Toxic And Anti-Cancerogenic Effect Of Brown Seaweed Cystoseira Tamariscifolia

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Abstract: Cystoseira tamariscifolia is a brown alga which represents an important natural biomass on the Moroccan Atlantic coast. Study of the acute toxicity of this species was carried out on Swiss albinos mice. The LD50 was determined through the Spearman-Karber method by injecting the aqueous algal extract to 36 mice organized in lots of 6 animals each for 6 concentrations and the experiments were conducted in triplicates. A dose of 0.5ml of each concentration of the aqueous extract were injected to the mice intraperitonally while the 0.5 ml of saline at 0.9% was injected to the control lot. The anticancerogenic effect study was carried out on murin myelom cells P3/X63-Ag8.653, using different concentrations of the aqueous extract of the alga. The results obtained show a toxic effect of the alga on the mices with LD50 of 738.61±34 µg of alga/ g of body weight. A significant anticancerogenic effect of the extract was also detected on myelom cells; it is propotionnel to the used concentrations.

Index Terms: Acute toxicity, anticancerogenic effect, brown marine algae, Cystoseira tamariscifola, extraction, median lethal dose LD_{50},

1 Introduction

Brown marine algae are found almost all over the world thanks to their adapting capacity though their reproduction and their response to different ecological conditions. It's supposed that these algae are releasing chemical material against different dangers to which they could be exposed (moving predators and invading microorganisms) [1]. Thus, many research teams studied the defensive capabilities of the brown alga in order to find natural material with antibiotic activities [2],[3], [4], [5] or even anti-cancerogenic activities [6]. Several species were reported to play a role in the preventing of biofouling phenomena; others are involved in the chemical, pharmaceutical and food industries. The species Cystoseira tamariscifolia is a brown alga belonging to the pheophycea class; fucals order. The geographic distribution of this alga on the Moroccan Atlantic Ocean coast is between Larache and Agadir with an important algal biomass around Rabat's area [7], [8]. It is a strong plant of 10 to 15 cm height, olive green color, rough on the surface. In fact, little is known about this species compared to other marine brown alga species such as Bifurcaria bifurcata [9], [10], [11], [12], [13], [14] or Cystoseira crinita [3], [15], [16], [17]. Some studies have been carried out on Cystoseira tamariscifolia such as antimicrobial effect [18], [19] or antioxidant effect [20]. However, the toxic and anticancerogenic effect of Cystoseira tamariscfolia has never been studied. Our study was initiated according to this fact in order to promote this alga. It was realized in a nutritional valorisation goal for the species Cystoseira tamariscifolia for economic exploitation as an added value both in human and animal food, following the example of several edible seaweed. For this a study of its toxic and cytotoxic effect has proved necessity through the study of its toxic effect on mices and its anticancerogenic capabilities on murine myelom cells.

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2 Materiel and methods

2.1 Harvesting

Cystoseira tamariscifolia was harvested during the spring from the Atlantic Ocean coast around Temara in the south of the Rabat (Oued Ykem). A total of 15 kg was harvested from different sites, depending on the algae availability. The fresh material is packed in plastic bags and selected with hands to avoid contamination. In laboratory the algae were washed with tap water to remove salt and thereafter with distillated water, next they were cut in small pieces, and dried during 48 hours at an ambient temperature about 35°C away from sunlight, in a shaded area exposed to the air stream onto a wooden grid support, then the dry alga was kept in paper bags closed away from the humidity and from the light.

2.2 Extraction

5 g of the dried alga is pounded in a mortar with 100 ml of sterile distilled water. The material is mixed with a 3 dimension vortex during one night, and then centrifuged at 3000g for 30 min, the liquid phase is recovered with a filter syringe (Millipore 0.22µm). The filtrate is aliquoted in eppendorf tubes and conserved at -20°C shielded from light until use.

2.3 Animals

126 Adult Swiss albinos mice (18-20g), aged from 6 to 8 weeks, of either sex were obtained from experimental center of the Pasteur Institute of Morocco. They were housed in groups of 6 per cage for seven days prior to experimentation in an ideal laboratory environment (Table1). Each experimental group consisted of six animals. Seven groups containing six mice in each were used in this study. Six groups were treated once intraperitoneally by algal aqueous extract, one group received saline at 0.9%. Before treatment the animals were subjected to a fast for 12h and they were weighed. All the animals were kept under continuous observation for 48 hours after the administration of dose, for any change in behavior or physical activities. Each animal was used only once. For ethical reason all animals were sacrificed at the end of study (AVMA Guideline, 2013) [21]. Experimental protocol were followed according to Guidelines for Care and Use of Laboratory Animals in Biomedical Research (2010).

TABLE 1: *Laboratory Conditions were maintained As Per OECD 423, 2001*

No	Condition Requirement
1	Room Temperature 23ºC
2	Humidity 50 to 60%
3	Light and Dark Period 12/12 Hours
4	Bedding Clean Sterilized Husk changed daily
5	Oral Feed Conventional Laboratory Diets, Like Standard Pellet Chow.
6	Distilled Drinking Water Ad libitum

2.4 Calculation of Median Lethal dose (LD_{50})

To allow a better bioavailability of active principle, the test of acute toxicity was realized by intra peritoneal injection of the algal extract to a group of 42 Swiss albinos mice. Animals were distributed in 7 lots, of six mice each, every lot of mouse received an injection of 0.5ml from the aqueous extract of the alga in a determined concentration. The concentrations were chosen to encadre the LD50, we start from a concentration of 100% of mortality to a concentration of 0 % of mortality [22]. The concentrations used were 5 %, 4.16 %, 3.47 %, 2.89 %, 2.41 % and 2.01 %, they were obtained after successives dilutions using a 1.2 dilution factor from the reference aqueous extract at 5g/100ml prepared as described previously. Every concentration was 3 times tested. One group of 6 mice was used as a control it receive 0.5 ml of saline at 0.9 %. For each mice the observation were made for 48 h and symptoms of toxicity and rate of mortality in each group were noted. At the end of study period expired animals were counted for the calculation of LD_{50}. The arithmetic method of Spearman-Karber was used for the determination of LD_{50} [22].

$$\log LD_{50} = \log LD_{100} + \log (df) (0.5 - \Sigma M/N)$$

LD_{100}: Lethal dose causing the 100% death of all test animals.
Df: Dilution factor
M: The average number of dead animals/ dose
N: Total number of animal in lot

Hodge and Sterner scale [23] was used for the evaluation of toxicity.

2.5 Cellular toxicity determination

The used cells are the myelomateuse lineage P3 / X63-Ag8.653, stemming from the Balb Balb/C mice provided by the blood transfusion establishment of Aquitaine-Bordeaux . These are tumoral cells which multiply indefinement in the time by escaping the apoptosis. Cells were cultivated in plates of culture of six wells each (Corning, Etats Unis) at a concentration of 0,1.106 cells / ml in RPMI medium 1640 (Eurobio, France) containing 15% Fetal veal serum (SVF, Eurobio, France), 2mM L-Glutamine (Eurobio, France), 100 UI/ml penicilline (Eurobio, France) and 100µg/ml streptomycine (Eurobio, France). 12 days incubation is carried out at 37°C and 5% of CO2. The cells can only be used in the tests when they reached an exponential growth phase which is a cell concentration from 0.5 to 0.7 106 cells/ml. Three sample concentrations were studied 1mg/ml, 2mg/ml and 5mg/ml. In fact, out of the 5g/100ml alga extract 100µl , 200 µl and 500 µl are withdrawn respectively and added to a RPMI medium with murine myeloma cells in order to have a 5ml final volume. The six wells plate containing the two samples (culture myeloma cells + aqueous extract) and the control (culture myeloma cells + distilled water) is labeled and incubated at 37°C. The cells were observed daily with an optic microscope in statif inverted (Hund, wetzlar), the cells concentrations were evaluated every 3 days, they were counted using Malassez cell according to trypan blue exclusion principle. All tests were realized in triplicate.

2.6 Statistical analysis

The statistical analysis of the alga effect on cell cultures of murin myeloma treated in comparison with the control cultures was carried out by comparing the cellular viability results using the X2 test at a probability threshold of 0.05. The concentration effect of the algal extract on cell cultures was analyzed by comparing the mean of cellular viability by the ANOVA test at a probability threshold of 0.05.

3 RESULTS AND DISCUSSION

3.1 LD_{50} determination

The results of the calculation of median lethal dose (LD_{50}) carried out 3 times in mice receiving 0.5 ml of algal extract at different concentrations by intra-peritoneal administration are shown in Table 2. The mean of LD50 calculated by the formula of Spearman-Karber is: 738.61 ± 34.03 mg of alga/kg of body weight, the lethal dose 100 (LD100) is 1250 mg / kg of body weight and a maximum tolerated dose (MTD) is 502,34mg/Kg of body weight. According to the scale of Hodge and Sterner [23] the value of the Lethal Dose 50 obtained can conclude after this study that Cystoseira tamariscifolia alga is slightly Toxic in white mice albino Swiss type. There must be some toxins in the alga extract that need to be determined (table 3). From the experiment the results reveal that the aqueous extract of Cystoseira tamariscifolia intraperitoneally have been found toxic with LD_{100} at 1250 mg/kg body weight of experimental animals as in the first 4 hours of observation 100% morbidity was observed. All the animals received 1250 mg/Kg i.p. were suffering from twitching, increase rate of respiration, sedation, abdominal muscle contractions, diarrhea, nasal bleeding. At the 2nd hour they were drowsy, less responsive and dyspnoeic before death. However, at 4th hour all mice had convulsion and expired. After the administration of aqueous extract of the alga mice were suffering from different symptoms which the severity was proportional of the dose.

TABLE 2: *Toxicological study of different doses of aqueous extract of Cystoseira tamariscifolia administered intraperitoneally at 0.5 ml in mice*

Groups	Dose mg/kg (mice)	Number of died mice by lot for every test		
		Test 1	Test 2	Test 3
Lot 1	C1 =1250	6/6	6/6	6/6
Lot 2	C2 =1041.66	5/6	5/6	5/6
Lot 3	C3 =868.05	3/6	5/6	4/6
Lot 4	C4 =723.27	3/6	4/6	3/6
Lot 5	C5 =602.81	2/6	2/6	2/6
Lot 6	C6 =502.34	0/6	0/6	0/6
Lot 7 (control)	C7= 0	0/6	0/6	0/6
	LD_{50}	LD1= 768.61	LD2= 701.63	LD3= 745.60

For every concentration of algal aqueous extract the test was realized on three lots with 6 mice each. For a concentration of 5g/100ml we obtain 100% of mortality and for 2.1g/100ml we obtain 0% mortality, between the two concentrations we obtain a propotional mortality to the concentration

TABLE 3: *Hodge and Sterner Toxicity Scale [23]*

Toxicity class	Term	LD_{50}
1	Extremely Toxic	Less than 1mg/Kg
2	Highly Toxic	1-50 mg/Kg
3	Moderately Toxic	50-500 mg/Kg
4	Slightly Toxic	500-5g/Kg
5	Practically Non Toxic	5-15g/Kg

However, aqueous extract at lower limit dose of 502.34 mg/kg body weight, was not found to cause mortality and non-significant changes were observed in wellness parameters used for evaluation of toxicity. Behavioral pattern, salivation, sleep of the treated as well as the control animals were found to be normal, diarrhea and coma did not occur in any of the mice.

3.2 Cytotoxicity determination

Table 4 and fig 1 show the results of the effect of different concentrations of aqueous extract on the viability/time of the murin myeloma cells. The cytotoxicity test shows a very significant decrease of viable cells in the culture with presence of algal aqueous extract compared to the control (with distilled water) for all days D3, D6, D9 and D12. On the other hand, myeloma murin cells were cultivated at the concentration of $0{,}1.10^6$ cells/ml, after malassez cell counting it was observed that the lethal effect of the alga extract on myoloma cells was proportional to the concentration of this extract in the medium. In fact for a concentration of 1mg/ml the number of D3 (day 3) viable cells was 340.000 compared to 280.000 and 100.000 for 2mg/ml and 5mg/ml concentrations respectively. This decrease of cells viability through the increase of the extract concentration is also seen for D6 and D9. The comparison of the viable cells in the cultures treated by three algal concentrations showed a significant difference between the concentrations 1mg/ml and 5mg/ml ($p<0.05$) and between the concentrations 2mg/ml and 5mg/ml for days D3, D6, D9 and D12 ($p<0.05$). However the difference of the viability for the concentrations 1mg/ml and 2mg/ml are not significant for day D6 ($p>0.05$). The alga extract could have an active compound with anti-tumoral effect. In fact, Kotaké et al [24] demonstrated that fucoxanthin, a carotenoid extracted from the brown algae, has an important anti-tumoral effect on man prostatic cancerous cells. Also, Ayyad et al [25], [26] purified a diterpene, from the Sargassium crispum and Cystoseira myrica brown algae, which has a cytotoxic effect, this active compound was also purified from the brown alga Cystoseira baccata by Mokrini et al. team [27]. Other similar studies on the Bifurcaria bifurcata, have shown that this brown alga contains cytotoxic compounds efficient on several Human tumoral cells [6].

TABLE 4: *Mean number of myeloma cells ($x10^3$) / ml according to time after their culture with the different concentration of the algal aqueous extract*

Days D_0 Algal extracts		D 3	D 6	D 9	D12
Control	100	740± 45^a	1000± 25^a	700±20^a	300± 8^a
Extract (1 mg/ml)	100	340± 35^b	318± 6^{bc}	350± 27^b	70± 3^b
Extract (2 mg/ml)	100	280 ± 16^c	318± 3^{bc}	250± 10^c	60± 2.7^c
Extract (5mg/ml)	100	100± 7^d	194± 1.5^d	120± 4^d	20± 1.85^d
Signification level		P<0.05	P<0.05	P<0.05	P<0.05

a, b, c and d correspond to the significant differences between the means at the level of the same column

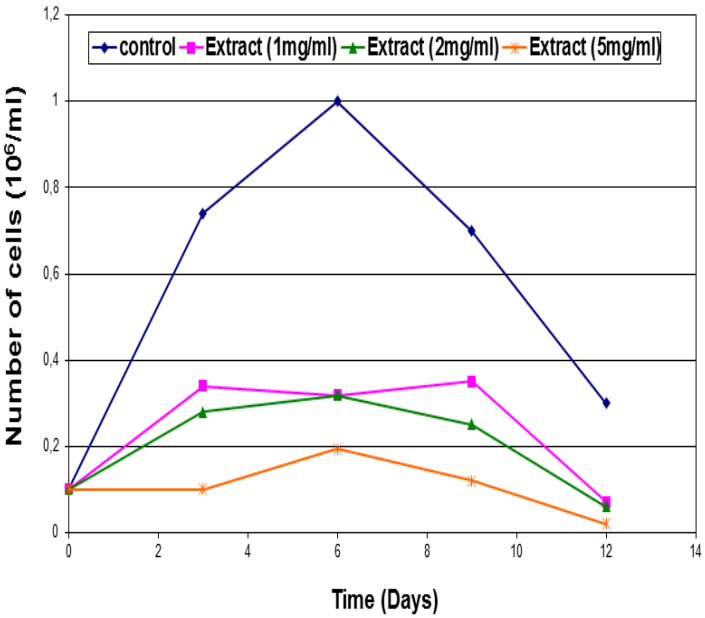

FIGURE 1: *Viability according to the time of myeloma cells cultured in the presence of algal aqueous extract at different concentrations*

4 CONCLUSION

According to the obtained results we can conclude that the alga Cystoseira tamariscifolia presents a significant toxicity on the mouse and thus it would be impossible to exploit it as an added value in human or animal food. However its cytotoxicity on the tumoral cells of the murin myeloma remain encouraging for an in vivo antitumoral study and a research for a possible active compound with anticancer character.

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