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Antiproliferative and antibacterial activity evaluation of red microalgae *Rhodosorus marinus*

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Antiproliferative activity of three extracts obtained from red microalgae *Rhodosorus marinus* was evaluated against cervical (HeLa), colon (HCT 116), lung (A549), prostate (22Rv-1) and breast (HCC38 and MDA-MB-231) cancer cell lines. Antibacterial activity of these extracts was also tested against *Salmonella choleraesuis*, *Listeria monocytogenes* and *Staphylococcus aureus*. All extracts were obtained from lyophilized biomass of red microalgae. Extract A was obtained using 40% ammonium sulfate precipitation and gel filtration chromatography with G-25 sephadex. Extract B was subjected to a similar process, but 60% ammonium sulfate precipitation was used. Extract C was obtained by methanol extraction and hydrophobicity chromatography using amberlite XAD-2. Protein concentration was determined in two extracts and total phenols in one extract, using Bradford and Folin techniques. Antiproliferative activity was evaluated at extract concentrations ranging from 0.125 to 1 mg/ml, using the spectrophotometric technique MTT (3 - (4,5 - dimetiltiazolyl - 2) - 2,5 - diphenyltetrazolium bromide). The antibacterial activity was evaluated by the impregnated disk test. Extract C showed antiproliferative activity against almost all cancer cell lines with an IC_{50} of 0.5 (HCT 116), 0.8 (HeLa), 0.9 (MDA-MB-231), 0.1 (HCC38), and 0.4 (22Rv-1) mg/ml, whereas none of the tested extracts showed antibacterial activity under experimental conditions.

Key words: *Rhodosorus marinus*, red microalgae, antibacterial activity, antiproliferative activity, methanolic extract.

INTRODUCTION

Infectious diseases and cancer are important public health problems worldwide. Both types of diseases are different, but share a common characteristic: uncontrolled growth of a cell type (Alberts et al., 2002; Bhunia, 2008). There are drugs whose function is to prevent the growth of harmful cells; antibiotics are used to control bacterial

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populations while antiproliferatives are used in the control of cancer cells growth (Alexander and Hong, 2008; Chang et al., 2003; Granier et al., 2011).

Nowadays, several pathogenic bacteria have shown an increase in antibacterial resistance against some antibiotics commonly used in therapy (Whichard et al., 2007). A similar situation has been observed in cancer treatments, where some antiproliferative drugs gradually decrease their function against cancerous cells (Gottesman, 2002). Drug resistance might be addressed by different strategies, such as prescribed treatment compliance, alternated treatments with different drugs, and the search for new antibacterial and antiproliferative drugs.

A very common source of antibacterial and antiproliferative drugs is vegetable organisms (Aneiros and Garateix, 2004). Plants and other marine organisms have a secondary metabolism, from which many molecules obtained have shown biological activity over several organisms (Saunders, 2009). Actually, this secondary metabolism is also helpful for different activities in the producer organisms, including defense against other organisms and adaptations to special environmental characteristics. Secondary metabolism is also present in marine organisms, but they have been only briefly studied (Jha and Zi-rong, 2004).

Based on the fact that immobile marine organisms inhabit densely populated environments surrounded by predatory mobile organisms, they need to produce inhibitory substances which are released into the marine environment in order to keep away mobile predators (Aneiros and Garateix, 2004; Debbab et al., 2010; Li and Vederas, 2009). However, a high concentration of the released metabolites is required to minimize the dilution effect in the aqueous marine medium. In this way, marine algae among other organisms might be good candidates for searching bioactive molecules capable of inhibiting cell multiplication in the predators and/or competitors.

The presence of pigments other than chlorophylls that allow light absorption in depths where chlorophyll cannot be absorbed is a common characteristic of red algae. Absorbed light is transferred to chlorophyll and, by several reactions, converted into chemical energy that is used by the algae (Eroglu et al., 2013; Lee, 2008). Additional steps required for light absorption before chlorophyll participation include several reactions and the subsequent production of metabolites (secondary metabolism) that might possess biological activities of interest.

Several types of biological activities have been reported in some red seaweed and microalgae species. A *Gracilaria verrucosa* ethanolic extract and its fractions, for example, obtained with ether and ethyl acetate and evaluated for antioxidant activity using the DPPH method, showed significant antioxidant activity *in vitro* tested (Abou and Shalaby, 2009). In addition, an aqueous extract of *G. corticota* showed cytotoxic activity against leukemic cell lines Jurkat and Molt-4 at concentrations of 9.336 and 9.726 µg/ml, respectively (Zandi et al., 2010). Moreover, three extracts from *Gelidium amansii* obtained using phosphate buffer, methanol and dimethyl sulphoxide, were evaluated for their activity against Hepa-1 (murine liver cancer), HL-60 (human leukemia) and NIH - 3T3 (murine embryonic fibroblasts) cells lines. All the extracts showed antiproliferative activity against Hepa - 1 and NIH - 3T3, but not against HL - 60 (Chen et al., 2004).

Phycocyanin is an accessory pigment to chlorophyll, which is present in red algae and some other organisms like cyanobacteria (Gantar et al., 2012; Martelli et al., 2014). For example, the antioxidant activity of *Spirulina* has been highly correlated with phycocyanin (Estrada et al., 2001). Phycocyanin obtained from *S. platensis* has already shown to induce apoptosis in murine cancer cells AK-5, through induction of reactive oxygen species and inhibition of cyclooxygenase-2 (COX-2). COX-2 catalyzes arachidonic acid conversion into prostaglandins; and overexpression of prostaglandins stimulates cell proliferation and Bcl-2 inhibition. The protein Bcl-2 inhibits cell apoptosis, Bcl-2 is also inhibited by phycocyanin (Pardhasaradhi et al., 2003).

The flavonoids compounds are present in phenolic extracts. The flavonoids are compounds that have shown antibacterial and antiproliferative activities in several organisms (Liu et al., 2013; Talib et al., 2012). According to previous information, protein and phenolic extract could be a potential source of bioactive compounds.

The red microalga *Rhodosorus marinus* which belongs to Rodophyta division and Rhodophyceae class is easy to culture in the laboratory under controlled laboratory conditions such as ambient temperature and synthetic culture medium elaborated with seawater. This microalga presents the majority of red seaweeds metabolic characteristics, and, being a unicellular organism, it is an optimal model to study red algae for biological evaluations.

The aim of this research work was to evaluate protein and phenolic extracts obtained from *R. marinus* for antiproliferative activity against cervical, colon, lung, prostate, and breast cancer cell lines, as well as for antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella choleraesuis*.

MATERIALS AND METHODS

Biomass obtention from microalgae

Red microalga *R. marinus* strain UTEX 1723 was acquired from Texas University collection. Microalgae were cultured for 60 days in modified Erdschreiber medium ($NaNO_3$ 0.02%, NaH_2PO_4 0.003%, $FeCl_3$ 1.16 mg/L, $MnCl_2$ 0.5 mg/L, $ZnCl_2$ 0.06 mg/L, $CoCl_2$ 0.024 mg/L, Na_2MoO_4 0.048 mg/L, cyanocobalamine 0.01 mg/L and thiamine 0.2 mg/L, dissolved in sterilized seawater (salinity of 35 g/L), with continuous air bubbling, 12/12 h dark/light cycles, and a temperature of 25 ± 2°C. The culture was then centrifuged at 3600

x *g*, 4°C, during 10 min (Eppendorf centrifuge 5804 R); supernatant was discarded and the pellet was washed twice with distilled water and then lyophilized at - 90°C, low pressure during 70 ± 5 h (Labconco Freeze zone 6 plus) and dried biomass was collected for further analyzes. Unless otherwise stated, all chemicals used were purchased from Sigma Aldrich Co.

Protein extracts

Protein extracts from *R. marinus* (Extracts A and B) were obtained as previously reported by Básaca-Loya et al. (2009). This method was slightly modified as follows. Lyophilized biomass (2 g) was re-suspended in 50 ml of PBS (0.01 M Na_2HPO_4, 0.1 M NaCl, pH 7.0), and the *R. marinus* suspension was maintained during 30 min at -80°C, and then it was manually macerated during 5 min using mortar and pestle. The macerated solution was sonicated during 30 min using Cole Parmer 8890 equipment, and centrifuged at 5000 × *g*, during 15 min, at 4°C. The supernatant was filtered through a Whatman # 1 filter paper, saturated with 40% $(NH_4)_2SO_4$, and maintained under gentle agitation during 3 h, at 4°C. After that, the solution was centrifuged as previously described, the pellet was collected (Extract A), and the supernatant was saturated until 60% $(NH_4)_2SO_4$ was reached. This last solution was maintained under gentle agitation during 12 h, at 4°C, and centrifuged again under the same conditions, the supernatant was discarded, and the pellet was collected (Extract B) for further use.

Both extracts were fractionated by gel filtration chromatography, using sephadex G-25 as the stationary phase and PBS as the eluent, at 1 ml/min flow rate. Elution of the components of the extracts was monitored at 280 nm (Beckman coulter spectrophotometer DU 530). After elution, each extract was collected and dialyzed. Dialysis process was performed using a membrane tubing (Spectrum 132655, MWCO 6 - 8 kDa) that was sealed and placed in 5 successive water baths containing deionized water at 4°C and gentle stirring at time 0, 4, 16, 20 and 24 h, respectively; then, both extracts were lyophilized under the conditions previously described.

Protein quantification

Protein quantification was performed following the method described by Bradford (Bradford, 1976). A standard curve was prepared using bovine serum albumin in concentrations from 62.5 to 2 mg/mL. Color development was measured at 595 nm, 5 min after reagent addition. Additionally, SDS-PAGE chromatography was performed, as described by Grabski and Burgess (2010).

Phenolic extract

Phenolic extract (Extract C) was obtained according to the techniques previously described (Martos et al., 2000), with the following variations: lyophilized *R. marinus* biomass (2 g) was suspended in 50 ml of acid water (deionized water, pH 2.0). The suspension rested at -80°C, during 30 min; then it was manually macerated, during 5 min. After a 30 min-period of sonication, the solution was centrifuged at 5000 × *g*, at 4°C, during 10 min, and the pellet was discarded. The supernatant was passed through an Amberlite XAD-2 (Fluka, pore size 9 nm, particle size 0.3 to 1.2 mm) column (25 × 2 cm).

Phenolic compounds remained in the column, while sugars and other polar compounds eluted with the aqueous solvent. The column was washed with acid water (pH 2.0, 125 ml) and subsequently with deionized water (125 ml). The whole phenolic fraction was finally eluted with methanol (250 ml) and taken to dryness under reduced pressure (145 Torr, 45°C). Finally, the extract was suspended in 10 ml of water and lyophilized under the same conditions described above.

Total phenolic compound quantification

Total phenolic compounds content was measured using Folin - Ciocalteu´s phenol reagent (Waterhouse, 2003). Fifty microliters of extract were mixed with 3 mL of water and 250 µL of 1 N Folin reagent. After 5 min, 750 µL of 20% sodium bicarbonate and 950 µL of water were added and maintained for 30 min at room temperature. Finally, absorbance was measured at 765 nm. A standard curve was obtained using gallic acid (G7384) in concentrations from 80 to 400 µg/ml.

Antiproliferative evaluation

HeLa (ATCC CCL2), HCT 116 (ATCC CCL247), A549 (ATCC CCL185), and ARPE 19 (ATCC CRL2302) cells were cultured in MEM medium (D7777) supplemented with 10% bovine fetal serum (BF6178). For MDA-MB-231 cells (HTB 26), MEM medium was also used, but 15% bovine fetal serum, 1% L-glutamine (G6392), and 1% non-essential aminoacid solution (M7145), were added. Finally, for HCC-38 cells (ATCC CRL2314) and 22Rv-1 (ATCC CRL2505), RPMI medium (R4130) supplemented with 10% bovine fetal serum and 1% L-glutamine, was used. All cell cultures were maintained at 36 ± 1°C, 5% CO_2 atmosphere and 85% of moisture, until 95% of confluence was reached. Cells were detached with trypsin-EDTA solution (T4049) and a cellular suspension of $2x10^5$ cells/ml was prepared.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (ATCC 30-1010K) was performed as previously described (Mhadhebi et al., 2011). Briefly, 50 µL of cellular suspension were placed in microtiter plate wells (in triplicates) and incubated overnight at 36 ± 1°C, 5% CO_2 atmosphere and 85% of moisture. After that, 50 µL of extract, dissolved in the same medium used for culture, was added. The tested concentrations included serial dilutions from 1 to 0.125 mg/ml of each extract in the appropriate culture medium. Cell cultures in microtiter plates were incubated during 48 ± 1 h under the same previously described conditions. Then, 10 µL of MTT solution were added to each well and incubated for another 4 h-period. Finally, 150 µL of acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol) were added to dissolve formazan crystals. The absorbance was measured at 570 nm. Fresh medium and 25 mmol cis-diammineplatinum (II) dichloride (P4394) solutions were used as experimental controls. The experiment was repeated three times.

Antibacterial evaluation

Antibacterial activity was determined using the disk diffusion assay (Ravaei et al., 2013). Bacterial strains used for the experiment were *S. choleraesuis* (ATCC 14028), *L. monocytogenes* (ATCC 19111), and *S. aureus* (ATCC 25923). All the bacteria were grown in tryptic soy broth (BD 257107), 36 ± 1°C, for 24 h. Blank disks (5 mm diameter, 1 mm thickness) were impregnated with 10 µL of extract and let to dry; then, they were impregnated again with the same amount of extract. The concentration used of each extract was 1 mg/ml. Blank disks, impregnated with PBS and penicillin - streptomycin solution (P0781), were used as negative and positive control, respectively. Twenty milliliters of molten Mueller-Hinton agar (BD 221275) were poured into 8 cm sterile Petri dishes and let to solidify. One-hundred microliters of the overnight broth culture of each bacterial strain (previously adjusted to 0.5 McFarland turbidity), were spread on the plates. A disk impregnated with the test extract and two control disks were placed on the surface of the

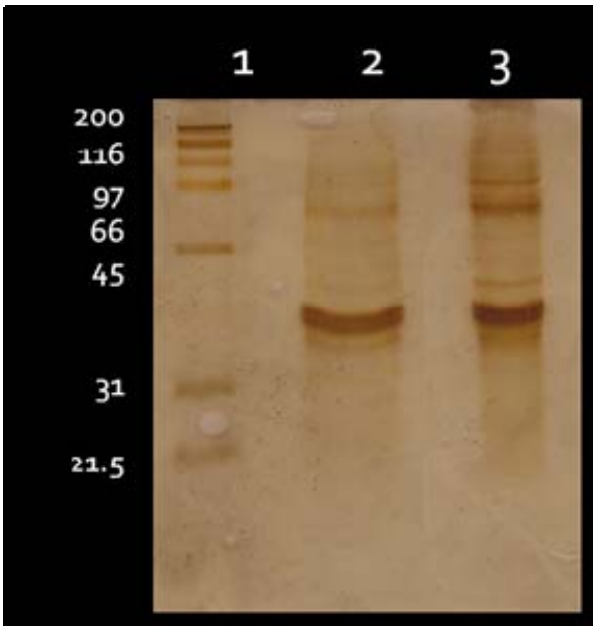

Figure 1. Electrophoretic profile obtained for protein extracts using SDS-PAGE and silver stain. Molecular weights are expressed in kDa. Lane 1, molecular weight marker; lane 2, extract obtained with 40% ammonium sulfate precipitation; and lane 3, extract obtained with 60% ammonium sulfate precipitation.

agar and incubated at 36 ± 1°C, for 24 h. The experiment was carried out in triplicates. An inhibition zone around the disks was interpreted as a positive qualitative antibacterial activity.

Statistical analysis

Cell proliferation graphs were obtained using Graphpad prism version 3.02. Tukey means comparison test (95%) was used to determine difference between cells proliferation under different extracts concentrations. For IC_{50} a linear regression was performed. Means comparisons and linear regression were analyzed using NCSS 6.0 software.

RESULTS

Quantification of extracts

Two calibration curves were required for the quantification of protein and phenolic extracts. For protein extract, a calibration curve was made with bovine serum albumin obtaining the following equation: $A = 0.7129C - 0.0042$ ($R^2 = 0.9982$); where, A represents absorbance and C the test extracts concentration. Using the previous equation, protein concentration in the extract A was 0.697 mg/ml, whereas for extract B, the protein concentration was 1.103 mg/ml. The electrophoretic profiles for both protein extracts obtained by SDS-PAGE are shown in Figure 1.

For the quantification of total phenolic compounds in extract C, the calibration curve was made using gallic acid, obtaining the following equation: $A = 0.9621C + 0.0006$ ($R^2 = 0.9984$). According to this equation, the concentration of total phenolic compounds in the extract C was 7.67 mg equivalents of gallic acid / g in dry weight basis.

Antiproliferative evaluation

Two different effects were observed in the cancerous cell lines when exposed to the test extracts. In Figure 2, these two different effects could be observed; extract C was able to inhibit CCL247 cell proliferation showing a dose-response type of relationship, while HTB26 cell line was not inhibited by the extract B. The statistical analysis confirmed previous observations.

The IC_{50} value, defined as the extract concentration that resulted in 50% of cell growth inhibition, was calculated for each extract versus each cell line (Table 1). A relationship between IC_{50} value and proliferation response was observed in our experiment. When an inhibition of cell growth was obtained the IC_{50} parameter was a positive value, whereas in non-effect or slightly promotion effect, a negative IC_{50} value was obtained.

Table 1 shows all the IC_{50} calculated for the effect of the three extracts against each tested cell line.

Antibacterial evaluation

No antibacterial effect was observed when bacterial species *S. aureus*, *L. monocytogenes* and *S. choleraesuis* were exposed to the extracts, under previously described conditions.

DISCUSSION

Electrophoretic patterns (Figure 1) of extracts A and B were quite similar, although extract B (1.103 mg/ml) had almost twice protein compared to extract A (0.697 mg/ml). The intensity of the bands in electrophoretic gels was consistent with the protein concentration. A previous report (Básaca-Loya et al., 2009), proposed that extract A included a set of proteins different from the ones obtained in extract B. According to this previous report, just extract B contained phycobiliproteins, and extract A was just a set of proteins different of phycobiliproteins that could be discarded. However, electrophoretic profiles showed similar patterns with apparently higher protein concentration in extract B. Previous observation was confirmed using Bradford to determine protein content and, in this way, inferring that both extracts might include the same set of proteins, just with different concentration, was possible. No further characterization was performed because neither extract A nor extract B showed biological activity under tested conditions.

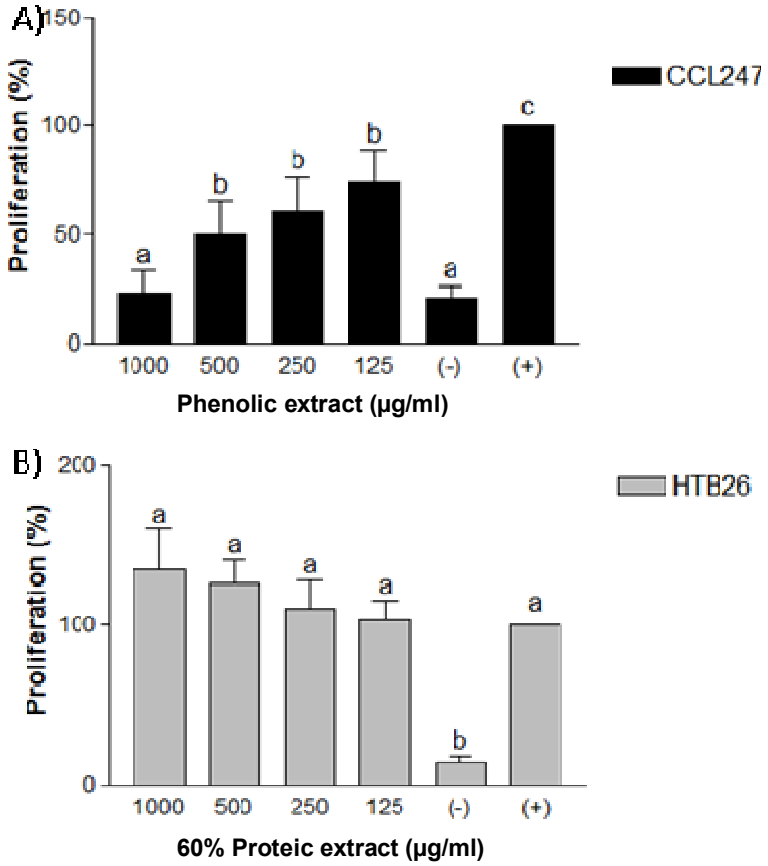

Figure 2. Different effects of tested extracts in cancer cells proliferation. A) an inhibition in growth of CCL247 by the concentration effect of phenolic extract. B) non effect in HTB26 proliferation due to different concentrations of 60% protein extract addition. Different literal in bars means a statistical difference ($p<0.05$). (-) 25 mmol cis-diammineplatinum (II) dichloride, a commercial antiproliferative drug and (+) fresh culture medium.

Table 1. IC_{50} value in mg/mL (the total extract concentration that resulted in 50% of cell growth inhibition) for the three tested extracts and Cis-Pt (commercial antiproliferative drug used as control) against the different cell lines. ND means not detected, because no relationship could be observed between extract concentration and cell proliferation.

Cell line	Extract A	Extract B	Extract C	Cis-Pt
CCL2 (HeLa)	ND	-1.0921	0.8010	0.0080
CCL247 (HCT 116)	ND	ND	0.5031	0.0073
HTB26 (MDA-MB-231)	ND	-1.4006	0.9401	0.0068
CRL2314 (HCC38)	-0.2175	-1.1862	0.1776	0.0127
CCL185 (A549)	-0.1214	-0.6520	ND	0.0096
CRL2505 (22Rv-1)	-0.2436	-0.5836	0.4088	0.0079
CRL2302 (ARPE19)	-1.1623	-1.1201	ND	ND

Reports of total protein content in red microalgae are not common; however, when phycocyanin was obtained from *R. marinus* in a previous work (López, 2011); a total protein concentration of 0.478 mg/mL was reported for the 60% ammonium sulfate precipitation fraction (similar to extract B). In our work, 1.103 mg/ml were obtained, which is a higher concentration than 0.478 mg/ml. This could be explained by the modifications proposed to the original extraction technique.

Total phenolic compounds have been reported for microalgae. In a previous study for *S. maxima,* 4.51 mg/g dry weight in phenolics (gallic acid equivalents) were

reported (El-baky et al., 2009). This value is higher than that obtained in the present work for *R. marinus*. Both species, *S. maxima* and *R. marinus,* are unicellular organisms, but the auxiliary pigments to chlorophyll differ from each other, since *R. marinus* is considered a red microalga and *S. maxima* is classified as a cyanobacterium. These considerations might in part explain the differences in the content of phenolic compounds.

In addition, total phenolic compounds content has been studied in some red macroalgae when biological activity was studied (Echavarria et al., 2009), with values for phenolics ranging from 0.15 to 1.98 mg equivalents of gallic acid / g in a dry weight basis, these values being lower than those obtained in present research work.

Although red seaweeds have been studied in a very limited way as a source of antiproliferative compounds, there are still some studies where antioxidant activity of phenolic extracts has been tested, and strong antioxidant activity was observed in *G. verrucosa* and some other red seaweed from the Gulf of Thailand (Abou and Shalaby, 2009; Boonchum et al., 2011). It was proposed that some antioxidant molecules, such as vitamins, enzymes, isolated from marine organisms, including microalgae, could also show antiproliferative activity (Debbab et al., 2010).

The main phycobiliprotein present in *R. marinus,* phycoerythrin, was already isolated and tested for its antiproliferative activity against HeLa cells (Rascon-Durán, 2009). A negative response was obtained; no antiproliferative activity of phycoerythrin against HeLa cells was verified. These results are similar to those obtained in the present study, where both biliprotein extracts (A and B), which include phycoerythrin and other phycobiliproteins, showed no antiproliferative activity against all cancer cell lines tested. Phycocyanin (phycobiliprotein also present in *R. marinus*) was also tested against murine cancer cells, with phycocyanin in lower concentrations than concentrations used in this work showing antiproliferative activity (Pardhasaradhi et al., 2003). This difference in the results could be due to the fact that all cancer cells used in our work were from human isolates; this seems to be important, since species specificity in antiproliferative activity was previously referred (Alberts et al., 2002).

As previously mentioned, few studies aimed on antiproliferative activity evaluation using red seaweeds have been reported. One of these reports evaluated an aqueous extract from red macroalga *G. corticota* against human leukemic cells using higher concentrations than concentrations used in present work showing the better inhibition activity at 9.3 mg/ml extract concentration (Zandi et al., 2010). Actually, these authors found a small antiproliferative activity but IC_{50} was not reported.

The potential of red macroalgae as a source of antiproliferative compounds needs to be studied in a broader way, since some investigations have shown an activity against normal cells. The aqueous and methanolic extracts obtained from *Ge. amansii* showed antiproliferative activity against murine hepatoma cells, but also against murine embrionary fibroblasts (Chen et al., 2004).

On the other hand, the phenolic extract (Extract C), obtained from *R. marinus* showed antiproliferative activity against CCL2, HTB26, CCL247, CRL2505 and CRL2314 (cancer cells lines), but not against CRL2302 (non-cancerous cell line) and CCL 185 (also a cancer cell line) (Table 1). It is important to consider that a positive value in IC_{50} indicates the concentration required to inhibit the proliferation of 50% of exposed cells, whereas a negative value indicates a non-effect in antiproliferative activity or a slightly promoter effect. As in this case, when a screening study is performed in order to find a possible biological activity, tested values with antiproliferative activity needs to be smaller than 1 mg/ml total extract concentration, higher concentrations could affect all the exposed cells (cancer and normal cells) by osmotic pressure of the extract (Picot et al., 2006). Due to this reason, the value obtained for CRL2302 (IC_{50} = 3.9321 mg/ml) is not considered as having an important biological activity. In other words, the required amount of phenolic extract in order to reach at least a 50% antiproliferative effect in CRL2302 would be almost 4 mg/ml, and usually a commercial antiproliferative drug is administrated in concentrations smaller than 1 mg/ml. In Table 1 it is possible to observe that IC_{50} obtained for extract C is much bigger than for Cis-Pt (commercial antiproliferative drug used as control), it is important to recall that Cis-Pt is an isolated kind of molecules and the extract is a rich mixture of different kinds of molecule, for this reason an extract could have IC_{50} bigger than isolated molecules (until 1 mg/ml as previously mentioned).

In *Cystoseira crinita*, a brown seaweed, the antiproliferative activity of a methanolic extract of phenolic compounds was obtained by a process quite similar to the one used in this piece of work and a IC_{50} of 58 and 80 µg/ml were reported by Mhadhebi et al. (2011) against colon and breast cancer cell lines, respectively (Mhadhebi et al., 2011). Compounds other than phycobiliproteins might be the responsible for this biological activity.

Based on the above, *R. marinus* could be a good source of new molecules with a potential to be used as antiproliferative agents against some human cancer cells. In terms of the kind of extract tested in this study, the phenolic extract was a better choice than protein extracts. None of the tested extracts showed antibacterial activity, but their antiproliferative potential is promising. The characterization of the phenolic extract and the addition of other test, are needed to perform a more complete description of its activity. For the purpose of this research work, red microalgae have a potential for being further studied as a source of novel molecules with biological

activity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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