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CYTOTOXIC AND APOPTOTIC ACTIVITIES OF MARINE SPONGE *Stylissa Massa* HEXANE AND METHANOL EXTRACTS AGAINST BREAST CANCER CELL

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Abstract: Breast cancer is common among Filipino women. Statistical results indicate that one in every 13 Filipino women is expected to develop breast cancer in today's generation, as stated by the Philippine Society of Medical Oncology (2015). It is associated with the failure of the body to maintain its normal homeostasis and its exposure to harmful chemicals that can result in the proliferation of abnormal cells. The marine environment has abundant potential marine sponges that may play significant roles in drug development for their uniquely structured and unexploited novel compounds. Hence, the in-vitro cytotoxic and apoptotic activity of marine sponge *Stylissa massa* extract collected from the aquatic resources of Barangay Tando, Nueva Valencia, Guimaras, Philippines, was investigated. The freshly chopped marine sponge was extracted using methanol and partitioned with n-hexane. The extracts were tested on MCF-7, a breast cancer cell line, for their cytotoxicity using MTT Assay. The apoptotic activities were detected using Annexin V-FITC assay kit by fluorescence microscopy and Caspase-Glo 3/7 assays. Both extracts contain alkaloids and amino acids. They inhibited the growth of MCF-7 cells in a dose-dependent manner with an IC_{50} values of 25 µg/mL and 90 µg/mL for hexane and methanol extract, respectively. These results showed significant cytotoxic and apoptotic activity of hexane and methanol extracts of *S. massa*. Thus, this marine sponge should be given attention as a potential local source of compounds with apoptotic activity for cancer.

Keywords: *Stylissa massa, MTT assay, MCF-7, thin layer chromatography*

1. INTRODUCTION

Breast cancer is common among Filipino women. One in every 13 Filipino women is expected to develop breast cancer, as stated by the Philippine Society of Medical Oncology in 2015. The Philippines has been identified as having an annual mortality rate of 12.8 per 100,000 with breast cancer in Asia (DOH, 2020). Chronic morbidity can develop in any individual due to certain factors such as heredity, poor diet, sedentary lifestyle, and constant exposure to various toxic elements (Castellsagué et al., 2014; Ledesma et al., 2011). Cancer is also associated with the failure of the body to maintain its normal homeostasis, which can result in the proliferation of abnormal cells and loss of apoptosis. Apoptosis is a form of controlled cell death that typically happens if the cell is damaged or abnormally produced. A deregulated apoptosis usually results in cell proliferation commonly observed in cancer.

The marine environment has abundant species that play a significant role as sources of bioactive compounds that can be used for drug discovery and development. These

uniquely structured and unexploited novel compounds are responsible for the adaptive nature of aquatic creatures such as sea cucumbers, marine algae, marine-derived fungi, and sea sponges (Abraham et al., 2010). Due to the nature of the environment where aquatic organisms are being exposed, the marine body, such as marine sponges, produces chemical compounds called secondary metabolites that researchers have studied for their pharmacological activities against anticancer. The extracts from Philippine marine sponge *S. massa are* one of the potent inhibitors of mitogen-activated protein kinase-1 or the MEK-1 associated with cell proliferation events (Tasdemir et al., 2002). The study of its antibacterial activity showed that the butanol fraction yielded the bromo-pyrrole alkaloids associated with inhibition of biofilm formation (Sun et al., 2018). The complexity of pyrrole alkaloids synthesized from the marine sponge has been used to study pharmacologic activity, metabolomics, and biotechnology (Mohanty et al., 2020).

The majority of the sponges live in the marine environment. They are sessile animals that cannot move and are filter feeders that dissolve material passing through their porous bodies. They feed through suspended food and particles like bacteria, viruses, and even pathogenic organisms. Sponges have a vast selection of weapons which include toxins they produce. The compounds from marine sponges protect them from predators and thus serve as a defense mechanism. The toxic substances, some bioactive compounds produced by marine sponges, could be a gateway for researchers to investigate further novel chemical compounds for potential pharmaceutical applications against cancer. The present study evaluated the cytotoxic and apoptotic activities of *S. massa* collected from Guimaras Province against breast cancer cells MCF-7. The mode of action and component *S. massa* hexane and methanol extracts were studied using Apoptotic Assay kits and Thin-layer Chromatography.

2. METHODOLOGY

2.1 Sample collection and extraction

The fresh marine sponge samples were collected from Barangay Tando, Nueva Valencia, Guimaras. The collection was accompanied by permits from Barangay Tando, Municipality of Nueva Valencia, and Guimaras Province Veterinary Quarantine Permit. One of the most critical properties of a marine sponge is fast degradation. Thus, samples were washed with distilled water to remove the seawater and then placed in a thermo-cooler with ice. The Philippine National Museum Spongiology Department authenticated the sponge. The specimen was identified as *Stylissa massa* and provided a specimen number of NMSO118 (Figure 1). The collected samples were stored and then frozen at -20ºC at the University of San Agustin Research Laboratory until used.

The samples were cut into small pieces using a sterile suture scissor. A 300-gram sample was extracted three times with 500 mL of 100% methanol for 24 hours. The residual or excess water in methanol extract was removed using anhydrous sodium sulfate. The methanolic crude extract was filtered and partitioned with n-hexane to extract the non-polar compounds. The extracts were evaporated to dryness using a rotary evaporator at 37ºC and then vacuum concentrated. Lastly, the fractions were spontaneously dried with nitrogen gas (Biegelmeyer et al., 2015). The collected fractions were stored in a dark-colored amber bottle and kept at -20ºC before assays.

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Figure 1. Thallus of freshly collected *S. massa* (Lumpy orange sponge) from Barangay Tando, Nueva Valencia, Guimaras.

2.2 Culture of human breast adenocarcinoma cells (MCF-7)

MCF-7 cells were obtained from the Mammalian Cell Culture Laboratory headed by Dr. Sonia D. Jacinto of the University of the Philippines-Diliman. The cells were cultured in a growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) in an incubator at 37ºC and 5% CO_2 until it became 80% confluent. The cells were subcultured once 80% confluent (Koca et al., 2013). The culture medium was supplemented with 10% fetal bovine serum (FBS) (Duarte et al., 2010). The assays were performed at the Mammalian Cell Culture Laboratory of the University of the Philippines-Diliman and the Mammalian Tissue Culture Laboratory of the Research Center for the Natural and Applied Sciences (RCNAS-UST).

2.3 MTT assay

The assay was conducted following Mosmann's (1983) procedure with slight modifications. Briefly, cells were seeded at 6×10^4 cells/mL in sterile 96-well microtiter plates. The plates were incubated overnight at 37 °C and 5% CO_2. Eight concentrations of *S. massa* extracts (0.78 µg/mL to 100 µg/mL) were prepared for the treatment. Doxorubicin (DOX) was used as the positive control and 1% dimethyl sulfoxide (DMSO) for the negative control. Following incubation, cells were treated with the extracts using the determined concentrations. The DOX concentrations used varies from 0.04 µg/mL to 6.25 µg/mL. Treated cells were incubated for 72 hours at 37°C and 5% CO_2. The extract with an IC50 of < 30 µg/mL was considered significant. Percent inhibition was computed using the equation below.

$$\text{Equations\% Inhibition} = \frac{\text{Absorbance of cells treated with DMSO} - \text{Absorbance of cells with Samples}}{\text{Absorbance of cells treated with DMSO}} \text{x100\%}$$

2.4 Thin layer chromatography

2.4.1 Annexin V-FITC assay

The apoptotic activity of the extracts was tested using the Annexin V-FITC kit (BioVision, CA, USA). MCF-7 cells (1 x 105 cells/100 µL) were plated in sterile 96-well

white-walled, clear-bottom plates. After 24 hours, MCF-7 cells were treated with 25 µg/mL and 90 µg/mL, respectively; the computed IC50 values of hexane and methanol extract were obtained from the MTT assay. After 24 hours and 48 hours of treatment, the cells were collected by centrifugation and resuspended in 500 µL of 1X binding buffer. Then, 5 µL Annexin V-FITC was added, and the plate was incubated at room temperature for 5 minutes in the dark chamber. The cell suspension was placed on a glass slide and covered with a cover slip. The cells were observed under a fluorescence microscope using a FITC filter at 200X magnification.

2.4.2 Caspase- Glo 3/7 assay

The effect of marine sponge extracts on caspase 3/7 activity was determined using Caspase-Glo 3/7 kit (Promega, Madison, WI). Briefly, MCF-7 cells (10,000/100 µL/well) were plated in 96-well white-walled, clear-bottom plates. After 24 hours, cells were treated with 200 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL of hexane and methanol extracts. At 24 hours post-treatment, 100 µL assay reagent was added to each well. The plate was then incubated in the dark for 0.5-hour, 1 hour, 2 hours, and 3 hours. After incubation, the luminescence was measured by the Glomax Luminometer. The blank reaction (reagent and media) was used to measure background luminescence associated with the cell culture system and Caspase-Glo® 3/7 reagent. The activity value was obtained by subtracting the luminescence of the blank reaction from experimental values. Data were presented as mean ± SEM of 3 trials with triplicates.

2.5 Thin layer chromatography

Thin-layer chromatography was performed to determine the components in the extracts that displayed more than 50% inhibition in the MTT assay. The silica gel plate was used as the stationary phase, and a combination of dichloromethane and methanol as the mobile phase. The solvent system was equilibrated in the developing chamber at room temperature. Then, 10 µL of the extract was spotted on the silica gel plate using a capillary tube. The spotted plates were placed in the developing chamber using forceps. The plates were removed from the chamber, and the spots were visualized at UV254nm and UV365nm. Then, it was sprayed with ninhydrin and dragendorffs reagents (Agatonovic-Kustrin et al., 2019).

2.6. Statistical analysis

Results of in-vitro assays were reported as mean ± SEM of 3 trials with triplicates. Statistical significance was then analyzed using the One-way Analysis of Variance followed by a Tukey-Kramer multiple comparison test. A *p*-value less than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Cytotoxic activity

The cytotoxic effect of *S. massa* extracts on MCF-7 was determined by MTT assay. The assay was used because it is a gold standard in measuring the toxic effect of a given compound on living cells. As shown in Figure 2A, hexane extract displayed notable

cytotoxic activity against MCF-7 in a concentration-dependent manner ranging from 2% to 80% inhibition. Hexane extract at 100 µg/mL exhibited the highest cytotoxic effect with an IC_{50} of 25 µg/mL, which is comparable to the standard doxorubicin (6.25 µg/mL) with an IC_{50} of 1.15 µg/mL ($p = 0.92$). This result is in good agreement with cycloheptapeptide stylissatins B isolated from *Stylissa massa* with an IC_{50} ranging from 2.4 µM to 9.8 µM (Sun et al., 2016). The obtained IC_{50} values are indications of notable cytotoxicity as IC_{50} values less than 30 µg/mL are considered active (Jokhadze et al., 2007). On the other hand, the methanol extract did not exert a cytotoxic effect against MCF-7; percent inhibition was less than 20% in all treatments (Figure 2B). To determine the potential toxic effect of hexane extract on normal cells, it was tested on normal neonatal fibroblast cells. The results showed that hexane extracts are non-cytotoxic up to 100 µg/mL (data not shown). The treatment with both extracts resulted in alteration of morphology, including numerous flake structures and disrupted cell structures (Figure 2C1.2). Most cytotoxic agent kills cancer cells by altering the cell membrane causing cellular death and leakage of cellular contents (Stockert et al., 2012).

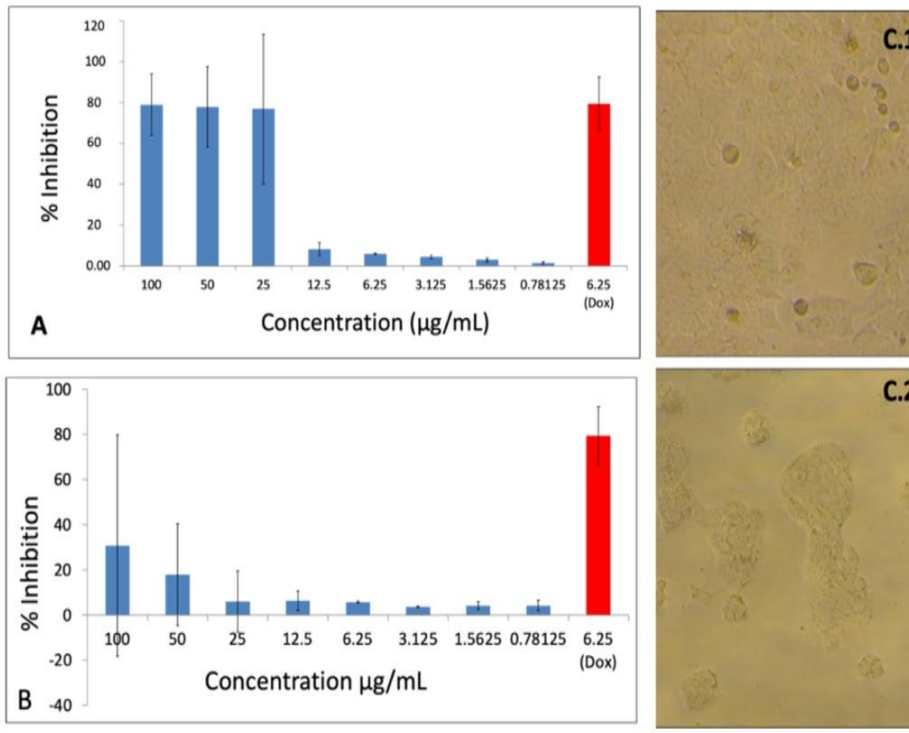

Figure 2. Inhibitory effect of *S. massa* extracts on MCF-7. Cells were treated with various concentrations of (**A**) hexane extract and (**B**) methanol extract for 72 hours. **C.** Photomicrographs of MCF-7 treated with *S. massa* hexane extract. (**1**) MCF-7 cells before treatment showing intact morphology and (**2**) MCF-7 cells 72 hours after the treatment showing altered morphology and leaked contents (n = 3 with triplicates).

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3.2 Apoptotic activity of S. massa

The *S. massa* extracts at 25 µg/mL (hexane) and 90 µg/mL (methanol) were used in this assay because these concentrations showed acceptable IC_{50} values from the MTT assay. Annexin V-FITC was used in order to confirm the initiation of apoptosis. Phosphatidylserine translocation from the inner to the outer portion of the cell membrane of mammalian cells is a sign of apoptosis. The apoptotic activity can be detected by Annexin V-FITC calcium-dependent binding protein with affinity to the translocated phosphatidylserine (Mannarreddy et al., 2017). Results showed that methanol and hexane extract induced apoptosis in MCF-7 cells (Figure 3). Methanol extracts exerted minimal apoptotic activity as only a few cells were observed with fluorescence (Figure 3C). Strong fluorescence indicates the death of MCF-7 cells treated with 25 µg/mL and 90 µg/mL hexane extracts (Figure 3E-F). Annexin V is a cellular protein that binds to phosphatidylserine at the outer portion of the plasma membrane produced by phospholipids. Furthermore, annexin V is a calcium-dependent phospholipid-binding protein. Usually, the phosphatidylserine is located in the inner portion of the plasma membrane or the cytosolic portion. Once the phosphatidylserine is translocated to the outer surface of the plasma membrane, it will be a marker of apoptosis. Under those circumstances, Annexin V will bind to the translocated phosphatidylserine at the outer portion of the plasma. Finally, green fluorescence is shown, indicating that the cells are undergoing apoptosis (Wu et al., 2017).

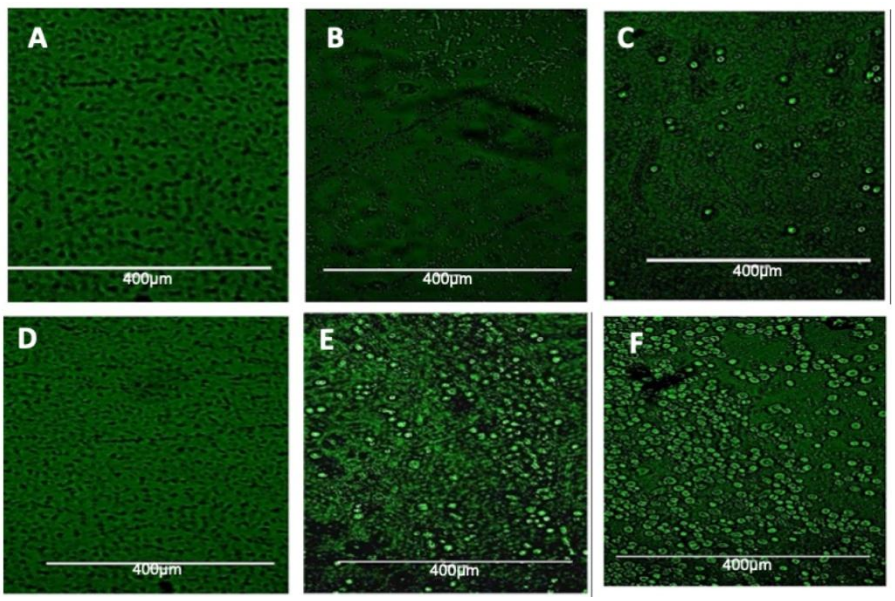

Figure 3. Apoptotic activity of *S. massa* extracts on MCF-7 as detected by Annexin V-FITC **A.** Untreated; **B.** 24 hr-treatment with 25 µg/mL methanol extract; **C.** 48 hr-treatment with 90 µg/mL methanol extract; **D.** Untreated; **E.** 24 hr-treatment with 25 µg/mL hexane extract; **F.** 48-hr after treatment with 90 µg/mL hexane extract.

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The Caspase-Glo 3/7 activity was measured in MCF-7 cells treated with *S. massa* extracts. Results showed that both extracts significantly induced activity of Caspase 3/7 enzymes as seen in higher luminescence from 0.5 hour to 3 hours compared to baseline measurement at 0 hours (Figure 4). In all treatment groups, the highest luminescence was observed at 0.5 hour but gradually decreased over time. The activity of *S. massa* (25 µg/mL) hexane extract was comparable to the effect of DOX (6.25 µg/mL) at 0.5 hr ($p = 0.996$) and 1 hour ($p = 0.852$) (Figure 4A). At concentrations above 25 µg/mL, both extracts activated caspase 3/7 activity as seen in higher luminescence than baseline measurement. However, the activity is lower than the standard DOX drug (Figure 4B-D). DOX was used as standard because it is medically accepted as an anti-cancer agent due to its apoptotic property. It was reported to induce apoptosis in cultured cardiomyocytes *in vitro* by activating caspase-3 and *in vivo* in cardiac ventricles in rats (Ueno et al., 2006). Caspases are a class of proteases that are instrumental in carrying out many cellular functions, including cell differentiation, remodeling, and death. Classically, the caspase cascade is initiated via cleavage of the so-called initiator caspases (caspase-2, caspase-8, caspase-9, and caspase-10), most likely by autoproteolysis, while the initiator caspases, in turn, cleave and activate the executioner caspases 3, 6, and 7. As a result, they will be the effectors of apoptosis once activated inside the cell (Dhar et al., 2009).

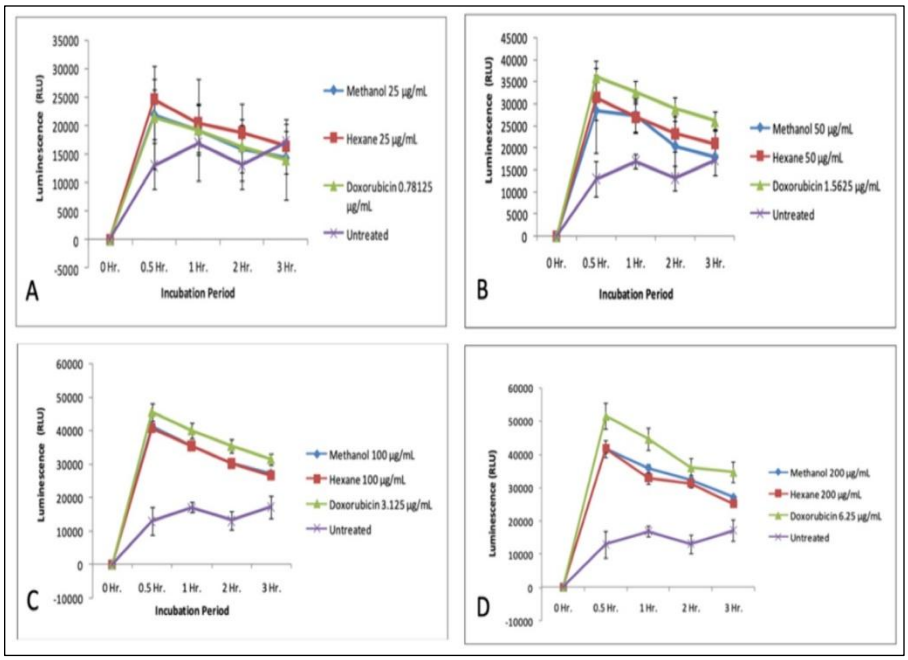

Figure 4. Apoptotic effect of *S. massa* hexane and methanol extracts on MCF-7. Cells were incubated with (A) 25 µg/mL of extracts in comparison with 0.78 µg/mL DOX; (B) 50 µg/mL of extracts in comparison with 1.56 µg/mL DOX; (C) 100 µg/mL of extracts in comparison with 13.125 µg/mL DOX; (D) 200 µg/mL of extracts in comparison with 6.25 µg/mL DOX. The luminescence was examined using a caspase 3/7 assay kit at indicated time points.

Apoptosis can be induced by either extrinsic or intrinsic pathways. In the extrinsic pathway, the signal molecule comes from outside of the cell. The sequence is mediated by the Fas-associated death domain (FADD), which results in the formation of DISC (death-inducing signaling cascade). Procaspase 8 interacts with the DISC and is converted into the active form of caspase 8. The procaspase 3/7 receives activation signals from caspase 8 to become an active form of caspase 3/7 (Nair et al., 2014). On the other hand, the intrinsic pathway of apoptosis is initiated within the cell. Pro-apoptotic proteins in the mitochondrial membrane, such as BAX and BAK, maintain apoptosis by piercing the outer mitochondrial membrane. This results in mitochondrial outer membrane permeabilization and triggers the leaking of the cytochrome-c. The cytochrome-c forms a complex with APAF-1 proteins, and procaspase 9 is activated into active caspase 9 that initiates the caspase 3/7 proteolytic cascade. The extrinsic and intrinsic pathways both lead to cell death via caspase 3/7, which is considered a key executioner pathway of apoptotic cell death (Shin et al., 2018).

3.3 Thin layer chromatography

Figure 4 shows the components of marine sponge *S. massa* that may have the potential for inducing cytotoxic activity on MCF-7 cells. The spots were carefully observed for the presence of secondary metabolites (Table 1 and Figure 5). The extracts were positive for amino acids and alkaloids, which most likely contributed to the observed cytotoxic activity of *S. massa* extracts (Mannarreddy et al., 2017). The same results were obtained from the chromatographic fraction of CH_2Cl_2 extract of *S. massa* aldisine alkaloids and *stylissatin* B-D amino acid (Sun et al., 2016). Literature also shows that marine sponges have a variety of secondary metabolites such as strigolactones, peptides, alkaloids (Indole), ethers (including ketals), phenols (including quinones), and steroids (including steroidal saponins) and terpenoids (Ullah et al., 2022; Hu et al., 2011).

Table 1. Thin layer chromatography for *S. massa* extracts with the solvent used, Rf values, and positive results for components.

Extract	Mobile Phase	Observation	Rf value	Staining	Components
Hexane	DCM:MeOH 20:01	3 blue spots at 254 and 365 nm	0.80, 0.10, 0.08 at 254 and 365 nm	(+) ninhydrin (+) dragendorffs	amino acids alkaloids
Methanol	DCM:MeOH 10:01	4 blue spots at 254 nm 2 blue spots at 365 nm	0.96, 0.70, 0.54, 0.50 at 254 nm 0.70, 0.54 at 365 nm	(+) ninhydrin (+)dragendorffs	amino acids alkaloids

DCM= dichloromethane; MeOH=methanol

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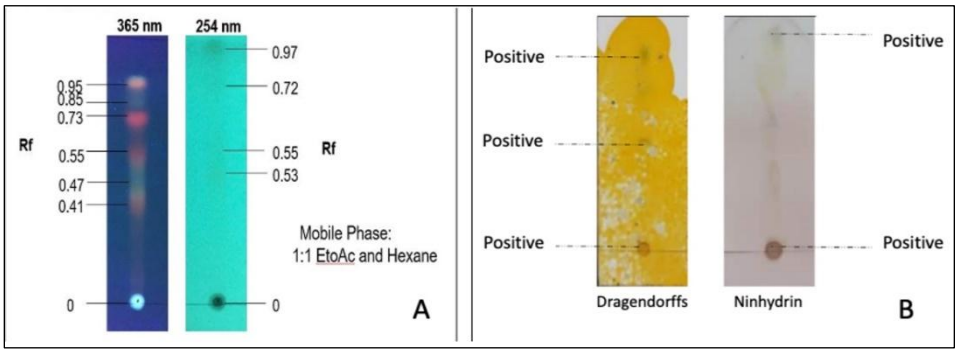

Figure 5. Thin layer chromatography results of *Stylissa massa* hexane fraction. **A**. Optimized TLC chromatogram viewed at 365 nm and 254 nm; **B**. TLC plate sprayed with visualizing agents.

4. CONCLUSIONS

The marine sponge *S. massa* was extracted using 100% methanol and was defatted with n-hexane and methanol. The hexane extract significantly inhibited the growth of MCF-7 cells in a concentration-dependent manner with an IC_{50} value of 25 µg/mL. Methanol extract exhibited minimal cytotoxic effect with an IC_{50} value of 90 µg/mL. Hexane extract displayed more pronounced apoptotic activity than methanol extract as shown in the fluorescent photomicrograph of Annexin V-FITC. Both extracts activated Caspase- 3/7 activity, as shown in the high mean luminescence results. Both extracts from *S. massa* were positive for the presence of amino acids and alkaloids. These findings indicate that the marine sponge *S. massa* is a potential source of active compounds with apoptotic activity. However, there is still a need to further evaluate these extracts in terms of their safety in *vivo* animal models and panels of normal human cells. The isolation of the specific active constituents responsible for the observed bioactivities is also needed to elucidate the mechanism of action.

5. ACKNOWLEDGMENT

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