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STEROL COMPOSITION AND ANTI-ATHEROSCLEROSIS EFFECTS OF *XESTOSPONGIA MUTA* EXTRACTS BY INCREASING TRANSCRIPTIONAL ACTIVITY OF SR-B1 PROMOTER

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ABSTRACT: Methanolic crude extract, diethyl ether fraction and sterol composition of *Xestospongia muta* were screened for cytotoxicity activity against HepG2 (liver hepatocellular) cells by MTS assay. Potential sterol mixture was profile using Gas Chromatography-Mass Spectrometry (GC-MS analysis), and luciferase assay were performed to further analyze its potentially on anti-atherosclerosis effects by increasing transcriptional activity of SR-B1 promoter. The result indicates that methanolic crude extract, diethyl ether fraction and sterol mixture (F2) does not exhibit cytotoxicity activity against HepG2 cell line with IC_{50} (>100 µg/mL, 94.45 ± 1.506 µg/mL and 55.30 ± 3.761 µg/mL). Cisplatin was used as a positive control, and the IC_{50} values against HepG2 cells were 3.22 ± 1.342 µg/mL. Sterol composition determines using GC-MS from diethyl ether fraction revealed 3 major sterols; cholesterol, stigmasterol, and ethanethioic acid. The mixture of different sterol analyses using luciferase assay showed potential on the ability to act as a ligand to activate the SR-B1 promoter. These preliminary findings suggested that sterol mixture from diethyl ether fraction is potential to be further investigated to develop other alternatives of anti-atherosclerosis agent.

INTRODUCTION: Marine organisms have proved to be potential sources of bioactive compounds in therapeutics importance with sponges, bryozoans, and tunicate being the most promising organism for sources of new bioactive compounds. Sponges from the phylum Porifera are the oldest metazoan that still extent on our planet and contain perhaps the greatest diversity of sterols [1, 2]. Sterol is a subgroup of steroid with a hydroxyl group at the 3- position of the A-ring and play essential roles in the physiology of eukaryotic organisms [3].

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Sponges of the genus *Xestospongia* (family, Petrosiidae) have been an interesting and important source of biologically active natural products. This genus has received extensive consideration since the discovery of few sterol compounds; aragusterol A and C in cytotoxic and antitumor activity [4], haplosamates A and B, ibisterol B and C and polyhydroxysterol for HIV protease inhibitory activity [5, 6] and others prominent biological activities including antimicrobial [7] and inhibitors of histamine [8, 9]. Xestospongine B, ageliferine and sceptin has been isolated from *Xestospongia sp.* [10] and believed to have relation in somatostatin. Somatostatin receptor subtype-2 (SSTR-2). SSTR-2 was found specifically expressed on human macrophages/monocytes. Macrophages have been reported can be used to evaluate the inflammatory activity in unstable plaques that build up along the arteries [11].

Atherosclerosis or hardening of the arteries is a condition whereas the deposition of lipid along the inner lining of the blood vessel [12]. Usage of statin to lower the level of lipid may lead to adverse side effects when used in the long term. In previous studies, [13] had evaluated *Xestospongia sp.* collected from Archipelago of Bidong Islands against HepG2 cell lines and found the *Xestospongia sp.* diethyl ether extracts as well as sterol mixtures shows a bright potential as anti-atherosclerotic agent due to its promising activity in increasing the expression of transcriptional regulation of pGL3-PPRE in the transfected HepG2 cells expressed. The increased expression of pGL3-PPRE reflects the ability of these samples from *Xestospongia sp.* To act as a ligand that can activate endogenous PPARy and subsequently increased the transcription of SR-B1 gene involved in reverse cholesterol transport (RCT).

Thus, objectives of this study are to analyze the cytotoxicity activity of the methanolic crude extract, diethyl ether fraction and sterol mixture of *Xestospongia muta* against HepG2 (hepatocellular liver cells) and further identified the composition of the sterols and analyze it potentially on anti-atherosclerosis effects by increasing transcriptional activity of SR-B1 promoter using luciferase assay.

MATERIAL AND METHODS:

Species Collection and Preparation of Fractions: Sample was collected at Pantai Vietnam, Pulau Bidong N 05º36.783' E 103º03.506 through scuba diving at a depth of 15-20 m. Samples were photographed *in-situ* for better species characterization and identification are done by Dr. Jasnizat Saidin as *Xestospongia muta*. Freshly collected sponge specimen was cleaned from any debris, chopped and stored at 80 ºC freezer before lyophilization using a freeze drier FD-550 (EYELA, Japan) to remove water.

The powder form of *X. muta* sample 1.25 kg was exhaustively macerated with 100% methanol in the ratio of 10 g dried sample to 100 ml methanol and repeated for three times to maximize the extraction. After maceration, the solution was filtered and evaporated to dryness using a rotary vacuum evaporator below 40 ºC to obtain methanol crude extract (51.975 g). Dry methanol crude extract was reconstituted in diethyl ether, transferred into a separation funnel and added up with distilled water in 2:1 ratio of diethyl ether to water respectively [14].

The separation funnel was then shaken up vigorously before the active compounds were allowed to settle down according to its polarity. The partitioning processes were repeated for at least three times or until the diethyl ether phase turned colorless. To further obtain the polar compound from the separation, *n*-butanol was added to the separation funnel in exchange of diethyl ether and was shook vigorously together with the distilled water used in non-polar extraction beforehand. Both diethyl ether (16.402 g) and *n*-butanol fractions (1.752 g) were dried completely using rotary evaporator, and the metabolic profile was checked *via* Thin Layer Chromatography (TLC) for isolation of bioactive compound from the sample.

Column Chromatography was further used to separate the molecules in the fractions so that the isolation and characterization work can be done efficiently. In this study, the diethyl ether fraction of *Xestospongia muta* was subjected to Column Chromatography for isolations of bioactive compounds based on the facts that the fractions were non-toxic and the yield of the fraction was higher compared to the *n*-butanol fraction. The diethyl ether fraction of the sample was subjected to series of the chromatographic column (23cm × 3cm) using hexane and ethyl acetate and subsequently followed by ethyl acetate and methanol in different ratios as a solvent system while silica gel 60, 0.040-0.063 mm (Merck) as the stationary phase.

The fractions were collected about 100 ml each, evaporated, labeled and monitored by TLC using hexane: ethyl acetate (7:3) as a solvent system. The fractions that showed the similar band was combined while fractions that form crystal-like structure after the evaporation process were taken as fractions of interest DEF-F2.

High- Performance Thin Layer Chromatography: HPTLC protocol was adapted from (Shafaei, 2012) [15] with some modification. The extracts were spotted using Automatic TLC Sampler 4 (CAMAG, Switzerland) on aluminium TLC plate gel F_{254} (layer thickness of 0.2 mm)

(Merck). The samples were streaked in the form of narrow bands with a length of 10.0 mm at a constant rate of 100 nL/sec using nitrogen aspirator. The migration distance was 9 cm, with a migration time of 25 min. The selection of the solvent system was based on the separation of the bands. The mobile phase was hexane: ethyl acetate (7:3). The developed plate for extracts and fractions was visualized using CAMAG TLC Visualizer 4 (CAMAG, Switzerland) under the wavelength of 254 nm, 366 nm, and anisaldehyde derivatization 366 nm.

Derivatization: 1 mg of sample was mixed with 100 µl of CH_2Cl_2, vortexed, and dried with nitrogen gas. The residue was mixed with 50 µl N-Methyl-N- trimethylsilyl- trifluoroacetamide (MSTFA), heated at 80 ºC for 15 min, cooled and a volume of 1 µl was diluted in 1 ml of hexane for Gas Chromatography-Mass- Spectrometry (GC-MS) analysis.

Gas Chromatography- Mass- Spectrometry Analysis: Fraction of *Xestospongia muta* was analyzed by GC-MS Shimadzu (QP2010SE Ultra) equipped with Wiley Library software. Condition: BP 5 MS column (30 m × 0.25 mm, 0.25 µm film thickness). The oven was programmed as follows: the initial temperature was 50 ºC (hold 1 min) to 300 ºC (rate 5 ºC /min, hold 5.0 min). The carrier gas was helium (purity 99.9%) and the flow rate 4.9 mL/min. Significant MS operating parameters: ionization voltage, 70eV; ion source temperature, 200 ºC scan mass range, 50-600 u. Identification of chemical components was based on comparison of their mass spectral data with existing Wiley Library.

Cell Culture: The human hepatocellular carcinoma liver cell line (HepG2) and the stable transfected human hepatoma cell line (HepG2) containing SR-B1 promoter (HepG2-SRB1) were maintained as exponentially growing culture in modified eagle medium (MEM) supplemented with 1% amino acid, 1% sodium pyruvate, 1% antibiotic (Penicillin and Streptomycin) and 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2 at 37 ºC. The cell was passaged upon reaching 75% confluency using 0.25% trypsin-EDTA.

Cytotoxicity Assay: The cytotoxicity activity of the sponge extracts has been evaluated on the HepG2 cell using MTS assays according to (Barltrop, 1991) [16]. The assay has been conducted using the cell titer 96® aqueous one solution cell proliferation assay (Promega USA). HepG2 cells with 80-90% confluency were seeded in a 96 well-plate at 8×10^3 cells / well, 24 h before treatment. Before addition to the cultured cell, the crude extracts and fractions were dissolved in DMSO and were subsequently diluted in 2-fold dilution ranging from 0.39 µg/mL to 100 µg/mL.

Cells treated with DMSO only act as negative control while cisplatin act as a positive control. The 96 well-plate was incubated for 72 h at 37 ºC with the presence of 5% CO_2. After incubation, 20 µl MTS solution was added into each well in a dark condition, and the plates were further incubated for 90 min. The absorbance used was 495 nm on a Glomax spectrophotometric plate reader (Promega USA) in which the intensity of light produced is directly proportional to the number of cells available in the well plate. The cytotoxicity activity of the sample was calculated as below:

$$\text{Percentage of Inhibition} = (Tz - Tc) / (Tz) \times 100$$

Where, Tz: absorbance reading from negative control, Tc: test in which the crude concentration is a presence at the different concentration levels.

Assay of Luciferase Activity Using ONE-Glo™ Luciferase Assay System: HepG2-SRB1 cell were seeded into 96 wells optical bottom with polymer base white plate (Thermo Scientific) for luciferase assay and seeded into 96 well plate cell culture plate F type (SPL Lifesciences) for cytotoxicity assay. The 96 well plates were then incubated for 24 h to obtain a uniform and monolayer cell distribution with approximately 30,000 cells per well. After 24 h, the complete media were discarded. Before the treatment process, 100 µl of new complete media with 10% fetal bovine serum (FBS) were prepared to contain the treatment diluted in two-fold dilution ranging from 1.56 µg/mL to 50 µg/mL. The 96 well-plate was incubated for 24 h at 37 ºC with the presence of 5% CO_2.

After incubation, 20 µl MTS solution the cell titer 96® aqueous one solution cell proliferation assay (Promega USA) was added at each well in a dark condition, and the plates were further incubated for

30 min. The absorbance at 495 nm was read on a Glomax spectrophotometric plate reader (Promega USA) in which the intensity of light produced is directly proportional to the number of cells available in the well plate. Then, 90 µl of ONE-Glo™ luciferase assay reagent (Promega USA) were added and mixed well to ensure that the reagent and the sample were homogenized evenly. After 5 min of incubation, the luciferase activities were measured using Glomax illuminometer (Promega).

RESULTS AND DISCUSSION:

HPTLC- Profiling: HPTLC analysis was performed to examine the difference in chemical constituents between methanol crude extracts and other fractions that been isolated from *Xestospongia muta* **Fig. 1**. The results indicated that hexane: ethyl acetate (7:3) is a good solvent for profiling the chemical constituents as showed by well separated, non-tailing, and non-diffuseness observed on HPTLC plate.

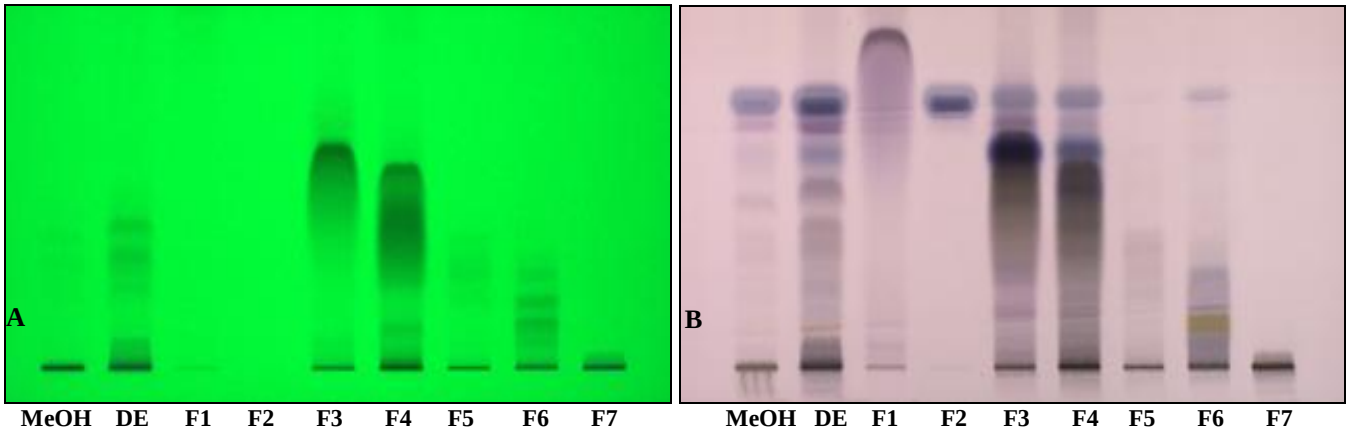

FIG. 1: HPTLC PROFILING OF METHANOL CRUDE EXTRACT OF *X. MUTA* (MeOH), DIETHYL ETHER FRACTION OF *X. MUTA* (DE), FRACTION 1 - 7 COLLECTED THROUGH COLUMN CHROMATOGRAPHY. VISUALIZED UNDER 254 nm (A) AND 366 nm AFTER DERIVATIZED WITH ANISALDEHYDE STAINING REAGENT (B)

The results showed that the chromatographic profiles of the extracts not visible under white light but few compound or substances can be viewed under 254 nm and 366 nm. Chromophore compounds that can be visualized under UV 254nm normally have conjugated double bonds, carbonyl, and aromatic ring. Example of sterol chromophore compound isolated from *Xestospongia sp.* are xestosaprol D and E. These two compounds contain carbonyl functional group and were conjugated to the chromophore responsible for the 255 nm absorption maximum in the UV spectrum [17].

Longwave usually consists of a compound that appeared as bright zones on a dark background including aflatoxins, polycyclic aromatic hydrocarbons, riboflavin, and quinine. Substances that cannot be seen in visible or ultraviolet (UV light) can be visualized with suitable visualization reagents such as anisaldehyde-sulphuric acid (98%) which form colored, fluorescent, or UV absorbing compound. **Fig. 1B** using derivatization reaction carried out post- chromatography [18]. The results showed that the major compounds observed were sterol, the intensity of the purple spot shows the high content of sterol mixture extracted. Almost all fractions demonstrated the closest R_f value for the purple spot (sterol mixture). The chromatographic isolation of F2 from *X. muta* yielded a substance with a crystal-like structure and whitish. Detection using anisaldehyde shows a violet color that represents detection of sterol [19] and the R_f value for the crystal-like structure was 0.48 (hexane: ethyl acetate (7:3). The composition of the substance was further analyzed using GC-MS.

Sterol Composition of F2: A high-resolution mass spectrum equipped with Wiley Library in combination with Gas Chromatography was used for the chemical analysis of active fraction, F2. Chemical characteristics of active fraction F2 were found to be a mixture of sterols. A total of 6 peaks were observed with relative percentage, retention times, and molecular weight as presented in **Table 1**. The sterol composition of active fraction revealed that the main sterol was stigmasterol (35.7%) followed by cholesterol (27.63%) and ethanethiol acid (14.01%).

These data were compared with sterol composition from previous studies by Gauvin (2004) showed that it was similarities in sterol mixtures contain in *Xestospongia sp.* which include skeleton C27 (cholesterol), C28 (campesterol) and C29 (stigmasterol).

TABLE 1: ANALYSIS OF STEROL COMPOSITION OF *XESTOSPONGIA MUTA* FROM F2 BY GC-MS

Peak area (%)	Retention time (min)	Compound name	Molecular weight
6.34	5.393	Methylamine	175
14.01	8.630	Ethanethioic acid	160
4.46	52.303	Pregn-5-en-3-ol	456
27.63	52.948	Cholesterol	458
35.7	53.123	Stigmasterol	488
11.85	54.691	Campesterol	472

Cytotoxic Activity: The cytotoxic effects of the methanolic crude extract, diethyl ether fraction and F2 of *Xestospongia muta* on HepG2 cell lines were examined in this study by MTS. A dose-response curve for the 50% of inhibition was plotted against the concentration of 1.56-100 µg/mL of the extracts, fractions and F2. The cytotoxicity of the sample depends on its IC_{50} value in which according to National Cancer Institute of America, IC_{50} is defined as the concentration of crude extract which able to inhibit 50 percent of cell proliferation *in-vitro* where for crude extract the concentration is 30 µg/mL and 4 µg/mL for the pure compound. All samples showed IC_{50} more than 30 µg/mL are potential and do not exhibit any cytotoxic effect on the liver cells **Table 2**. This is important to ensure the concentration used do not exhibit any cytotoxic effect on the HepG2 cells.

TABLE 2: INHIBITION CONCENTRATION AT 50% (IC_{50}) VALUES (µg/mL) FROM CYTOTOXICITY ASSAY

Extract / Fraction	IC_{50} value (µg/mL)
Methanol	>100
Diethyl ether	94.45 ± 1.506
Fraction 2	55.30 ± 3.761
Cisplatin (Positive control)	3.22 ± 1.342

Based on the result, all samples showed interesting potential to be further analyzed as the IC_{50} of F2 is more than 30 µg/mL. In comparison with the study done by Izzati (2014), she reported that sterol mixture extracted from *Xestospongia sp.* also shown low cytotoxicity towards the HepG2 cell line. Her finding supported F2 IC_{50} and show a bright potential to proceed with a luciferase assay.

Luciferase Assay: Fraction 2 has been further analyzed with luciferase assay to screen the potential of this fraction to induce the transcriptional activity of SR-B1 promoter by utilizing luciferase reporter gene **Fig. 2**. The assay was carried out in different 6 concentrations; ranging from 1.56 µg/mL to 50 µg/mL of final concentration. The inducibility effects of the extract were assessed by comparing the activity with a known compound that induces SR-B1 transcriptional activity, triscostation A (TSA) 0.05 µg/mL and DMSO (0.1%) was served as a negative control.

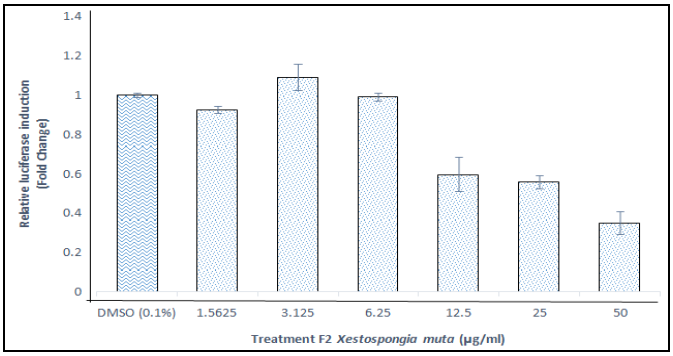

FIG. 2: LUCIFERASE ACTIVITY OF F2 FROM *XESTOSPONGIA MUTA* ON TRANSFECTED SR-B1 HepG2 CELLS. Each value represented mean ± SD of triplicates (n=3)

Fraction 2 showed the bell-shaped curve, which indicated by the transcriptional activity expression of SR-B1 promoter was increased as the concentration increased and decreased when the concentration gets higher. The results of luciferase activity were not in a dose-dependent manner but depend on the ability of F2 to acts as a ligand to activate the SR-B1 promoter. For future work, structural elucidation of the single sterol compound should be done to determine the type of sterols group do the compound belongs. Furthermore, by knowing the structure of the sterol compound, the relationship between structure-activity and bioactivity of the sponge sample can be determined as chemical structure, and specific bioactivity is related to the specific action of a compound. Besides, determination on the mechanism of action

on how the sterol mixture F2 binds to the ligands binding domain upon activation of the assays should also be carried out to have a full understanding on the mechanism of activation of an important pathway that is beneficial to drug discovery research and human health.

CONCLUSION: The methanol crude extract, diethyl ether, as well as sterol compound F2 shows a bright potential as anti-atherosclerotic agent due to its promising activity in increasing the expression of SR-B1 promoter in the transfected HepG2 cells expressed as luciferase activity after 24 h of the treatment. The increased expression of SR-B1 promoter reflects the ability of these samples from *Xestospongia muta* to act as the ligand.

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CONFLICT OF INTEREST: There is no conflict of interest

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