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Potential Anti-Atherosclerotic Compound Isolated from *Acanthaster planci.*

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ABSTRACT

Methyl benzoate was isolated from the the outer layer of *A. planci* (APOL) and tested towards cytotoxicity to HepG2 cells and Luciferase assay. The compound was not toxic towards HepG2 cells with IC_{50} value of 150.71 ±11.31 µg/ml. Methyl benzoate was screened with PPRE promoter to determine its ability as PPAR ligand and expressed the target gene with maximum fold change of range 3.5 to 4.0 at concentration 0.78 µg/ml. The confirmation as PPARγ ligand was carried out with SRB-1 promoter and the positive results were obtained. The compound showed optimum expression higher than positive control used at 3.12µg/ml with almost 5.0 fold change. In addition, expression of Luciferase genes was higher than positive control even at lower concentration 0.39 µg/ml. These results indicated that methyl benzoate from APOL have the potential as anti-atherosclerotic agent, might as well beneficial as pharmaceutical products to prevent atherosclerosis which should be further explored.

Keywords: Methyl benzoate, Acanthaster planci, SRB-1 promoter, anti-atherosclerotic agent

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INTRODUCTION

Atherosclerosis is the term used to describe the injured arteries and the deposition of cholesterols, called plague at the arteries wall [1]. Atherosclerosis develops during lifetime and the development of plaques could be separated into three phases; the formation of fatty streaks, development of a complex lesion and plaque rupture [1, 2]. Atherosclerosis is the dominant cause of cardiovascular disease (CVD) including myocardial infarction (MI), heart failure, stroke and claudication [3].

Recently, many researchers have focused on the root of this disease including the role of reverse cholesterol transport (RCT). The release of High Density Lipoprotein (HDL) ultimately reduces the formation of atherosclerosis by transporting the Low Density Lipoprotein (LDL) from the bloodstream to be deposited in the liver [4]. Scavenger receptor type B-1 (SRB-1) is one of the receptors in RCT that able to bind acetyl-LDL within reverse cholesterol transport. SRB-1 could be majorly found in liver and act as mediates or receptor in the uptake of lipids from HDL particles before the lipoprotein could be transported back to peripheral tissue [5]. In this study, transfected SRB-1 plasmid was studied in order to search for natural product compound that could activate the promoters as well as the expression of the luciferase gene. When the gene is expressed, the potential compound would act as ligand activator for RCT. *Acanthaster planci*, also known as the crown-of-thorns starfish, belongs to phylum Echinodermata, the family of Acanthasteridae. These starfish possesses various bioactivities that induce researchers to research and develop new marine drugs or therapies [6]. *Acanthaster planci* fed on coral polyps and caused corals bleached and died. Various biologically active compounds [7-9] and toxins [10-14] have been identified. Previously we reported the isolation of phenyl ethanone, stigmasterol and β–sitosterol from *Acanthaster planci* and their potential as peroxissome proliferator activated receptor (PPAR) ligand. As part of our ongoing interest in this species, herein, we reported the isolation, characterisation of methyl benzoate (**1**) and its potential as anti-atheroslerotic agent.

EXPERIMENTAL

General Experimental Procedures

Infrared spectra were recorded on spectrometer Model Perkin Elmer Spectrum 100 Fourier Transform Infra-red (FTIR) Spectrometer using KBr disc. Mass spectra were obtained on the GCMS (Shimadzu/QP5050A). NMR spectra were acquired on the Bruker Spectrospin-400 (400 MHz) and DMX 600 NMR spectrometers. Melting point was determined using instrument Model Perkin Elmer Pyris 6 Disc (Disc Scanning Calorimeter). Column chromatography was performed on silica gel 60 (230-400 mesh, Merck). The Chromatotron was carried out by preparing the plate with Silica Gel TLC –Gypsum binder.

Sample Preparation

The *A. planci* was collected from the islands of Terengganu in the East Coast of Peninsular Malaysia via scuba diving at the depth of 5 to 10 metres. The samples were kept in ice during transportation to the laboratory and furthered with samples processing. The *A. planci* was separated into two parts; the outer layer (skin and spines) and visceral organs. Subsequently, the samples were freeze-dried and ground to powder formed. The samples were stored at below -80 ^{o}C in labelled and tagged storage bottle.

Extraction and Isolation

The outer layer of *A. planci* (128.80g) were macerated with methanol at room temperature for several times. The solvent was evaporated under reduced pressure to give 7.56 g of methanolic crude extract. About 2.16 g of the crude extract was suspended in H2O and then successively partitioned with diethyl ether and n-BuOH. The n-BuOH extract was evaporated in vacuo, and the crude extract (981 mg) was subjected to a chromatotron, eluting with CHCl3-MeOH (9.5:0.5) to yield 10.60 mg of compound (**1**).

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Biological evaluation

Cytotoxic Screening Assay

The HepG2 cell was treated with a serial dilution of compound (**1**), from the highest concentration of 100µg/ml to the lowest concentration of 0.39µg/ml. The treatments were carried out in eight replicates to ensure the accuracy of the results. The negative control consist of 20% DMSO and 80% of Modified Eagle Media (MEM). Vincristine sulphate was used as positive control. The cells were incubated for 72 hours in 5% carbon dioxide incubator at 37°C. The cytotoxicity of the compounds were determined using CellTiter 96®AQueous One Solution Cell Proliferation Assay (MTS) where only 20µL of the solution were transferred in each well and left incubated for 1.5 hour in 37°C. The cell viability was measured using Glomax Multi-detection (Promega) at absorbance 490nm. The value of IC50, the effective concentration of drug that is required for 50% inhibition of the cell was determined with non-linear regression

Luciferase Assay

The assay was done with two steps which are the screening with response element of PPARɣ and the gene expression involving Scavenger Receptor Type B class 1 (SRB-1) as promoter. All the genes, peroxisome proliferator response element (PPRE) and SRB-1 promoters were transfected within the host, HepG2 cells. The maintenance and treatments of these transfected cells were identical to cytotoxicity assay. For luciferase assay involving the PPRE, the compound was diluted as in cytotoxicity assay but the cells were incubated only for 24 hours after treatment. The expressions were determined by adding 50µL of Dual-Glo® Luciferase Reagent (DGL) and incubated for 10 minutes. Then, the measurement was done with Glomax Multidetetction System followed with the addition of 50µl of Dual Glo® Stop & Glo® Reagent (DGLSG).

For expressions of Luciferase with SRB-1 promoters, the experiment above was repeated using One-Glo Luciferase Reagent. In addition, the incubation times was shortened for only 3 to 7 minutes.

RESULTS AND DISCUSSION

Chemistry

The IR spectrum of compound (**1**) showed the absorption bands of alkene sp^2, (~3000cm^{-1}), carbonyl group (1640cm^{-1}) and aromatic ring (~1600cm^{-1} and 1590cm^{-1}) functionalities. The ^{1}H NMR spectrum revealed signals of an OCH_3 at δ_H [3.33 (3H, s)], and three olefinic proton at δ_H [8.20 (2H, d, *J*=8.0 *Hz*), 7.63 (2H, t, *J*= 7.6 *Hz*) and 7.75 (1H, t, *J*= 6.8 *Hz*)]. The ^{13}C and DEPT spectra showed eight carbon resonance ascribed to an OCH_3 (δ 49.7 ppm), one carbonyl (δ 164.1 ppm), one quartenary carbon (δ 130.5 ppm) and three methine (CH) carbons atoms (overlapped) (δ 127.9, 128.8, 133.8 ppm). HMBC has revealed the correlation of aromatic proton δ 8.20 ppm with C=O at δ 164.1 ppm and aromatic proton at δ 7.63 ppm with Cq at δ 130.5 ppm. Based on all information from NMR, compound (**1**) was elucidated as methyl benzoate (Figure 1).

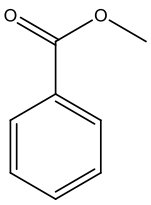

Figure 1: Methyl Benzoate isolated from *Acanthaster planci*

Biological Assay

Cytotoxicity Screening Assay.

The compound did not inhibit at 50% of the cell growth indicated that methyl benzoate did not toxic towards the cells. However, the compound constantly inhibited the cells when the concentration increased (Figure 2). The inhibition activity might give activity towards other cells but for HepG2 cells, the compound did

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not possess any anti-cancer properties. Since HepG2 cells were used as cell hosts in this study and the compound did not toxic towards the cells, Luciferase assay with peroxisome proliferator response elements (PPRE) and Scavenger Receptor Type B-1 (SRB-1) could be carried out.

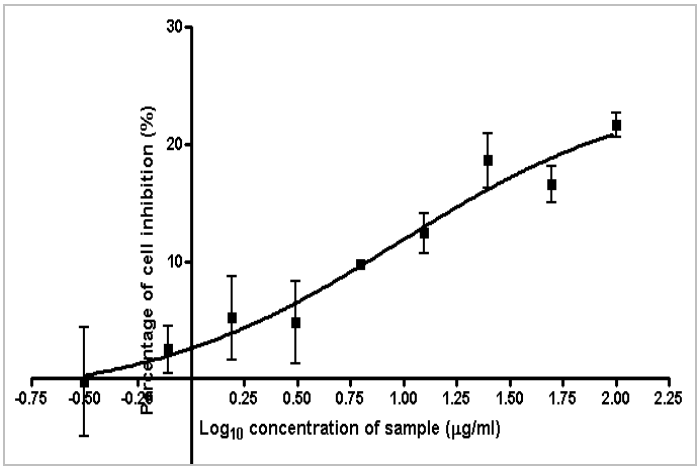

Figure 2: The percentage of HepG2 cells inhibition against various concentration of Methyl Benzoate

Luciferase Assay

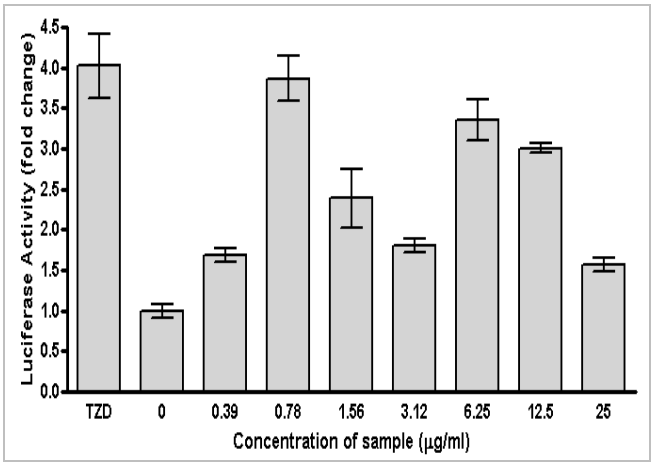

Figure 3: Luciferase activity of transfected HepG2 cells (PPRE) against concentration of Methyl Benzoate

The Luciferase assay was done with screening of the expression of Renilla Luciferase gene from the firefly within the transfected HepG2 cells. The cells were transfected with Peroxisome proliferator response element (PPRE) that responded to the Peroxisome proliferator-activated receptors (PPAR). The study has focused on the inhibition of atherosclerosis progression involving PPARɣ. Previous clinical studies approved the role of PPARɣ as atherosclerosis inhibitor with the present of PPARɣ ligand, thiazolidinedione (TZD) [15]. Thus, in this assay, TZD was used as positive control to indicate capability of methyl benzoate as PPARɣ ligand as well as to compare the fold change of the compounds with various concentrations. Generally, methyl benzoate gave significant results and comparable with TZD (Figure 3). The cut-off point of the expression was determined by comparing the standard deviation of the compound at concentration 0 µg/ml. When the expression was higher than three times of the standard deviation, it was considered to have ability as PPARɣ ligand. At lower concentration, the maximum activity was recorded. At the concentration 0.78 µg/ml, the expression was similar with the positive control but when the concentration was higher, only slight expression was detected. This might due to the availability of the cells within the plate. However, the activity at concentration 6.25µg/ml was increased and the activity decreased when the concentration was higher. The increasing activity might be caused by optimum substrate-enzyme like activity occur where at this concentration, compound had sufficient amount to bind as ligand and express the luciferase gene higher than previous concentration. The activity decreased after that might due to the cell death of HepG2 as cell hosts. As

a result, the expression decreased. Thus, methyl benzoate had the ability as PPARɣ ligand and had the potential to play role as inhibitor ligand to reduce the formation of atherosclerosis. The result showed significant activity that lead to the other assay as determination whether methyl benzoate could bind to SRB-1 promoter and increased the expression of renilla luciferase gene.

With the promising result from the screening study with PPRE, methyl benzoate was subjected to modified gene recombinant involving SRB-1 promoter. The result showed that methyl benzoate could increase the expression compared to positive control (Figure 4). At lower concentration, the expression was higher than control indicating that the compound could act as ligand activator for the promoter even at low concentration. The optimum concentration was 3.12µg/ml and at the highest concentration of 25µg/ml, no expression could be measured. The compound confirmed the ability to act as ligand in activating SRB-1 promoter. The expression was higher depended on the dose of treatment. Plus, the expression was higher than positive control used indicated that the compound was a better ligand in activating SRB-1 promoter. At lower concentration of 0.39µg/ml, the expression of Luciferase gene was already higher than LRH. The increment of dose still increased the expression. This might due to the possibilities that the excess binding site of SRB-1 promoter allowed the binding of compound molecules and activated the promoter.

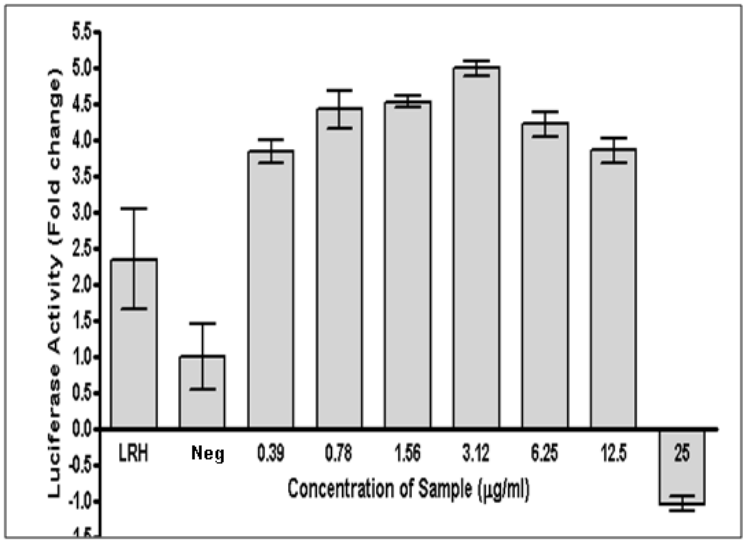

Figure 4: Luciferase activity of transfected HepG2 cells (PPRE) against concentration of Methyl Benzoate

CONCLUSION

Methyl benzoate had shown the potential as PPAR ligand and expressed the luciferase gene at optimum fold change higher than positive control used. Confirmation with SRB-1 promoter revealed its potential as ligand to activate the promoter ad expressed the gene higher than controls. With these promising results, it is believed that methyl benzoate is beneficial for atherosclerosis prevention and *Acanthaster planci* have proved to be the best candidate for drug discovery study.

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