Jurnal Teknologi

Full Paper

IN-VITRO ANTI-INFLAMMATORY ACTIVITIES OF EXTRACTS FROM BACTERIA ASSOCIATED WITH MARINE SPONGES: *THEONELLA* SP.

Siti Aisha Mohd Radzi[a], Yosie Andriani[a], Habsah M.[a], Tengku Sifzizul Tengku Mohamad[a], Jasnizat Saidin[a,b*]

[a]Institut of Marine Biotechnology, Universiti Malaysia Terengganu, Malaysia
[b]School of Marine & Environmental Sciences, Universiti Malaysia Terengganu, Malaysia

Article history
Received
29 June 2015
Received in revised form
8 September 2015
Accepted
25 October 2015

*Corresponding author
ijaxzt@umt.edu.my

Abstract

In recent years, high numbers of research focusing on secondary metabolites produced by terrestrial bacteria towards anti-inflammatory properties but, minority of them used bacteria associated to marine organism. In this study, 25 bacteria colonies were isolated from sponge *Theonella* sp. which was collected from Bidong Island, Terengganu. Cytotoxicity and anti-inflammatory activity of secondary metabolites produced by these bacteria were studied using macrophage cell lines: RAW 264.7 .Macrophage plays a role in mammalian immune system by providing defense against a foreign harmful substance for example Lipopolysaccharides (LPS) which induces production of pro-inflammatory mediators such as nitric oxide (NO). Inhibition of NO production in LPS stimulated RAW 264.7 cells is highly established assay to screen for anti-inflammatory activity. All isolates were cultured and supernatants were collected for the extraction of secondary metabolites using diaion HP-20 to obtain crude extracts. Evaluations of the cytotoxicity effect of the extracts on RAW 264.7 were done using MTT assay. The highest concentration sample producing lowest toxicity effect was used for anti-inflammatory assay. Macrophages were induced with LPS before treated with different concentrations of crude metabolites in anti-inflammatory assay. The effects of crude secondary metabolites extracted to the induced macrophage were monitored by observing the inhibition level of Nitric Oxide (NO) released by RAW 264.7 into supernatant and estimated from nitrate standard curve. A preliminary assay revealed that isolate label with TM1.8 and TM 1.9 possesses anti-inflammatory activities of 112.06% and 109.7% respectively for every 1ug/L sample. The result suggested that these two crude extracts were more effective in inhibiting NO production compared to positive control N-Monomethyl-L-arginine Monoacetate (L-NMMA) which showed an inhibitory activity at 87.41% for every 1ug/L sample.

Keywords: RAW264.7 cells, Lipopolysaccharides, anti-inflammatory, Nitrite Oxide

1.0 INTRODUCTION

Ocean is a rich source of biological and chemical diversities which may provide benefits to human. Marine life can be divided into two groups which are macro and micro-organism. Both organisms play major role in balancing the ecosystem/ habitats by forming symbiotic relationship which benefits each other. Marine lives have their own role for sustainability of marine environment especially marine microorganisms which play a role as primary producer in food chains, control flow of marine energy, nutrient and produce metabolites which are important nutrients to other marine organisms. Sponges have been identified as one of main sources for bioactive compounds with various activities such as, anti-bacterial, anti-cancer, anti-fouling, anti-inflammatory and immunosuppressive(Lam K. 2007). Large numbers of compounds were isolated every year and their activities were tested for drug discovery. Recent research reported that many bioactive natural products from marine organism have striking similarities to metabolites of their associated microorganism including bacteria(Mayer 1999). Thus it is important to explore the possible role of marine

bacteria associated with sponges in providing new therapeutic resources to many diseases nowadays. It is well known that sponges are filter feeder for microorganisms such as bacterium, phytoplankton and virus which will produce a good symbiotic interaction between them (Devi *et al*. 2011). It has been reported in some sponges species that the symbiotic to microorganism can take up to 50% of biomass(Fieseler *et al*. 2004) and consisting of hundreds to thousands taxa.It is also known that sponges produce secondary metabolites for survival and protection from the other marine organism(Devi *et al*. 2011). As previously mentioned, bacteria and other marine organisms closely associated to give benefit to each other. Since the relationship happen for a quite long time period, research has pointed out that good activity of compound may produce from this interaction which can leads to potential drug discovery with a good activities for mankind (Donia & Hamann 2003). Recently, recovery of metabolites produces by bacteria associated with marine organism become a growing field in pharmaceutical and the search for new potential drugs from marine organism have resulted in approximately 10,00 metabolites being isolated for detection of biological activities and identification (Kelecom 2002).Focus for marine natural products is important in discovery and development of drugs from marine organism. Various numbers of products have been isolated from different marine sources.

In this study, potential of secondary metabolites crude extract to inhibit inflammatory was measured. It is expected to exploit by product from bacteria symbiotic process to produce anti-inflammatory activities that can be useful for the long run. After inflammation occurs, recovering process is essentials for healing. Bacteria, either pathogenic, probiotic or symbiosis capable to evade the immune response by inhibit or reducing parts of inflammatory pathways (Hur *et al*. 2012). Level of nitric oxide in cultured supernatant was measured using Griess reagent and become a benchmark to evaluate level of inhibition. The Griess reaction is a method that widely used to measure amount of nitrite. NO is important molecule for host defense response to against abnormal substances such as: bacteria, viruses, fungi and parasites (Bogdan *et al*. 2000). Nitric Oxide (NO) is a small radical formed from terminal guanidine nitrogen atom of arginine. This free radical involved in many important biological functions. However, overproduction of NO may lead to tissues damage and can cause chronic inflammation (Vane & Botting 1987).

2.0 EXPERIMENTAL

Sample collection. Sample was collected at Pulau Bidong, Terengganu. (5°36'16.7"N 103°03'42.6"E) by scuba diving at a depth approximately 15 meters. Upon collection sponges tissues were cut into a small piece, rinsed with sterile distilled water and put into a sterile 1mL tube. 1mL of sterile distilled water was added and tissues sample were crushed using homogenizer. Solution from sample were serially diluted, spreaded on Marine Nutrient Agar (MNA).The plates were incubated at temperature 20-25℃ and observed daily for two weeks to pick up new colony onto new fresh plate based on colony size, color, morphology, and growth time interval. Colonies were isolated and cultured onto a new plate until pure colonies were obtained. Pure colonies were then stock into cryopreservation system and kept at -80°C prior further experiments.

Extraction of Secondary metabolites. Every single colony of bacteria was grown in 50 mL of Marine Nutrient Broth (MNB) using rotary shaker at temperature 20-25℃ for 48 hours. After 48hours, fermentation broth were centrifuge at 6000rpm for 15 minutes, supernatant were collected and extracted using Diaion HP-20 resin (Supelco,Bellefonte,PA,USA) by a modified method (Lee *et al*. 2003). Weight of secondary metabolites crude extract for each bacteria strains was recorded and crude extracts was stored at 4℃.(Pathak R. 2011)

MTT cell viability assay. RAW 264.7 cells were seeded for 24 hours with density at $5X10^4$ cell/ well in 96 wells plate. The cells then were treated with various concentrations of secondary metabolites crude extracts for 24 hours. After 24 hours (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, USA) was added to final concentration every well 0.5mg/ml and the cells then were incubate for 4 hours at 37℃. Medium were removed and formazan participate were solubilized with DMSO. Absorbance was measured at 550nm using microplate reader (Thermo Scientific, U.S) (Lee *et al*. 2003).

Sodium Nitrite ($NaNo_3$) Standard Curve. A Standard curve of Sodium Nitrite was used to estimate the concentration of Nitrites produced. Sodium Nitrite (Merck, U.K) was dissolved in culture media and a series of sodium nitrite concentration was prepared. The absorbance was measured by mixing sodium nitrite solution with Griess Reagent with ratio 1:1 and absorbance were read at 540 nm in micro plate reader. Straight line standard curves were plotted with the absorbance versus function of amount of nitrite(Yi *et al*. 2013).

Inhibition of Nitric Oxide (NO) production. RAW 264.7 were seeded at density $5X10^4$ cells/well in 96 wells plate and incubated for 24 hours at 37℃ . Cells then were stimulated with 1ug/ml LPS for 24 hours and treated with optimum concentration of secondary metabolites crude extracts. After 24 hours treatment, cell culture media were harvest and mixed with equal amount of Griess reagent (Sigma Aldrich, USA.) to determine the level of nitrite. Then, the absorbance was measured at 540nm in a micro plate reader. Amount of nitrite in the media were calculated from sodium nitrite ($NaNO_3$) standard curve (Lau *et al*. 2012).

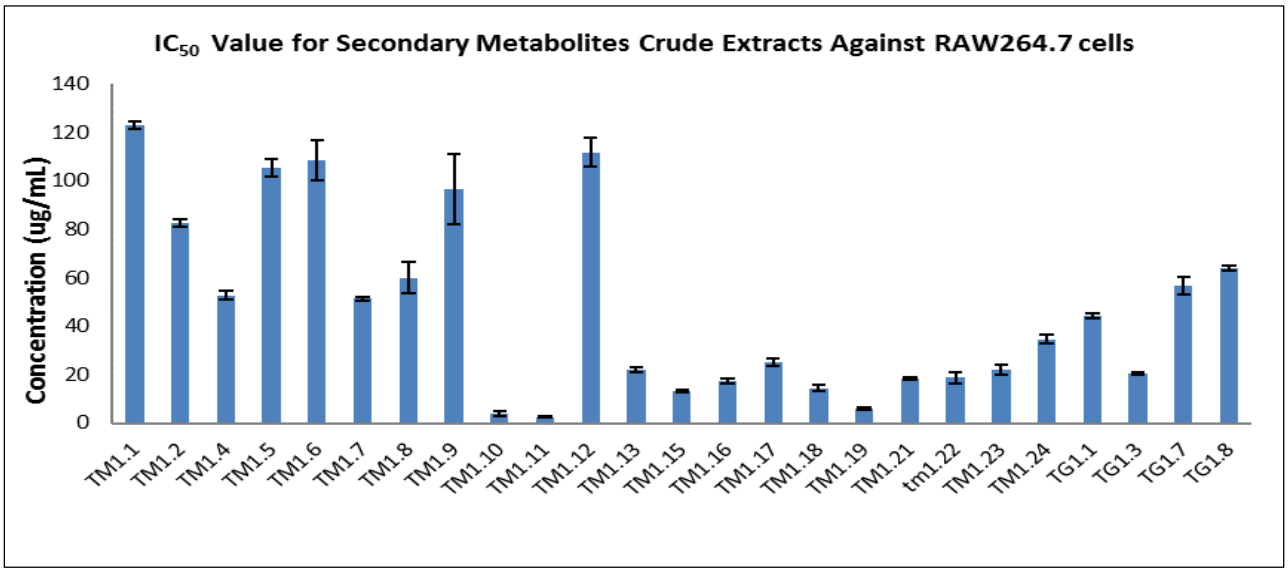

Figure 1 IC_{50} value for secondary metabolites crude extracts on Macrophages RAW 264.7

3.0 RESULTS AND DISCUSSION

A total number of 25 colonies bacteria were isolated from sponge *Theonella sp*. by spread plating on Marine Nutrient Agar (MNA). Majority of the colonies isolated were classified as gram negative bacteria after gram staining had been done. All isolated colonies were labelled with TM 1.1 until TM 1.24 and TG 1.1 until TG1.8 and stored in -80℃ for further analysis.

All secondary metabolites crude extracts were screened for MTT cell viability assay against macrophages RAW264.7 cell lines. The cells were treated with crude extracts to study the level of cytotoxicity produces by various concentrations of crude extracts. It was measured using MTT. Quantity of formazan product produced when MTT was added to the treated cells indicates the number of survived cell.

TM1.1, TM 1.5, TM 1.6, and TM 1.12 shows higher value in IC_{50} value which is 123 ug/mL ±1.73, 105.33 ug/mL ±3.53, 108.33 ug/mL ±8.33, and 111.67ug/mL ±6.01 respectively (Figure 1). IC_{50} value of more than 100 ug/mL indicates that crude extracts is not toxic to the cells because at a concentration more than 100ug/mL of extracts half of the cell died. Crude extracts of TM1.10 and TM 1.19 show the least IC_{50} value (4mg/mL ±1.15 and 6ug/ml±0.56 respectively). Low IC_{50} value indicates crude extracts are highly toxic. Low concentration of the crude applied to the cells killed half of the cells. Others crude extract showed moderate cytotoxic effect on RAW264.7 with IC_{50} value ranges from 14 ug/mL to 97 ug/mL.

Secondary metabolite plays an important role as antioxidant, free radical-scavenging, UV light-absorbing and anti proliferative (Mayer, 1999) A few crude extracts might affect the viability of the cell since it's contains different types of bioactive compounds therefore give different effects. Three main groups commonly found in secondary metabolites – terpenes, phenolics and nitrogen containing compounds(Devi *et al*. 2011). Every group give different activities and respond to cytotoxic assays. Crude extracts which shows highest value of IC_{50} indicates that the crude consist of high composition of bioactive compounds which lead to survival of the cell even at high concentration of secondary metabolites crude extracts were applied to treat the cells. While low value for IC_{50} indicates, secondary metabolites might cause toxicity to the cell lines it is due to crude extracts consists compounds which effected/killed the cell.

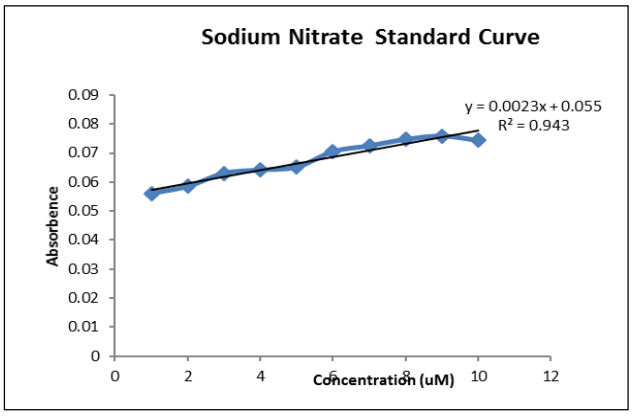

Figure 2 Standard curve of Sodium Nitrate

A Standard curve of Sodium Nitrate (Figure 2) is used for quantification of NO produced by RAW 264.7 cells treated with different samples. Since level of nitrite can be obtained from the standard curve, level of nitrites produced in the sample treated wells can be calculated using the regression plot obtained. Raw data from anti-inflammatory assay represent production of Nitric Oxide (NO) from cell supernatant. Production of NO from LPS treated wells were assumed as no inhibition.

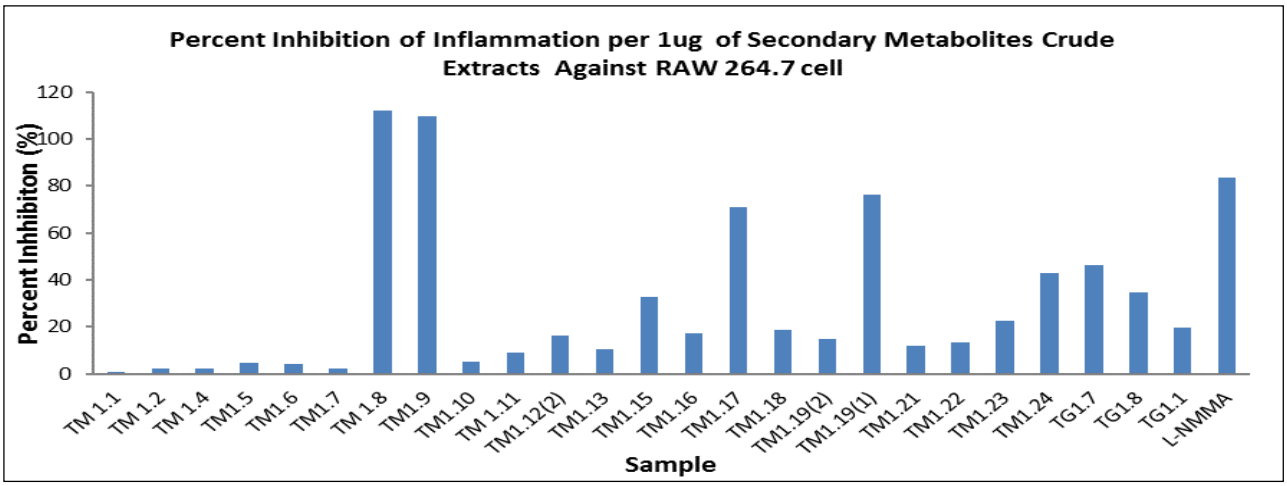

Figure 3 Percent Inhibition of Inflammation of LPS induced Secondary Metabolites Crude Extracts

Anti-inflammatory activities for all 25 secondary metabolites crude extracts were evaluated for the inhibition of NO production in the LPS-stimulated RAW264.7 cells. Nitrite accumulation in the cells increased due to the LPS treatment. Over accumulation of NO might affect inflammatory diseases including wound, hypertension, shock, arteriosclerosis and others(Pathak R. 2011). So, the proposed role of secondary metabolites crude extract is to reduce accumulation of NO which could cause chronic inflammation. Screening of anti-inflammatory activities among twenty five bacteria isolates revealed that all 25 crude extracts showed anti-inflammatory activities since it showed positive value in inhibitory activities. Figure 3 shows inhibition for positive control, L-NMMA is 83.52%± 0.01. Four secondary metabolites crude extracts showed higher inhibition activities than the positive control. Compared to positive control L-NMMA, two crude extracts TM1.8 and TM1.9 showed higher activity which was 112% ± 0.0037 and 109.7% ± 0.0028, respectively. Both of these crude extracts may consist bioactive compound which gave good responses in inhibiting inflammatory activities. These two extracts contain bioactive compound which shows positive inhibition of inflammatory response by reducing accumulation of NO when LPS was induced to the cells. Meanwhile, the rest of the extracts showed moderate or low inhibitory activities and these might be due to the bioactive compound which not playing a specific role to reduce inflammation or composition of anti-inflammatory compound are low and not enough to inhibit production of NO in the cells.

4.0 CONCLUSION

Five of bacteria isolates in this study showed good activities to cytotoxicity assay however, result for both assays (MTT assay and anti-inflammatory) were not interrelated. TM1.1 gives a highest IC_{50} value for cell viability assay, but when tested with anti-inflammatory, TM1.8 give highest inhibition. Secondary metabolites crude extracts of TM1.8 posses higher NO inhibitory activities among 25 secondary metabolites crude extracts tested. TM 1.8 consist moderately high number or composition of bioactive compound and leads to high anti-inflammatory activities. Recent studies have proved that, crude/compound possed anti-oxidant activity which may be effective inhibiting inflammatory process. Some of this might reacts due to the chemical reaction and due to excess of electronegative (oxygen) radical.

Acknowledgement

These studies were supported by a Grants Research Acculturation Grant Scheme (RAGS) 57103 and Institute of Marine Biotechnology, Universiti Malaysia Terengganu.

References

[1] Bogdan, C., Röllinghoff, M., & Diefenbach, A. 2000. Reactive Oxygen and Reactive Nitrogen Intermediates in Innate and Specific Immunity. *Current Opinion in*

Immunology. 12(1): 64-76. http://doi.org/10.1016/S0952-7915(99)00052-7.

[2] Devi, N. K. A., Rajendran, R., & Sundaram, S. K. 2011. Isolation and characterization of Bioactive Compounds From Marine Bacteria. *Journal of Natural Products*. 2(March): 59-64.

[3] Donia, M., & Hamann, M. T. 2003. Marine Natural Products and Their Potential Applications as Anti-Infective Agents. *Lancet Infectious Diseases*. 3(6): 338-348. http://doi.org/10.1016/S1473-3099(03)00655-8.

[4] Fieseler, L., Horn, M., Wagner, M., & Hentschel, U. 2004. Discovery of the Novel Candidate Phylum Poribacteria in Marine Sponges Discovery of the Novel Candidate Phylum "Poribacteria" in Marine Sponges. *Applied and Environmental Microbiology*. 70(6): 3724-3732. http://doi.org/10.1128/AEM.70.6.3724.

[5] Hur, K. Y., So, J.-S., Ruda, V., Frank-Kamenetsky, M., Fitzgerald, K., Koteliansky, V., … Lee, a.-H. 2012. IRE1 Activation Protects Mice Against Acetaminophen-Induced Hepatotoxicity. *Journal of Experimental Medicine*. 209(2): 307-318. http://doi.org/10.1084/jem.20111298.

[6] Kelecom, A. 2002. Secondary Metabolites from Marine Microorganisms. 74: 151-170.

[7] Lam, K. S. 2007. New Aspects of Natural Products in Drug Discovery. *Trends in Microbiology*. 15(6): 279-289. http://doi.org/10.1016/j.tim.2007.04.001.

[8] Lau, K. M., Lai, K. K., Liu, C. L., Tam, J. C. W., To, M. H., Kwok, H. F., … Lau, C. B. S. 2012. Synergistic Interaction between Astragali Radix and Rehmanniae Radix in a Chinese herbal formula to promote diabetic wound healing. *Journal of Ethnopharmacology*. 141(1): 250-256. http://doi.org/10.1016/j.jep.2012.02.025.

[9] Lee, J. C., Park, H. R., Park, D. J., Lee, H. B., Kim, Y. B., & Kim, C. J. 2003. Improved Production of Teicoplanin Using Adsorbent Resin in Fermentations. *Letters in Applied Microbiology*. 37(3): 196-200. http://doi.org/10.1046/j.1472-765X.2003.01374.x

[10] Mayer, A. M. S. 1999. Marine Pharmacology. *The Pharmacologist*. 41(4): 159-164.

[11] Pathak R. 2011. Assessment of Antibacterial and Free Radical Scavenging Activity in Pychrophilic Arthrobacter sp. *Pharmacologyonline*. 1: 344-355.

[12] Vane, J., & Botting, R. 1987. Inflammation and the Mechanism of Action of Anti-Inflammatory Drugs. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*. 1(2): 89-96.

[13] Yi, P. F., Bi, W. Y., Shen, H. Q., Wei, Q., Zhang, L. Y., Dong, H. B., … Wei, X. Bin. 2013. Inhibitory Effects of Sulfated 20(S)-Ginsenoside Rh2 on the Release of Pro-Inflammatory Mediators in LPS-induced RAW 264.7 cells. *European Journal of Pharmacology*. 712(1-3): 60-66. http://doi.org/10.1016/j.ejphar.2013.04.036.