RESEARCH ARTICLE

Cytotoxic Activity of Different Extracts from Brown Marine Macroalgae from the El Jadida Region (Morocco)

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ABSTRACT

The therapeutic use of extraordinary virtues of algae in healthcare is very ancient and has evolved throughout the history of humanity. However, low or high doses and/or prolonged administration of algae-derived biologically active substances may trigger adverse or even harmful effects such as hemolytic properties.

In this study, hemolytic activities of the brown algae *Cystoseira myriophylloides (F/Sargassaceae)* from El Jadida coast (Moroccan Atlantic coast) on human erythrocyte were investigated.

The toxicity of chloroform-methanolic, methanolic, chloroformic, isoprpanolic and butanolic extracts and fractions derived from *Cystoseira myriophylloides* on human erythrocytes was measured by *in vitro* hemolytic assay.

The results obtained show variable cytotoxicity of C. *myriophyloides* against the red blood cells. Indeed, the crude extract has a fairly high hemolytic activity that is very marked when the erythrocytes are incubated in the presence of the chloroformic extract. However, the cytotoxic activity is greatly increased in the presence of the butanolic fraction derived by partitioning of the isopropanolic extract. Moreover, the hemolysis activity is dose-dependent and depending on the evolution of incubation time. Thus, these results show that the majority of extracts and fractions of brown macroalgae *Cystoseira myriophylloides* manifest hemolytic activity.

Keywords: Hemolytic activity, Moroccan coast, Macroalgae, *Cystoseira myriophylloides*, Algal extracts, and fractions.

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INTRODUCTION

The understanding of the healing properties of algae and their use has rapidly spread around the world. Indeed, algae are widely used as a food source[1] and in traditional Chinese medicine for their pharmacological activities.[2]

The Moroccan coasts have an undeniable wealth in terms of diversity and quantity of macroalgae. The city of El Jadida is classified among the Moroccan maritime sectors which are characterized by an important specific richness. Furthermore, seaweed has been found as producers of compounds and substances to protect themselves from the exposure of external environmental factors (UV radiation, pollution, and mechanical stress). Some of those algal-derived natural products such as phenolic compounds, alkaloids, terpenes, saponins, steroids, and polysaccharides have received considerable attention in recent years due to their various biological properties.[3] Numerous reports show macroalgae to possess a broad spectrum of biological activities such as antiviral,[4] antiinflammatory, anticoagulant, antibacterial,[5,6] antitumor,[7] and antioxidant activities.[8] Those active compounds can act at different levels.[9,10]

However, these chemical substances, conferring their therapeutic power on the algae, may have a hemolytic or anti-hemolytic effect on human erythrocytes, at low or high doses and/or prolonged administration. Algal-derived biologically active substances may trigger adverse or even harmful effects such as hemolytic anemia.[11] It is therefore essential to determine their hemolytic power for rational adaptation, especially in the case of non-integrity of digestive mucous membranes (mouth, stomach, intestine), and to study the cytotoxic effect of these algae. Moreover, the toxicity of a bioactive molecule is a key factor in drug design, and hemolytic activity provides the

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first information on the interaction between molecules and biological entities at the cellular level. The hemolytic activity of a compound is, therefore, an indicator of its cytotoxicity towards the cells.[12]

Many species of *Sargassaceae* family were reported to have a broad range of biological activities such as antibacterial, antifungal, enzymatic and anti-tumor activities in *Cystoseira tamariscifolia*,[13] antioxidant activity in *Cystoseira crinita*.[14]

To our knowledge, the hemolytic activity of this macroalgae family has never been evaluated. Therefore, we thought that it would be of interest to evaluate the cytotoxic and hemolytic activities of extracts and fractions from the seaweed *Cystoseira myriophylloides* collected from the Moroccan Atlantic coast.

Our study is part of the program of the valorization of Moroccan marine natural resources; it aims to screen biological activities in macroalgae from Moroccan Atlantic coast to isolate and characterize bioactive products that were potentially used in therapeutics and cosmetics.

MATERIAL AND METHODS

Algal material

The algal material was collected by hands at low tide on the coast of Sidi Bouzid (3 km south of El Jadida) GPS coordinates (33° 09'-33° 16'N, 8° 30'-8° 45' W) in April 2015. Collected samples are first washed on-site with seawater to remove epiphytes and all other foreign matters. In the laboratory, the samples are rinsed with running water then washed with distilled water.

After taxonomic identification, the algae are photographed and voucher specimens are deposited in the herbarium (algothèque) of our laboratory of Biotechnology and Valorization of Plant Resources: Algae and Plants of the Faculty of Sciences, Chouaib Doukkali University, El Jadida, Morocco.

The sorted sample is frozen at – 80 °C and freeze-dried using a lyophilizer (Free Zone Plus 2.5 liters, 7644 2900 model, Labconco corporation).

Erythrocyte suspension

An erythrocyte suspension is prepared from human blood taken from healthy patients after their consent. The red blood cells are separated from the plasma by centrifugation at 2400 rpm for 10 min. After two washes, the cell pellet is re-suspended in a volume of PBS (phosphate buffered saline) identical to the volume of plasma removed. The erythrocyte suspension obtained is diluted 20 times in PBS.

Methodology

Preparation of algal extracts

Part of the lyophilized sample is milled, and the resulting powder is extracted with different solvents, chloroform-methanol (2:1, V/V), methanol, chloroform and isopropanol-water (70:30, V/V) with constant stirring for 1 hour at room temperature. After filtration, the residue is extracted a second time by ultrasound for 15 minutes using the same solvent. The extract obtained is filtered under the same conditions, as previously mentioned. The resulting filtrates were pooled and concentrated under reduced pressure (temperature ≤45 ° C) using a rotary evaporator (Buchi R-3000).

The isopropanolic extract was further partitioned with butanol to obtain a water-soluble fraction and a butanolic soluble fraction (BF).

The concentrated sample was reduced to powder by lyophilizing and stored in a desiccator until use.

Hemolytic activity test

In vitro hemolytic activity was performed by spectrophotometer method.[15] A volume of the cell suspension was mixed with of the algal extracts at different concentrations in 40% DMSO. The mixtures were incubated at 37°C at different times. After incubation, the mixture was centrifuged at 1500 rpm for 10 min. The free hemoglobin in the supernatant was measured in UV-Vis spectrophotometer at 548 nm. Two controls were prepared without extracts; the negative control where the erythrocyte suspension was supplemented with 40% DMSO (minimal hemolysis), while the positive control corresponds to the erythrocyte suspension supplemented with a 10% triton X100 solution (maximal hemolysis). Each experiment was performed in triplicate at each concentration.

The percentage of the hemolysis activity of the extracts was calculated according to the following formula:

x 100

At: absorbance at 548nm of the test sample at 60 min.

An: absorbance at 548nm of the control (negative control at 60 min)

Ac: absorbance at 548nm of the control (positive control at 60 min)

Statistical Analysis

All the tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). The results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

RESULTS

Hemolytic activity was evaluated on an erythrocyte suspension of human blood. The hemolytic effect in the presence of different concentrations of the different extracts derived from the algae was measured in UV-Vis spectrophotometer at 548 nm.

The activity was evaluated initially on the crude extract (CM extract) at different concentrations (Figure 1).

The addition of the CM extract of *C. myriophylloides* in the reaction medium is manifested by an increase in the absorbance, due to the lysis of red blood cells, in comparison with the negative control. This is clearly visible for the concentrations 50 and 100 mg/mL depending on the period of time. This absorbance is the result of the presence of hemoglobin in the reaction medium.

To target the hemolytic effect, taking into account the nature of the substances present in the extract tested (the polar and apolar metabolites), a sequential fractionation by

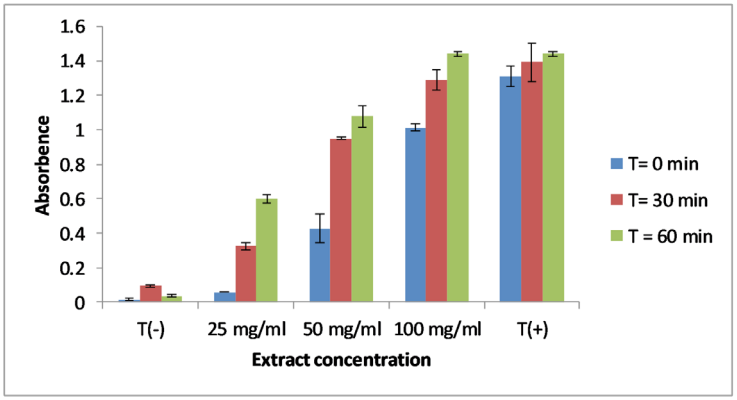

Figure 1: Hemolytic activity (Hemolysis rate) of chloroform-methanolic extract (CM) from *C. myriophylloides* tested at various concentrations and various time of incubation.
T (-): negative control; T (+): positive control. n = 3.

solvents was carried out to identify the fractions responsible for this effect.

At first, we tested the methanolic extract (MC) on blood cells; this extract has a very weak hemolytic effect (Figure 2). At 100 mg/mL, the hemolysis rate is 1.66% relative to the positive control (Table 1).

On the other hand, the presence of the chlorofomic extract (CH) results in an increase in the optical density from the concentration of 25 mg/mL reflecting significant lysis of the red blood cells (Figure 3).

The rate of hemolysis induced in the presence of the CH extract is more significant than in the presence of the CM extract. In fact, at 25 mg/ml, the rate of hemolysis increases from 39.27 to 95.14% in the presence of CM and CH extract, respectively (Table 1).

Several studies have shown a positive correlation between extracts containing compounds such as saponins and the hemolytic effect.[16] In this sense, we carried out an extraction to enrich our extracts in this type of compounds. The algal powder was extracted first with hexane. The resulting powder

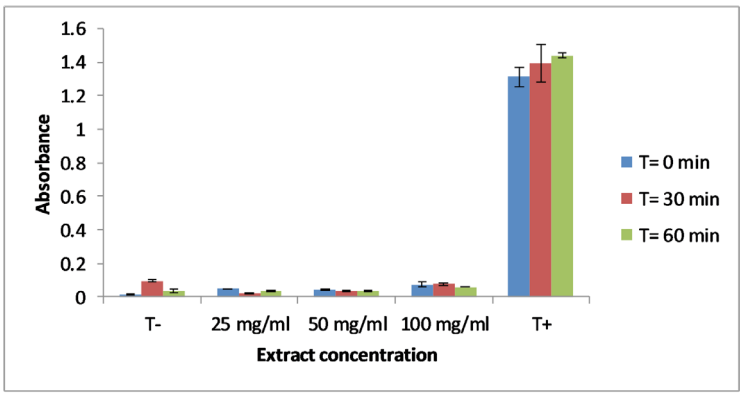

Figure 2: Hemolytic activity (Hemolysis rate) of methanolic extract ME) from C. *myriophylloides* tested at various concentrations and various time of incubation.
T (-): negative control; T (+): positive control. n = 3.

Table 1: Hemolytic activity (Hemolysis rate) of the different concentrations of *C. myriophyloides* extracts tested at various concentrations and various time of incubation (in percentage relative to the positive control (T +).

	Hemolysis rate (%)					
Nature of the extraction solvent	*20 (mg/mL)*	*25(mg/mL)*	*30(mg/mL)*	*50(mg/mL)*	*100(mg/mL)*	*T (+)*
Methanol/Chloroform Blend	NT	39.278	NT	72.241	97.432	97.502
Méthanol	NT	-0.243	NT	0.069	1.666	97.502
Chloroform	NT	95.142	NT	100.139	104.788	97.502
Isopropanol	NT	3.331	NT	7.911	27.272	97.502
Butanol	28,765	NT	42.644	NT	NT	97.502

NT = Not Tested; T (+) = positive control

were then extracted with isopropanol-water (70:30; v/v) like the same procedure described above. The isopropanolic extract (IP) obtained was further partitioned with butanol to obtain partial separation in two fractions, a water-soluble fraction and a butanolic soluble fraction.

The IP extract has a slight hemolytic effect at a concentration of 100mg/ml after 60 min of incubation (Figure 4). Indeed, the absorbance is 0.429 nm. The percentage of hemolysis is 27.27% compared to the positive control (97.50%) (Table 1).

On the other hand, the butanolic fraction (BF) reveals a hemoglobin release in the incubation medium reflecting an interesting hemolytic effect (Figure 5), at the concentration of 30 mg/mL after 60 min of incubation. The accorded absorbance is 0.650 nm, and the percentage of hemolysis is 42.62% compared to the positive control (97.50%) (Table 1).

DISCUSSION

Morocco is a country strongly influenced by the sea. In fact, the Moroccan coasts contain a vast algal diversity possessing a large repertoire of chemical compounds associated with various biological properties in various fields of interest for humans (food, cosmetic, human health, agri, and horticultural sectors).[17] Marine algae are of growing interest in the biomedical field, particularly thanks to their bioactive substances which have shown great potential for anti-inflammatory, antimicrobial, antiviral and anti-tumor drugs.[18]

The *Sargassaceae* family has a broad spectrum of biological activity: antibacterial, antifungal, enzymatic, anti-tumor, antioxidant and antiviral activity.[4,5,7,13,14]

Nowadays, this algal richness remains insufficiently exploited, and to our knowledge, no study has been conducted

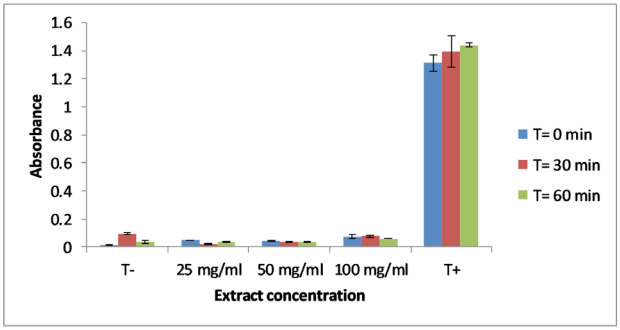

Figure 3: Hemolytic activity (Hemolysis rate) of chloroformic extract (CH) from *C. myriophylloides* tested at various concentrations and various time of incubation. T (-): negative control; T (+): positive control. n = 3.

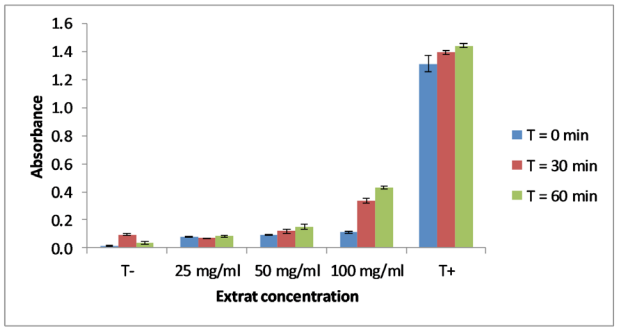

Figure 4: Hemolytic activity (Hemolysis rate) of isopropanolic extract (IP) from C. myriophylloides tested at various concentrations and various time of incubation. T (-) : negative control; T (+) : positive control. n=3.

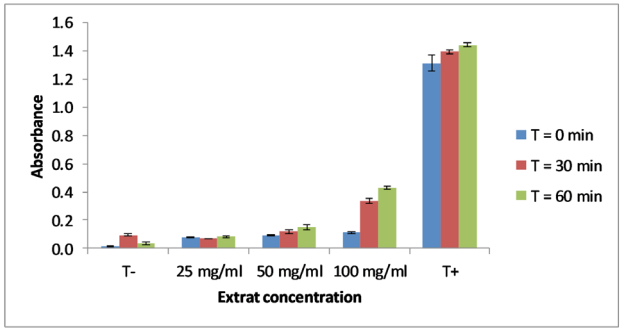

Figure 5: Hemolytic activity (Hemolysis rate) of butanolic fraction (BF) from *C. myriophylloides* tested at various concentrations and various time of incubation. T (-): negative control; T (+): positive control. n = 3.

on the cytotoxic activity of *C. myriophyloides* collected from the Atlantic coast of Morocco. This broad spectrum of biological activity has led us to evaluate the cytotoxic activity of various *C. myriophyloides* extracts and fractions, through the hemolytic test. This test was widely used because it highlights the toxicity of the extracts by the destruction of red blood cells resulting from the lysis of the membrane lipid bilayer.

The erythrocyte model is used in general for the hemolytic study because of its ready availability and its membrane similarity with other cell membranes.[19] The hemolytic activity was evaluated by the spectroscopic method allowing a quantitative measurement of hemolysis.[15]

In this study, the hemolytic activity was evaluated in various extracts and fractions of C. *myriophylloides*. At first, three extracts are prepared by varying the polarity of the used solvents. This approach was performed to highlight the presence of the activity by first testing the crude extract where the majority of the metabolites are represented (apolar compounds and polar compounds) and then to test the extracts enclosing mainly apolar compounds (chloroformic extract) or polar (methanolic extract). This first step of the work shows that the crude extract has significant hemolytic activity against erythrocytes of human blood. This crude extract is a complex mixture of compounds of highly variable chemical structure that may be responsible for the observed effect and these compounds can act individually or in synergy.

Unlike the methanolic extract where the effect on red blood cells is very low or even missing, the hemolytic activity of erythrocytes was found to a greater degree in the chloroformic extract which would seem to be more effective.

Indeed, several studies have been conducted on *cystoseira* using various solvents for extraction such as methanol, acetone, diethylether, ethanol, dichloromethane, and water. The results often differ as to the sought activity and the nature of the studied extract.[4,7,20]

Our first results suggest that the compounds responsible for the hemolytic activity are mainly apolar.

Saponins are glycosides compounds spread widely in plants and marine algae, which display a tremendous structural diversity and a wide spectrum of biological activities.[21] Among those activities, hemolysis is the most general one common of saponins.

Many works highlight the hemolytic effect of many structurally disparate saponins.[16,22–24] In this perspective, we sought to fractionate our seaweed material using solvents, allowing enrichment of saponins.

After delipidation with hexane and extraction with isopropanol 70% (IP fraction), a suspension of this extract was further extracted with butanol to give a butanol-soluble fraction.

The activity of isopropanolic extract results in the slight lysis of erythrocytes. However, the butanolic fraction obtained from this extract has a very important hemolytic activity. This activity reflects an enrichment of the fraction into active compounds. Moreover, the activity recorded at the level of this partially purified fraction could be largely attributed to polar compounds, and in particular to polar saponins.

However, our study shows that the activity is also observed when using the chloroformic extract, which usually contains apolar molecules. So, in this case, the observed activity could be due to the different compounds, including saponins.

The correlation between the structure of saponins and cytotoxic activity was studied by Wang *et al*.[16] It has been demonstrated that the hemolytic activity is highly dependent on their structures (sugar length, sugar linkage, the substitution on the sugar as well as the aglycone).

It is believed that those saponins can form complexes with sterols of the erythrocyte membrane, particularly cholesterol, thus causing an increase in permeability and the subsequent loss of hemoglobin.[25–27]

Indeed, saponins possess both water-soluble and lipid-soluble components. They consist of a lipid-soluble nucleus, having a steroid or a triterpenoid aglycone structure, with one or more of water-soluble carbohydrates.[28]

As a result, the hemolytic activity could be due to the steroid saponins and/or to the triterpenoid saponins and/or to other compounds soluble in different used solvents. Indeed, some research reports that hemolytic activity is attributed to both of the steroid saponins and triterpenoid saponins but to varying degrees.[29,30]

The hemolytic activity of each extract is related to their chemical composition. It's possible that the cytotoxic activity was not only or not related to lytic proprieties but perhaps due to membrane instability induced by the extracts.[30]

Furthermore, several reports indicate that the membranes of human erythrocytes from blood types have varying stability as determined from the mean corpuscular fragility.[31,32]

CONCLUSION

Cystoseira myriophylloides from the region of El Jadida in the Atlantic coast (Morocco) is studied for the first time for the hemolytic activity. Our results demonstrate the presence of a real cytotoxic activity in the crude extract of *Cystoseira myriophylloides* as well as in some sub-fractions. These preliminary results are worthy of interest and suggest that *Cystoseira myriophylloides* can constitute a source for the development of natural cytotoxic compounds which incites us to continue the seaweed material fractionation and screening operations in order to isolate the active compound(s) in particular at the extracts which induce the most pronounced hemolytic activity : chloroformic and butanolic.

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