100 and 200 mg mL^{-1}) of crude extracts and fractions were prepared in DMSO and loaded on sterile filter paper discs (6 mm in diameter, corresponding to 0.5, 1, 2 mg per disc, respectively), which were placed on the inoculated plates. A disc prepared in the same condition with only the corresponding volume of pure DMSO was used as a negative control. The inoculated plates were stored at 4 °C for 2 h to allow metabolite diffusion and then incubated at 37 °C during 24 h for bacteria and at 30 °C during 48 h for fungi. In the case of positive antimicrobial activity, the halo diameter around the disc was measured by using a luler and expressed in millimeters. Cefotaxime (CTX, 30 µg per disc), ampicillin (AMP, 10 µg per disc) and tetracycline (TE, 30 µg per disc) were used as positive controls against bacteria while nystatin (Ny, 30 µg per disc) was used as reference antifungal drug against yeast. Assays were performed at least in three independent experiments.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC values were determined for EA fractions that presented the highest activity in the agar disc diffusion test using microdilution method as described by Okusa et al. (2007), with some modifications. Ethyl acetate fractions of among tested algae were dissolved in DMSO (30

mg/250 µL) and diluted to 5 mL with Muller Hinton Broth, the final DMSO concentration being 5%. This solution was transferred in 96-wells plates (200 µL/well) and serially diluted (base 2 logarithmic dilutions) with Muller Hinton Broth. 24 h cultures of microbial strains were stirred with 0.9% NaCl to achieve 0.5 McFarland (10^8 cells mL^{-1}), diluted for 1/100 to achieve 10^6 cells/mL, and inoculated in the 96-wells plates (100 µL/well). The cultures were incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of extract showing no visible growth on the agar plates after incubation time. The MBC was determined by subculturing samples from the wells with concentrations above the MIC on fresh solid medium and further incubated at 37 °C for 18-24 h. The lowest concentration of MIC tubes with no visible bacterial growth on solid medium was regarded as MBC. Experiments were carried out under sterile conditions and performed at least in duplicate.

Antiparasitic activity

Ethyl acetate fractions, which were found to be the most active in antimicrobial activity, were assessed for their antiparasitic activities against *Trypanosoma brucei brucei* and *Plasmodium falciparum.*

Antitrypanosomal activity

Bloodstream forms of *T. b. brucei* strain 93 were cultured in HMI9 medium supplemented with 10% foetal calf serum at 37 °C under an atmosphere of 5% CO_2. In all experiments, log-phage cell cultures were harvested by centrifugation at 3.000 x g and immediately used. Drug assays were based on the conversion of a redox-sensitive dye (resazurin) to a fluorescent product by viable cells (Bosc et al. 2012). Drug stock solutions were prepared in pure DMSO. T. b. brucei bloodstream forms ($3x10^4$ cells mL^{-1}) were cultured as described above in 96-well plates (200 µL per well) either in the absence or in the presence of different concentrations of EA fractions with a final DMSO concentration that did not exceeded 1%. After a 72 h incubation, resazurin solution was added in each well at the final concentration of 45 µM. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths after a further 4 h incubation. The percentage of inhibition of parasite growth rate was calculated by comparing the fluorescence of parasites maintained in the presence of drug to that of in the absence of drug. DMSO was used as a control. The concentration causing 50% growth inhibition (IC_{50}) was obtained from the drug concentration-response curve. IC_{50} value is the

mean +/- the standard deviation determined from at least three independent experiments. Pentamidine was used as anti-trypanosomal drug control.

Antiplasmodial activity

The chloroquine-resistant strain FcB1/Colombia of *Plasmodium falciparum* was maintained *in vitro* on human erythrocytes in RPMI 1640 medium supplemented by 8% (v/v) heat-inactivated human serum, at 37 °C, under an atmosphere of 3% CO_2, 6% O_2 and 91% N_2. The *in vitro* antiplasmodial activity was evaluated by the radioisotopic method (Desjardins et al. 1979). This method determines the inhibition of parasite growth in culture in the presence of various concentrations of molecules by measuring the incorporation of [^{3}H] hypoxanthine into parasite nucleic acids. Assays were performed in 96-well plates as described by Labaied et al. (2004), EA fractions were prepared in DMSO at 50 mg mL^{-1} and serially diluted with culture medium before being added to asynchronous parasite cultures (1% parasitemia and 1% final hematocrite, 200 µL final volume per well) in 96-well microplates. Plates were maintained for 24 h at 37 °C. 0.5 µCi of [^{3}H] hypoxanthine was then added to each well, and parasites were maintained for another 24 h. Plates were frozen and thawed. Cell lysates were then collected onto glass-fiber filters and counted in a liquid scintillation spectrometer. Growth inhibition was determined by comparison of the radioactivity incorporated into the treated culture with that in the control culture maintained on the same plate. IC_{50} values were obtained from the drug concentration-response curve and the results were expressed as the mean values ± standard deviations determined from three independent experiments. Chloroquine was used as antimalarial drug control.

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Table S1. Antimicrobial activity of crude extracts and fractions of *S. vulgare*, *C. hirsutus* and *R. verruculosa* by agar disc diffusion (inhibition of growth expressed as mm diameter of inhibition zone).

Crude extracts and fractions		mg/disc	Inhibition zone diameter [a] *B. cereus* ATCC 10876	*S. aureus* ATCC 6538	*M. luteus* ATCC 9341	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 8739	*K. pneumoniae* ATCC 700603	*C. albicans* ATCC 10231
S. vulgare		0.5	6	7.5±0.4	6	6	6	6	6
	CE	1	6	8.75±0.9	6	6	6	6	6
		2	6	9.25±0.5	6	6	6	6	6
		0.5	6	7.25±0.3	6	6	6	6	6
	PE	1	6	7.25±0.3	6	6	6	6	6
		2	6	7.75±0.3	6	6	6	6	6
		0.5	7±0.0	8.37±0.7	6	6	6	6	6
	EA	1	7.16±0.2	9.62±0.4	6	6	6	6	6
		2	9±1.4	10.37±0.7	6	6	6	6	6
		0.5	7±0.0	7±0.0	6	6	6	6	6
	DCM	1	7.16±0.2	7±0.0	6	6	6	6	6
		2	7.33±0.5	7.83±0.2	6	6	6	6	6
	BOH	0.5-2	6	6	6	6	6	6	6
	RA	0.5-2	6	6	6	6	6	6	6
C. hirsutus		0.5	7.5±0.3	10±0.0	6	6	6	6	6
	CE	1	8.0±0.0	10.5±0.7	6	6	6	6	6
		2	8.5±0.7	10.5±0.7	6	6	6	6	6
		0.5	7.25±0.3	7±0.0	6	6	6	6	6
	PE	1	7.5±0.0	7±0.0	6	6	6	6	6
		2	7.5±0.0	7.5±0.7	6	6	6	6	6
		0.5	9.75±0.3	12±0.7	6	6	6	6	6
	EA	1	10.75±0.3	13±0.0	6	6	6	6	6
		2	10.75±0.3	14.5±0.7	6	6	6	6	6
		0.5	7±0.0	9±0.7	6	6	6	6	6
	DCM	1	7±0.7	9.5±0.7	6	6	6	6	6
		2	7±0.7	9.5±0.7	6	6	6	6	6
	BOH	0.5-2	6	6	6	6	6	6	6
	RA	0.5-2	6	6	6	6	6	6	6
R. verruculosa		0.5	6	7±0.0	6	6	6	6	6
	CE	1	6	7±0.0	6	6	6	6	6
		2	6	8±0.0	6	6	6	6	6
	PE	0.5-2	6	6	6	6	6	6	6
		0.5	7±0.0	8.5±0.7	6	6	6	6	6
	EA	1	7±0.0	9.5±0.7	6	6	6	6	6
		2	8±0.0	10.5±0.7	6	6	6	6	6
		0.5	7±0.0	7.75±0.7	6	6	6	6	6
	DCM	1	7±0.0	8.5±0.7	6	6	6	6	6
		2	7±0.0	9.25±0.3	6	6	6	6	6
	BOH	0.5-2	6	6	6	6	6	6	6
	RA	0.5-2	6	6	6	6	6	6	6
	AMP	10µg/disc	6	18	6	Nt	6	6	Nt
	CTX	30µg/disc	6	30	40	18	31	40	Nt
	TE	30µg/disc	21	33	29	13	31	29	Nt
	Ny	30µg/disc	Nt	Nt	Nt	Nt	Nt	Nt	16
	DMSO		-	-	-	-	-	-	-

CE: Crude extract; PE: Petroleum ether fraction; EA: Ethyl acetate fraction; DCM: Dichloromethane fraction; BOH: n-butanol fraction; AR: Aqueous residue; [a]The diameter of the filter paper discs (6 mm) is included; AMP: Ampicillin; CTX: Cefotaxime;TE: Tetracycline; Ny: Nystatin; DMSO : Dimethyl sulfoxide; - : Indicating no activity; Nt: Not tested; Values presented are the means +/- Standard deviations of three independent experiments.

Table S2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate fractions of the three algae.

	Algae	*S. aureus* ATCC 6538		*B. cereus* ATCC 10876	
		MIC (mg mL^{-1})	**MBC (mg mL^{-1})**	**MIC (mg mL^{-1})**	**MBC (mg mL^{-1})**
EA	*S. vulgare*	1.875	>15	3.75	>15
	C. hirsutus	0.937	>15	1.875	>15
	R. verruculosa	3.75	>15	7.5	>15

EA: Ethyl acetate fraction; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

Table S3. Antitrypanosomal activity of ethyl acetate fractions of the three algae.

Parasite	EA of algae	IC_{50} µg mL^{-1}
T. brucei brucei	*S. vulgare*	9.3±4.9
	C. hirsutus	27.2±5.0
	R. verruculosa	11.0±1.1
	Pentamidine (nM)	5.4±0.4

EA: Ethyl acetate fraction; IC_{50}: Concentration inhibiting 50% of parasite growth; Mean and standard deviation (SD) were determined from at least three independent experiments.