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METHYL BENZOATE FROM *ACANTHASTER PLANCI* EXHIBITS LIPID-LOWERING ACTIVITY IN HYPERCHOLESTEROLEMIC SPRAGUE-DAWLEY RAT'S HEPATOCYTE BY INCREASING SCAVENGER RECEPTOR CLASS B TYPE 1 (SR-B1) GENE EXPRESSION

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Keywords:

Methyl benzoate, Hypercholesterolemia, Scavenger receptor class-B type-1

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ABSTRACT: Hypercholesterolemia occurs when a high cholesterol level in the blood may lead to atherosclerosis. Scavenger receptor class B type 1 (SR-B1) is a protein found on the surface of various cells that facilitates the reverse cholesterol transport or efflux of cholesterol from peripheral tissues back to the liver. SR-B1 receives cholesterol from high-density lipoprotein (HDL), which is then transported and metabolized in the liver cells. In this study, the compound methyl benzoate isolated from *Acanthaster planci* was investigated for its ability to reduce hypercholesterolemia by increasing the expression of the SR-B1 gene. Using MTS assay, methyl benzoate showed a non-cytotoxic effect on hepatocellular liver carcinoma (HepG2) cells. The luciferase assay revealed that methyl benzoate increased SR-B1 promoter activity by 1.23-fold compared to the negative control. The expression of SR-B1 gene examined by real-time polymerase chain reaction (RT-PCR) increased the expression by 1.24-fold higher than that of the positive control. In addition, HDL uptake increased by 5% in the methyl benzoate-treated cells. As for the *in-vivo* model, methyl benzoate was administered to hypercholesterolemic Sprague-Dawley rats for 28 days *via* oral gavage. Blood withdrawn after 28 days revealed an improved lipid profile of the rats where the level of HDL was increased. In contrast, total cholesterol and low-density lipoprotein (LDL) levels were decreased. In conclusion, methyl benzoate isolated from *A. planci* reduced the total cholesterol, which may be mediated by SR-B1, making its potential for future therapeutic use.

INTRODUCTION: Hypercholesterolemia is a condition where the cholesterol level in the blood is higher than 200 mg/dL [1].

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An increase in cholesterol level can be either due to external factors such as an unhealthy diet or an internal factor such as a genetic disorder (familial hypercholesterolemia).

High cholesterol levels can lead to atherosclerosis, one of the lipid metabolism disorders and chronic diseases. According to Sirtori *et al.* [2], atherosclerosis occurs in large and medium arteries. The plaque forms due to the interaction between

inflamed smooth muscle cells, foam cells, calcified regions, necrotic cores, accumulated modified lipids, endothelial cells, and leukocytes, making this a very complex disease with many metabolic, vascular, and immune involvement throughout the process. One of the pathways that can reduce cholesterol and the incidence of atherosclerosis is reverse cholesterol transport, where free and accumulated cholesterol accumulated in the blood vessel is transported to the liver for metabolism [3]. The main parts involved in reverse cholesterol transport are the acceptor (lipoprotein), enzyme and cholesterol ester transfer protein responsible for transporting cholesterol, and triacylglycerol between lipoprotein, which determines the content of cholesterol in high-density lipoprotein (HDL). Cholesterol in HDL is transported into the liver cells through SR-B1, converted into bile acid, and later discarded through the gastrointestinal tract (feces).

SR-B1 is a protein that acts as a scavenging receptor. It can be found in numerous cell types, including the liver and small intestine, and consists of two parts which are apo-A1 and phospholipid. In peripheral tissues, the receptor facilitates cholesterol detachment from the cell membrane, diffuses into maturing HDL, and is transported to the liver [4]. HDL is crucial in removing cholesterol from the peripheral tissue and circulation to the liver through reverse cholesterol transport.SR-B1, also present in the liver cells, will bind HDL, and the cholesterol portion of HDL will be transported into liver cells for deposition and reduce the level of circulating cholesterol and the risk of atherosclerosis [5, 6]. Metabolism of cholesterol occurs in the liver, including synthesis, clearance of plasma, and lipid **Fig. 1.**

Statin was developed to reduce cardiovascular mortality in patients by reducing hypercholesterolemia. Statin functions by inhibiting hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase, an enzyme that limits the rate of cholesterol biosynthesis [7, 8] and subsequently reduces cholesterol levels in the blood [9]. However, this potent drug has its own side effects, such as myositis [10, 11], apoptosis [12, 13] and tendon rupture [14, 15]. Based on recent research, statin reduced the incidence from 20% to 40% [16]. Our previous group successfully isolated methyl benzoate from a marine echinoderm, *A. planci* [17]. Based on their findings, we further tested the suitability and ability of the compound as a potential agent in reducing hypercholesterolemia. The compound was further investigated for its potential in reducing hypercholesterolemia, initially using the *in-vitro* model to determine the cytotoxicity and gene expression and then in *in-vivo* model utilizing Sprague-Dawley rats to evaluate the effects on lipid profiles as well as on liver enzyme and changes in liver morphology.

MATERIALS AND METHODS:

Cytotoxicity of Methyl Benzoate: The human hepatoma cells (HepG2) were cultured in Modified Eagle Medium (MEM) supplemented with 1% amino acid (v/v), 1.5 g/L sodium bicarbonate, 1 mM of sodium pyruvate, 1000 U/mL penicillin-streptomycin and 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% (v/v) carbon dioxide supplementation for 24 hours. The total cell count used for each well was 8.0 x 104 cells/mL. After 24 hours, the cells were treated with methyl benzoate ranging from 1.56 to 200.00 µM. After 72 hours, the viability and proliferation of the cells were assayed by 3-(4, 5 – dimethylthiazol – 2 - yl) – 5 - (3-carboxymethoxyphenyl) – 2 - (4 - sulfophenyl) - 2H-tetrazolium (MTS) reagent [17].

Determination of Luciferase Reporter Assay: For luciferase activity assay, HepG2 cells harbouring SR-B1 promoter-reporter construct integrated into the genome were seeded at a cell density of 3x 105 cells/mL in 96-well plate for 24 hours. The cells were treated with various concentrations of methyl benzoate diluted with MEM ranging from 1.56 to 50 µM for 24 hours. After 24 hours of treatment, ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay kit (Promega, USA) was used to measure the fluorescence and luminescence for each well to obtain the luciferase activity.

SR-B1 Gene Expression: Gene expression study was carried out using a modified method by 18. Briefly, HepG2 cells were cultured in flask until 80% confluency. Then, it was treated with three concentrations of methyl benzoate (1.56, 3.125 and 6.25 µM), 0.1 µg/ml trichostatin-A (positive control) and 0.5% dimethyl sulfoxide (DMSO) (negative control) at 37°C for 24 hours. Then, total

RNA was extracted from cells using TRI-Reagent ® (Molecular Research Centre, USA), followed by removing of DNA contaminant using RQ1 RNase-Free DNase (Promega, USA).

RT-PCR was performed using iTaq ™ Universal SYBR® Green One-step kit (Bio-Rad, USA) in CFX Connect ™ Real-Time System (Bio-Rad, USA) with forward and reverse primers as follow; SR-B1 forward5'-CTGTGGGTGAGATCATGTGG-3', SR-B1 reverse5'-GTTCCACTTGTCCACGAGGT-3', β-actin forward 5'-TCACCCTGAAGTACCCCATC-3'and β-actin forward 5'-CCATCTCTTGCTCGAAGTCC-3'. The RNA first was reverse transcribed into cDNA at 50°C for 20 minutes. Initial PCR amplification starts at 95°C for 5 minutes and is followed by 40 steps at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds.

Determination of SR-B1 Protein on Cell Surface: Immunocytochemistry was carried out to determine the presence of SR-B1 protein on the surface of HepG2 cells after being treated with methyl benzoate following the method from [18]. HepG2 cells were cultured into the 6-well plate with glass slip at a density of 20000 cells/100 µl. After overnight incubation, the cells were treated with methyl benzoate for 24 hours. After the treatment, the medium was discarded, and 1 ml of 4% (w/v) paraformaldehyde was added to each well, and the plate was incubated at room temperature for 25 minutes in dark condition. After 25 minutes, 4% paraformaldehyde was removed, and cells were washed three times with 1 ml of PBS, with 5 minutes for each wash. Subsequently, 500 µl of universal protein blocking reagent (UPBR) was added into each well, and the plate was wrapped with aluminium foil and incubated at 37°C for one hour. After the incubation, UPBR was discarded and 1 ml of 1x immunostain wash buffer (ISWB) was added and the plate was agitated for 10 minutes. ISWB was then discarded and 500 µl of primary antibody (Scarb1 polyclonal antibody), diluted 500X, was added to each well and incubated for 24 hours at 4°C. After incubation, the antibody was discarded, and cells were washed three times with ISWB for 10 minutes for each wash. A secondary antibody (goat anti-rabbit IgG) diluted 1000X, was added and the plate was incubated for one hour at 4°C. Subsequently, antibody was discarded and cells were washed 4 times with 1 ml of ISWB, with 10 minutes for each wash. The plate was air-dried in the dark, and the cover slip was removed from the well. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). When DAPI was dried, the coverslip was placed on a glass slide with surface of cells facing the glass slide mounted with mounting medium. Slide was then viewed under confocal microscope at 40x magnification.

Animal Study: This animal study was carried out according to the guideline approved by the Universiti Malaysia Terengganu Animal Ethics Committee (Reference: UMT/JKEPHT/2018/14). Sprague-Dawley rats were used for this study to determine the effects of methyl benzoate in reducing hypercholesterolemia and at the same time producedno hepatotoxicity effect. Rats were fed a high-cholesterol diet to increase the cholesterol to a hypercholesterolemic level (100 mg/dL) before the methyl benzoate treatment. The high cholesterol diet was prepared by combining 1% (w/w) of cholesterol, 0.1% (w/w)of cholic acid, 5% (w/w)of corn oil and 15% (w/w)of starch to the food pellet. The mixture was moulded into smaller pieces and was dried at 70°C for 24 hours.

Commercially available methyl benzoate (Sigma-Aldrich) was used for the *in-vivo* study due to the low yield isolated from the A planci, which was insufficient for the treatment in rats. Rats were divided into two groups of methyl benzoate-treated and untreated hypercholesterolemic rats. For the treated group, the rats were treated with 625 µM of methyl benzoate via oral gavage for 28 days; for the untreated group, the rats were treated with normal saline for the same time. During this period, the rats were handled gently to minimize the stress. After 28 days of treatment, the lipid profiles (total cholesterol, HDL, LDL) were determined. Determination of serum aminotransferase: aspartate transaminase (AST) and alanine transaminase (ALT) were done using kits from Elabscience (USA) according to manufacturer's protocols. The rats were euthanized, and a histological analysis of the liver was carried out. The livers were stained using haematoxylin and eosin to observe any changes caused by the treatment, according to the previous study by [19].

Statistical Analysis: All data were expressed as Mean ± Standard deviation (SD). The data was analyzed using Bonferroni-Holm post hoc test using Daniel's XL Toolbox version 7.3.4 software. The p-value was less than 0.05 was considered a statistically significant difference.

RESULTS AND DISCUSSION:

Methyl Benzoate Reduces the Expression of SR-B1 Gene: Methyl benzoate was investigated for its cytotoxicity effect against HepG2 cells **Fig. 2** *via* MTS assay. Based on the results obtained, the viability and growth of HepG2 cells significantly increased with the methyl benzoate concentrations ranging from 1.56-100 µM, and remained unchanged at 200µM. The US National Cancer Institute has set a criterium of IC_{50} value above 20 µg/ml for extract and fraction and 4 µg/ml for the compound to be categorized as non-cytotoxic [20].

Generally, IC_{50} value represents the concentration of the test compound required for 50% inhibition against biological processes [21]. Since, methyl benzoate isolated from A. planci17 did not produce any inhibition of cell growth of more than 50% of the cell population, therefore, the compound was not toxic against HepG2 cells. Methyl benzoate is a non-cytotoxic compound used as a healing agent [22, 23].

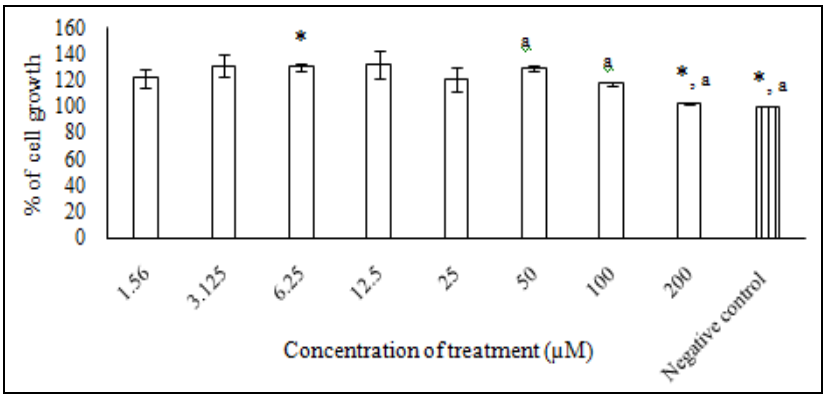

FIG. 2: PERCENTAGE OF CELL GROWTH OF HEPG2 AFTER TREATED WITH METHYL BENZOATE WITH VARIOUS CONCENTRATION FROM 1.56 TO 200 µM. Data presented as mean ±SD with n=3 and P<0.05. Asterisk and letters above the bars indicate significantly different between treatments according to the Benferroni-Holm test.

Luciferase assay was carried out to provide insight on the effects of methyl benzoate in regulating SR-B1 promoter activity. Mat-Lazim *et al.* [17] demonstrated that methyl benzoate increased the transcriptional activity of peroxisome proliferator responsive element (PPRE), the binding site of a transcription factor known as peroxisome proliferator-activated receptor (PPAR). Interestingly, PPRE was also shown to be present on the SR-B1 promoter, and the interaction between PPAR and PPRE induced the expression of SR-B1 in hepatocytes [24]. Interestingly, methyl benzoate was also shown to increase SR-B1 promoter activity [17].

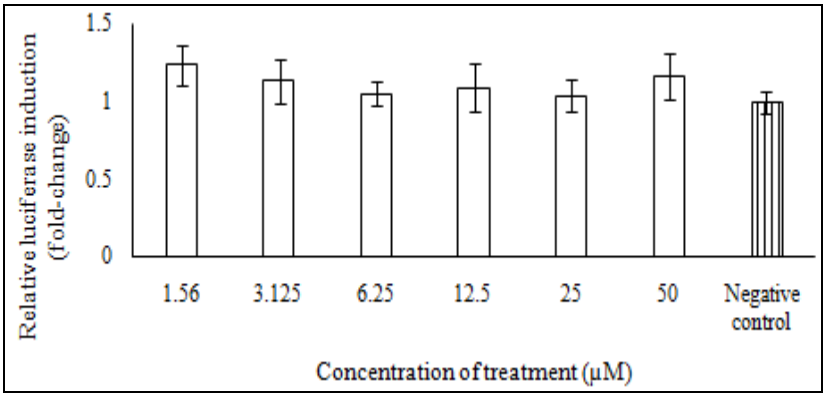

FIG. 3: TRANSCRIPTIONAL ACTIVITY OF LUCIFERASE TRANSFECTED WITH SR-B1 GENE IN HEPG2 CELLS AND THEN TREATED WITH METHYL BENZOATE FOR 24 HOURS. Data presented as mean ±SD with n=3 and P<0.05. However, there is no significant difference between treatments according to the Benferroni-Holm test.

Correspondingly, our study also produced an increase in SR-B1 transcriptional activity when stably transfected HepG2 cells harbouring SR-B1 promoter-luciferase gene were treated with methyl benzoate with concentration of 1.56 µM was found to significantly upregulated the promoter activity by 20% as compared to control **Fig. 3.** Since, methyl benzoate was found to induce SR-B1 promoter activity, the compound was then investigated for its effect in regulating the SR-B1 mRNA expression. Based on the results obtained **in Fig. 4, the concentration of 6.25 µM significantly increased SR-B1 mRNA expression by 20%** compared to the control.

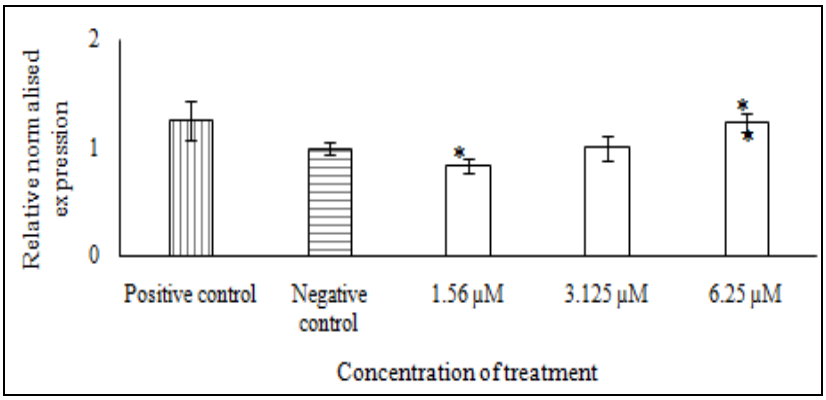

FIG. 4: THE EXPRESSION OF SR-B1 GENES IN HEPG2 CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF METHYL BENZOATE. THE MRNA TRANSCRIPT LEVELS WERE REPRESENTED AS RELATIVE NORMALISED EXPRESSION AGAINST REFERENCE GENE (B-ACTIN) AND UNTREATED HEPG2 (NEGATIVE CONTROL). ASTERISK ABOVE BARS INDICATE STATISTICALLY SIGNIFICANT DIFFERENCE AT *P*<0.05 ACCORDING TO BONFERRONI-HOLM POST HOC TEST. Data presented as mean±SD with n=3.

As shown in **Fig. 5**, HepG2 cells treated with methyl benzoate for 24 hours produced a higher intensity of green fluorescence staining representing SR-B1 protein presence on the cells was clearly visible as compared to untreated group. It clearly indicates that an increase in SR-B1 promoter activity, correspondingly increased SR-B1 mRNA level, which in turn, correspondingly induced the level of SR-B1 protein present in liver cells. It is interesting to note that methyl benzoate induced PPRE transcriptional activity, which may due to the activation of its corresponding transcription factor, PPAR.

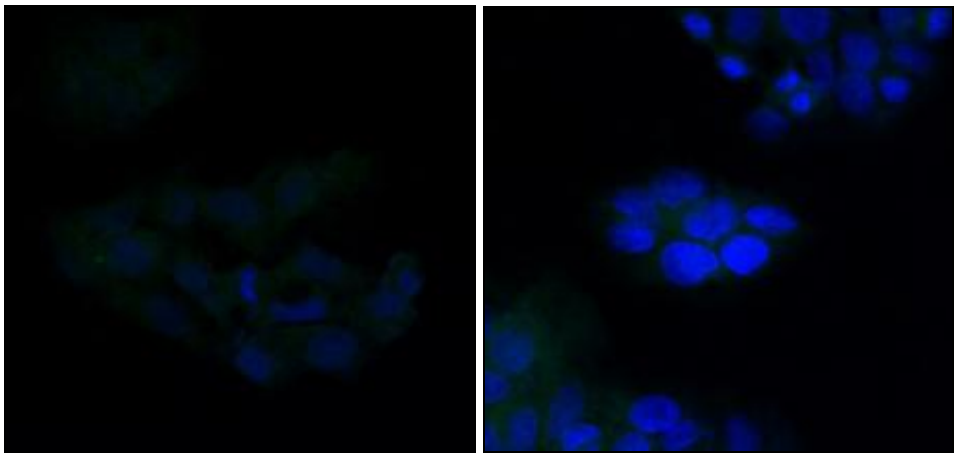

FIG. 5: IMMUNOCYTOCHEMISTRY OF HEPG2 CELLS AFTER TREATMENT WITH A) METHYL BENZOATE AND B) UNTREATED CELLS (NEGATIVE CONTROL) SHOWING LOCALIZATION OF SR-B1 STAINED WITH GREEN FLUORECEIN. NUCLEI (BLUE) WERE STAINED WITH DAPI

PPAR is activated by two classes of ligands, natural and synthetics [25]. Examples of natural ligand (endogenous) are linoleic acid, palmitic acid, 8-hydroxy eicosapentaenoic acid (8-HEPE) and 12-hydroxyeicosatetraenoic acid (12-HETE), while synthetic ligands (exogenous) are clofibrate, fenofibrate, gemfibrozil and thiazolidinediones [26].

Thiazolidinediones were demonstrated to induce SR-B1 gene expression by activating the binding of PPAR to its binding site, PPRE on SR-B1 promoter [5, 24]. Therefore, it is interesting to speculate that methyl benzoate may regulate the gene expression of SR-B1 by acting as a natural ligand for PPAR. In addition, it was proposed that a natural product, catechins from green tea upregulated SR-B1 via inducing the activity of PPAR [27]. Ren *et al.* [28] reported that following treatment with quercetin, a natural compound, increases SR-B1 expression and, at the same time, increase HDL uptake by HepG2 cells which may have vast potential in reducing the development of cardiovascular disease. It was widely demonstrated that an increase in HDL uptake was attributed to SR-B1, which plays an important role in reverse cholesterol transport [29]. Therefore, it is interesting to investigate whether methyl benzoate may exert a similar effect on HDL uptake by liver cells.

Lipid Profile and Serum Aminotransferase Effect of Methyl Benzoates in Hyper-cholesterolemia-induced Sprague Dawley Rats: To further investigate the effect of methyl benzoate in lowering the level of plasma lipid profiles, an *in-vivo* model of hypercholesterolemia-induced Sprague-Dawley rats were used. Rats that were fed with high cholesterol diet increased their total cholesterol level from 81 mg/dL (day 0) to 162 mg/dL (day 7) by 50% and to 203 mg/dL (day 14) with another 20% increase from day 7, which indicate that the condition of hypercholesterolemia was successfully induced [1]. Total cholesterol, HDL and LDL were recorded before and after the treatment with methyl benzoate after 28 days. As shown in **Fig. 6**, there was a significant decrease in total cholesterol and LDL by 30, 48 and 27%, respectively, from the initial value. Interestingly, HDL showed an increase of 30% higher than the initial value after 28 days of treatment.

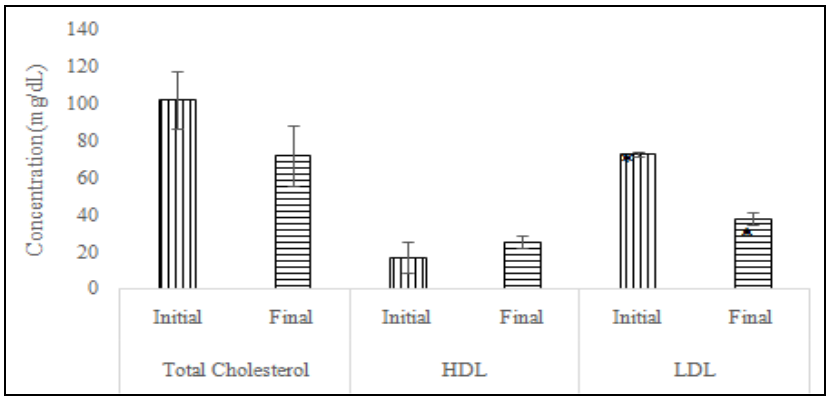

FIG. 6: CHANGE IN LIPID PROFILE AFTER 28 DAYS OF TREATMENT WITH METHYL BENZOATE. Asterisk above bars indicate statistically significant difference at $p<0.05$ according to Bonferroni-Holm post hoc test. Data presented as mean±SD with n=3.

According to Li *et al.* [30], PPAR plays an important role in regulating the expression of genes involved in increasing HDL via the binding to PPRE presence on the gene promoters. An increase in HDL observed after 28 days of treatment with methyl benzoate indicates an improvement in lipid profile which led to a decrease in the level of total cholesterols. A possible activation of PPAR by the compound may explain an increase the expression of SR-B1 which promoteda reverse cholesterol transport, not only inducing the cholesterol efflux from peripheral tissues[4]to form HDL, but more importantly, increased the levels of SR-B1 on liver cells, which in turn, induced the uptake of cholesterol portion of HDL and return the free-HDL to the circulation, which led to an increase to the level of HDL. It was reported that an increase in the expression of SR-B1 and other genes involved in reverse cholesterol transport is a result of the activation of PPAR [31] which caused a reduction in cholesterol levels in mice [32]. According to Trigatti [33], the inactivation of SR-B1 in mice can disrupt reverse cholesterol transport and increase cholesterol and the risk of atherosclerosis. Interestingly, methyl benzoate also decreased LDL levels in this study and correlated well with the finding by [34]. After the treatment with olive and sunflower oil as a dietary supplement, the level of LDL was also decreased. A decrease in plasma LDL was postulated due to an increase in the uptake of LDL-cholesterol in the liver by the LDL receptor.

It was widely demonstrated that an enzyme known as PSCK9 is responsible for mediating the degradation of LDL receptor in liver cells, thus, reducing the receptor levels on the cell surface, and decreasing the uptake of LDL-cholesterol by liver cells [35]. PCSK9 was also reported to have PPRE presence on its promoter [36] and together with its transcription factor, PPAR, plays a pivotal role in inhibiting the gene expression of PCSK9 which leads to an increase in LDL receptors on liver cells. Thus, it is tempting to speculate that methyl benzoate may exert similar action mechanisms in reducing the plasma LDL level. Combining the action of methyl benzoate in regulating HDL and LDL levels, the compound also concomitantly decreased the circulating level of total cholesterols. One way to evaluate hepatotoxicity is by measuring enzyme activity that involves metabolism. AST and ALT are the antioxidant enzymes that are presence in animal, function in defence against wide variety of toxicant ranging from oxidative stress, metabolites and chemicals [37]. The levels of liver enzymes AST and ALT were usedas biochemical markers for early detection of acute hepatotoxicity [38] due to their sensitivity and fairly exclusive for liver [39]. When there are changes in the liver cells (for example, injury and stimulus), the cell membrane becomes permeable or even destroyed, releasing the enzymes into the blood. Thus, ALT and AST can be used to evaluate liver damage 40 quantitatively. According to Arafa [41], on the effect of curcumin in lowering hyper-cholesterolemia in rat, AST and ALT concentrations were relatively higher in untreated groups, while in treated groups, both AST and ALT concentrations were decreased significantly. Similarly, in our study, both ALT and AST concentrations were decreased by 10 and 18%, respectively, as compared to the untreated group when treated with the compound methyl benzoate **Fig. 7**. Specifically, the levels of ALT and AST were reduced to 26.7 and 23.7ng/ml, respectively. The levels of enzymes determined in high cholesterol rats treated with methyl benzoate were still within the normal reference range, respectively [42].

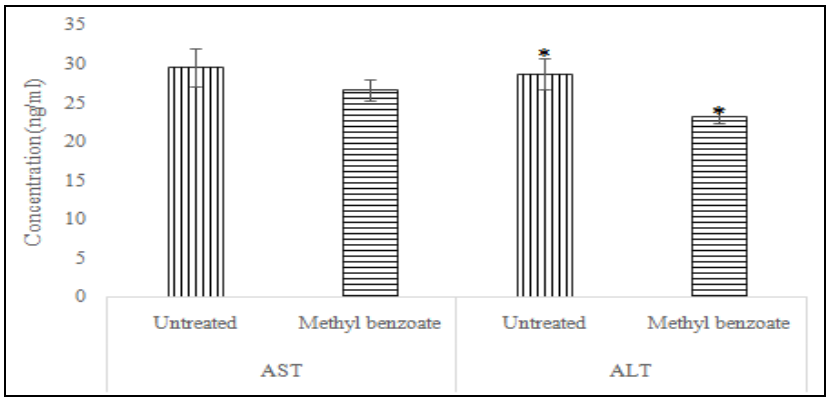

FIG. 7: AST AND ALT CONTENT IN RATS AFTER 28 DAYS OF TREATMENT WITH NORMAL SALINE (UNTREATED) AND METHYL BENZOATE. Asterisk above bars indicate a statistically significant difference at $p<0.05$ according to Bonferroni-Holm post hoc test. Data presented as mean±SD with n=3.

Histology Analysis: The effect of methyl benzoate in reducing hypercholesterolemia was also observed in histological slides of the extracted liver. Methyl benzoate treatment improves lipid profile by transporting cholesterol to liver to be further metabolized. Steatosis can be observed clearly within the hepatocytes, where steatosis was higher in the treated group as compared to the control **Fig. 9.** An increase in the cholesterol uptake was observed in the liver due to more cholesterols were deposited in the liver before it was converted into bile which was due to an increase in SR-B1. According to Xu *et al.* [43], steatosis can be defined as the accumulation of excessive triglyceride and cholesterol. All these later will be converted into bile acid and secreted *via* fecal secretion [44]. The present findings showed that cholesterol droplets were found within or in between the cells. According to [45] cholesterol-enriched diets can lead to hypercholesterolemia and later steatosis. It also has no severe effect to liver function. In this study, an increase in steatosis was associated with decrease in cholesterol level in the blood as an evident increase in cholesterol uptake by liver cell due to increase in SR-B1. However, Helsley 46 on the effect of ritonavir showed an increase in hepatic

steatosis after treatment with ritonavir, an activator for PXR. However, the treatment has a different effect on the lipid profile, increasing plasma cholesterol level.

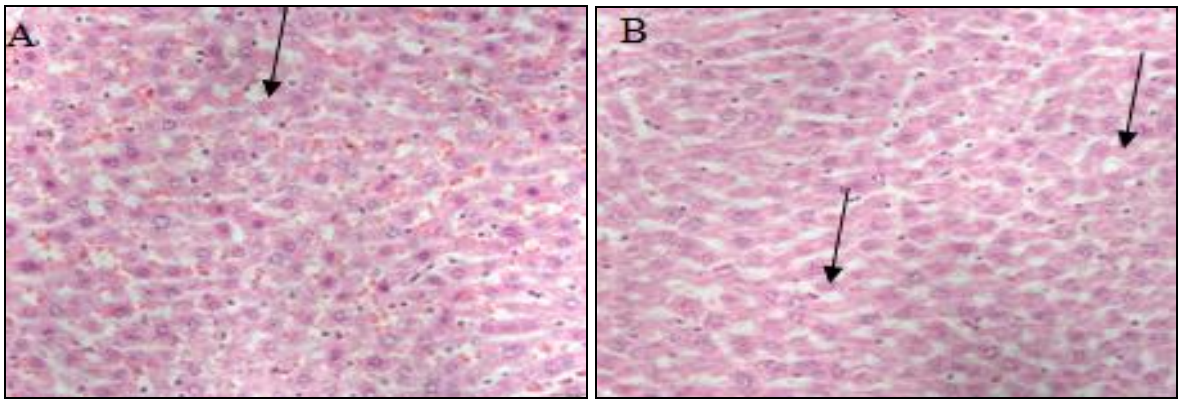

FIG. 8: HISTOLOGY OF THE LIVERS AFTER 28 DAYS OF TREATMENT WITH A) NORMAL SALINE (UNTREATED) AND B) METHYL BENZOATE. IMAGES WERE OBTAINED USING A STANDARD LIGHT MICROSCOPE AT 40× MAGNIFICATION. ARROWS INDICATE CHOLESTEROL DEPOSITING IN AND WITHIN THE CYTOPLASM

CONCLUSION: Methyl benzoate exhibited low toxicity and successfully increased the SR-B1 expression. The evidence of up-regulation observed at the cell surface correlates with increased gene expression and can be a strong justification for using methyl benzoate *in-vivo* stage. Methyl benzoate reduced hypercholesterolemia based on the lipid profile after 28 days of treatment.

Reduction in total cholesterol and LDL increase in HDL further confirmed methyl benzoate's ability to reduce hypercholesterolemia. Furthermore, the non-toxicity effects of methyl benzoate on the liver were further proven based on the serum aminotransferases and histological analysis of the liver as well as an increase of SR-B1 expression in the liver. These findings showed the potential of methyl benzoate isolated from A. planci that may pave the way in achievingnew therapeutic strategies for treating high cholesterol-related diseases.

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CONFLICTS OF INTEREST: Nil

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