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Full Length Research Paper

Antibacterial, antioxidant and cytotoxic activities of extracts from the thermophilic green alga, *Cosmarium* sp.

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Unicellular green algal strains, identified as *Cosmarium* (*Chlorophyta*), were isolated from Aïn-Echeffa hot spring in north Tunisia. Different extracts (methanol, hexane, acetone, acetone: methanol and water) obtained from both biomass and extracellular polysaccharides (EPS) were evaluated for their antibacterial, antioxidant and cytotoxic activities. First, extracts were tested *in vitro* for eventual antibacterial activities against a collection of Gram positive and negative bacteria. Most extracts (biomass and EPS) showed significant antibacterial effects, with minimum inhibitory concentrations (MIC) ranging from 28 to 85 µg/ml for biomass and 50 to 150 µg/ml for EPS. Moreover, based on the capacity of each sample to scavenge the ABTS radical cation, we revealed that the EPS aqueous extract presented a moderate antioxidant activity (24.97%). Finally, the toxicity of the biomass extracts was evaluated using the brine shrimp *Artemia salina*, as test organism. All extracts were identified as non-toxic (LC_{50} > 400 µg/ml).

Key words: *Cosmarium*, biomass, extracellular polysaccharides (EPS), cytotoxicity, antibacterial, antioxidant.

INTRODUCTION

Survival and growth of microalgae living in extreme natural environments (habitats characterized by extreme levels of temperature, salinity, pH or mineral deficiency or excess) is an interesting topic from both biochemical and physiological points of view (Fogg, 2001). However, little is known about the mechanisms allowing algal adaptation to such extreme conditions. In general, microalgal species might survive in stressed environments as a result of two different processes: physiological adaptation, usually resulting from modifications of gene expression; and, if appropriate genetic variability is available, classic evolutionary changes occur in populations subjected to a consistent stress, due to genetic mutations that confer tolerance (Bradshaw and Hardwick, 1989; Fogg, 2001). Several microalgae produce bioactive metabolites in response to the ecological pressure. As a consequence of the increasing demand in new drugs from natural products, there is, nowadays, a greater interest towards microalgae of extreme environments, as they are able to produce a wide range of active substances with antibacterial, antiviral, antifungal, enzyme-inhibiting, cytotoxic and antioxidant activities (Ghasemi et al., 2004).

Microalgae living in geothermal waters might be of particular interest in Tunisia, where there are not less than 70 hot springs across the country, mainly in the north-east (N-E), north-west (N-W) and south (Ben Dhia and Meddeb, 1990). These geothermal waters are used directly for agricultural irrigation in the south of the country; whereas, in the N-E and N-W, they are widely utilized to support establishments for therapeutic hot bathing. It should be noted that, in the last decades, geothermal waters have been also exploited in geo-

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thermal energy production (as a source of heat) (Chaibi and Bourouni, 2005).

The *Cosmarium* genus is a freshwater member of *Chlorophyta* phylum (green algae), *Desmidiales* order (desmids) (Brook, 1981). There are more than 1000 species of this genus (Gerrath, 1993), most of them are cosmopolitan, with more than 400 found in North America. Although, this algal genus is more frequent in acidic, oligotrophic, aquatic habitats, it may also be found in sub-aerial environments, alkaline, eutrophic ponds and lakes, and in thermal waters (Wehr and Sheath, 2003).

As a member of the desmids order, *Cosmarium* species secrete significant amounts of extracellular polymeric substances (EPS) that form an extensive sheath, external to the cell wall; and function in adhesion and ensheathment within the biofilm complex. Kiemle et al. (2007) showed that desmids EPS had significant uronic acid (3 to 29%) and protein (2 to 10%) content and polysaccharides sulfated to various degrees. Recently, several studies (Daneshavar et al., 2007; Srinivasan and Viraraghavan, 2010) were conducted on *Cosmarium* species, from the standpoint that they represent a biomaterial potentially useful for biological treatments, such as the decolorization of textile dye. However, there is presently, an increasing interest in these algae, as a natural source of bioactive molecules. In this context, Abdo et al. (2012a, b) studied the antibacterial and antiviral activities of *Cosmarium*, from non thermal sources; and did not detect significant antibacterial or antiviral activities. Taking into consideration the lack of studies dealing with the biological activities of thermophilic strains of *Cosmarium* species, the aim of this study was to evaluate a set of antibacterial and antioxidant activities and the toxicity of aqueous and organic extracts from biomass and EPS of *Cosmarium* sp. sampled from hot springs in Tunisia.

MATERIALS AND METHODS

Microalgae

Cosmarium sp. was isolated from geothermal water samples collected in “Aïn-Echeffa”, in the N-E of Tunisia (36° 49’ N, 10° 34’ E). Samples were identified as *Cosmarium* sp., based on microscopic morphological traits (Kanetsuna, 2004). *Cosmarium* was grown in standard flasks containing a Bold’s growth medium (Bischoff and Bold, 1963) at 60°C, under 20 $\mu mol.m^{-2}.s^{-1}$ light intensity, in order to obtain a stock algal culture to be used during the experiments. Aqueous and organic extracts were prepared from lyophilized biomass and EPS.

Biomass and EPS extraction

The extraction of soluble compounds from *Cosmarium* biomass was performed using a soxhlet, with methanol, acetone and hexane as solvents. Organic solutions of acetone : methanol (1:1) were then prepared. For the water-soluble biomass fraction, a mixture of 10 ml distilled water and 40 mg of biomass, was sonicated (30 W, 10 x 30 s), stirred at 4°C during 2 h, and then centrifuged (4000 rpm, 20 min, 4°C, 320R, Hettich Zentrifugen Universal, Germany). The extract was finally concentrated using a freeze dryer (Telstor Lyoalfa 6, Spain). All the concentrated biomass extracts were weighed and new extract solutions were prepared by addition of 1% dimethyl sulfoxide (DMSO). Extracts were preserved at 4°C.

The released EPS were purified, following the method described by Trabelsi et al. (2009). The *Cosmarium* culture, at the stationary phase, was filtered through millipore filters with a 25 µm pore diameter. The resulting supernatant was filtered again through a Whatman filter paper no. 2 (Whatman International Ltd., UK), to separate the culture medium from the cells. The resulting filtrate, which contained the culture medium and the released EPS was concentrated using a tangential ultrafiltration cell (Millipore, USA) and low-molecular-weight substances were eliminated by washing membranes with deionised water. Finally, the purified EPS were freeze-dried and fractionized, using the same solvents previously used for biomass extraction. Following extraction, EPS were concentrated, using a Rotavapor RE100 (Bibby, France).

Antibacterial bioassay

Antibacterial activity of the different extracts of biomass and EPS (methanol, hexane, acetone, 1:1 acetone : methanol and water) was assayed qualitatively by the paper disk agar diffusion method, following Ghasemi et al. (2007), then quantitatively by the determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), as described in Challouf et al. (2011). In order to determine the mechanism of antibiosis, in active extracts, the ratio of MBC/MIC was calculated. As described in Nurul et al. (2010), the extract is considered as bactericidal when MBC/MIC≤2; bacteriostatic when 2<MBC/MIC<16; and ineffective when MBC/MIC≥16. Three Gram+ (*Micrococcus luteus* NCIMB8166, *Staphylococcus aureus* ATCC25923 and *Staphylococcus epidermis* NCIMB885) and 3 Gram- (*Pseudomonas aeruginosa* ATCC27853, *Escherchia coli* ATCC25922 and *Salmonella typhimurium* LT2) bacterial strains were used as test organisms. All strains were kindly provided by the “Laboratoire d’Analyse et de Contrôle des Polluants Chimiques et Microbiologiques de l’Environnement” (Faculty of Pharmacy of Monastir, Tunisia).

Cytotoxicity assay with brine shrimp larvae

Only biomass extracts displaying antibacterial activity were screened for toxicity with larvae (nauplii) of *Artemia salina* (brine shrimp) as described by Solis et al. (1993). The extracts were dissolved in seawater. Water-insoluble extracts were primarily dissolved in dimethyl sulfoxide (DMSO; final concentration, 1%). The test was performed in triplicate in microwell plates, with extract concentrations of 1000, 100 and 10 µg/ml. *Artemia* eggs were incubated for 48 h in artificial seawater. The nauplii were collected and brought into contact with the test substances. After 24 h of incubation at room temperature, the number of surviving nauplii in each well was determined. As controls, *A. salina* nauplii were submitted to 95% ethanol (100% lethality) and seawater containing 1% DMSO (100% survival). The 50% lethal concentrations (LC_{50}) of the extracts were determined. The number of survivors was counted and the percentage of death was calculated. Larvae were considered dead when they did not exhibit any internal or external movement during several seconds of observation. Values of LC_{50} that were greater than 100 µg/ml were considered to represent an inactive compound or extract (Moshi et al., 2009).

Antioxidant assay

The antioxidant capacities of biomass and EPS extracts were

Table 1. Antibacterial activity of *Cosmarium* sp. biomass extracts against bacterial strains, based on the inhibition zone diameter (DIA), minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC).

Extract	Parameter	*M. luteus NCIMB8166*	*S. aureus ATCC25923*	*S. epidermis NCIMB885*	*E. coli ATCC25922*	*S. typhimurium* LT2	*P. aeruginosa ATCC27853*
Acetone : methanol	DIA*	19.5±3.5	14.5±0.7	13.5±0.7	8.5±0.7	-	-
	MIC	42.5	28	28	28	-	-
	MBC	>85	>85	>85	>85	-	-
Methanol	DIA	-**	-	-	-	-	-
	MIC	-	-	-	-	-	-
	MBC	-	-	-	-	-	-
Acetone	DIA	13.5±2.12	-	12.5±0.7	8±0	-	-
	MIC	85	-	42.5	28	-	-
	MBC	>85	-	>85	>85	-	-
Hexane	DIA	-	-	11±1.4	8.5±0.7	-	-
	MIC	-	-	85	28	-	-
	MBC	-	-	>85	>85	-	-
Water	DIA	19.5±2.12	23.5±0.7	20±8.4	-	14.5±2.12	-
	MIC	85	85	85		85	-
	MBC	>85	>85	>85		>85	-
Kanamycine (1 mg/disk)***	DIA	34.9±0.14	35.5±0.7	29.7±0.2	34.5±0.7	30.1±0.14	12.5±0.7

Mean diameter of inhibition zone ± SD (mm)/microorganism strain; **no activity; ***positive control.

evaluated, using the Trolox equivalent antioxidant activity (TEAC) method, which measures the ability of a compound to scavenge $ABTS^{\cdot+}$ radical (Re et al., 1999).

RESULTS

Antibacterial activity

The tested biomass extracts exhibited different levels of antibacterial activities against the used microorganism strains (Table 1). All extracts, except methanol, were active against at least one bacterial strain. For active extracts, there was no differential inhibition, based on the bacterial type (Gram+ or -). The aqueous extract was characterized by the largest active scope; against four bacterial strains (Table 1), in association with comparatively high diameters of inhibition, in particular against *M. luteus* (≈20 mm), *S. aureus* (>20mm) and *S. epidermis* (>20mm). All active extracts were characterized by values of MBC, equal to or higher than 85 µg/ml.

Results of the paper disk agar diffusion assay, MIC and MBC, for the different extracts of EPS, are illustrated in Table 2. All EPS extracts showed an inhibitive effect against at least one bacterial strain. The acetone : methanol (1:1), hexane and aqueous extracts presented an antibacterial effect only on Gram +ve strains, whereas methanol and acetone extracts were active regardless of the Gram+/- criterion. The acetone extract had the largest active scope; against four bacterial strains (Table 2). In most cases, values of MIC were equal to or higher than 150 µg/ml, with the exception of the acetone extract (50 µg/ml against *E. coli*). MBC values were equal or higher than 150 µg/ml. Besides, the acetone extract was the unique fraction that expressed clear bactericidal effects against both *S. typhimurium* (MBC/MIC = 1) and *E. coli* (MBC/MIC = 3).

Cytotoxicity assay

LC_{50} values were as follows: water (406 µg/ml), acetone (438 µg/ml), hexane (500 µg/ml) and methanol: acetone (500 µg/ml). All the LC_{50} values were greater than 100 µg/ml. Consequently, water, acetone, hexane and methanol: acetone extract were considered inactive against brine shrimp.

Antioxidant activity

When compared with Trolox, percentages of inhibition of

Table 2. Antibacterial activity of *Cosmarium* sp. EPS extracts against 6 bacterial strains based on the inhibition zone diameter (DIA), minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC).

Extract	Parameter	*M. luteus NCIMB8166*	*S. aureus ATCC25923*	*S. epidermis NCIMB885*	*E. coli ATCC25922*	*S. typhimurium* LT2	*P. aeruginosa ATCC27853*
Acetone : methanol	DIA*	17.5±3.5	14.5±0.7	11±0	-	-	-
	MIC	150	150	150	-	-	-
	MBC	>150	>150	>150	-	-	-
Methanol	DIA	29±1.4	-	-	-	13.5±2.12	-
	MIC	150	-	-	-	150	-
	MBC	>150	-	-	-	>150	-
Acetone	DIA	10±0	-	10±1.4	8.5±0.7	14.5±0.7	-
	MIC	150	-	150	50	150	-
	MBC	>150	-	>150	150	150	-
Hexane	DIA	-	-	12±0	-	-	-
	MIC	-	-	150	-	-	-
	MBC	-	-	>150	-	-	-
Water	DIA	-	24±5.6	-	-	-	-
	MIC	-	150	-	-	-	-
	MBC	-	>150	-	-	-	-
Kanamycine (1 mg/disk)***	DIA	34.9±0.14	35.5±0.7	29.7±0.2	34.5±0.7	30.1±0.14	12.5±0.7

Mean diameter of inhibition zone ± SD (mm)/microorganism strain; **no activity; ***positive control.

most extracts (Table 3) were quite low (<20%). Although, the aqueous EPS extract displayed a slightly higher percentage (24.97%), it remained moderate when compared with Trolox.

DISCUSSION

In recent years, the efficiency of several antimicrobial drugs has decreased, as a consequence of phenomena of drug-resistance expressed by numerous pathogen strains (Kandhasamy and Arunachalam, 2008). Therefore, enhancing research efforts towards the identification of new natural antimicrobial substances would be of great interest for the establishment of alternative therapies in difficult handling infections, such as diarrhea, mastitis, abortion and upper respiratory complications, usually caused by *Escherichia, Staphylococcus*, *Salmonella* and *Pseudomonas*. Recent trends in drug research from natural sources have shown that algae are promising organisms to furnish novel biochemically active compounds (Cardozo et al., 2007). The main active substances biosynthesized by algae include vitamins, pigments, fatty acids, sterols and polysaccharides (Cardozo et al., 2007).

In the present investigation, extracts obtained by methanol, acetone, hexane, acetone : methanol and water, exhibited variable degrees of antimicrobial activity on the tested bacterial strains, regardless of their Gram classification. Eloff (1998) reported that soxhlet extraction of dried materials only work well for compounds that can withstand the high temperature; but cannot be used for thermolabile compounds as it will affect their biological activity. Therefore, the antibacterial active extracts identified from *Cosmarium* sp. in our study, probably contain thermostable compounds.

Abdo et al. (2012a, b) screened *Cosmarium* isolated from fresh water sources in Egypt, for antibacterial activities and found that methanolic and aqueous extracts had lower antibacterial activity, as compared to other species investigated (*Spirulina*, *Chrococcus*, etc.). In our study, the biomass aqueous extract and the EPS acetone extract were clearly active against a wide range of bacterial strains, making them interesting to purify and characterize the corresponding active principles, in the future. The fact that *Cosmarium* strains from thermal sources (case of our study) exhibited higher activity than strains living in non thermal habitats (Abdo et al., 2012a, b) was expected because expression of active metabolites represents usually a physiological and

Table 3. Percentage of ABTS·+ reduction, for different crude biomass and EPS extracts.

Parameter	Inhibition (%)
Reference solution	
Trolox	72.66
Biomass fractions	
Methanol	17.94
Acetone	2.77
Hexane	5.11
Acetone : methanol	3.42
Water	13.07
EPS fractions	
Methanol	10.71
Acetone	4.64
Hexane	2.34
Acetone : methanol	11.25
Water	24.97

evolutionary response to stressful conditions (Bradshaw and Hardwick, 1989; Fogg, 2001).

As for antioxidant activity, water extract from EPS showed the highest free $ABTS^{\cdot+}$ scavenging activity. Nevertheless, this same extract was characterized by an antibacterial activity only against *M. luteus*. Several similar studies have demonstrated that various EPS released by different algae, bacteria and fungi are potent antioxidants (Asker et al., 2009; Leung et al., 2009).

Conclusion

This study shows that the crude extracts of *Cosmarium* sp. biomass and excreted EPS, exhibit antibacterial activity against Gram +ve and -ve bacteria. Besides, results of brine shrimp lethality show that biomass extracts are nontoxic. The present study confirms the traditional therapeutic use of hot springs, demonstrating that some thermophilic microalgae (here *Cosmarium* sp.) are potential sources of bioactive compounds, and thus, should be investigated for natural antibiotics. To achieve this goal, the present study will be further extended to identify and purify the corresponding active compounds.

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