ABSTRACT

Anticholesterol Activity of *Anacardium occidentale* Linn. Does it Involve in Reverse Cholesterol Transport?

(Aktiviti Antikolesterol *Anacardium occidentale* Linn: Adakah Pengangkutan Kolesterol Berbalik Terlibat)

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Anacardium occidentale belongs to the Anacardiaceae family. It had been scientifically proven to have anti-hypercholesterolemia effect in high cholesterol diet induced animal laboratory study. However there is no study regarding the mechanisms involves in cholesterol reducing effect by A. occidentale leaves extract. In this study, cytotoxic assessment and anti-cholesterol activity of A. occidentale leaves aqueous extract (AOE) were investigated. Cytotoxic study was performed by exposing hepatoma cell (Hep G2) towards AOE with concentration ranging from 0.002 to 20 mg/mL for 24 h. Anacardium occidentale extract was found to be not toxic to the cell. Then, the highest and not toxic AOE concentrations (20, 10, 5 and 2.5 mg/mL) were selected for anti-cholesterol study. The ability of AOE to reduce cholesterol in cell culture experiment was carried out by pretreating Hep G2 with selected concentrations of AOE in 6-well plate before the cell was exposed to low density lipoprotein (LDL). The concentration of farnesyl-diphosphate farnesyltransferase (FDFT1), apolipoprotein A1 (Apo A1), lecithin-cholesterol acyltransferase (LCAT), low density lipoprotein receptor (LDLR), scavenger receptor B1 (SR-B1), ATP binding cassette transporter A1 (ABCA-1) and hepatic lipase (HL) were determined from the 6-well plate media. The results showed that AOE did not significantly increase the concentration of LDLR. However, AOE significantly increased the concentration of FDFT1, APO A1, LCAT, SRB-1, ABCA-1 and HL. The HMGR activity experiment showed that all selected AOE concentrations cannot significantly reduce the HMGR enzyme activity. These findings suggested that AOE may involve in reverse cholesterol transport process to reduce cholesterol metabolism in Hep G2 cell.

Keywords: Anacardium occidentale; cholesterol metabolism; cytotoxic; Hep G2; reverse cholesterol transport

INTRODUCTION

Nowadays, the metabolic diseases such as atherosclerosis and cardiovascular disease are increasing among the people in developing countries around the world (Mensah 2003; Pearson 1999). Hypercholesterolemia related diseases have been listed as the number one killer that causes death...
among Malaysian (MOH 2011). This phenomenon is due to several factors such as unhealthy food consumption and sedentary lifestyle (Cruz 2000; Wu et al. 2012).

Hypercholesterolemia is characterized by elevated plasma low density lipoprotein (LDL) (Laurence et al. 2003). Exogenous cholesterol which is 15% from total cholesterol in human body is gained from daily dietary intake. Endogenous cholesterol which is 85% from total cholesterol is synthesized internally by the body especially liver (Radhakrishnan et al. 2001).

Liver is the major organ which plays a central role in regulating plasma lipoprotein and cholesterol concentrations (Ness & Chambers 2000; Ott & Lachance 1981). Cholesterol is produced endogenously by mevalonate pathway which involves HMG CoA reductase (HMGR). In mevalonate pathway, farnesyl-diphosphate farnesytransferase (FDFT) is considered as an important enzyme that contributes to the formation of squalene which is exclusively routed into cholesterol formation after multi-step pathway (Horvat et al. 2011).

In order to maintain cholesterol level in human body, cholesterol is regulated by reverse cholesterol transport (RCT) in which cholesterol from peripheral tissues were returned back to the liver to be diminished (Ghosh 2012; van der Velde et al. 2010). High density lipoprotein (HDL) play a major role in RCT and the process also involves some protein molecules and enzymes such as apolipoprotein A1 (Apo A1) (Getz & Reardon 2011), lecithin-cholesterol acyltransferase (LCAT) (Zannis et al. 2006), scavenger receptor B1 (SRB1) (Zannis et al. 2006) and hepatic lipase (HL) (Brown et al. 2010). Besides that, low density lipoprotein receptor (LDLR) will be expressed by liver to facilitate the endocytosis of cholesterol-rich LDL (Carneiro et al. 2012).

Currently, Statin family drug such as Pravastatin is used as cholesterol-reducing treatment because of their ability to bind with HMGR, thus, slowing down the process of cholesterol metabolism (Sasaki et al. 2012; Wilding 2010). However, previous studies have reported that statin medication can cause many side-effects including muscle and kidney damage (Reinhart & Woods 2012). Nowadays, most of researchers re-examine the abilities of common herbs to treat hypercholesterolemia.

Herbs and nutraceuticals are commonly used in many households in Malaysia. In Malay communities, A. occidentale is one of the side dishes that often eaten raw with rice (Faridah et al. 2006; Nurhanani et al. 2008). Previous studies have shown that the leaves of A. occidentale contain antioxidant activities (Abas et al. 2006; Runnie et al. 2004). This also supported by previous phytochemical studies of A. occidentale that discovered the presence of compounds such as flavonoids (Laurens & Paris 1977), which have been reported to offer biological benefits, in reducing the rate of the oxidation process. Furthermore, a study by Fazali et al. (2011) reported that aqueous extract of A. occidentale possess the ability to increase the antioxidant enzymes in high cholesterol diet induced rabbit.

This study was done to investigate the involvement of A. occidentale in the process of reverse cholesterol transport to reduce cholesterol metabolism.

**MATERIALS AND METHODS**

**CELL LINES AND CHEMICALS**

Human liver hepatocellular carcinoma (Hep G2) cell line was bought from American Type’s Tissue Culture (ATCC). Penicillin-streptomycin, trypan blue, fetal bovine serum (FBS), dimethyl sulphoxide (DMSO), 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), phosphate buffer saline (PBS) were bought from Sigma, USA.

**PREPARATION OF PLANT EXTRACT**

Fresh leaves of the A. occidentale were provided by Forest Research Institute of Malaysia (FRIM). It was identified and authenticated by a plant taxonomist in Institute of Bioscience, Universiti Putra Malaysia (Voucher Specimen Number: SK233). The leaves were cut into small pieces, around 1 cm in length and dried in an oven for 24 h at 40°C. The dried A. occidentale leaves were ground into powder. Then, 100 g of A. occidentale powder was soaked in 900 mL of distilled water for 24 h at room temperature. The mixture was incubated in the shaking water bath at 60°C for 6 h. The mixture was filtered and freeze-dried. The crude extract of A. occidentale (AOE) was kept at -20°C until use.

**CELL CULTURE AND MAINTENANCE**

Hep G2 was cultured in RPMI 1640 media, supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. Hep G2 cell was cultured on 75 cm² flask in a humidified atmosphere containing 5% CO₂ incubator at 37°C. Cells at passage 8 were grown to confluences before treatment of AOE.

**CYTOTOXICITY STUDY: DETERMINATION OF AOE IC₅₀**

Hep G2 cell was seeded in a 96-well plate at the density of 1 × 10⁴ cell per well. The plate was incubated in 37°C with 5% CO₂ atmosphere for 24 h. The cell was allowed to grow and spread overnight prior incubation with 200 μL of AOE extracts at various concentrations (0.02 - 20 mg/mL) except for control group. After 24 h of incubation with the extract, 20 μL of MTT (final concentration 0.5 mg/mL) solution was added into the 96-well plate and the plate was further incubated into a CO₂ incubator at 37°C for 4 h. After that, the media was discarded and 100 μL of DMSO was added to each well to dissolve formazan crystals. The plate was read at 570 nm by using microplate reader. The experiment was done in triplicate. The percentage of cells viability was calculated as:

\[
\text{Percent of viability (\%)} = \frac{\text{Absorbance of the treated cell}}{\text{Absorbance of control cell}} \times 100.
\]
DETERMINATION OF APO A1, LCAT, LDL R, SRB – 1, ABCA-1, FDFT1 AND HL USING CELL CULTURE

This experiment was carried out after the effective concentration of AOE had been identified. The four highest AOE concentrations (2.5, 5, 10, 20 mg/mL) were selected according to cytotoxicity assay which showed that it was not toxic to the Hep G2 cell. The cell was plated in 6-well plate at the concentration of 1 × 10⁶ cells/well followed by incubation in CO₂ incubator at 37°C for 48 h. The cells were divided into 3 groups: Untreated control group (Hep G2 + 10 μL LDL), Pravastatin control group (Hep G2 + 10 μL LDL + 250 nM Pravastatin) and treatment groups (Hep G2 + 10 μL LDL + AOE (20, 10, 5, 2.5 mg/mL)). The plate was incubated in a 5% of CO₂ atmosphere at 37°C for 24 h. After 24 h of incubation, the media was collected into falcon tube and centrifuged at 1000 rpm. The supernatants were collected for the determination of Apo A1 (AssayMax, Catalog No: EA5301-1), LDLR (Wuhan EIAAB science Co., LTD, Catalog No: E91008Hu), LCAT (Wuhan EIAAB science Co., LTD, Catalog No: E98516 Hu), SRB – 1 (Wuhan EIAAB science Co., LTD, Catalog No: E1530Hu), ABCA-1 (Wuhan EIAAB science Co., LTD, Catalog No: E91242Hu), FDFT1 (Wuhan EIAAB science Co., LTD, Catalog No: E1708Hu) and HL (Wuhan EIAAB science Co., LTD, Catalog No: E0769Hu). The procedures of the experiments were according to the instruction provided in the manual kit. The experiment was done in triplicate.

DETERMINATION OF HMG-COA REDUCTASE INHIBITION ACTIVITY USING ENZYME ASSAY KIT

This test was done in order to study the ability of AOE to directly inhibit HMGR enzyme activity. The test was performed according to the procedure provided in the kit’s manual (CS1090, Sigma). A. occidentale extract (2.5, 5, 10 and 20 mg/mL) was freshly prepared. AOE samples or pravastatin was added with nicotinamide adenine dinucleotide phosphate (NADPH) and HMG-CoA substrate solution in 1 mL cuvette. Blank was added with the same substrate and enzymes solution, without sample or pravastatin. The reaction was started by adding HMG-CoA Reductase (HMGR) to all cuvettes. The absorbance reading was performed kinetically at every 1 min for 10 min by using UV spectrophotometer at 340 nm wavelength. The experiment was done in triplicate. The calculation of HMGR activity was done according to equation:

\[
\text{Units/mgP} = \frac{\Delta A_{340/min}^{\text{control}} - \Delta A_{340/min}^{\text{sample}}}{\text{Control}}
\]

STATISTICAL ANALYSIS

The results of all experiments were expressed as mean ± standard deviation for three independent experiments. The data were statistically analysed using SPSS version 18. One way-analysis of variance (ANOVA) with Tukey HSD post hoc was performed to compare the mean between groups. Significance level was set at \( p < 0.05 \).

RESULT

MTT ASSAY

Figure 1 shows that the treatment of AOE at concentration of 0.002 to 20 mg/mL did not kill 50% of Hep G2 cell population. The IC50 value of AOE was considered more than 20 mg/mL.
EFFECT OF AOE ON FDFT1 CONCENTRATION

Figure 2 shows that FDFT1 was significantly reduced \((p<0.05)\) in Pravastatin group compared with untreated control. FDFT1 concentration in Hep G2 treated with AOE at 2.5 and 5 mg/mL were not significantly different compared with untreated cells. Unexpectedly, AOE at 10 and 20 mg/mL significantly increased \((p<0.05)\) the concentration of FDFT1 compared to untreated control.

EFFECT OF AOE ON APO A1, LCAT AND LDLR CONCENTRATIONS

Figure 3 demonstrated that Pravastatin group has clearly increased (61\%) Apo A-1 concentration in Hep G2 compared with untreated group. AOE treatment at all tested concentrations significantly increase \((p<0.05)\) the Apo A-1 concentration compared with untreated control at the same level with Pravastatin group.

Figure 4 shows that Pravastatin increased 74.9\% of LCAT concentration \((p<0.05)\) compared with untreated group. Treatment with AOE from 20 to 2.5 mg/mL increased the LCAT concentration compared with untreated group.

Figure 5 shows that treatment of Pravastatin effectively increased the LDLR concentration compared with untreated group. However, treatment of AOE at indicated concentrations showed no effect on LDLR concentration compared with untreated group.

EFFECT OF AOE ON SR-B1, ABCA-1 AND HL CONCENTRATIONS

Figure 6 shows that Hep G2 cell released SR-B1 at a basal level in untreated condition. However, Pravastatin treatment significantly increased \((p<0.05)\) the concentration of SR-B1. Treatment of AOE at indicated concentrations also increased SR-B1 but it was not comparable to Pravastatin treatment.

Data expressed as Mean ± SD. The treatment groups with the different letter indicate that the respective concentration treatments were significantly different from one the other \((p<0.05)\)

FIGURE 2. Human farnesyl-diphosphate farnesyltransferase 1 (FDFT1) against concentration of *A. occidentale*

FIGURE 3. Human Apolipoprotein A1 (Apo A1) against concentration of *A. occidentale*
Data expressed as Mean ± SD. The treatment groups with the different letter indicate that the respective concentration treatments were significantly different from one the other (p<0.05)

FIGURE 4. Human Lecithin Cholesterol Acyltransferase (LCAT) against concentration of *A. occidentale*

FIGURE 5. Human low density lipoprotein receptor (LDLR) against concentration of *A. occidentale*

FIGURE 6. Human scavenger receptor class B member 1 (SRB-1) against concentration of *A. occidentale*
Figure 7 shows that Pravastatin has markedly significantly increased ($p<0.05$) ABCA-1 concentration in Hep G2 compared with untreated group. Treatment of 2.5 and 5 mg/mL AOE also demonstrate a similar level as compared with Pravastatin group. Surprisingly, treatment of AOE at higher concentrations (10 and 20 mg/mL) demonstrated a lower level of LDLR concentration which were at similar level with untreated group.

Figure 8 shows that Pravastatin treatment has markedly increased HL concentration compared with untreated group. Treatment of AOE at all indicated concentration has also showed significant increase of HL concentration compared with untreated control in a dose-dependent manner. Interestingly, treatment of AOE at 10 and 20 mg/mL AOE showed significantly higher ($p<0.05$) secretion of HL compared to Pravastatin treatment.

**EFFECT OF AOE ON HMGR ACTIVITY**

The result of HMGR activity assay (Figure 9) showed that Pravastatin significantly inhibit HMGR activity compared with untreated control with 56.4% of inhibition. However, AOE showed no inhibitory action on HMGR activity. In turn, AOE at 20, 10 and 2.5 mg/mL markedly increase the HMGR activity while AOE at 5 mg/mL slightly increased compared with untreated control.

**DISCUSSION**

Hep G2 was used as human liver model since it still retain normal cholesterol metabolism like normal hepatocytes cell (Dashti 1992; Yanagita et al. 1994 ) and expressed several genes involved in cholesterol homeostasis including LDLR, HMGR, FDFT1 and SRB1 genes. Hep G2 cell line was also reported to synthesize cellular triglycerides and cholesterol and has been widely used on cholesterol synthesis and metabolism study (Funatsu et al. 2001; Scharnagl et al. 2001).

Plasma cholesterol is synthesized hepatically or extra-hepatically. It also described as biliary sources (endogenously) or absorbed from the intestine derived from dietary (exogenously) (Mackay & Jones 2011).
Endogenous cholesterol production is controlled by HMG-CoA reductase (HMGR), a rate-limiting enzyme which involves in the biosynthesis of cholesterol by mevalonate pathway. There are many published data demonstrated that cholesterol level can be managed by inhibiting the activity of HMGR (Dansette et al. 2000; Dhingra & Bansal 2006). Another enzyme involved in cholesterol biosynthesis is FDFT1. The enzyme is implicated in the formation of squalene which is exclusively routed into cholesterol formation after multi-step pathway (Horvat et al. 2011). In this study, the ability of AOE to reduce the activity of HMGR and FDFT1 were investigated. The results of this study showed that AOE had no inhibitory properties in both HMGR and FDFT1 enzymes activity. Inability of AOE to control both enzymes showed that AOE cannot control the cholesterol production through mevalonate pathway.

One of the strategies to manage cholesterol level in human body is by regulating reverse cholesterol transport (RCT). Reverse cholesterol transport pathway proposed the mechanism by which the excess cholesterol from peripheral tissues were transported back to the liver when it is not been used by cells. In the process of RCT, cholesterol is transported by HDL from atherosclerotic plaques or other lipids back to liver to be excreted (Stephen & Matthew 1997). The cholesterol is absorbed into HDL particles, esterified with a long chain fatty acid by LCAT (Milada & Jiri 1999). After that, cholesterol is taken up in the liver and excreted in bile (Sander et al. 1989). This RCT is regulated by several protein molecules and enzymes such as Apo AI, LCAT, SRB1, HL and LDLR.

Apo A-I is essential for nascent HDL formation (Hiromitsu et al. 2002). High secretion of Apo A-I is desired in order to increase the reverse cholesterol transport and normalize the LDL molecules inside the lumen of vessel. From the result (Figure 3), pravastatin can stimulate the Hep G2 cell to express Apo A-I. This result was similar to the previous report by Paul et al. (1997) that showed statin drug can increase the Apo A-I concentration in blood. Meanwhile, AOE treatment showed similar activity compared with pravastatin control which means these treatments can also increase the concentration of Apo A-I in the Hep G2.

From the liver, hepatic cholesterol is released to the circulation as very-low-density lipoprotein (VLDL) and metabolized to remnant lipoproteins after HL removes triglyceride (TG) (Brown et al. 2010). The remnant lipoproteins was removed by LDL receptors (LDL-R) or further metabolized to LDL (Carneiro et al. 2012). Figure 8 shows that AOE increased the HL release by Hep G2. This will contribute to the acceleration of cholesterol metabolism by changing the lipoprotein density. By action of HL, the VLDL molecules will be actively transformed to become LDL. Then, LDL can be carried out from the media by LDLR at the surface of Hep G2 membrane.

In controlling the concentration of cholesterol level in the blood, LDL is partly cleared from plasma through the action of the LDL receptor (Ghanaia et al. 2010) by both the liver and peripheral cells (Laurence et al. 2003). Any substance that can increase the LDLR is considered very beneficial in order to lowering down the LDL in the blood (Kevin 2007). A study done by Masayoshi et al. (2006) showed that statin drug can increase the concentration of LDLR and LDL uptake by liver. The result for pravastatin control was similar compared with the previous study (Polisecki et al. 2008). However, the treatment with AOE did not increase the concentration of LDLR by Hep G2.

Scavenger receptor class B type I (SR-B1) is a cell-surface HDL receptor that selectively takes up the cholesteryl ester (CE) of HDL (Acton et al. 1996). The binding process HDL and SR-B1 at the liver cell will mediate the selective uptake of CE and the bi-directional transfer of free cholesterol from HDL into the liver cell to be breakdown (Kinoshita et al. 2004). From the result, it showed that the treatment of 5 and 10 mg/mL of AOE can increase SR-B1. Since SR-B1 has a significant role for HDL to transport cholesterol into the liver, it was suggested that AOE has an
anti-hypercholesterole role of SR-B1 in relation to CE uptake and reverse cholesterol transport.

ABCA1 transports excess cholesterol from cells to lipid-poor apoA-I to form nascent HDL and provide an efficient pathway for cells to reduce plaque atheroma formation (Liu & Tang 2012). From the result, it showed that at concentration of 2.5 and 5 mg/mL of AOE can increase ABCA-1 which proves that at certain concentration, AOE may mediate the RCT process in order to unload excess cholesterol in the tunica intima.

Lecithin cholesterol acyl transferase (LCAT) is synthesized in the liver (Frohlich et al. 1982) The esters that are formed move into the core of HDL, enabling the HDL particles to acquire more free cholesterol from other lipoproteins and cell membranes. The activity of LCAT requires interaction with apo A-I, which is found on the surface of HDLs (Myoungsook et al. 2001). The ability of AOE to increase the LCAT concentration showed that this sample might have strong effect to up-regulate the reverse cholesterol transport.

Collectively, the results suggested that AOE has mild cholesterol lowering effects by demonstrating a significant increased of molecules that involve in reverse cholesterol transport (APO A-I, HL, SR-B1 and ABCA-1) in HepG2 cell. However, AOE at indicated concentrations has no effect in controlling mevalonate pathway. From this study, it was identified that the pathway of AOE aqueous extract action in lowering the total cholesterol might be more on reverse cholesterol transport.

This study was done on cell culture which has certain limitation and weakness. In vivo study need to be done to understand the anticholesterol effect of AOE in actual biological system. Besides that, molecular study must be done to investigate the molecules that involve in the anticholesterol metabolism pathways.

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Received: 29 January 2015
Accepted: 4 June 2015