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THE CYTOTOXIC ACTIVITIES OF PHILIPPINE MARINE SPONGES *STELODORYX PROCERA* TOPSENT 1904 AND *RHABDASTRELLA* SP. AGAINST COLORECTAL CANCER CELL LINE HCT116

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ABSTRACT: Marine sponges have become a promising source of secondary metabolites with diverse structures and have been shown to have a wide range of biological activities. In search of bioactive compounds, a preliminary cytotoxic assessment of marine sponge extracts was conducted. The cytotoxic activities of the hexane, dichloromethane (DCM), ethyl acetate, and aqueous extracts of the marine sponges, *Stelodoryx procera* Topsent 1904 and *Rhabdastrella* sp., collected off the coast of Zamboanga City, Philippines were tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The hexane extract, ZP12NPh, from *S. procera* and DCM extract, ZP07NPd, from *Rhabdastrella* sp. exhibited moderate toxicity against colorectal cancer (HCT116) cell line at 30 µg/mL. Further purification of the hexane extract from *S. procera* has afforded two (2) sub-fractions, ZP12NPh4 and ZP12NPh5, which have shown to be highly cytotoxic against HCT116 at 30 µg/mL. While the DCM extract from *Rhabdastrella* sp., has afforded fourteen (14) highly cytotoxic sub-fractions (ZP07NPd2, ZP07NPd3, ZP07NPd4, ZP07NPd5, ZP07NPd9, ZP07NPd10, ZP07NPd13, ZP07NPd15, ZP07NPd16, ZP07NPd17, ZP07NPd18, ZP07NPd19, ZP07NPd20 and ZP07NPd21) against HCT116 at 30 µg/mL. These results warrant further detailed investigation to determine and characterize the structure of the compounds responsible for their bioactivity.

INTRODUCTION: The majority of the reported natural products originate from terrestrial habitats. However, marine bioprospecting represents a vast and relatively untapped area that is likely to be intensified.

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Marine organisms have been shown to be a very rich source of unique and biologically active secondary metabolites that have attracted the interest of both chemists and pharmacologists. Some of these compounds are extremely potent, which are produced in response to the harsh conditions that occur in the marine environment [1–3].

In recent years, marine natural products have been a source of new drugs with diverse and often unique structures [4], many associated with interesting biological properties that are in pre-

clinical development and on the market, with others making significant contributions to our understanding of cellular processes at the biochemical level [5-7]. In particular, the richness of unique metabolites produced by marine sponges has attracted the attention of those trying to develop new drugs [8]. Several articles have been published on the biological activities of marine sponges, but the mechanisms responsible have yet to be elucidated [9, 10]. As of 2018, there are 296 new reported compounds from marine sponges, with pharmacological activities such as anticancer, antifungal, antiviral, anthelmintic, antiprotozoal, anti-inflammatory, immunosuppressive, neuro-suppressive, and antifouling properties [11, 12].

Human cancer-derived cell lines are fundamental models used in laboratories to study the biology of cancer and to test the therapeutic efficacy of anticancer agents [13]. These cultured cancer cell lines are the most widely used *in-vitro* models and have formed the basis of cell-based assays that are used for screening collections of compounds to determine its effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death [14, 15]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). This technology has been widely adopted and remains popular in academic labs, as evidenced by thousands of published articles [16-19].

The Indo-Malay archipelago and South China sea have approximately 1,200 described species, with the Philippines having less than 500 species documented [20]. In the past two decades, bio-activities of Philippine marine organisms were reported, [21, 22] leading to the isolation of compounds that are cytotoxic to a variety of cancer cell lines and act on specific molecular targets in major cell signaling pathways implicated in various diseases [23, 24]. In this study, the hexane, dichloromethane (DCM), ethyl acetate and aqueous crude extract of two marine sponges collected off the coast of Zamboanga City, Philippines, were evaluated for potential cytotoxicity against cancer cell line using MTT Assay. The two marine sponges were *Stelodoryx procera* Topsent 1904 and *Rhabdastrella* sp. *S. procera* belongs to the family Myxillidae [25,] and marine sponges belonging to this family are not extensively studied. *Rhabdastrella* sp. is a genus of marine sponges belonging to the family Ancorinidae. They are mostly found in Indo-West Pacific, Asian tropical oceanic shallow waters and are hermaphroditic [26]. There are numerous compounds isolated from *Rhabdastrella* sponges, mostly are isomalabaricane triterpenoids which have been shown to be toxic against a panel of cancer cell lines [27].

MATERIALS AND METHODS:

Chemicals: The solvents and reagent chemicals used in this study are analytical and HPLC grade of brands from Scharlau (USA), RCI-Lab Scan (Thailand). The Thin Layer Chromatography (TLC) profile monitoring followed using plastic-backed TLC plates (Merck, Silica gel 60 F_{254}). The *in-vitro* cytotoxicity of the fractions against HCT116 (colon) cell line was tested using CellTiter96® Non-radioactive Cell Proliferation Assay Kit (Promega G4100).

Collection of Sponges: The marine sponges *Stelodoryx procera* Topsent 1904 and *Rhabdastrella* sp., were collected off the coast of Brgy. Sinubong, Zamboanga City, Philippines on April 2015 by hand scuba at a depth of 5-10 meters by local sea divers. The samples were stored in sterile containers and brought to the laboratory. Taxonomic identification of the marine sponges is credited to Dr. Ephrime B. Metillo of the Department of Biological Sciences, MSU-IIT.

Sample Preparation and Solvent Partitioning: The freeze-dried and pulverized sponge sample was soaked in 1:1 EtOAc-MeOH solution for three (3) days, filtered *in-vacuo* to obtain the nonpolar extracts of *Stelodoryx procera* Topsent 1904 (ZP12NP) and *Rhabdastrella* sp. (ZP07NP). The said nonpolar crude extracts underwent a series of solvent partitioning using appropriate solvents. The nonpolar extracts were dissolved in methanol and centrifuged. The supernatant liquid was regarded as the methanol fraction, and the residue underwent another round of solvent partitioning. The methanol extract was partitioned with twice the volume of hexane. The upper layer was collected and concentrated *in-vacuo* and regarded as the hexane fraction (ZP12NPh, ZP07NPh). The upper portion was mixed with twice the volume of water and

partitioned with twice the volume of dichloro-methane. The lower layer was concentrated *in-vacuo* and labelled as the dichloromethane (DCM) fraction (ZP12NPd, ZP07NPd). The upper layer was then concentrated *in-vacuo* and freeze-dried to obtain the aqueous fraction (ZP12NPa, ZP07NPa). The residue collected from the centrifugation of the nonpolar extract was dissolved in ethyl acetate and concentrated *in-vacuo* to afford the ethyl acetate fraction (ZP12NPea, ZP07NPea).

Fractionation of *Rhabdastrella* sp. DCM Fraction, ZP-07NPd: About 280.0 mg of the DCM (ZP-07NPd) fraction was subjected to initial purification on silica column (70-230 mesh) through gravity column chromatography with a gradient concentration of hexane/ethyl acetate and ethyl acetate/methanol solvent system in 5% increments, which were monitored with thin layer chromatography. Fractions were then pooled according to the TLC profile to afford twenty-one (21) sub-fractions.

Fractionation of *S. procera* Hexane Fraction, ZP-12NPh: The hexane (ZP-12NPh, 190.0 mg) fraction was fractionated on a silica column (70-230 mesh) through gravity column chromatography with gradient concentration of hexane/ethyl acetate and ethyl acetate/methanol solvent system in 10% increments which were monitored with thin layer chromatography. Fractions were then pooled according to the TLC profile to afford eight (8) sub-fractions.

TLC Analysis of Sub-fractions: Plastic-backed (Silica gel 60 F_{254}) TLC plates were used in all TLC analyses. For this study, solvent systems comprising of hexane, chloroform, and methanol were used for all the TLC analyses with the following ratios: hexane: $CHCl_3$ (in 0-100% in 25% increments), $CHCl_3$: MeOH (in 0-100% in 25% increments). Visualization of TLC plates was done first by short and long wavelength (254 and 365 nm λ) UV detection. TLC spot detection was using vanillin-H_2SO_4 spray reagent (1.5 g vanillin in 25 mL ethanol + 2.5 mL conc. sulfuric acid) for general application after UV detection. The color development was accomplished by heating the treated TLC plates in an oven at 110 °C for 5-10 min.

Cell Viability Assay (MTT): Human colon carcinoma cells (HCT116) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% antibiotic-antimycotic (Invitrogen) under a humidified environment with 5% CO_2 at 37 °C.

The cytotoxicity of the samples was measured in triplicate wells, each at 30 μg/mL concentration. A 100μL cell suspension containing 20,000 cells was seeded in a 96-well microplate except for the blank wells and incubated overnight at 37 ºC and 5% CO_2 atmosphere. The next day, 0.5μL of test samples were added to their corresponding wells in a 96-well plate. The culture medium in negative control were replaced with 100μL culture medium + 1% sterile DMSO. Then, the 96-well plate will be incubated overnight at 37 ºC and 5% CO_2 atmosphere. A 15μL MTT reagent was then added to all wells and incubated for 2 hours at 37 ºC and 5% CO_2 atmosphere. After incubation, a 100μL of solubilization solution/stop mix were added to all wells and incubated for 24 h at 37 ºC and 5% CO_2 atmosphere. Then, the absorbance at 570nm wave-length was recorded using SpectraMax® 250 (Molecular Devices) Spectrophotometer with microplate reader. The results were calculated as follows:

Corrected Absorbance = Absorbance of Sample - Absorbance of Blank

Cytotoxicity, % Viability = Corrected Absorbance of Sample / Corrected Absorbance of Negative Control x 100

RESULTS AND DISCUSSION:

Cytotoxic Activity of Marine Sponge *Rhabdastrella* sp.: The solvent partitioning of the nonpolar crude extract from *Rhabdastrella* sp (ZP07NP) afforded hexane (ZP07NPh, 141.3 mg), DCM (ZP07NPd, 316.1 mg), aqueous (ZP07NPa, 1088.3 mg), and ethyl acetate (ZP07NPea, 109.6 mg) fractions. The bioactivity of the said fractions was tested against colon (HCT116) and liver (HePG2) cancer cell lines using MTT assay. Cytotoxicity was classified based on the cell viability values obtained as follows: < 25%, high cytotoxicity; 25-70%, moderate cytotoxicity; and >70% were qualified as low cytotoxicity [28]. The results are tabulated and compared in **Table 1** and **Fig. 1**. All four fractions exhibited moderate cytotoxicity against HCT116, with ZP07NPd having the most considerable activity at 30 μg/mL.

Based on such results, ZP07NPd was subjected to initial fractionation on silica gel through gravity column chromatography to afford twenty-one (21) sub-fractions. Cytotoxicity screening of the sub-fractions was done using MTT assay against colon (HCT116) cancer cell line at 30 µg/mL. **Table 2** and **Fig. 2** summarize the results showing ZP07N-Pd2, ZP07NPd3, ZP07NPd4, ZP07NPd5, ZP07N-Pd9, ZP07NPd10, ZP07NPd13, and ZP07NPd15 - ZP07NPd21 as the sub-fractions with high toxi-cities against HCT116. A generalization that can be drawn from these results is that *Rhabdastrella* sp. could be a rich source of bioactive compounds based on literature [27].

TABLE 1: CYTOTOXICITY OF PARTITION FRACTIONS FROM THE NONPOLAR CRUDE EXTRACT OF *RHABDASTRELLA* SP. AGAINST HCT116 AT 30 µg/mL

Samples	%Viability	Remarks[28]
ZP-07NP	3.930 ± 0.008	High Cytotoxicity
ZP-07NPh	61.38 ± 0.14	Moderate Cytotoxicity
ZP-07NPd	56.67 ± 0.08	Moderate Cytotoxicity
ZP-07NPea	72.36 ± 0.07	Moderate Cytotoxicity
ZP-07NPa	79.86 ± 0.02	Moderate Cytotoxicity
PC	1.31 ± 0.00	High Cytotoxicity

Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

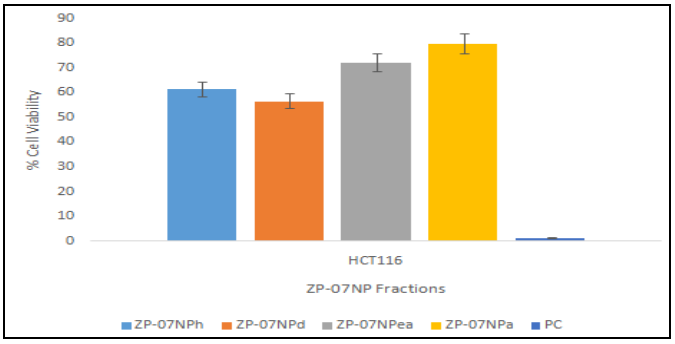

FIG. 1: CYTOTOXICITY OF PARTITION FRACTIONS FROM THE NONPOLAR CRUDE EXTRACT OF *RHABDA-STRELLA* SP. AGAINST HCT116 AT 30 µg/mL. Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

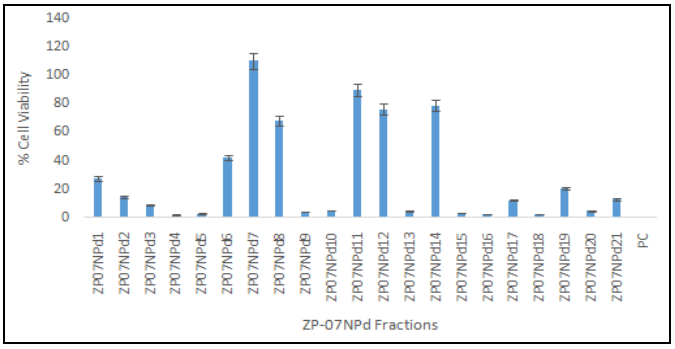

FIG. 2: CYTOTOXICITY OF DCM SUB-FRACTIONS (ZP07NPD) FROM THE NONPOLAR CRUDE EXTRACT OF *RHABDASTRELLA* SP. AGAINST HCT116 AT 30 µg/mL. Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

TABLE 2: CYTOTOXICITY OF DCM SUB-FRACTIONS FROM THE NONPOLAR CRUDE EXTRACT OF *RHABDASTRELLA* SP AT 30 µg/mL

Sub-fractions	%Viability	Remarks[28]
ZP07NPd1	27.46 ± 0.14	Moderate Cytotoxicity
ZP07NPd2	14.72 ± 0.04	High Cytotoxicity
ZP07NPd3	8.76 ± 0.08	High Cytotoxicity
ZP07NPd4	2.04 ± 0.00	High Cytotoxicity
ZP07NPd5	2.82 ± 0.01	High Cytotoxicity
ZP07NPd6	42.13 ± 0.18	Moderate Cytotoxicity
ZP07NPd7	110.22 ± 0.17	Low Cytotoxicity
ZP07NPd8	68.22 ± 0.17	Moderate Cytotoxicity
ZP07NPd9	4.17 ± 0.03	High Cytotoxicity
ZP07NPd10	5.22 ± 0.00	High Cytotoxicity
ZP07NPd11	89.78 ± 0.32	Low Cytotoxicity
ZP07NPd12	76.28 ± 0.15	Low Cytotoxicity
ZP07NPd13	4.59 ± 0.01	High Cytotoxicity
ZP07NPd14	78.55 ± 0.15	Low Cytotoxicity
ZP07NPd15	3.06 ± 0.00	High Cytotoxicity
ZP07NPd16	2.46 ± 0.02	High Cytotoxicity
ZP07NPd17	12.41 ± 0.00	High Cytotoxicity
ZP07NPd18	2.55 ± 0.03	High Cytotoxicity
ZP07NPd19	20.76 ± 0.36	High Cytotoxicity
ZP07NPd20	4.82 ± 0.21	High Cytotoxicity
ZP07NPd21	12.88 ± 0.10	High Cytotoxicity
PC*	0.88 ± 0.00	High Cytotoxicity

Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

Cytotoxic Activity of Marine Sponge *Stelodoryx procera* Topsent 1904: The solvent partitioning of the nonpolar crude extract from *Stelodoryx procera* Topsent 1904 (ZP12NP) has afforded hexane (ZP12NPh, 199.1 mg), DCM (ZP12NPd, 284.8 mg), aqueous (ZP12NPa, 1076.9 mg) and ethyl acetate (ZP12NPea, 32.5 mg) fractions. The MTT assay results of these fractions are tabulated and compared in **Table 3** and **Fig. 3**. All four fractions exhibited moderate cytotoxicity against HCT116 in which ZP12NPh had the most considerable activity at 30 µg/mL. The hexane fraction **(ZP12NPh)** was subsequently purified through silica column chromatography to give eight (8) sub-fractions. MTT assay results revealed high toxicity of fractions ZP12NPh4 and ZP12NPh5 against HCT116 at 30 µg/mL, while ZP12NPh6 expressed low cytotoxicity (**Table 4** and **Fig. 4**).

The toxicity of fractions 7 and 8 was not tested due to very low weights. These results suggest that bioactive compounds could be present in *S. procera*, worthy of further investigation since the literature survey has revealed no reported compounds from this particular marine sponge to date.

TABLE 3: CYTOTOXICITY RESULTS OF ZP12NP PARTITION FRACTIONS AGAINST HCT116AT 30 µg/mL

Samples	%Viability	REMARKS[28]
ZP12NP	15.583 ± 0.035	High Cytoxicity
ZP12NPh	51.00 ± 0.17	Moderate Cytotoxicity
ZP12NPd	65.56 ± 0.13	Moderate Cytotoxicity
ZP12NPea	59.63 ± 0.21	Moderate Cytotoxicity
ZP12NPa	67.57 ± 0.07	Moderate Cytotoxicity
PC	1.31 ± 0.00	High Cytoxicity

Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin.

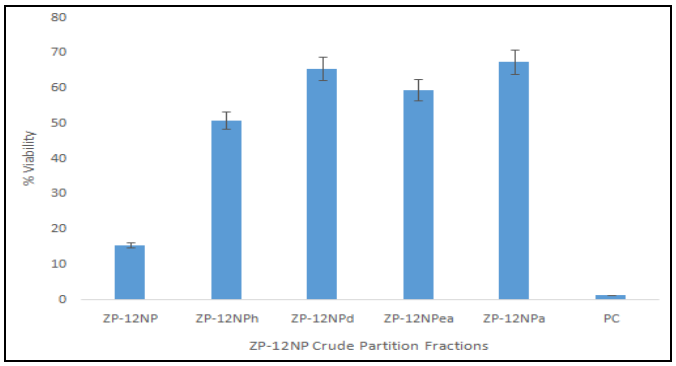

FIG. 3: CYTOTOXICITY RESULTS OF ZP-12NP PARTITION FRACTIONS FROM *S. PROCERA* AGAINST HCT116 AT 30µg/mL. Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

TABLE 4: CYTOTOXICITY RESULTS OF ZP12NPH SUB-FRACTIONS AGAINST HCT116 AT 30 µg/mL

Samples	% Viability	Remarks[28]
ZP12NPh1	98.0 ± 0.10	Low Cytoxicity
ZP12NPh2	93.57 ± 0.09	Low Cytoxicity
ZP12NPh3	95.79 ± 0.10	Low Cytoxicity
ZP12NPh4	13.43 ± 0.05	High Cytoxicity
ZP12NPh5	8.18 ± 0.02	High Cytoxicity
ZP12NPh6	88.67 ± 0.06	Low Cytoxicity
*PC	1.40 ± 0.00	High Cytoxicity

Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

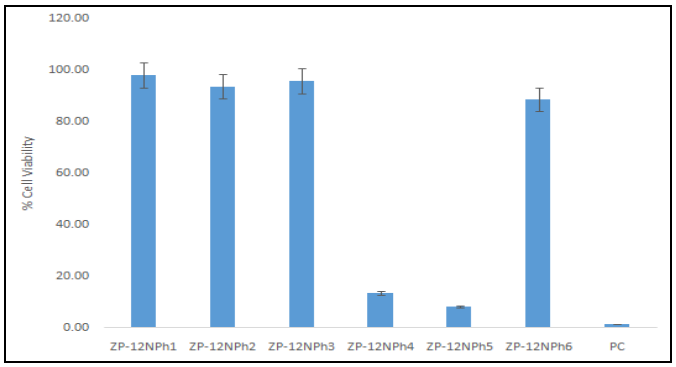

FIG. 4: CYTOTOXICITY RESULTS OF ZP12NPH SUB-FRACTIONS AGAINST HCT116 AT 30 µg/mL. Values are expressed as Mean ± SD (n = 3); PC – positive control, Digitonin

CONCLUSION: The MTT assay of the crude extracts of the marine sponges, *Stelodoryx procera* Topsent 1904 and *Rhabdastrella* sp., from the coast of Zamboanga City, Philippines, proved that the said assay could be a reliable method in screening compounds with medicinal properties from natural sources. The purification of nonpolar crude extracts from and *Stelodoryx procera* Topsent 1904 and *Rhabdastrella* sp., had produced sub-fractions that could be considered cytotoxic and hence could be a source of drug leads. The results suggest that a more rigorous screening should be done in all fractions of the same sponge sample in order to have a more effective search of compounds with potential medicinal values. Furthermore, the data from this study were used as the basis of a thorough investigation of the chemical constituents of these marine sponges.

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