

Marine algae extracts effects on cell proliferation on a malignant melanoma cell line and an immortalized fibroblast cell line

Zohreh Ramezanpour (✉ zohreh66@gmail.com)

International Sturgeon Research Institute, Agricultural Research Education and Extension Organization

Fateme Ghanbari Pirbasti

Laboratory of Biotechnology, Clean Nature Explorer (CNE) Company

J. Robert Waaland

University of Washington

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Abstract

Methanol extract fractions from three Persian Gulf seaweeds were tested for their effects on cell proliferation in a human malignant melanoma cell line (A375) and immortalized human foreskin fibroblast cell line (Hu02-KP). The seaweeds *Gracilaria salicornia*, *Padina boergesenii*, and *Polycladia myrica* were collected from the intertidal zone of Qeshm Island. Cell survival and proliferation were assessed by ELIZA and MTT assays. Some fractions from *G. salicornia* and *P. boergesenii* showed significant inhibition of proliferation in the melanoma cell line (41-58% and more than 85% respectively) but not the fibroblast cell line. The most abundant components identified in both species were 1, 2 –benzenedicarboxylic acid, diisooctyl ester and hexadecanoic acid, methyl ester.

Introduction

The diversity of chemicals produced by marine organisms has stimulated exploration of their potential as sources of useful chemicals for a variety of purposes (Ramezanpour et al., 2021). Here we report the effects of extracts of three Persian Gulf Seaweeds, *Gracilaria salicornia* (C. Agardh) E. Y. Dawson, *Padina boergesenii* Allender & Kraft, and *Polycladia myrica* (S.G. Gmelin) Draisma, Ballesteros, F. Rousseau & T. Thibaut on proliferation of malignant and non-malignant human cell lines.

Materials And Method

Sampling

G. salicornia, *P. boergesenii*, and *P. myrica* (formerly *Cystoseira myrica*) were gathered from the intertidal zone of Qeshm Island (26°56'N, 55°57'E) in the Persian Gulf, Iran. Immediately after collection, they were washed with seawater to remove epiphytes, rinsed with cold water, and transferred in an ice box to the laboratory where they were frozen in liquid nitrogen, freeze-dried, and stored in labeled plastic tubes at – 19°C until extraction in the laboratory. *G. salicornia* and *P. boergesenii* were identified and recorded in NCBI GenBank KJ801830.1. *P. myrica* was determined by Michael Wynne.

Extract preparation

For each sample 10 g of algal tissue wet weight (ww) were extracted four times with 100 mL of methanol in a soxhlet extractor. Then, the extract was filtered through a 0.2 µm Whatman GF/C filter. Finally, it was concentrated in a rotary vacuum evaporator at 40°C for 3 h (Buchi, Flawil, Switzerland) to obtain a semi-solid extract, followed by weighing and storing at – 19°C for further use.

Chemical composition determination through applying gas chromatography–mass spectrometry (GC-MS)

An Agilent 5975c mass selective detector was used in the present study. Additionally, an ion source temperature of 300°C and electron energy of 70 eV were used to acquire an electron ionization mass spectrum. The gas chromatograph was equipped with a HP-5MS capillary column and helium carrier gas at the rate of 3 mL min⁻¹. In ethanol extract analysis, the oven temperature program was initially set at 50°C and maintained for 2 min, followed by a steady climb to 120°C for 10 min and an increase to 270°C for 5 min. GC-MS analysis was performed in triplicate.

Isolation of Major compounds of extracts by column chromatography

The residue of methanol extract of *G. salicornia* was dissolved in methanol and then subjected to silica gel 60 (0.063-0.200 mm) column chromatography and eluted in sequence with 20 mL of each composition of hexane:chloroform (6:12), hexane:chloroform:acetone (4:6:12) and finally more polar compounds were separated with hexane:methanol (4:12). Finally three major fractions were obtained (G1-G3).

The residue of methanol extract of *P. boergesenii* was dissolved in methanol and then subjected to silica gel 60 (0.063-0.200 mm) column chromatography and eluted with chloroform:methanol in gradient (9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 3:7) to give four fractions (P1-P4).

The residue of methanol extract of *P. myrica* was dissolved in methanol and then subjected to silica gel 60 (0.063-0.200 mm) column chromatography and eluted in sequence with composition of hexane:chloroform:acetone (1:6:3), following by increasing the

proportion of acetone to separate polar compounds. Finally four major fractions were obtained (F1-F4).

NMR

The ^1H NMR spectra were obtained on a Bruker Avance 500 spectrometer. CDCl_3 was used as solvent.

Effect of the extract components on the cell proliferation of human malignant melanoma cell line (A375) and immortalized human foreskin fibroblast cells (Hu02-KP)

The effect of aflibercept (AF) and bevacizumab (BV) was evaluated on cell proliferation and viability using an MTT assay. Briefly, the cells were seeded in a 96-well plate (5×10^3 cells well $^{-1}$), cultured, and incubated at 37°C overnight. Further, the supernatant was replaced with a previously-described serum free medium without 10% FBS. Furthermore, VEGF, AF, and BV were added into the wells according to the following instructions.

The control group included the cells and serum free medium, while the VEGF one consisted of the cells, serum free medium, and 100 ng mL $^{-1}$ VEGF. Regarding the AF and BV groups, 100 $\mu\text{g mL}^{-1}$ AF and BV were respectively poured into the wells containing the cells, serum free medium, and 100 ng mL $^{-1}$ VEGF.

After 72 h, MTT solution (250 g mL $^{-1}$ as final concentration) was added to each well and the plate was incubated at 37°C for 3 h. Then, 100 μL of DMSO was poured into each well to dissolve insoluble formazan.

The cell proliferation was measured based on the cell metabolism activity, as well as the mitochondrial conversion of MTT into formazan. The quantity of the formed formazan was determined by using a microtiter plate reader at 570 nm. Finally, the cells exposed to the extracts (2.5 mg mL $^{-1}$ to 10 $\mu\text{g mL}^{-1}$) for 12 h were used for MTT assay to assess the in vitro cytotoxicity of A375 and Hu02-KP.

Results

The bioactive methanol extracts of *G. salicornia*, *P. boergesenii*, and *P. myrica* were fractionated by polar compounds elution. A total of 22 bioactive compounds were obtained from the peaks of fractions by GC-MS analysis (Table 1).

Table 1
GC-Mass analysis of major fractions of methanol extract of *Polycladia myrica*, *Gracilaria salicornia* and *Padina boergesenii* separated by column chromatography

Compound	<i>P. boergesenii</i>				<i>G. salicornia</i>			<i>P. myrica</i>			
	P1	P2	P3	P4	G1	G2	G3	F1	F2	F3	F4
<i>Cyclopentasiloxane, decamethyl-</i>											3.73
<i>Dianhydromannitol</i>							3.05				
<i>1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone</i>						2.77					
<i>1,4-Benzenedicarboxylic acid, dimethyl ester</i>			1.95	0.96				3.53	7.88	4.97	
<i>Hexadecane</i>											2.44
<i>Cyclododecene, 1-methyl-</i>							2.37				
<i>Methyl tetradecanoate</i>		2.60			4.25	5.01	7.41	4.59	6.34	7.70	3.01
<i>Tridecanoic acid, 12-methyl-, methyl ester</i>			1.25								
<i>Octadecane</i>											2.65
<i>Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-(PINANE)</i>						7.67	5.47				
<i>2-Pentadecanone, 6,10,14-trimethyl-</i>					4.89		5.02	5.72	5.27	4.10	3.03
<i>1,2-Benzenedicarboxylic acid, butyl octyl ester</i>								1.89			
<i>9-Hexadecenoic acid, methyl ester</i>							3.36				
<i>Cyclohexadecane</i>					2.54			1.89			
<i>Hexadecanoic acid, methyl ester</i>	64.31	25.43	10.07	3.33	28.79	35.30	39.23	33.22	53.62	62.62	27.61
<i>Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester</i>			0.98								3.35
<i>Ethylhexylmethylphthalate (1,2-Benzenedicarboxylic acid, butyl methyl ester)</i>			1.16	2.10				1.87			
<i>9-Octadecenoic acid, methyl ester</i>	12.08	2.38	1.43			6.43	4.36				
<i>9,12-Octadecadienoic acid, methyl ester</i>		2.63			2.57	2.17	2.80	2.32	3.85	4.99	
<i>Octadecanoic acid, methyl ester</i>	9.08	2.88	2.35	1.08	3.29	2.96		3.45	9.87	5.22	3.75
<i>1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester</i>						2.05	2.88	14.06			13.20
<i>1,2-Benzenedicarboxylic acid, diisooctyl ester</i>		64.07	78.77	92.53	8.78						

Based on the results, the cell toxicity of the fractions of *G. salicornia* (G1, G2, and G3) exhibited a minimum cell survival of 19% at 15 mg mL⁻¹ concentration on fibroblasts after 24 h, as well as a maximum at 1 mg mL⁻¹ concentration. However, the cell survival of G1 and G2 fractions showed no significant difference in fibroblasts and melanoma cell lines. Further, G3 showed no significant difference on melanoma and fibroblast cells except at concentrations of 0.5–2.5 mg mL⁻¹, it was not observed to be the same as in fibroblast cells with significant mortality, but in melanoma cells the survival was 41–58% (Fig. 1).

From *P. boerghesensis*, four fractions with different colors (P1, P2, P3, and P4) were separated and are presented in Fig. 2. No significant difference was observed in the effect of fractions P1, P2, P3, and P4 in the same concentrations on melanoma cells A375 and Hu-02 fibroblasts.

Significant results were obtained for the effect of fraction P3 after 24 and 48 h so that the 2.5

mg mL⁻¹ concentration of P3 led to the highest inhibition of A375 melanoma cells (> 85%) and Hu-02 fibroblast cells (88%). Fraction P2 averages about 55% of A375 melanoma cells and about 88% of Hu-02 fibroblasts.

Finally, the effects related to the fractions of *P. myrica* (F1, F2, F3, and F4) were not significantly different on melanoma cells and fibroblasts (Fig. 3).

¹H NMR spectra

The ¹H NMR spectrum was recorded for the two fractions isolated from chromatography column which had better anticancer activity. Based on the ¹H NMR spectrum of the fraction separated from *G. salicornia* in deuterated chloroform, no signal was detected in the 6–9 ppm range assigned to aromatic protons (Fig. 4). All peaks appeared at the chemical shift of 0.84–5.1 ppm, which are attributed to aliphatic protons. Additionally, three peaks were observed respectively at 5.11 (broad), 4.85, and 4.18–4.60 ppm (broad) with the peak area of 2, 1, and around 3, which may be related to the protons on the carbon unsaturated bond and/or those adjacent to electron-withdrawing group. The other peaks emerged at the chemical shift of 2.28 (peak area: 0.2), 2.00 (2, triplet), 1.74–1.84 (1.5, two broad merged ones), 1.53 (0.4), 1.23 (2.8, sharp and tall), and 0.86 (0.6, triplet).

Further, the ¹H NMR spectrum of the fraction from *P. boerghesensis* (Fig. 5) was measured in deuterated chloroform, which exhibited no signal corresponding to aromatic protons (6–9 ppm). Furthermore, two broad peaks appeared at 5.13 and 3.99 with the peak area of 13 and 7, respectively. Finally, a sharp and a triplet peak were detected at 1.24 (3) and 0.86 (1), respectively.

Discussion

Many chemicals extracted from marine organisms have different mechanisms of antitumor and cell growth effects (Lichota and Gwozdziński 2018). In the present study, methanol was used to maximize the potential for extracting organic components from each sample.

Cancer starts when cells begin to grow out of control. Melanoma one of the most problematic cancers starts in certain skin cells (melanocytes) and can develop anywhere on the skin.

Ning and Andl (2013) reported the significant roles of microRNAs on the pathogenesis of some skin cancers such as squamous cell carcinoma (SCC) and melanoma.

In melanoma, the over expression of miR-182 promotes survival, migration, and metastasis repressed by the tumor suppressor FOXO3 and microphthalmia-associated transcription factor-M, while its expression increases with the development from primary to metastatic melanoma (Segura et al. 2009).

The most active anticancer compounds include the psammoplin isolated from marine microalgae, cyanobacteria, and heterotrophic bacteria (Lichota and Gwozdziński 2018). In addition, the alkaloids from marine algae produce anticancer compounds (Güven et al. 2010; Tohme et al. 2011). Polyphenols and polysaccharides are extracted from marine flora (Boopathy and Kathiresan 2010). Further, bryostatins, halichondrins, spongistatin, discodermolide, hemiasterlins, and salinosporamides derived from the marine organism metabolites exhibit cytostatic activity. Some researchers outlined the antiproliferative activity of the 3-Epi-29-hydroxystelliferin E derivative of stelliferin against melanoma cells (MALME-3M) (Meragelman et al. 2001). In fact, this compound is an isomalabaricane-

type triterpenoid, which is extracted from the sponge *Jaspis stellifera* (McCabe et al. 1982). According to Berge et al. (2002), the sulfolipid classes (SLs) in the total lipids of *Porphyridium cruentum* (S.F.Gray) Nägeli show inhibitory effects on malignant melanoma (M4 Beu) cancer cells. The sulfolipids in algae were considered to be high in palmitic acid (C16:0) in *Galaxaura cylindrica* (J. Ellis & Solander) J.V. Lamouroux and *Taonia atomaria* (Woodward) J. Agardh. Furthermore, sulfoquinovosyl-diacylglycerol and sulfoquinovosylacylglycerol (SQAG) have been recognized as the main sulfolipids in algae (El Baz et al., 2013).

The results of our GC-MS analysis demonstrated several important sources of anticancer agents. The compounds were effective in vitro due to their anti-proliferative activities against melanoma and fibroblast cell lines. In addition, 1,4-benzenedicarboxylic acid, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, diisooctyl ester, ethylhexyl methyl phthalate (1,2-benzenedicarboxylic acid, butyl methyl ester), and butyl octyl ester were found in the fractions from *P. boergesenii* and *P. myrica*. The compounds have been reported in many medicinal plants (Save et al. 2015; Beulah et al. 2018) and in red algae such as *Jania rubens* (L.) Lamouroux, *Corallina mediterranea* J.E. Areschoug and *Pterocladia capillacea* (S.G. Gmelin) Bornet (El-Din and El-Ahwany 2016).

1,2-benzenedicarboxylic acid and diisooctyl ester have found in *Allium autumnale*, which is associated against two human breast cancer cell lines MDA-MB-231 and MCF-7 (Isbilen et al. 2018). Furthermore, 1,2-benzenedicarboxylic acid showed effectiveness on human prostate, breast, and colon cancers with strong immunomodulatory B-cell stimulation (Save et al. 2015).

Wang and Tao (2009) suggested the antitumor activities of 1, 4-benzenedicarboxylic acid, dimethyl ester, as the volatile component of *Stigmatella WXNJ-B*, on mouse melanoma cell lines. According to Kannabiran et al. (2014), 1,2-benzenedicarboxylic acid, mono 2-ethylhexyl ester from marine *Streptomyces* sp. VITSJK8 exhibits above 80% the cytotoxic activity on the growth of mouse embryonic fibroblast cancer cell lines and normal human keratinocytes.

The human melanoma cell lines are more resistant to the toxic effect of fatty acids. Cytotoxicity is observed in SK-Mel 23 cells after treatment with arachidonic, linoleic (LA), palmitic, and palmitoleic acids (Andrade et al. 2005). Palmitic acid or hexadecanoic acid is the most common saturated fatty acid in the fractions of *G. salicornia* and *P. myrica*, and the second most common compound in the fractions of *P. boergesenii*.

Octadecanoic acid, methyl ester, 9, 12-octadecadienoic acid, methyl ester, and 9-octadecenoic acid, methyl ester were identified also in the detected fractions.

Altuner et al. (2018) emphasized the antimicrobial bioactivity of 9,12-octadecadienoic acid. Additionally, 9,12-octadecadienoic acid plays an inhibitory role in the metabolites of cancer metastasis (Horrobin and Ziboh 1997, Maggiora et al. 2004). Researchers proposed different anticancer activities for 9,12-octadecadienoic acid. In low concentration it stimulates cell proliferation in the human breast cancer and lung cancer cell lines in vitro, as well as promoting colon and prostate tumorigenesis, and tumor growth in animal models (Xu and Qian 2014). Horrobin and Ziboh (1997) reported the endogenous conversion of 9, 12-octadecadienoic acid into various downstream ω -6 PUFAs. Thus, the effects of 9, 12-octadecadienoic acid on cancer growth can actually be related to a combination of the effects of its downstream products.

Lu et al. (2010) showed that the relative resistance of colorectal cancer cell lines to the cytotoxic action of 9, 12-octadecadienoic acid is related to its concentration and a reduction in caspase-3 activation which induces cancer cell apoptosis. Kachhap et al. (2000) observed a synergistic effect of linoleic acid and endogenous estrogen in a diet rich in ω -6-polyunsaturated fatty acid which may modulate BRCA1 gene expression thereby promoting breast cancer.

9-octadecenoic acid or oleic acid has the potential of antibacterial and antifungal activities (McGraw et al. 2002). Regarding the present study, it was the second most abundant component of *P. boergesenii* (P1) and *G. salicornia* (G2 and G3) although no significant effect was obtained for the fractions P1 of *P. boergesenii* and G3 of *G. salicornia* on melanoma cell lines.

Based on the ^1H NMR spectra of the third fraction (P3 and G3) in the methanol extract of *P. boergesenii* and *G. salicornia*, all protons appeared at high field and low chemical shifts, which can be attributed to aliphatic protons rather than aromatic ones. Finally, the compounds detected in the two fractions possessed aliphatic protons and were devoid of any aromatic substitution.

The present research has shown that additional and continued exploration of the diverse chemicals produced by the metabolism of marine algae may continue to result in discovery and identification of new resources of potential benefit to human health and welfare.

Conclusions

Some methanol extracted fractions of *Gracilaria salicornia*, *Padina boergesenii*, and *Polycladia myrica* inhibited proliferation and viability of human malignant melanoma cell lines (A375) and human foreskin fibroblast (Hu02-KP). Fractions G3, *G. salicornia* and P3 of *P. boergesenii* showed mortality in the melanoma cell line A375, but no significant toxicity was observed in fibroblast Hu02-KP cells. The most abundant components detected in the effective methanol extracts were 1, 2-benzenedicarboxylic acid, diisooctyl ester and hexadecanoic acid, methyl ester were respectively detected as the most abundant components in effective fractions of *P. boergesenii* and *G. salicornia* on A375 cell lines.

Declarations

Declarations: Conflict of interest the authors declare no competing interests.

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Availability of data and material: All data analyzed during this study are included in the article.

Author contribution: Z.R. and J.R.W. wrote the main manuscript text and Z.R. prepared figures 1-5. F.G. prepared table 1. All authors reviewed the manuscript. J.R.W. edited the manuscript. **Acknowledgements:** We thank Dr. Michael Wynne for determination of *Polycladia myrica* and Dr. Alireza Fadaeei for his recommendations. We thank Somaye Rasoli for contributing to sampling of the algae.

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Figures

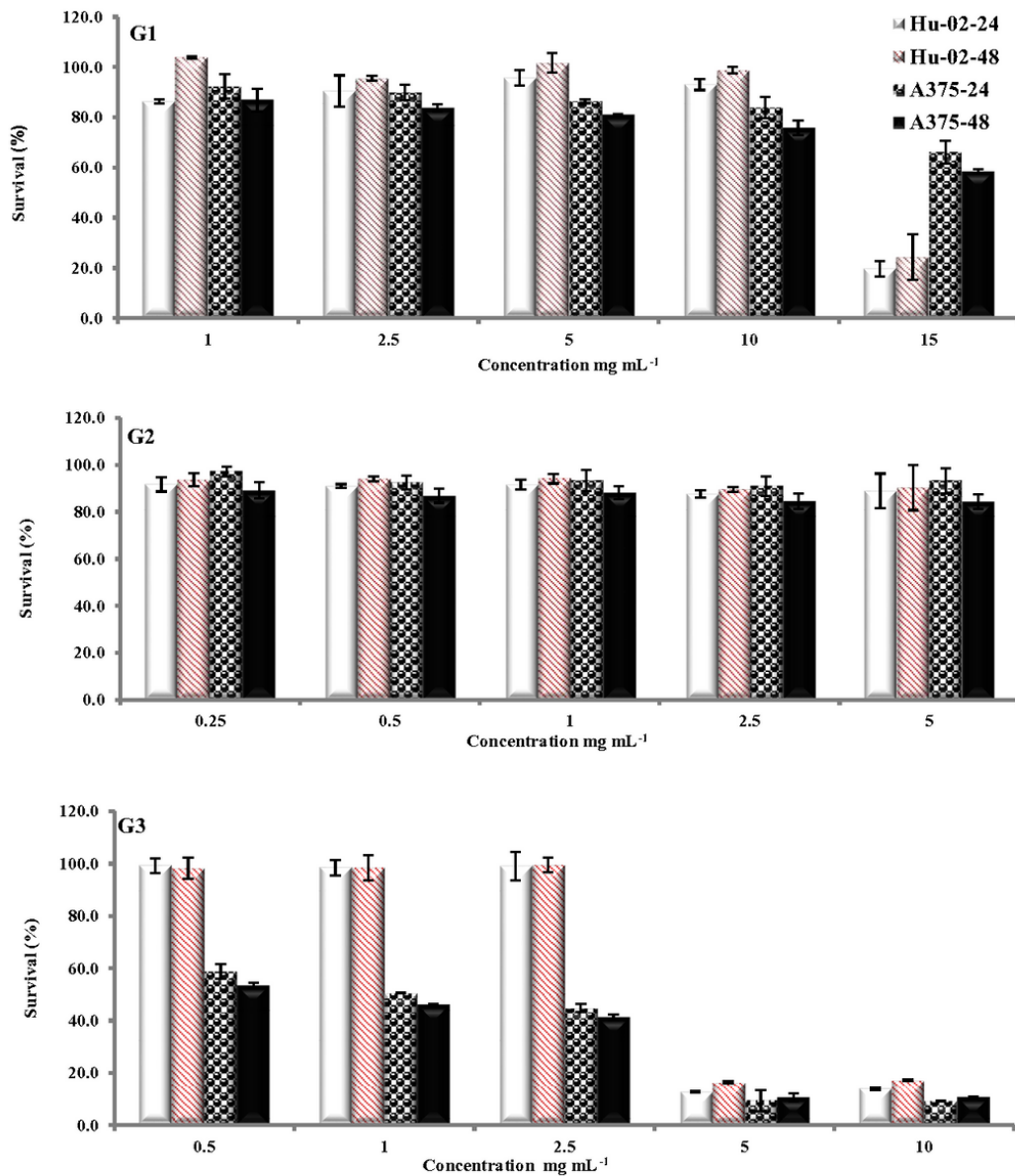


Figure 1

Survival percentage (Mean $\pm$ standard deviation, $n=3$) of melanoma cells (A375) and healthy skin cells (Hu-02) after 24 and 48 hours of exposure to the material isolated from the chromatographic column from the material extracted from the alga *Gracilaria salicornia* (G1, G2 and G3)

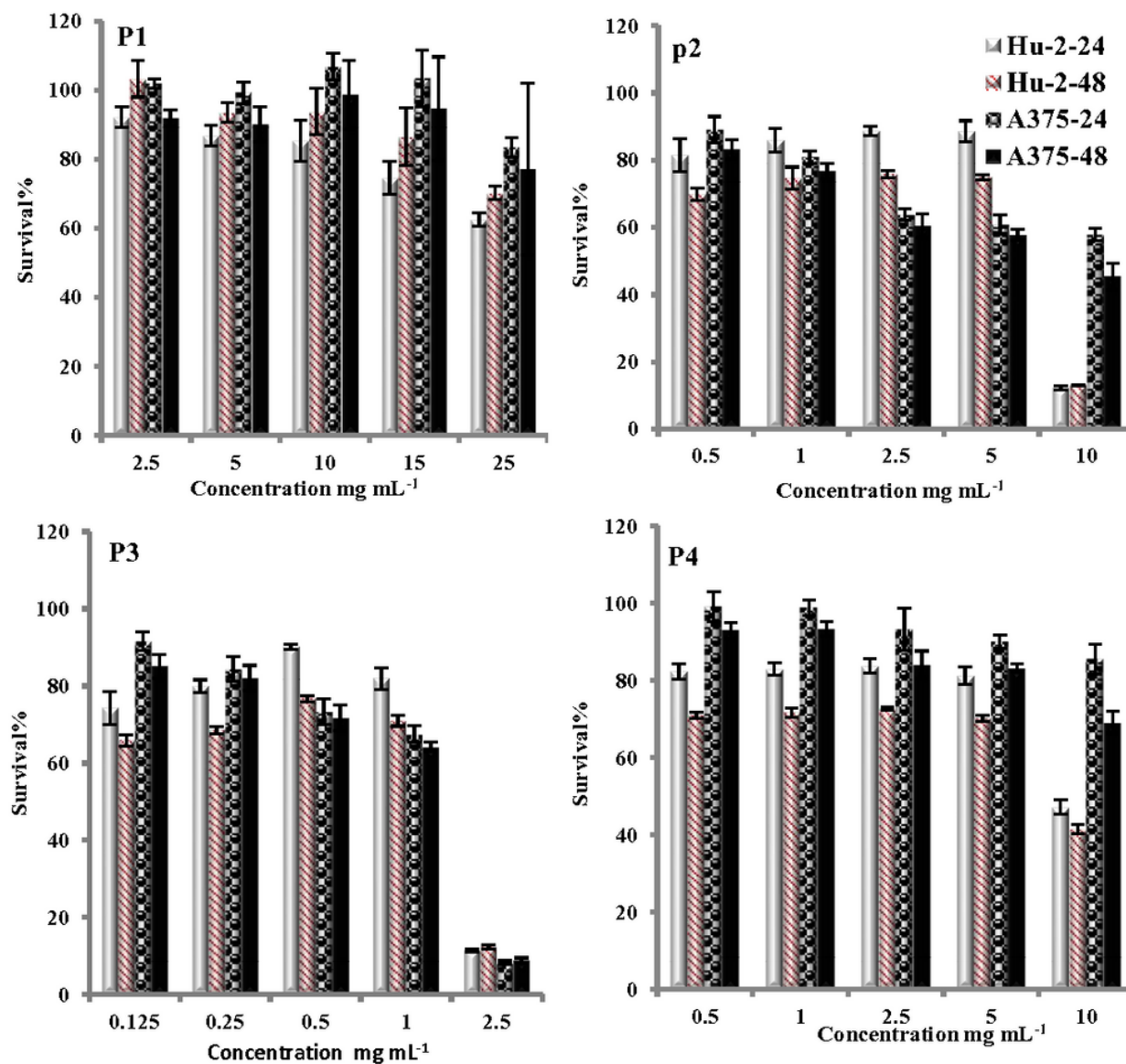


Figure 2

Survival percentage (Mean $\pm$ standard deviation, $n=3$) of melanoma cells (A375) and healthy skin cells (Hu-02) after 24 and 48 hours of exposure to the material isolated from the chromatographic column from the material extracted from the alga *Padina boergesenii* (P1, P2, P3 and P4)

Figure 3

Survival percentage (Mean $\pm$ standard deviation, $n=3$) of melanoma cells (A375) and healthy skin cells (Hu-02) after 24 and 48 hours of exposure to the material isolated from the chromatographic column from the material extracted from the alga *Polycladia myrica* (F1, F2, F3 and F4)

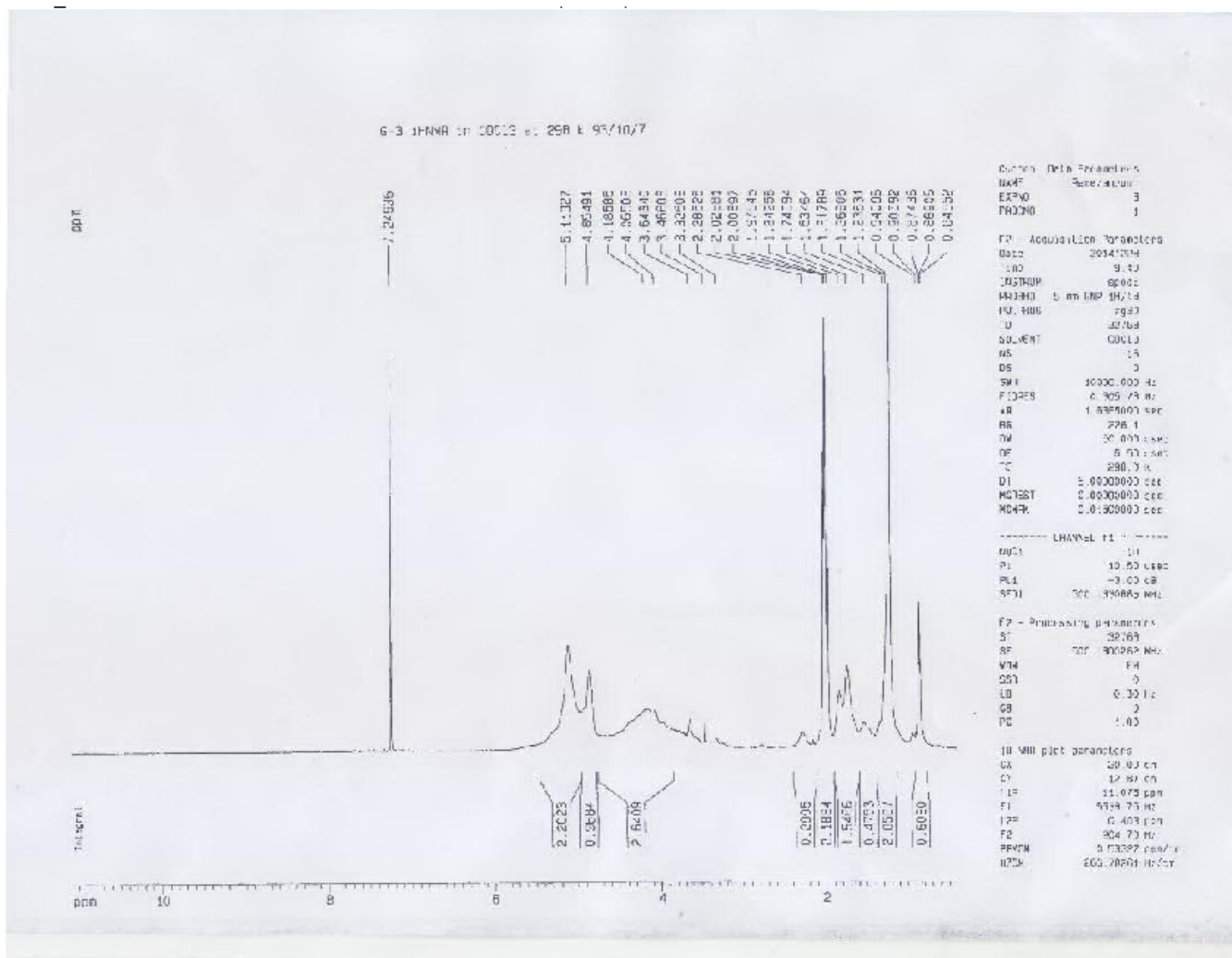


Figure 4

H-NMR spectrum of third fraction (G3) of methanol extract of *Gracilaria salicornia*

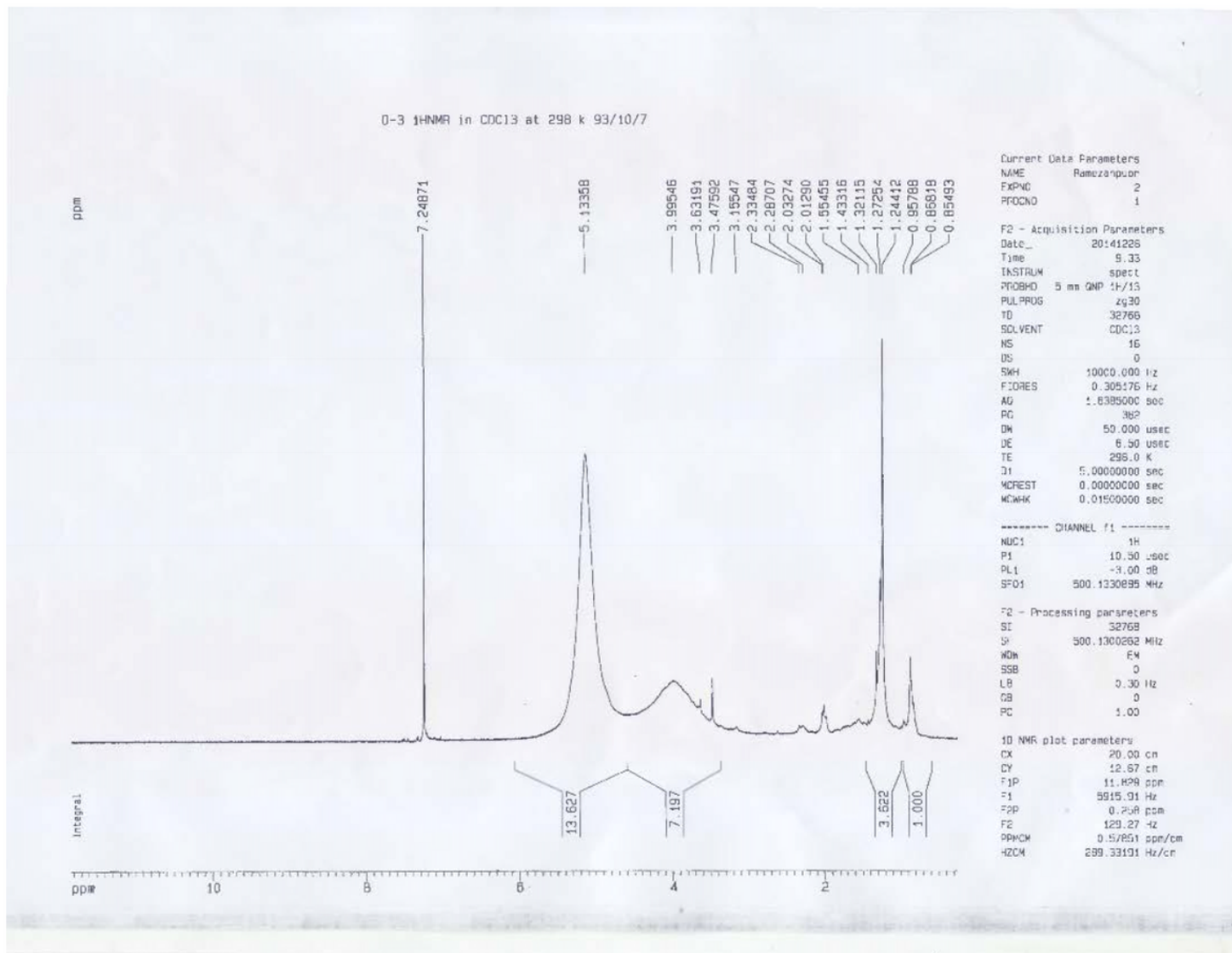


Figure 5

¹H-NMR spectrum of third fraction (P3) of methanol extract of *Padina boergesenii*