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ARTIGO ORIGINAL

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In vitro interaction of the native lectin isolated from the green seaweed *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae, Bryopsidales) against cancer HL-60 cells

Interação *in vitro* da lectina nativa isolada da alga marinha verde *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae, Bryopsidales) contra células cancerígenas LH-60

Ismael Nilo Lino de Queiroz[1], José Ariévilo Gurgel Rodrigues[2], Renata Line da Conceição Rivanor[2], Gabriela Cunha Vieira[3], Edfranck de Souza Oliveira Vanderlei[4] & Norma Maria Barros Benevides[2]

[1]Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro - UFRJ
[2]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará - UFC
[3]Departamento de Farmacologia e Fisiologia, Universidade Federal do Ceará - UFC
[4]Faculdades Nordeste - Fanor

*Email: (arieviloengpesca@yahoo.com.br)

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Abstract Seaweeds have structurally diverse metabolites with biotechnological importance, including lectins, considered a variable class of proteins which bind reversibly to specific carbohydrates. The *Caulerpa cupressoides* (Chlorophyta) lectin (CcL) exhibiting *in vivo* pharmacological actions has been described; however, CcL-based diagnostic tool for cancer has not been investigated. This study analyzed molecular weight, cytotoxicity and the *in vitro* interaction against human promyelocytic leukemia cell lines (HL-60). Extraction of CcL was performed in 25 mM Tris-HCl buffer (TB) (4 h stirring, pH 7.5) and then CcL was purified by ion-exchange chromatography on DEAE-cellulose column using TB containing 0.5 M NaCl, which eluted protein peak exhibiting haemagglutinating activity against trypsin-treated rabbit erythrocytes. Polyacrilamide gel electrophoresis in the presence of β-mercaptoethanol revealed to be a dimeric protein (17 kDa) by comparison with the markers trypsin inhibitor and α-lactoalbulmin. CcL did not cause cytotoxicity up to 500 μg mL^{-1} in HL-60 cells using MTT reduction assay; but, showed agglutination (125, 250 and 500 μg mL^{-1}) in comparison to Concanavalin A (50 μg mL^{-1}) based on photomicrographic analysis. The results allow us to consider CcL as a promising diagnostic tool for HL-60 cells in further studies.

Keywords: green alga, agglutinin, cytotoxicity, diagnostic tool.

Resumo As algas marinhas possuem metabólitos diversos estruturalmente com importância biotecnológica, incluindo lectinas, consideradas uma classe variável de proteínas as quais reversivelmente se ligam a carboidratos específicos. A lectina de *Caulerpa cupressoides* (Chlorophyta) (LCc) tem mostrado ações farmacológicas *in vivo*. Entretanto, não tem sido investigada como ferramenta diagnóstica para o câncer. Analisou-se o peso molecular, citotoxidade e a interação *in vitro* com células de leucemia promielocítica da linhagem (LH-60). A extração de LCc foi desenvolvida em tampão Tris-HCl 25 mM (TT) (4 h sob agitação; pH 7,5) e em seguida, a LCc foi purificada por cromatografia de troca iônica em coluna de DEAE-celulose usando TT contendo NaCl 0,5 M, da qual pico protéico eluído apresentou atividade hemaglutinante contra eritrócitos de coelho tratados com tripsina. A eletroforese em gel de poliacrilamida, na presença de β-mercaptoetanol, revelou uma proteína dimérica (17 kDa) por comparação com os marcadores inibidor de tripsina e α-lactoalbumina. A LCc não mostrou, em ensaio de redução pelo MTT, citotoxicidade até 500 μg mL^{-1} em células LH-60. Contudo, na análise fotomicrográfica, mostrou aglutinação (125, 250 e 500 μg mL^{-1}) em comparação a Concanavalina A (50 μg mL^{-1}). Os resultados permitem a considerar LCc como uma ferramenta diagnóstica promissora em estudos futuros realizados com células LH-60.

Palavras-chave: alga verde, aglutinina, citotoxicidade, ferramenta diagnóstico.

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Introduction

Cancer is currently recognized as the first-leading cause of death worldwide (Nader et al., 2001) and its incidence and prevalence has been mainly attributed to environment and lifestyles factors (e.g., tobacco smoke, infections, obesity and alcohols) (Parkin, Pisani & Ferlay, 1999). Some diseases, such as malignant tumors of the oral cavity (Leite, Nunes, Moreira, Couto & Teixeira, 2010) and leukemia (Efficace et al., 2014), have became of considerable interest for clinical and research studies concerning cell differentiation and neoplastic cell identification methods (Hauert, Martinelli, Marone & Niggli, 2002; Silva et al., 2011). Carbohydrates are directly involved in interactions between tumor cells, between tumor cells and the extracellular membrane, or between tumor cells and endothelial cells (Shanron & Lis, 1993; Nangia-Makker, Conklin, Hogan & Raz, 2002). Although in some cases may be possible identify some functional significance of disease-associated changes in glycosylation, is difficult to better understand the process of malignancy (Dube & Bertozzi, 2005).

Some potentially bioactive functional metabolites derived from different marine organisms, including sponges, cnidarians, tunicates, fishes and macroalgae, have been isolated and studied for cytotoxicity as a suggestion of novel therapeutic and diagnostic strategies against cancer cells and for use in cancer chemotherapy (Costa-Lotufo et al., 2006). Among the diverse classes of biopolymers, lectins (also known as haemagglutinins or agglutinins) have become the subject of special interest due to their applications in various fields, such as biology, cytology, biochemistry, medicine and food science and technology (Smit, 2004; Cardozo et al., 2007).

Lectins are a structurally heterogeneous class of proteins of non-immune origin that have the property to bind reversibly to carbohydrates present in different cell surfaces, recognizing and agglutinating specific oligosaccharides or glycoconjugates (Peumans & Van Damme, 1995). Land plants (Peumans & Van Damme, 1995; Araújo et al., 2013), seaweeds (Rogers & Flangu, 1991; Benevides et al., 2001; Cardozo et al., 2007) and invertebrates (Sun et al., 2007; Melo et al., 2014) are sources in lectins having capacity to control nociception, inflammation (Silva et al., 2010a), healing (Nascimento-Neto et al., 2012), inhibit the growth of bacteria (Holanda et al., 2005; Melo et al., 2014) and insect larvae (Leite et al., 2005) and to participate in innate immune response of invertebrates by inducing bacterial agglutination (Sun et al., 2007). In the field of tumor lectinology, they play an important role to identify differences between normal tissues and tumor cells, like potential diagnostic tools (Bakalova & Ohba, 2003; Silva et al., 2011). However, there are still few biological descriptions on the marine algal lectins. To the best of our knowledge, no datum concerning the actions of lectins isolated from seaweeds on human promyelocytic leukemia cells (HL-60) has been reported.

A number of algae species is found along Brazilian coastal line; however, there are still few records concerning their natural metabolites pharmacological effects (Costa-Lotufo et al., 2006). Recently, Carneiro, Rodrigues, Teles, Cavalcante & Benevides (2014) analyzed some chemical components in four tropical seaweeds (*Hypnea musciformis*, *Solieria filiformis*, *Caulerpa cupressoides* and *C. mexicana*), in which proteins and carbohydrates were the most frequently polymers isolated. *C. cupressoides* var. *lycopodium* C. Agardh (Caulerpaceae, Bryopsidales) contains a lectin (glycoprotein) (CcL) which was previously purified by α-lactose agarose-affinity chromatography, with molecular mass of 23.158±1.010 kDa (a dimer), when determined by SDS-PAGE in the presence of β-mercaptoethanol, and exhibiting haemagglutinating activity against erythrocytes from humans and various animals (Benevides et al., 2001). This isolated CcL was further tested in animal models of nociception and inflammation and exhibited antinociceptive and anti-inflammatory effects (Vanderlei et al., 2010). In another research, CcL induced T helper 2 immune responses in mouse splenocytes and produced high levels of cytokines (interleukins-10 and -6) (Abreu et al., 2012).

Based on these considerations, the aim of this study was to evaluate a novel purification protocol of CcL and its *in vitro* actions on HL-60 cells.

Material and Methods

MARINE ALGA

Samples of the green seaweed *C. cupressoides* were manually collected by hand from the northwestern Brazilian coast (Flecheiras beach, Ceará State). The material was then cleaned of macroscopic epiphytes, washed with distillated water and finally stored at -20 °C until use. A voucher specimen (4977) was archived at the Prisco Bezerra Herbarium (Department of Biology, Federal University of Ceará, Brazil).

OBTAINING AND PURIFICATION OF CCL

CcL was obtained according to the procedure previously reported by Benevides et al. (2001) where the alga (500 g fresh tissue) was initially ground to a fine powder under liquid nitrogen, and then stirred for 4 h with two volumes of 25 mM Tris-HCl buffer (pH 7.5) (TB) (1:4 w/v). The mixture was filtrated through nylon, and centrifuged 6.000 × g for 30 min at 4 °C. A sample of the crude extract (50 mg) was dissolved in TB and then CcL was purified by ion-exchange chromatography on DEAE-cellulose column (30 × 2 cm), previously equilibrated and washed with the same TB, followed by elution of unbound proteins and pigments, adsorbed proteins were obtained using TB containing 0.5 M NaCl. Fractions were collected and monitored at 280 nm (Amersham BiosciencesUtrospec 1100 spectrophotometer) and tested for hemagglutinating activity based on Benevides et al. (2001) using small glass tubes a series of 1:2 dilutions (0.1 mL) of the fractions which was mixed with 0.1 mL of a 2% suspension of trypsin-treated rabbit erythrocytes and the degree of agglutination was monitored visually after centrifugation (3400 × g, 2 min). The activity was expressed as hemagglutination units (H.U.), which was defined as the inverse of the highest dilution still capable of causing agglutination and specific activity was expressed as heamagglutination units per mg of protein. The 0.5 M NaCl-eluted active fraction were pooled, dialyzed, free-dried and used for *in vitro* assays.

POLYACRILAMIDA GEL ELECTROPHORESIS (SDS-PAGE)

This procedure was performed to determine the molecular weight of the CcL based on Laemilli method as reported by Hames & Rickhood (1983), using a vertical system. In this experiment, CcL was applied to a 15% 1-mm-thick polyacrylamide slab gel in 25 mM Tris-HCl, 20 mM glycine, pH 8.9, with 0.1% sodium dodecyl sulfate and run for 30 min at 100 V. Samples and standards were prepared in TB, pH 6.8, containing SDS and β-mercaptoethanol. A standard picrate-Coomassie-blue method, as described by Stephano et al. (1986), was used for staining the gel following electrophoresis. The molecular mass of the CcL was estimated by comparison with the electrophoretic mobility of standards: β-phosphorilase (97 kDa) albumin bovine serum (66 kDa), ovoalbumin (45 kDa), carbonic anhydrate (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa).

HUMAN PROMYELOCYTIC LEUKEMIA CELL LINES (HL-60)

The HL-60 cells were obtained from National Institute of Cancer (USA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin). Cells stocks of HL-60 were maintenance at 37°C and 5% CO_2. Further, they were titrated by plaque forming assay.

IN VITRO CYTOTOXICITY ASSAY

This assay was based on Abreu et al. (2012) by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by incubating triplicate HL-60 cell line monolayers cultured in 96-well microplates in RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 g mL^{-1} of penicillin/streptomycin (Novafarma, Anápolis, GO, Brazil). CcL was tested at three different concentrations (125, 250 and 500 μg mL^{-1}), triplicate. The cultures were incubated for 24 h at 37 ºC and 5% CO_2. The toxicity of the CcL was determined by comparing the percentage of 3H-thymidine incorporation (as an indicator of cell viability) of CcL-treated wells in relation to untreated wells. Concanavalin A (Con A) was used as positive control and 0.9% saline as negative control. Non-cytotoxic concentrations were defined as those causing a reduction of [3H]-thymidine incorporation below 30% in relation to untreated control. Cellular viability was evaluated by the neutral red dye-uptake method according to Borenfreund and Puerner (1985). The 50% cytotoxic concentration (CC_{50}) was calculated as the dilution that caused a reduction of 50% in number of viable cells.

MICROSCOPIC ANALYSIS

The purity of culture was monitored by Borenfreund and Puerner' method. The cell morphological alterations and agglutination in the presence of CcL (125, 250 and 500 μg mL^{-1}) and Con A (50 μg mL^{-1}) were observed in light microscopy (400 ×).

Results and Discussion

ISOLATION AND CHARACTERIZATION OF CCL

The crude extract from the green seaweed *C. cupressoides* var. *lycopodium* was obtained in TB from the algal tissue and then fractionated on DEAE-cellulose column which separated the material into two protein peaks (P I and P II). The first protein peak eluted with equilibrium TB resulted in an unbound material without haemagglutinating activity and the second one was eluted at 0.5 M NaCl-containing TB which exhibited retained proteins containing haemagglutinating activity), as illustrates in figure 1A.

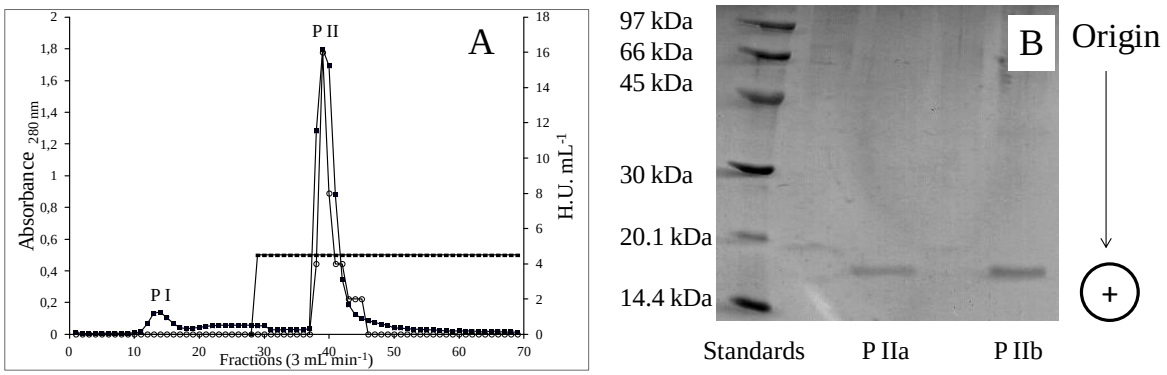

Figure 1. (A) Ion-exchange chromatography on DEAE-cellulose column of the lectin from Caulerpa cupressoides var. lycopodium (CcL). Fractions were collected and checked by 280 nm (■—■) and haemagglutinating activity (○—○). (-) NaCl concentration. (B) SDS-PAGE of purified CcL (P IIa: 10 µL, P IIb: 20 µL) and standards (β-phosphorilase (97 kDa) albumin bovine serum (66 kDa), ovoalbumin (45 kDa), carbonic anhydrate (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa)) present on gel were stained by Stephano et al.' picrate-Coomassie-blue method.

The employment of different matrixes (e.g., Sepharose 6MB, mannan-Sepharose 4B, fetuin-agarose, Sephadex G-100 and guar) have been reported for obtaining of lectins from different organisms, including seaweeds (Holanda et al., 2005; Abreu et al., 2012), animals (Sun et al., 2007) and land plant seeds (Araújo et al., 2013).

The chromatographic profile obtained by DEAE-cellulose was similar to that observed by affinity chromatography on α-lactose agarose column, of which two protein fractions was also obtained. The protein yield of CcL, which was eluted with 0.5 M NaCl-containing TB and had haemagglutinating activity in the presence of rabbit erythrocytes (P II-DEAE) (Figure 1A), was 36% from the crude extract, similar to that obtained from α-lactose agarose (38%); but, followed by gel filtration on Bio Gel P-100, only 6% yield was also previously obtained (Benevides et al., 2001). The purified CcL also exhibited agglutination for trypsin-treated rabbit erythrocytes, confirming the same properties to bind reversibly to carbohydrates present in this type of cell (Benevides et al., 2001).

As CcL presented haemagglutinating activity (Figure 1A), a SDS-PAGE procedure was performed according to Laemilli method as reported by Hames & Rickhood (1983) in order to estimate its molecular weight. The apparent molecular mass, in the presence of β-mercaptoethanol, revealed a single protein band and its presence was also verified doubling the volume of the sample on gel (17 kDa, a dimeric protein by comparison with the markers trypsin inhibitor and α-lactoalbulmin) (Figure 1B). This result was not in accordance to that found for this same algal species by Benevides et al. (2001), who previously described its molecular weight of~ 23 kDa, but is in concordance with other green seaweed species (Rogers & Flangu, 1991; Sampaio, Roger & Barwell, 1998) and in the red seaweeds *Solieria filiformis* (29 kDa) (Holanda et al., 2005) and *Bryothamnion seaforthii* (9 kDa) (Nascimento-Neto et al., 2012), which suggested low molecular weight algae lectins. Possibly, the time of the year in which of this algal species was collected and the protocol of this present study could perhaps explain the difference concerning its molecular weight (Cardozo et al., 2007).

Therefore, the isolated CcL by DEAE-cellulose was confirmed by both haemagglutinating activity and SDS-PAGE analyses (Figure 1), which revealed an alternative strategy to reduce its purification steps (Benevides et al., 2001; Chan & Ng, 2012). Based on these previous findings, CcL was further assayed against HL-60 cell lines (human promyelocytic leukemia cells), based on an *in vitro* citotoxicity assay, as well as evaluate its interaction with these same cells.

CCL HAS NO CYTOTOXIC EFFECT TO HL-60 CELL LINES

The CcL, when tested by MMT reduction assay for 24 h, had no inhibitory effect on the cell viability (HL-60 cells) at CC_{50} up to 500 μg mL^{-1} (Table 1). No significant change in the cellular morphology was observed at any treatment based on Borenfreund & Puerner (1985) and Abreu et al. (2012) (data not shown). No cytotoxicity was found for saline (negative control) (data not shown). Con A (positive control), which is widely utilized as a reference for immunological assays and known for its cytotoxicity which lead to cell death, did not exhibit toxic effect below a concentration of 50 μg mL^{-1} (data not shown). Other concentrations of CcL were not tested due to the lack of *in vitro* inhibitory effect.

Table 1. Cytotoxicity assay with HL-60 cell lines in the presence of CcL and Con A.

Compounds	leukemia cells	Cytotoxicity (CC_{50}, μg mL^{-1})
CcL	HL-60	> 125
		> 250
		> 500
Con A		< 55

CC_{50} - 50% cytotoxic concentration; Con A – not exhibit toxic effect below a concentration of 55 μg mL^{-1}.

Literature reports some lectins with *in vitro* cytotoxic effects on cancer cell lines (Ohba, Bakalova & Muraki, 2003; Araújo et al., 2013). By contrast from our findings, the lack of cytotoxicity on HL-60 cells is in accordance with CcL-treated Swiss mice for 7 consecutive days. No toxicological significance at level of some biochemical parameters of blood (AST, ALT and urea) and morphological alteration of organs (liver, kidney and heart) was observed (Vanderlei et al., 2010). In another study, Abreu et al. (2012) tested CcL on Balb/c mouse splenocytes and any cytotoxic effect was found 24 h after treatment.

IS THE NATIVE CCL INTERACTING WITH HL-60 CELLS?

Based on hypothesis that lectins have the ability to bind to specific carbohydrates expressed on different cell surfaces (Peumans & Van Damme, 1995), CcL (125, 250 or 500 μg mL^{-1}) was tested *in vitro* against HL-60 cells and compared to the control groups (Con A - 50 μg mL^{-1} – positive control; 0.9% saline - negative control) (Figure 2).

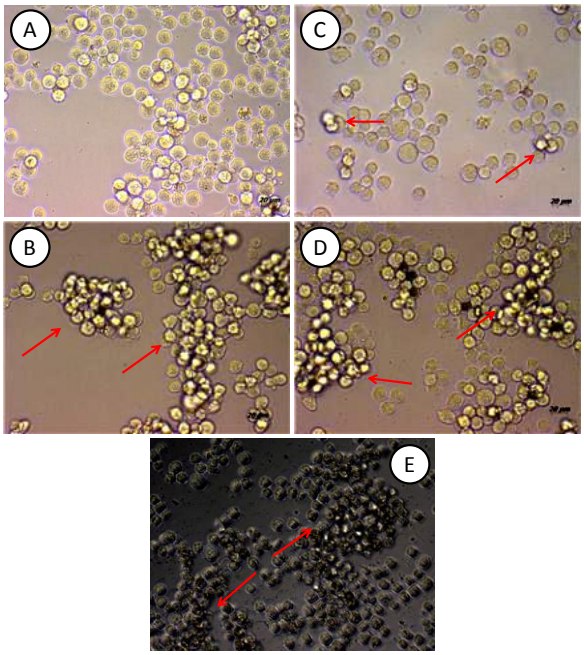

Figure 2. Photomicrography of the human promyelocytic leukemia cells (HL-60) in the presence of saline (A), Con A (50 μg mL^{-1}) (B) and CcL (125 (C), 250 (D) and 500 (E) μg mL^{-1}) after 24 h. Native CcL is capable of agglutining HL-60 cells compared to Con A (red arrows). The samples were observed under a light microscope at 400 ×. Scale bar 20 μm.

The degree of CcL-leukemic (HL-60) cells agglutining was observed as a function of the concentration (Figure 2). CcL (250 and 500 μg mL^{-1}) showed a higher capacity to induce agglutination in HL-60 cells (Figures 2D, E) than that observed for Con A (Figure 2B). Notably, native CcL-cells binding were reduced at 125 μg mL^{-1} (Figure 2C), being almost similar to the saline (Figure 2A). No cell damage significance was found in all the concentrations tested with CcL; therefore, further observations must still be done regarding our partial conclusions. Con A suggested induce cell depletion (Hatano et al., 2008) and apoptosis because of its mitogenic effects (Büssinga et al., 1996).

It has been recognized that blood cells become more sensitive to agglutination when they are previously treated with some enzymes, such as trypsin (Holanda et al., 2005). Benevides et al. (2001) observed that CcL had preference for human blood group A trypsin-treated erythrocytes (due to the presence of the sugar N-acetylgalactosamine which is the terminal immunodominant sugar of the A antigen) and then the rabbit erythrocytes. More recently, Sun et al. (2007), investigating the lectin isolated from the haemolymph of

white shrimp *Litopennaeus vannamei*, tested its ability to agglutining against three types erythrocytes (human, mouse and chicken) and discovered, after treatment with trypsin, a strong agglutination titre towards chicken erythrocytes. The presence of terminal N- and O-acetyl groups in the oligosaccharide chain of glycoconjugates appeared as a requirement for such property.

This study showed that CcL interacted in different degrees with leukemic cells and manifesting cytoagglutinating (Figure 2) (Ohba, Bakalova & Muraki, 2003; Chan & Ng, 2012), but no cytotoxic effect (Table 2). Ohba, Bakalova & Muraki (2003) verified the relationships between lectin-cells binding, cytotoxicity activity and cytoagglutinating activity against normal lymphocytes and cultured leukemic cell lines. It was observed that lectins, when analyzed by fluorescent confocal microscopy, strongly interacted with all cells tested, being an inverse correlation found between cytotoxicity and degree of lectin-cells binding (leukemic cells).

In a previous study, CcL displayed a strong anti-inflammatory effect by reduction the rat edema induced by lambda-carrageenan-type sulfated polysaccharide which is extracted from red seaweeds leading to an inflammatory response (leukocyte migration) by indirect mechanism via activation of macrophages (Silva et al., 2010b). Observation from this inflammation test postulated that CcL possibly interacted with cell-receptors, such P- and L-selectins, leading a reduction on the leukocyte recruitment (Silva et al., 2010a; Vanderlei et al., 2010). It is also emerged the hypothesis that differentiated HL-60 cells, when stimulated with chemotectic peptide, would represent an efficient strategy to investigate molecular mechanisms of neuthophil emigration (Hauert, Martinelli, Marone & Niggli, 2002). Previous reports have demonstrated that certain seaweeds lectins evoke stimulatory responses in some biological systems (Smit, 2004; Holanda et al., 2005).

An intriguing result of this present study was a lack of anti-tumor effect in HL-60 cells (Table 1), but with *in vitro* haemagglutinating activity of CcL (Figure 2) because a number of lectins exhibit both properties at the same time throught their carbohydrate binding activity (Araújo et al., 2013). However, it is also reported that the haemagglutinating activity of lectins would represent an adverse consequence due to red blood cell agglutination in patients during cancer treatment (Chan & Ng, 2013). A 70 kDa galactose-specific lectin from the tubers of *Dioscorea opposite* cv. Nagaimo was capable of inhibiting the growth of some cancer cell lines (breast cancer MCF7 cells, hepatoma HepG2 cells and nasopharyngeal carcinoma CNE2 cells), but when was introduced galactose, the effect was not related to the carbohydrate biding specificity of the lectin (Chan & Ng, 2013). In fact, there is a lack of studies concerning the disease-associated changes in glycosilation (Shanron & Lis, 1993) because the structural complexity of carbohydrates which became difficult to better understands the process of malignancy (Dube & Bertozzi, 2005).

The search for specific agents from different origins for treatment and prevention of diseases and disorders have been urgently needed (Smit, 2004; Costa-Lotufo et al., 2006), especially for control of cancer (Hauert, Martinelli, Marone & Niggli, 2002; Ji, Shao, Zhang, Hong & Xiong, 2008; Silva et al., 2011; Pomin, 2012) and painful and inflammatory reactions (Vanderlei et al., 2010; Rodrigues et al., 2012). CcL revealed agglutination power in HL-60 cells, but studies are needed to find out what are the mechanisms involved in this agglutination and how this feature would take part in cancer diagnosis. However, these observations do not exclude its use potential as cytotoxic agent on other cancer cell lines (Chan & Ng, 2013).

Conclusion

The green seaweed *Caulerpa cupressoides* var. *lycopodium* lectin is purified by DEAE-cellulose which presents, when submitted to polyacrylaminde gel electrophoresis in the presence of β-mercaptoethanol, average molecular weight of 17 kDa (a dimeric protein). It is devoid of *in vitro* cytotoxic effects on human promyelocytic leukemia cells, but could it reveals as an interesting diagnostic tool in cancer research.

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