

ANTI-INFLAMMATORY ACTIVITY OF BACTERIA ASSOCIATED WITH MARINE SPONGE (*HALICLONA AMBOINENSIS*) VIA REDUCING NO PRODUCTION AND INHIBITING CYCLOOXYGENASE-1, CYCLOOXYGENASE-2, AND SECRETORY PHOSPHOLIPASE A2 ACTIVITIES

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Received: 20 May 2017, Revised and Accepted: 13 July 2017

ABSTRACT

Objective: This study aimed to investigate the anti-inflammatory activity of methanol extract and fractions of bacteria associated with sponge (*Haliclona amboinensis*) and to evaluate their effect in reducing NO production and inhibiting cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and secretory phospholipase A2 (sPLA2) activity.

Methods: All bacterial isolates were cultured and supernatants were collected for the extraction of secondary metabolites using diaion HP-20 to obtain methanol extracts. Evaluation of cytotoxicity property was carried out on macrophage cell lines (RAW264.7) by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazoliumbromide assay. Anti-inflammatory screening was done by inducible nitric oxide assay on RAW264.7 cell lines with lipopolysaccharide (LPS) stimulation. Dianion HP-20 was used to remove salt content. A selected methanol extract was subjected to further fractionations by C-18 reverse phase and their anti-inflammatory potential was evaluated by COX-1 and COX-2, and sPLA2 enzymatic assay.

Results: Seven methanol extracts showed no cytotoxic property against RAW 264.7 cell line (inhibitory concentration 50% > 30 µg/ml) and selected for anti-inflammatory screening assay. Result showed methanol extract HM 1.2 reduced NO production >80% and it has been selected for phytochemical screening, further fractionations and assay. Phytochemical screening showed alkaloids and terpenoids present in the HM 1.2. The HM 1.2 and its fractions (F1, F2, F1C1, F1C2, F1C3, and F1C4) were proven to inhibit COX-1, COX-2, and sPLA2 activity in the range of 60.516-116.886%, 20.554-116.457%, and 70.2667-114.8148%, respectively.

Conclusions: This study revealed that bacteria associated with *H. amboinensis* have produced anti-inflammatory activity via reducing NO production and inhibiting COX-1, COX-2, and sPLA2 activity.

Keywords: *Haliclona amboinensis*, Associated bacteria, Anti-inflammatory, Inducible nitric oxide, Cyclooxygenase-1, Cyclooxygenase-2, Secretory phospholipase A.

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INTRODUCTION

Activation of macrophage plays an important role in the initiation and propagation of inflammatory responses by production of cytokines, interleukin 1 β , tumor necrosis factor-alpha, NO, cyclooxygenase-2 (COX-2), and other inflammatory mediators. Over-expression of the inflammatory mediators in macrophage is involved in many inflammation related diseases, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis, and pulmonary fibrosis [1]. A number of drugs have been designed to treat inflammation in the human body, and three major groups of these drugs commonly used in the treatment of inflammatory disease are corticosteroids, nonsteroidal anti-inflammatory drugs, and disease-modifying anti rheumatoid drugs, which include biological agents. These drugs are widely used and are effective in treating inflammatory diseases but give side effects to the body, such as gastrointestinal ulceration, bleeding, and platelet dysfunction. This study investigated a new source from nature, namely sponges, as a potential alternative medicine that can reduce inflammation. There are some active compounds from sponges that have proven beneficial to humans such as anti-bacteria, anticancer, antidiabetic and antioxidant, antifouling, and anti-inflammatory [2-7].

Due to these benefits, many researchers have been interested in studying and exploring the sponges. The large exploitation of

sponges will interfere with their sustainability and their habitat. For example, to produce 1 g of anticancer compound, one or more ton of sponges are needed [8]. Therefore, sponge-associated bacteria can be used as an alternative source to replace the raw sponge specimens to help sponge population recovery. Although many specimens of sponges have been studied for their potentials to cure many diseases, anti-inflammatory potential of sponge-associated bacteria, especially (*Haliclona amboinensis*) - associated bacteria on RAW254.7 cells has not been studied yet and its mechanism of action as anti-inflammatory agent has never been investigated. Moreover, *H. amboinensis* sponge was chosen since this genus sponge was abundant at the selected sampling area and it was reported to possess many bioactivities. Thus, this study aimed to investigate the anti-inflammatory activity of methanol extracts and fractions of bacteria associated with marine sponge (*H. amboinensis*) and to evaluate their effect in reducing NO production and inhibiting COX-1, COX-2, and secretory phospholipase A2 (sPLA2) enzymes activity.

MATERIALS AND METHODS

Materials

2,2-diphenyl-1-picrylhydrazyl, quercetin, and NG-monomethyl-L-arginine were purchased from Sigma-Aldrich (MO, USA). Kit-560131

for COX-1 and COX-2, and kit-10004883 for sPLA2 were purchased from Cayman Chemical (MI, USA). All of other reagents and organic solvents were analytical grades from Sigma-Aldrich.

Sampling process and bacteria isolation

The sample of *H. amboinensis* was collected on April 2014 in Bidong Island, Terengganu, Malaysia. After the collection, the sponge was cut into small pieces (about 2 mm²) using sterile technique, and the pieces were transferred into a sterile Eppendorf tube and washed three times using sterile seawater (1000 µL) with gentle shaking (approximately for 1 minute). At the end of the washing processes, sponge pieces were crushed using a sterile pipette tip and 200 µL were seeded onto marine nutrient agar (MNA). Plates were incubated at 20°C and observed daily for 2 weeks. Bacteria colonies were picked up daily, marked and streaked onto fresh appropriate media. Bacteria were selected based on color, colony morphology and consistency, growth time, and pigment secreted in the media. The pure colonies were then stocked into 50% of glycerol and marine broth and kept in -80°C before further experiments.

Preparation of methanol extract from *H. amboinensis*-associated bacteria

Each isolated bacteria was cultured in marine nutrient broth (MNB) for 24 hrs, and then supernatants were separated from bacteria cells via centrifugation (6000 rpm, 15 minutes). Furthermore, Dianion HP-20 was used to remove salt content and yielded methanol extract. Before being used, the dianion beads were activated first by soaking them in methanol for 15 minutes. Then, they were placed into glass column chromatography, and methanol solvent was removed. Methanol was replaced with deionized water and left aside for 10 minutes. The supernatant free of bacteria was added into column chromatography and run through at a flow rate of 5 ml/minutes. Then, deionized water was passed through the column to remove excessive salt in retained crude extract. Methanol was used to elude the retained crude extract. The solvents were removed by rotary evaporator. Fractionation of selected methanol extract was done by C-18 reverse phase by column chromatography using water:acetonitril (6:4) as a solvent system, followed by gradient elution of sequential water and acetonitril.

Phytochemical screening

Phytochemical analysis of the chemical constituents of the methanol extract was adapted from Yadav and Agarwala [9].

Screening of cytotoxicity property of methanol extracts from sponge-associated bacteria on RAW264.7 cell line viability

The RAW cell line was obtained from the American Type Culture Collection. The RAW cell lines were kept in DMEM media (Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin-streptomycin at 37°C under 5% CO₂. The suspension cells were dispensed (100 µl) in triplicates into 96-well plates at optimized concentrations of 20000 cells/well in complete medium of DMEM (Sigma) as described previously [10]. The sample was prepared in varying concentration by serial dilution in DMEM medium with concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µg/ml in 96 well plates. Then, the plates were incubated for 72 hrs at 37°C, 5% CO₂, 90% humidity.

3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazoliumbromide (MTT) assay

The assay of each concentration was performed in triplicates, and untreated well cell population was performed as control wells. After 72 hrs, colorimetric MTT method assay was used to measure the living cells (slightly modified from Andriani *et al.* [11]). The MTT was dissolved at 5 mg/ml in phosphate-buffered saline. 20 µl of MTT solution was added into each well, and the plates were further incubated at 37°C in the incubator for 4 hrs. Supernatants were then discarded and 100 µl of dimethyl sulfoxide (DMSO) was added to the cultures and mixed thoroughly to dissolve the dark blue crystals formazan. The formazan quantification was then read by spectrophotometer at 571 nm. The

optical density (OD) of the samples was compared with that of the control to obtain the percentage viability, as stated below:

$$\text{Cells viability (\%)} = \left(\frac{\text{OD}_{571}(\text{sample})}{\text{OD}_{571}(\text{control})} \right) \times 100\% \quad [12]$$

Anti-inflammatory screening by inducible nitric oxide (iNOS) assay on RAW 264.7 cell line with lipopolysaccharide (LPS) stimulation

Standard nitrite assay

The stock solution of sodium nitrite was prepared using DMEM. A series of sodium nitrite concentrations were used to estimate the concentration of nitrites produced, starting from a concentration of 0-80 µM. 100 ml of each standard solution was prepared on 96 well plates. Furthermore, 100 µl of Griess reagent was added into each well and mixed. After incubation for 30 minutes at room temperature, the absorbance was read at 540 nm in micro plate reader [13].

Measurement of NO production on RAW264.7 cell line with LPS stimulation after treated by selected sample from bacteria associated with *H. amboinensis*

The RAW264.7 cells were plated at 2×10⁵ cells/well in 96 well plates and then incubated 24 hrs, and then cells were stimulated with LPS (1 µg/ml) and treated with sample to each well with the different concentration (two fold dilution from 100 µg/ml) then incubated for 24 hrs. Nitrite levels in cultured media were determined using Griess Reagent assay and presumed reflect NO levels. Briefly, 100 µl of cell culture media was mixed with 100 µl of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine dihydrochloride), incubated at room temperature for 10 minute and then the absorbance was measured at 540 nm. The nitrite concentrations were calculated using sodium nitrite standard curve. The methodology was slightly modified from Lau *et al* [14].

Effect of methanol extract and fractions from bacteria associated with *H. amboinensis* on COX-1, COX-2, and sPLA2 activities [15]

COX-1 and COX-2 enzymatic assay

The stock solution was prepared with 10 mg/ml of selected methanol extract dissolved in 100% DMSO. The extract was tested in duplicates of 5 µg/ml using a commercial COX inhibitory screening assay kit (Cayman test kit - 560131, Cayman Chemical Company) following the manufacturer's protocol. The COX inhibitor screening assay directly measured the amount of prostaglandin (PG) 2α produced in the COX reaction. Arginine was run as the positive control for the inhibition of COX-1 and COX-2. A volume of 10 µl each of sample and vehicle was diluted to 20 µl with 0.1 M Tris-HCl pH 8.0 and pre-incubated with the enzyme at 37°C for 15 minutes before the addition of arachidonic acid (AA). The reaction was initiated by the addition of 10 µl 10 mM AA and the tube was incubated at 37°C for another 2 minutes. Reaction was terminated by the addition of 50 µl 1 N HCl and saturated stannous chloride. Assays were performed using 100 units of ovine COX-1 and human recombinant COX-2. An aliquot was removed and the prostanoïd produced was quantified spectrophotometrically via enzyme immunoassay.

sPLA2 enzymatic assay

The selected methanol extract was tested in triplicates at 5 µg/ml using the sPLA2 (Type V) inhibitor screening assay kit (Cayman test kit - 10004883, Cayman Chemical Company) following the manufacturer's protocol. This assay kit contained a human recombinant Type V sPLA2 and reaction mixtures. Arginine was used as the positive control. A total of 10 µl each of test extract and vehicle were preincubated with 25 mM Tris-HCl buffer pH 7.5 containing 10 µl enzymes in a 96-well plate. The reaction was initiated by addition of 200 µl 1.66 mM diheptanoylthio-PC, and the plate was shaken for 30 seconds and incubated at 25°C for 15 minutes. Further, 10 µl 5,5'-dithio-bis-(2- nitrobenzoic acid) was added to stop the enzymatic reaction and to allow for color development. The plate was placed on a shaker for 1 minute to mix, and absorbance was measured at 405 nm using plate reader.

Statistical analysis

All the experiments were conducted in triplicates, and the data were presented as mean values ± standard deviation.

RESULTS AND DISCUSSION

Isolation of bacteria

A total of 19 bacteria from the sample *H. amboinensis* were isolated on MNA. These bacteria were observed and selected based on the color, colony morphology, and pigment secreted in the media. The results showed that almost all of the bacteria were stained as Gram-negative bacteria except one sample (HM 1.3) which was stained as Gram-positive bacteria (Table 1). The Gram staining has become a common diagnostic staining reaction in bacteriology. The gram type is an important criterion for describing bacteria and their taxonomy. Each of these bacteria was cultured in MNB, and the supernatant-free bacteria were extracted via Diaion HP-20, then evaporated. A total of 19 methanol extracts were yielded and used for further assay.

Screening of cytotoxicity property of methanol extracts from sponge-associated bacteria on macrophage cell lines (RAW264.7) viability

In this study, the cell viability of methanol extracts was determined according to the cytotoxicity property using MTT assay against RAW264.7 cell lines. The cytotoxicity property of methanol extracts of sponge-associated bacteria is shown in Fig. 1. The results showed the cytotoxicity property of 19 samples of methanol extracts according to their inhibition concentration value. There were seven out of nineteen samples considered nontoxic against RAW264.7 cell lines (inhibitory concentration 50% [IC₅₀] > 30 µg/ml), namely, HM 1.2, HM 1.3, HM 1.4, HM 1.5, HM 1.10, HM 1.11, and HM 1.3 (2). Cytotoxicity property of the samples were interpreted according to

the range of their inhibition value and interpreted as follows; IC₅₀ value <1.0 µg/ml is defined as highly toxic, value between 1.0 and 10.0 µg/ml is toxic, value between 10.0 and 30.0 µg/ml is moderately toxic, and value >30.0 µg/ml is defined as nontoxic (Adapted from Andriani et al. [16]). Therefore, sample extracts with IC₅₀ value more than 30 µg/ml were considered nontoxic on RAW264.7 cell lines and were chosen for further anti-inflammatory assay. Cytotoxicity activities of all samples were analyzed first to confirm that our samples were not cytotoxic against tested cell lines. This is important in the NO assay to indicate that the reduction of NO release is due to the anti-inflammatory activity, not derived from cell death caused by cytotoxic activity of the samples.

The macrophage cell line was chosen for cytotoxicity and anti-inflammatory studies since the macrophages are generally important components in the immune defense mechanism. During the progress of inflammation, macrophages actively participate in inflammatory responses by releasing proinflammatory cytokines and mediators [17].

Table 1: Bacteria stain and morphology

Number of sample	Sample's code	Gram	Morphology of bacteria
18	HG 1.4, HG 1.5, HG 1.6,	Negative	Bacillus
	HM 1.12, HM 1.1,	Negative	Bacillus
	HM 1.4, HM 1.5,	Negative	Bacillus
	HM 1.8, HM 1.9,	Negative	Bacillus
	HM 1.10, HM 1.11,	Negative	Bacillus
	HG 1.1, HG 1.2, HG 1.3,	Negative	Bacillus
	HG 1.7, HM 1.3 (2)	Negative	Bacillus
	HM 1.2, HM 1.6	Negative	Bacillus
1	HM 1.3	Positive	Bacillus

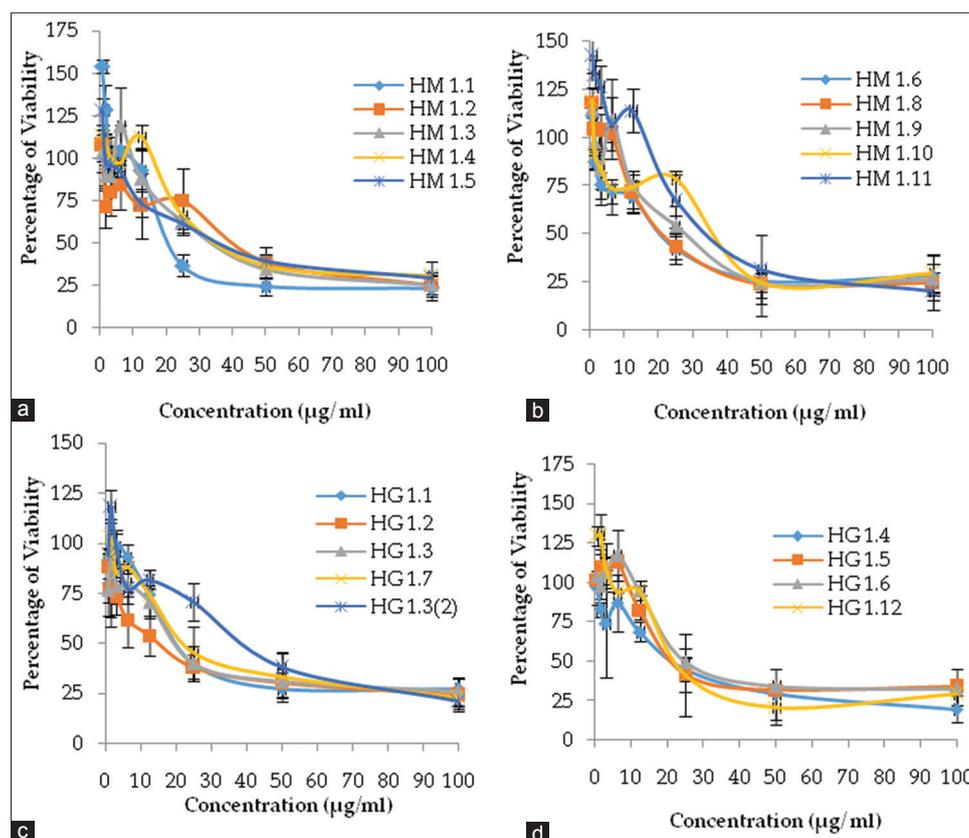


Fig. 1: Percentage of cell viability from methanol extracts of bacteria associated *Haliclona amboinensis* on RAW264.7 cell lines. (a) HM 1.1, HM 1.2, HM 1.3, HM 1.4 and HM 1.5, (b) HM 1.6, HM 1.8, HM 1.9, HM 1.10 and HM 1.11, (c) HG 1.1, HG 1.2, HG 1.3 and HG 1.7, (d) HG 1.4, HG 1.5, HG 1.6 and HG 1

Anti-inflammatory activity of methanol extracts and fractions from bacteria associated with *H. amboinensis*

Inhibition of NO production on macrophage cell lines (RAW264.7) with LPS stimulation

A standard curve of sodium nitrate with very strong coefficient correlation (R^2) of 0.9713 (Fig. 2) was used for quantification of NO produced by RAW 264.7 cells treated with different samples. Since the level of nitrite can be obtained from the standard curve, the level of nitrites produced in the sample treated wells can be calculated using the regression plot obtained [18]. Raw data from the anti-inflammatory assay represent the production of NO from the cell supernatant.

LPS is a nitrite synthase inducer, and when macrophages are activated with the LPS, there is an increase in the nitrite production by the independent NOS enzyme (iNOS). Yoon *et al.* [19] stated that LPS stimulation alone in murine macrophage RAW264.7 cells can induce iNOS transcription and protein synthesis and subsequent NO production. Inhibitory activity of NO production with LPS stimulated on RAW264.7 by extract HM 1.2 and its fractions from bacteria associated with *H. amboinensis* is shown in Fig. 3. There were five out of seven methanol extracts that showed potential as anti-inflammatory agents through inhibition of NO production (Fig. 3a). Three extracts showed high inhibition of NO production of more than 80%, namely, HM 1.3, HM 1.2, and HM 1.4. The lowest inhibition was shown by HM 1.3 (62.353%). All results were compared to the positive control N^G-monomethyl-L-Arginine, with the percentage of NO production at 62.305%.

Active methanol extract HM 1.2 was chosen for further fractionation since it was extracted from the bacteria which grew easily compared to the other active methanol extracts (HM 1.3 and HM 1.4). The process yielded eight combined fractions, and all of these fractions were further used for screening anti-inflammatory activity by iNOS assay. The results in Fig. 3b revealed that all of the fractionations from extract HM 1.2 showed high inhibitory activity on NO production (more than 80%). The highest percentage came from fraction 6 (83.952%), and the lowest percentage was obtained by fraction 5 (61.452%) at the concentration of 0.78 $\mu\text{g/ml}$ for both fractions. The data showed that these fractions reduced NO production at a level higher than positive control (NG-monomethyl-L-arginine). Fraction 1 (fr1), fraction 2 (fr2), fraction 6 (fr6), fraction 7 (fr7), and fraction 8 (fr8) were the most active fraction compared to fraction 3 (fr3), fraction 4 (fr4), and fraction 5 (fr5). They also showed higher activity than positive control (NG-monomethyl-L-arginine) with the percentage of the inhibition of more than 80%. Further fractionation was done for fraction 1 since it had more weight than the other fractions.

Moreover, further fractionation of fr1 yielded four fractions, namely, F1C1, F1C2, F1C3, and F1C4 (Fig. 3c). All fractions possessed anti-inflammatory activity on RAW264.7 cell lines. In the lowest concentration (30 $\mu\text{g/ml}$), the results showed that the activity was quite similar compared to positive control with values of 61.202%, 61.202%, 57.452%, 57.452%, and 62.924%, respectively, as shown in Fig. 3c. Their activity was also almost the same in different concentrations (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 $\mu\text{g/ml}$). All samples reduced NO production on RAW264.7 cell line. This study suggested that methanol extracts and fractions of sponge (*H. amboinensis*) - associated bacteria have potential as anti-inflammatory agents. Thus, methanol extract HM 1.2 and its fractions (fr1, fr2, F1C1, F1C2, F1C3, and F1C4) were selected for further assay to analyze their role as anti-inflammatory agents in inhibiting COX-1, COX-2, and sPLA2 enzyme activity.

Phytochemical screening

Phytochemical studies were carried out on selected methanol extract (HM 1.2) from *H. amboinensis*-associated bacteria since this extract revealed high anti-inflammatory activity and it was extracted from the bacteria which grew easily compared to the other active methanol extracts. The results showed that alkaloids and terpenoids were two chemical constituents present in this extract (Table 2). From results

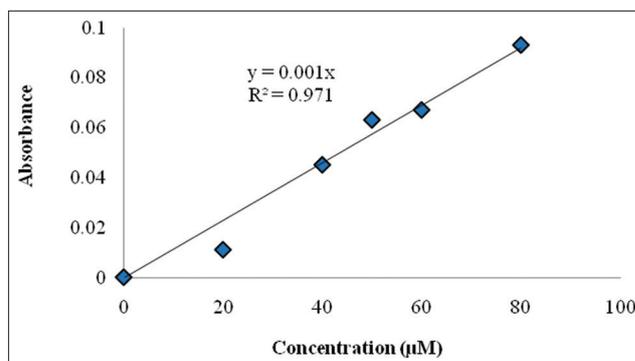


Fig. 2: Nitric oxide standard curve

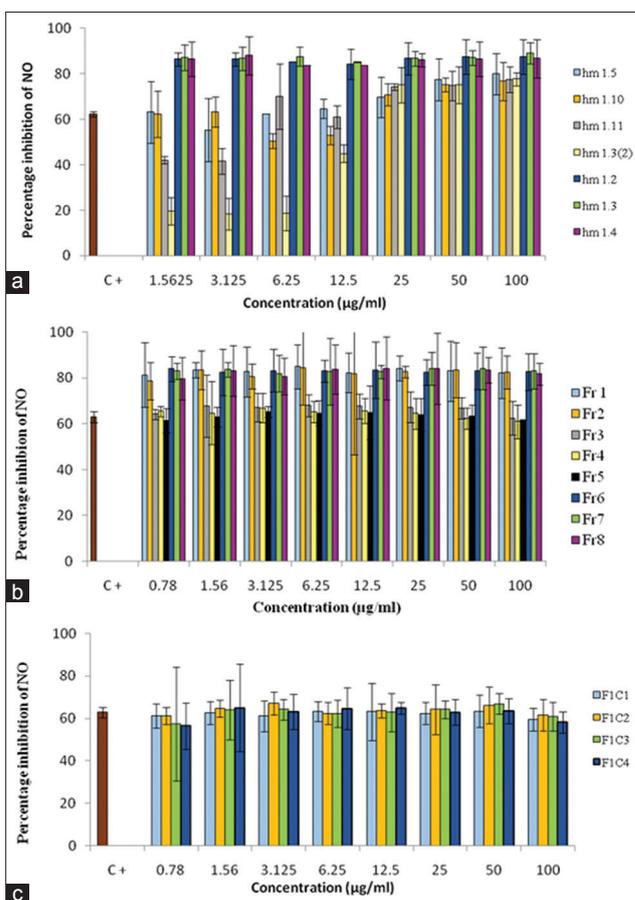


Fig. 3: Inhibitory activity of NO production with LPS stimulated on RAW264.7 by all methanol extracts (a) and HM 1.2 fractions (b and c) of bacteria associated with *Haliclona amboinensis*

above, all samples reduced NO production on RAW264.7 cell line. Alkaloids and terpenoids on the methanol extract HM 1.2 could have responsible on reducing NO production. This study suggested that methanol extracts and fractions of sponge (*H. amboinensis*) - associated bacteria have potential as anti-inflammatory agents. Then, Hm 1.2, fr1, fr2, F1C1, F1C2, F1C3, and F1C4 were selected for further assay to analyze their role as anti-inflammatory agents in inhibiting COX-1, COX-2, and sPLA2 enzyme activity.

Alkaloids and terpenoids are produced by many organisms, such as bacteria, fungi, plants, and animals. These compound groups have a wide range of therapeutic properties, including antimicrobial, antioxidant, antihypertensive, anticoagulant, anticancer, anti-inflammatory, wound healing and immune modulator, and other medicinal properties [20].

Phytochemical properties of the samples had a correlation with their therapeutic properties. Some studies have reported that alkaloid and terpenoids compounds have many potentials as antibacterial [2,3], anticancer and antitumor [4], antidiabetic and antioxidant [5], and anti-inflammatory [6,7] agents. Moreover, alkaloids from marine invertebrates such as sponges are reported to be anti-inflammatory potentials [21].

Effect of methanol extract and fractions in inhibiting COX-1 activity

The highest inhibition on COX-1 was shown at the concentration of 2.5 µg/ml by fr1 (116.886±4.698%), followed by extract HM 1.2 (114.646±0.235%), fraction F1C1 (114.646±0.235%), fr2 (77.001±3.759%), fraction F1C3 (76.299±3.033%), fraction F1C2 (66.405±0.136%) and fraction F1C4 (60.516±0.282%). At the concentration of 5 µg/ml, positive results were only shown by fr1 (73.986±0.194%). The results in Table 3 revealed that all samples possessed anti-inflammatory activity by inhibiting COX-1 enzyme activity. High activity on all samples could be attributed the group of alkaloids and terpenoids compounds on the methanol extract (Table 1) were present. Synergistic effect between chemical constituents in fraction 1 compared to its fractions (F1C1-F1C4) could have had an effect on its activity, thus fraction 1 possessed anti-inflammatory activity through inhibiting the COX-1 enzyme activity higher than its fractions.

Effect of methanol extract and fractions on inhibiting COX-2 activity

Table 4 shows the effect of the methanol extract and its fractions on inhibiting COX-2 enzyme activity.

At the concentration of 2.5 µg/ml, the highest activity was yielded by fraction F1C4 with the percentage of inhibition of 116.457±3.289%, followed by fraction F1C2 (107.362±0.235%), fraction F1C3 (50.495±0.658%), and fraction F1C1 (20.554±0.182%). At the concentration of 5 µg/ml, higher inhibition was yielded by fraction 2, while the other samples showed low inhibition activity compared to standard Arginine with the percentage of inhibition ranging from 6.104±1.856% to 23.001±0.507%. According to Milugo *et al.* [22], antagonistic relationship among phytochemicals would affect the efficacy of crude extracts. In our present study, antagonistic relationship between chemical constituents in extract HM 1.2 and its first fraction (fr1) could have had an effect on inhibiting COX-2 enzyme activity, thus higher inhibition of its second fraction (F1C2 and F1C4) was obtained. Alkaloids and terpenoids compounds in the samples could have also contributed to their activity since these compound groups were detected in the phytochemical study (Table 2).

Effect of methanol extract and fractions in inhibiting sPLA2 activity

The effect of the active methanol extract and fractions on inhibiting sPLA2 enzyme activity is shown in Table 5. All samples inhibited sPLA2 enzyme activity, with the highest inhibition obtained by fraction F1C2 (114.8148±0.940 %), followed by fraction F1C4 (105.5556±0.282%), fraction Fr2 (88.8889±0.507%), fr1 (85.1852±0.235%), fraction F1C3 (81.4815±0.003%) and extract HM 1.2 (70.2667±1.175%).

Alkaloids and terpenoids could be responsible for giving anti-inflammatory activity through inhibiting sPLA2 enzyme activity pathway. Yedgar *et al.* [23] suggested that the inhibition of PLA2 isoforms (such as sPLA2 and cPLA2) became a mean to block the synthesis of inflammation mediators resulting from AA metabolism, thus reduce the inflammation. However, there have been no studies reporting their anti-inflammatory potential activity as well as their mechanisms action in inhibiting COX-1, COX-2 and sPLA2 activity.

Some studies on antibacterial and anticancer activity of bacteria associated with sponge have been reported. Tropodithietic acid compound produced by *Pseudovibrio* sp. correlated sponge-associated bacteria was identified to be responsible for the antibacterial activity. These bacteria also produced phenolics chemical constituents [24].

Table 2: Phytochemical screening on active methanol extract HM 1.2 of bacteria associated with *H. amboinensis*

Phytochemical test	Phytochemical result
proteins and amino acids	-
Carbohydrates	-
Flavonoids	-
Phenols	-
Steroids	-
Glycosides	-
Terpenoids	+
Alkaloids	+

+: Phytochemical presence in the sample, *H. amboinensis*: *Haliclona amboinensis*

Table 3: Percentage of inhibition on COX-1 enzyme activity

Sample	Percentage of inhibition	
	COX-1 (5 µg/ml)	COX-1 (2.5 µg/ml)
HM 1.2	ND	114.646±0.235
fr1	73.986±0.194	116.886±4.698
fr2	ND	77.001±3.759
F1C1	ND	77.497±2.936
F1C2	ND	66.405±0.136
F1C3	ND	76.299±3.033
F1C4	ND	60.516±0.282
Arginine	100±0.000	100±0.000

Result were expressed as the means±SD of three independent experiments (n=3), SD: Standard deviation

Table 4: Percentage of inhibition on COX-2 enzyme activity

Sample	Percentage of inhibition	
	COX-2 (5 µg/ml)	COX-2 (2.5 µg/ml)
HM 1.2	20.511±0.940	ND
fr1	23.001±0.507	ND
fr2	46.927±2.805	ND
F1C1	21.344±1.391	20.554±0.182
F1C2	6.945±3.524	107.362±0.235
F1C3	0.874±2.349	50.495±0.658
F1C4	6.104±1.856	116.457±3.289
Arginine	100±0.000	100±0.000

Result were expressed as the means±SD of three independent experiments (n=3). ND: Not detected, SD: Standard deviation

Table 5: Percentage of inhibition on sPLA2 enzyme activity

Sample (1 µg/ml)	Percentage of inhibition
HM 1.2	70.2667±1.175
fr1	85.1852±0.235
fr2	88.8889±0.658
F1C1	88.8889±0.507
F1C2	114.8148±0.940
F1C3	81.4815±0.003
F1C4	105.5556±0.282
Arginine	100±0.000

Result were expressed as the means±SD of three independent experiments (n=3), SD: Standard deviation

Moreover, melanin from Marin *Bacillus* spp. BTCZ31 showed anti-inflammatory activity via inhibited the activity of COX and LOX enzyme [25]. Some new aminophenoxazinones alkaloids isolated from a marine bacteria, *Halomonas* sp. have shown antibacterial and cytotoxic activities against some tumor cell lines, HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma) [26]. Moreover, some new terpenoids, namely aspergiterpenoid A, (-)-sydonol, and (-)-sydonic acid have been isolated

from bacteria *Aspergillus* sp. associated with marine sponge *Xestospongia testudinaria*. These terpenoids showed very good antimicrobial activity against *Escherichia coli* and *Micrococcus tetragenus* [27].

Secondary metabolites from bacteria associated with *H. amboinensis* which contained alkaloids and terpenoids could possess anti-inflammatory activity via some roles. Inhibition of NO synthesis by inhibitors (alkaloids and terpenoids from bacteria associated with *H. amboinensis* will be inhibited of PGs production. According to Issa *et al.* [28], inhibiting PGs consequently will decrease all pro-inflammatory mediators such as PGE2 and NO, thus the inflammation will be reduced. In addition, releasing of PGs by inhibiting COX-1 and COX-2 enzymes activity through inhibiting sPLA2 activity could have also contributed on reducing the inflammation. Yedgar *et al.* [23] have reported that the inhibition of PLA2 isoforms (such as sPLA2 and cPLA2) as a mean to block the synthesis of inflammation mediators resulting from AA metabolism.

CONCLUSIONS

This study revealed that bacteria associated with *H. amboinensis* could have anti-inflammatory potential via reducing NO production and inhibiting COX-1, COX-2, and sPLA2 enzymes activity. Further study to identify the name of bacteria isolates from *H. amboinensis*-associated bacteria which produced anti-inflammatory agents and isolate the bioactive compounds are needed to support their activity.

ACKNOWLEDGMENTS

The authors wish to thank the Ministry of Higher Education for the fund provided under the RAGS Fasa II/2013 (Vote No.57103). Appreciation goes to Institute Marine Biotechnology, UMT, Kuala Nerus, Malaysia, for running all assays.

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