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Antiproliferative and pro-apoptotic properties against colon carcinoma cell line of the marine sponge haliclona sp. From poblacion, kauswagan, lanao del norte, Philippines

Mylene Mondarte Uy[1,2], Rajane Faith Taga Bautista-Ladrillo*[2]

[1] Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, Iligan City, Philippines

[2] Department of Chemistry, College of Science and Mathematics (CSM), Mindanao State University – Iligan Institute of Technology (MSU-IIT), Iligan City, Philippines

Corresponding author: Rajane Faith T Bautista Ladrillo ✉rajanefaith.bautista@g.msuiit.edu.ph, **Orcid Id**: https//orcid.org/0009-0008-2888-7471

Department of Chemistry, College of Science and Mathematics (CSM), Mindanao State University – Iligan Institute of Technology (MSU-IIT), Iligan City, Philippines

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ABSTRACT

Marine sponge *Haliclona* sp. was examined for its anticancer potential focusing on the antiproliferative and pro-apoptotic properties against HCT116 (colon) cancer cell line. The 16 fractions obtained from *Haliclona* sp. were subjected to antiproliferative and pro-apoptotic evaluation via MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and Caspase-Glo 3/7 assay, respectively, at 30 µg/mL concentration. Fractions 10, 13, 14, and 15 exhibited a significant decrease ($p < 0.05$) in cell number compared to the negative control but possessed moderate cytotoxicity (> 50% cell viability) against HCT116. Only fraction 8 showed a significant increase ($p < 0.05$) in relative light unit (RLU) and fold change (FC) (1809.78 and 2.42), more than two times larger, compared to the negative control (754.89 RLU and 1.00 FC). This result demands further study on fraction 8 to identify and characterize the structure of the compounds responsible for its bioactivity and hence could be a source of drug leads.

Keywords: Cancer, Marine sponge, *Haliclona* sp., Antiproliferative, Apoptosis

INTRODUCTION

Cancer is a major public health problem worldwide. The most common cancers are breast, lung, colon and rectum, and prostate accounting for nearly 10 million deaths in 2020, as reported by World Health Organization (WHO) [1]. This illness involves abnormal cell and tissue proliferation or growth potentially spreading to other parts of the body [2]. All types of cancer share certain traits referred to as hallmarks that give its malignant properties. Hanahan and Weinberg proposed six major biological hallmarks of cancer and two of these are sustaining proliferative signaling and resisting cell death [3]. As a result, the majority of traditional anticancer medications have been created to specifically target one of these markers [4]. Although conventional cancer treatment has been successful to some extent, high-dose requirements, adverse drug reaction (ADR), and the development of multiple drug resistance are some of the main drawbacks of it. Thus, the discovery and development of new anticancer drugs based on natural products which can be more effective with fewer side effects have been the focus of much research [5,6].

The investigation of natural products (chemical substances derived from animals, plants, and microbes) has been the single most productive source of leads for the development of drugs [7,8]. Natural products have long been used as a conventional source of therapeutic compounds, particularly secondary metabolites from terrestrial plants and microbes [9]. Currently, approximately 80% of the approved

chemotherapeutic drugs are based on bioactive natural products since they are usually less toxic, effective, inexpensive, and easily available than traditional chemotherapy drugs [10,11].

The marine environment offers considerable promise for finding novel organisms that can help with the prevention and treatment of cancer [11]. It has been confirmed that marine organisms contain highly rich sources of distinctive and biologically active secondary metabolites, where some compounds are extremely potent and are created in response to the harsh growth and competitive conditions that occur in the marine environment [12]. Marine sponges have been an abundant and chemically diverse source of natural compounds that produce bioactive metabolites that are involved in their defense mechanisms. They have been shown to exhibit a wide range of biological activities against human diseases with potential applications in medicinal chemistry and the pharmaceutical industry, particularly in relation to the process of developing new drugs [13,14]. As of 2020, there are five USA Food and Drug Administration (FDA) approved and one additional drug registered in Europe (EU). The first - USA FDA approved marine-derived drug is Cytarabine (Ara-C) which is approved in 1969 and is isolated from the Caribbean sponge *Tethya crypta* [15,16]. Marine sponge *Haliclona* sp. produces a wide range of secondary metabolites, most commonly bioactive alkaloids. Salicylihalamide A, a novel salicylate macrolide with a highly unsaturated amide side chain, found to possess a potent and unique differential cytotoxicity profile in the NCI 60-cell line human tumor assay was isolated from an Australian sponge *Haliclona* sp. [17]. In this study, marine sponge *Haliclona* sp. is evaluated for its anticancer potential specifically on its antiproliferative and pro-apoptotic properties against the HCT116 colon carcinoma cell line.

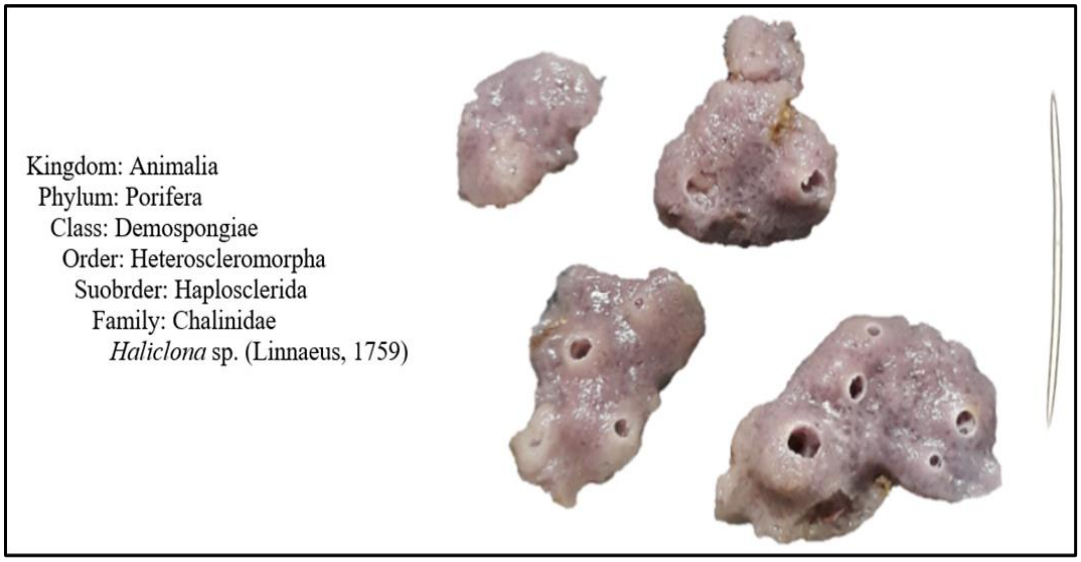
MATERIALS AND METHODS

Materials

The marine sponge *Haliclona* sp. (Figure 1) was collected from Poblacion, Kauswagan, Lanao del Norte, Philippines by the experts, and marine biologist collaborators, followed necessary procedure based on Bureau of Fisheries and Aquatic Resources (BFAR). Morphological identification of the sponge sample was made by the same collaborator. Voucher specimen of *Haliclona* sp. with voucher specimen number of PK10 is kept at Natural Products and Drug Discovery Laboratory, Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, Iligan City, Philippines.

The HCT116 (colon cancer) cells were purchased from American type culture collection. The McCoy's 5A media and the fetal bovine serum were obtained from Gibco. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (CellTiter96® Non-radioactive Cell Proliferation Assay) and Caspase-Glo® 3/7 Assay kits were purchased from Promega Corporation. Liquid chromatography grade of ethyl acetate, methanol, and ethanol from Sigma-Aldrich were used in sample extraction. Dimethyl sulfoxide (DMSO), digitonin, and sodium butyrate were purchased from Sigma-Aldrich and were used in the assay.

Figure 1: Image of the collected marine sponge *Haliclona* sp. from Poblacion, Kauswagan, Lanao del Norte, Philippines.



Preparation of *Haliclona* sp. Crude Extract

The sponge sample was then processed, freeze-dried, and weighed. The freeze-dried sponge sample was chopped and steeped in a 1:1 ethyl acetate-methanol mixture for 3 days to obtain the nonpolar extract. The sponge residues were then soaked in a 1:1 ethanol-water mixture for 3 days to finally obtain the polar extract. After solvent extraction, all of the extracted samples obtained were concentrated *in vacuo* and/or freeze-dried to obtain the weights of the sponge extracts.

Fractionation of Polar Crude Extract of *Haliclona* sp.

Fractions of polar crude extract of *Haliclona* sp. were obtained using flash chromatography (BUCHI Pure C-18 Flash,

cartridge: FP ID C 18-WP 80g). A 3.8 g dried polar crude extract was weighed and dissolved with 2 mL water: methanol (1:1). It was then mixed with Wakogel® with a 1:25 ratio (crude extract:Wakogel®) and placed in the cartridge. Water, methanol, and ethyl acetate were used as solvents. The flash chromatography resulted in 16 pooled fractions (F1 to F16). All of the fractions were concentrated in vacuo and/or freeze-dried, and stored in at least -15ºC prior analysis.

Cell Culture

The colon carcinoma cells (HCT116) were grown in McCoy's (Gibco) culture media with 10% Fetal Bovine Serum (FBS). The cells were incubated and sustained at 37ºC under 5% CO_2 atmosphere.

Antiproliferative Evaluation via MTT Assay

The MTT Assay (CellTiter96® Non-radioactive Cell Proliferation Assay) was conducted to determine the cytotoxicity of the *Haliclona* sp. on the HCT116 cell line through percent cell viability. The protocol provided by the manufacturer (Promega G4000) was followed.

A 90 µL cell suspension containing 20,000 cells was seeded in a 96-well microplate (excluding for the blank wells) and incubated overnight at 37ºC and 5% CO_2 atmosphere. The following day, 10 µL of 300 µg/mL test sample and positive control were added to their corresponding wells in a 96-well plate to yield a 30 µg/mL final assay concentration of test solutions. Digitonin was used as a positive control. For the negative control, a vehicle 0.3% DMSO was added instead of the test sample. 0.3% DMSO and culture medium without the cells were used as a blank. Incubation at 37ºC and 5% CO_2 atmosphere was done overnight prior to the addition of 15 µL MTT dye solution and incubated further for 4 hours at 37ºC under 5% CO_2 atmosphere. A 100 µL of solubilization solution/stop mix was added to all wells and then incubated for an hour at room temperature and in the dark. The absorbance was then measured at 570 nm using a spectrophotometer (Perkin Elmer Victor X3 multimode spectrophotometer). Percent viability was calculated as follows:

$$Cell\ viability, \% = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Blank}{Absorbance\ of\ Negative\ Control - Absorbance\ of\ Blank} \times 100$$

Pro-apoptosis Evaluation via Caspase-Glo 3/7 Assay

The Caspase-Glo 3/7 Assay was conducted to determine the induction of apoptosis by the *Haliclona* sp. extract on the HCT116 cell line. The protocol provided by the manufacturer of the kit (Promega G8091) was followed with slight modification on the amount of Caspase-Glo 3/7 reagent used.

A 90 µL cell suspension containing 10,000 cells was seeded in a 96-well microplate (excluding for the blank wells) and incubated for 6 hours at 37ºC and 5% CO_2 atmosphere. After 6 hours of incubation, 10 µL of 300 µg/mL test sample was added to their corresponding wells in the 96-well plate to produce a 30 µg/mL final assay concentration of test solutions. For positive control, 5 mM sodium butyrate was used as a positive control. For the negative control, 0.3% DMSO was added instead of the test sample. A vehicle 0.3% DMSO and culture medium without the cells were used as a blank. The 96-well plate was then incubated for 20 hours at 37ºC and 5% CO_2 atmosphere. After incubation, 10 µL of Caspase-Glo 3/7 reagent was added to each well and was then placed in an orbital shaker, where the plates were covered with foil while shaking, for 2 hours. The mixtures were then transferred into 96-well opaque white plates. Luminescence was measured using a luminometer (Perkin Elmer Victor X3 multimode spectrophotometer). The measured luminescence was recorded and expressed as Relative light units (RLU). Hence, the pro-apoptotic property of the *Haliclona* sp. fractions in terms of caspase 3/7 activity was reported as mean RLU. The higher the RLU values are, the higher the caspase 3/7 activity is. Furthermore, to evaluate the pro-apoptotic property of the sample compared to the untreated cells (negative control), fold change (FC) was calculated.

$$Fold\ change\ (FC) = \frac{RLU\ of\ test\ sample\ or\ positive\ control}{RLU\ of\ negative\ control}$$

Statistical Analysis

All experiments were done with three trials and three replicates per trial format. Results are reported as mean values and significant differences between the negative control and the sample fractions were determined by T-test at a 95% confidence level.

RESULTS AND DISCUSSION

Antiproliferative Evaluation

Cell culture is strongly tied to the creation of novel medications. Large molecule libraries, natural extracts, or isolates are examined in cytotoxicity studies such as antitumor activity in high-throughput screening methods. In order to identify that these chemicals or compounds are beneficial, differentiating viable and dead cells is important. There are many methods to determine cell number and viability, one of this is using tetrazolium salt assay, specifically, MTT assay [18]. The MTT assay relies on the mitochondrial enzyme succinate-dehydrogenase to convert the yellow tetrazolium salt (MTT) into a blue-colored formazan. The assumed advantage of this test is that only living cells with functional mitochondria can only undergo this reaction and that the number of viable cells per well is directly proportional with the amount of formazan formed. Therefore, any decrease or increase in the number of viable cells can be determined by measuring formazan concentration by means of a plate reader [19, 20].

Table 1: Summary of results in antiproliferative evaluation via MTT assay after treatment of 30 µg/mL *Haliclona* sp. fractions against HCT116 carcinoma cell line.

Test Samples	Cell Viability, %			
	Trial 1	Trial 2	Trial 3	Average
Negative Control[a]	100.00	100.00	100.00	100.00 ± 0.00
Positive Control[b]	26.69	14.03	23.34	**21.35*** ± 6.56
Fraction 1	106.47	81.17	82.36	90.00 ± 14.28
Fraction 2	107.92	84.96	85.66	92.85 ± 13.06
Fraction 3	98.11	82.92	80.06	87.03 ± 9.70
Fraction 4	95.97	96.78	78.68	90.48 ± 10.22
Fraction 5	115.81	95.18	87.77	99.59 ± 14.53
Fraction 6	110.95	97.66	75.70	94.77 ± 17.80
Fraction 7	133.44	127.19	114.47	125.03 ± 9.67
Fraction 8	113.43	83.02	90.08	95.51 ± 15.92
Fraction 9	96.63	69.75	84.06	83.48 ± 13.45
Fraction 10	73.76	61.93	81.08	**72.26*** ± 9.66
Fraction 11	102.86	98.47	102.50	101.28 ± 2.44
Fraction 12	105.96	96.78	100.22	100.99 ± 4.64
Fraction 13	95.45	86.21	81.95	**87.87*** ± 6.90
Fraction 14	96.04	84.09	87.93	**89.35*** ± 6.10
Fraction 15	94.94	80.68	76.70	**84.11*** ± 9.59
Fraction 16	97.83	81.93	59.03	79.60 ± 19.50

[a] culture medium with 0.3% DMSO

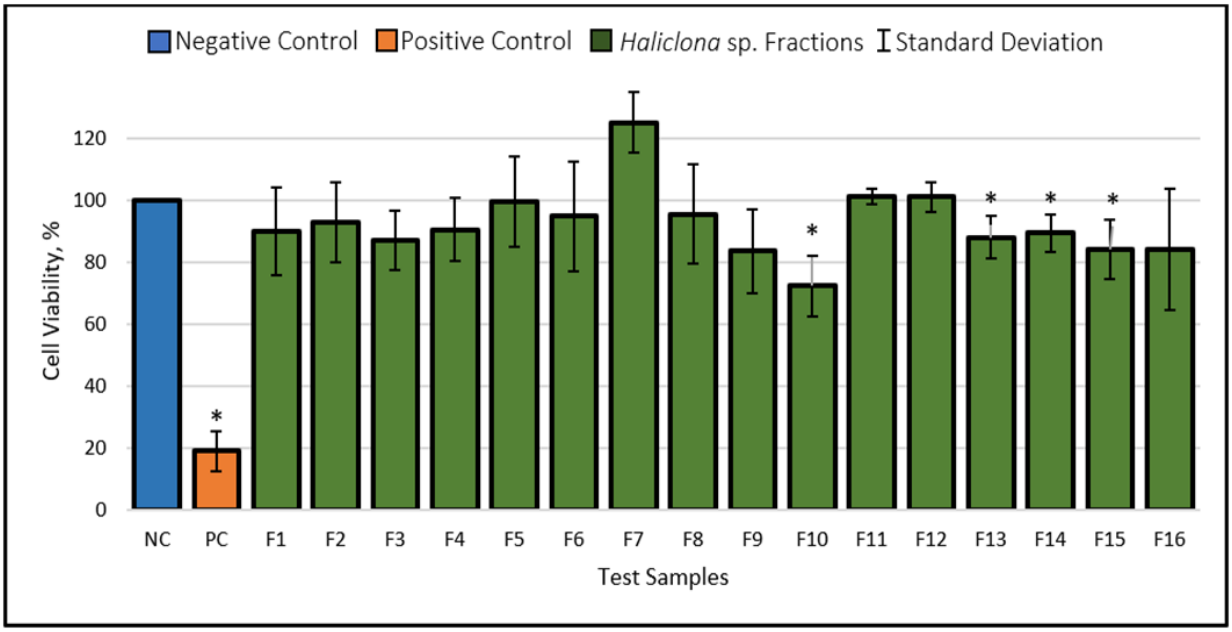
[b] Digitonin

***** Significantly lower percent viability than the negative control based on T-test ($p \leq 0.05$)

± standard deviation (SD)

The results are summarized in Table 1. *Haliclona* sp. fractions showed cytotoxicity against HCT116 cells at 30 µg/mL concentration except for fractions 7, 11, and 12 (Figure 2). However only fractions 10, 13, 14, and 15 exhibited a significant decrease ($p \leq 0.05$) in cell number compared to the negative control, with fraction 10 having the lowest percent cell viability of 72.26%. Moreover, the result obtained showed that the cell viability of all fractions is more than 50%, thus, contributing only a minimal cytotoxic effect against HCT116. The same result was observed in the study of Al-Massarani et al., (2016) which reported that most of the isolated compounds from *Haliclona* sp., collected from the Eastern coast of the Red Sea, exhibited weak cytotoxic activity against HepG-2. Daoy, and HeLa cancer cell lines [21]. Moreover, all steroids isolated from the marine sponge *Haliclona simulans*, collected from the Irish Sea, possessed low cytotoxicity against Hs27 cell line [22].

Figure 2: Graphical presentation of percent cell viability of HCT116 using the MTT assay after treatment of 30 µg/mL *Haliclona* sp. fractions. Digitonin was used as positive control. Test samples with asterisk (*) shows significantly lower percent viability than the negative control based on T-test ($p \leq 0.05$).



Pro-apoptosis Evaluation

It is widely acknowledged that anticancer drugs trigger apoptosis [23]. Apoptosis, or programmed cell death, plays a crucial part in regulating cell quantity in a variety of physiological and developmental settings. Many human malignancies have reduced apoptosis thus disruption of apoptotic activity plays a significant role in the development of tumor cells from normal cells [24]. The transformation of tumor cell occurs after a cascade of cell signaling and caspase-mediated events. The initiator caspases (caspases 8, 9, and 10) activates and initiate the apoptosis while the executioner caspases (caspases 3, 6, and 7) carry out the mass proteolysis that leads to apoptosis [23]. The Caspase-Glo 3/7 assay measures the caspase-3 and -7 activities in the cell. Once the Caspase-Glo 3/7 reagent was added, this results in cell lysis. There is then a caspase cleavage of the substrate and generation of a luminescent signal. The amount of luminescence produced is proportional to the amount of caspase activity present [25]. This luminescence is then measured to evaluate the pro-apoptotic property of *Haliclona* sp. fractions against HCT116 cell line. Moreover, the FC was calculated to evaluate the pro-apoptotic property of the sample compared to the negative control.

The measured RLU and calculated FC are summarized in Table 2.

Only fraction 8 of *Haliclona* sp. and sodium butyrate showed pro-apoptotic property against HCT116 cells at 30 µg/mL concentration with 2.42 and 4.35 FC, respectively (Figure 3). Furthermore, aside from sodium butyrate, fraction 8 exhibited significant increase ($p \leq 0.05$) in RLU and FC compared to the negative control. Based on the result, fraction 8 displayed pro-apoptosis property that was more than two times higher than that of negative control, indicating that the treatment of this fraction may have caused apoptosis by activating caspase-3/7.

Table 2: Summary of results in pro-apoptosis evaluation via Caspase-Glo 3/7 assay after treatment of 30 µg/mL *Haliclona* sp. fractions against HCT116 carcinoma cell line.

Test Samples	RLU				Fold Change			
	Trial 1	Trial 2	Trial 3	Average	Trial 1	Trial 2	Trial 3	Average
Negative Control[a]	822.67	689.33	752.67	754.89 ± 38.51	1.00	1.00	1.00	1.00 ± 0.00
Positive Control[b]	3402.00	3012.00	3420.00	**3278.00*** ± 133.10	4.14	4.37	4.54	**4.35*** ± 0.21
Fraction 1	452.00	337.33	432.00	407.11 ± 35.36	0.55	0.49	0.57	0.54 ± 0.04
Fraction 2	532.00	446.00	527.33	501.78 ± 27.92	0.65	0.65	0.70	0.67 ± 0.03
Fraction 3	602.00	481.33	438.00	507.11 ± 49.07	0.73	0.70	0.58	0.67 ± 0.08
Fraction 4	548.67	422.67	326.67	432.67 ± 64.28	0.67	0.61	0.43	0.57 ± 0.12
Fraction 5	464.00	387.33	442.00	431.11 ± 22.79	0.56	0.56	0.59	0.57 ± 0.01
Fraction 6	402.67	423.22	562.00	462.63 ± 50.04	0.49	0.61	0.75	0.62 ± 0.13
Fraction 7	400.67	430.00	527.33	452.67 ± 38.28	0.49	0.62	0.70	0.60 ± 0.11
Fraction 8	1535.33	1718.67	2175.33	**1809.78*** ± 190.29	1.87	2.49	2.89	**2.42*** ± 0.52
Fraction 9	665.33	730.67	743.33	713.11 ± 24.17	0.81	1.06	0.99	0.95 ± 0.13
Fraction 10	1199.33	477.33	613.33	763.33 ± 221.51	1.46	0.69	0.82	0.99 ± 0.41
Fraction 11	484.00	506.67	399.33	463.33 ± 32.66	0.59	0.74	0.53	0.62 ± 0.11
Fraction 12	404.67	295.33	491.33	397.11 ± 56.71	0.49	0.43	0.65	0.52 ± 0.12
Fraction 13	456.00	483.33	418.00	452.44 ± 18.94	0.55	0.70	0.56	0.60 ± 0.08
Fraction 14	375.33	367.33	341.33	361.33 ± 10.26	0.46	0.53	0.45	0.48 ± 0.05
Fraction 15	422.67	498.00	456.00	458.89 ± 21.79	0.51	0.72	0.61	0.61 ± 0.10
Fraction 16	354.67	530.00	342.67	409.11 ± 60.54	0.43	0.77	0.46	0.55 ± 0.19

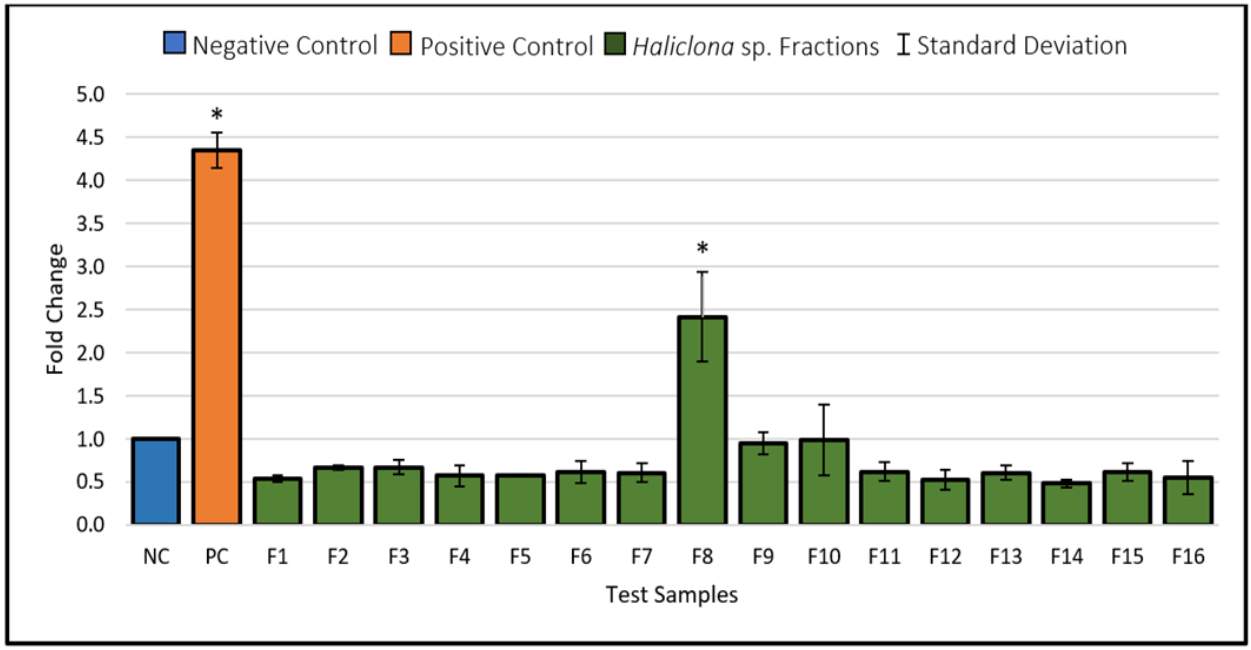
[a] culture medium with 0.3% DMSO

[b] Sodium butyrate

***** significantly lower percent viability than the negative control based on T-test ($p \leq 0.05$)

± standard error of the mean (SEM) for RLU and standard deviation (SD) for FC

Figure 3. Bar graphical presentation of the pro-apoptotic property of *Haliclona* sp. fractions in terms of fold change. Sodium butyrate was used as positive control. Test samples with asterisk (*) shows significantly higher fold change than the negative control based on T-test ($p \leq 0.05$).



CONCLUSION

The results from the MTT assay and Caspase-Glo 3/7 assay of the fractions sample of marine sponge *Haliclona* sp., collected from Poblacion, Kauswagan, Lanao del Norte, Philippines, verified that these methods could be a reliable procedure to evaluate two characteristics of cancer – sustained proliferation (anti-proliferative evaluation) and resistance to apoptosis (pro-apoptosis evaluation). These methods can also be used for examining the therapeutic qualities of natural sources. The fractions of *Haliclona* sp. showed cytotoxic effects however only fractions 10, 13, 14, and 15 show

significant results. Furthermore, all fractions exhibited weak cytotoxicity against HCT116 since the calculated cell viability was greater than 50%. Only fraction 8 of *Haliclona* sp. showed significant pro-apoptotic property against HCT116 with more than two times higher than that of negative control. The findings imply that fraction 8 should be subjected to a more thorough screening in order to more effectively find molecules with potential therapeutic use. The results of this study's data may serve as the foundation for a comprehensive analysis of the chemical components of the sea sponge *Haliclona* sp. as lead for the development of new anticancer drug and contribute to the growing information of marine sponges being a possible source of naturally-occurring bioactive compound.

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Conflict of interest

The authors declare no conflicts of interest for this study.

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