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Effects of *Tinospora crispa* aqueous extract in regulating cholesterol metabolism in human hepatoma cancer cell line (Hep G2)

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In this study, the ability of *Tinospora crispa* aqueous extract (TCAE) to regulate cholesterol metabolism in human hepatoma cancer cell line (Hep G2) was determined. Cytotoxic study was performed by exposing hepatoma cell (Hep G2) towards TCAE with concentration ranging from 0.002 to 20 mg/ml for 24 h at 37°C and with 5% CO_2 atmosphere. Result revealed that TCAE was not toxic to the cell. The ability of TCAE to reduce cholesterol in cell culture experiment was carried out by pre-treating Hep G2 with selected concentrations of TCAE (10, 5, 2.5, 1.25 and 0.625 mg/ml) in 6-well plate before the cell was exposed to low density lipoprotein (LDL). The concentration of apolipoprotein A1 (Apo A1), lecithin-cholesterol acyltransferase (LCAT), low density lipoprotein receptor (LDLR), scavenger receptor B1 (SRB1) and hepatic Lipase (HL) which involve in reverse cholesterol transport (RCT) pathway were determined from the 6-well plate medium. The direct pathway of cholesterol synthesis was performed according to the instruction provided in HMG-CoA Reductase Assay Kit manuals. The results showed that TCAE significantly increase ($p<0.05$) the concentration of Apo A1, LCAT, LDLR, SRB-1 and HL. The efficacy of these activities is appreciably good when compared with standard drug simvastatin. However, TCAE showed moderate effect in controlling mevalonate pathway. These findings suggested that TCAE has the potential to reduce cholesterol metabolism in Hep G2 cancer cell lines and the pathway of TCAE action possibly more on RCT.

Key words: *Tinospora crispa*, cholesterol metabolism, reverse cholesterol transport, cytotoxic, Hep G2.

INTRODUCTION

Liver is a very important organ which plays a pivotal role in cholesterol metabolism. Cholesterol is synthesis in hepatocytes through mevalonate pathway with the help of HMG CoA Reductase (HMGR) (Petras et al., 1999) and secreted into bloodstream as a forward pathway in order to supply cholesterol to peripheral cells (Friedman et al., 2009). Conversely, reverse cholesterol transport (RCT), the process by which excess cholesterol is effluxed from

cells into high density lipoprotein (HDL) particles, helps to carry out excess cholesterol and returned to the liver for excretion from the body (Ghosh, 2010; van der Velde et al., 2010). This process is crucial in regulating cholesterol homeostasis by facilitating the prevention of lipid accumulation, particularly, in atherosclerotic lesions and therefore, RCT is considered an atheroprotective process.

RCT is a complex process that encompasses some protein molecules and enzymes such as apolipoprotein A1 (Apo A1) (Getz and Reardon, 2011), lecithin-cholesterol acyltransferase (LCAT) (Zannis et al., 2006), scavenger receptor B1 (SRB1) (Zannis et al., 2006), hepatic Lipase (HL) (Brown et al., 2010) and low density lipoprotein receptor (LDLR) (Carneiro et al., 2012). Stimulation of reverse transport of cholesterol from cells of the arterial wall to the liver is considered the mechanism by which HDL exerts its anti-atherogenic properties. In addition, interaction of HDL with the arterial wall directly protects against oxidative stress and vascular inflammation (van der Velde et al., 2010). Therefore, low level of HDL is considered as an important risk factor for the development of atherosclerosis (Ghosh, 2010).

Tinospora crispa aqueous extract (TCAE) cholesterol reducing abilities has been explored earlier and it is proven that TCAE can reduce the plasma low density lipoprotein (LDL), total cholesterol (TC), and malondialdehyde (MDA) level on hypercholesterolemic-induced models. It was reported that, TCAE exerted significant results in decreasing the cholesterol level similar to what statin does (Zulkhairi et al., 2009). Despite the promising evidences of its ability in lipid lowering activity, information on the underlying mechanism of action which contributes to the effect is not well documented so far. Thus, it is important to investigate the effects of this plant in regulating the cholesterol metabolism through their essential biocomponents involved in either forward or reverse cholesterol transport pathways including HMGR, SRB1, Apo A1, LDLR, HL, ACAT, and LCAT in order to determine its specific mechanism of action. Therefore, the aim of this study is to investigate the TCAE effects in regulating cholesterol metabolism in Hep G2 cells.

MATERIALS AND METHODS

Cell lines and chemicals

Experimental Hep G2 cell line was purchased from ATCC (American Type's Tissue Culture, USA) and maintained as recommended. Simvastatin, penicillin/streptomycin, trypane blue, fetal bovine serum (FBS), dimetyl sulfoxide (DMSO), 3-4,5 dimetyl tiazol-2, 5 difenyl tetrazolium bromide (MTT) and phosphate buffer saline (PBS) were purchased from Sigma, USA. Instruments used in this experiment were CO_2 incubator (Shelab, German), hemocitometer (La Fontaine, Perancis), vacuum pump, multiple pipet (RAININ, USA), homogenizer (Hettich, Zentrifugen, German), water bath (Jeiotech, Korea), microplate reader (UVM 340, German), reverse microscope (Nikon Gerhana TS100) and micro centrifuge.

Preparation of plant extract

Preparation of TCAE was done according to the method by Kamarazaman et al. (2012) with slight modification. About 10 kg of fresh stem part of *T. crispa* were collected from Forest Research Institute Malaysia (FRIM) at Kepong, Selangor. The plant was authenticated by FRIM botanist (Voucher number: SBID009/15). The stems were cleaned, washed, cut and dried using an oven dryer with operating temperatures of about 55°C. The weight of the samples was monitored every day until constant weight was obtained. Subsequently, the dried stems of the plant are ground to a particle size of about 1 to about 4 mm by using a 20 hp pilot scale grinder. Then about 100 g dried *T. crispa* was soaked in 900 ml of distilled water ratio 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 TCAE 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 cm^2 flask in a humidified atmosphere containing 5% CO_2 incubator at 37°C. The cells were grown to confluences before treatment of TCAE.

Cytotoxicity screening: Determination of TCAE IC_{50}

Cytotoxic assessment of TCAE were performed according to the method by Ibrahim et al. (2010) by exposing the Hep G2 cell G2 (ATCC, HB8065TM, USA) to TCAE (ranging in concentration from 0.002 to 20 mg/ml) for 24 h at 37°C and 5% CO_2 atmosphere, in order to determine the inhibition concentration 50 (IC_{50}) of TCAE; concentration that killed 50% of cell population in well plate, that can jeopardize the cholesterol metabolism due to the cell death. 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 CO_2 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:

Percent of viability (%) = Absorbance of the treated cell / Absorbance of control cell

Determination of TCAE HMGR inhibition activity using enzyme assay kit

In this study, the ability of TCAE to reduce cholesterol synthesis

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was performed according to the instruction provided in HMG-CoA Reductase Assay Kit manuals (Catalog Number CS1090) obtained from Sigma Aldrich. *T. crispa* extract (5 and 10 mg/ml) was freshly prepared. The TCAE samples or simvastatin 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 simvastatin. 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 conducted according to the equation:

$$\text{Units/mgP} = \Delta A_{340}/\text{min}_{\text{control}} - \Delta A_{340}/\text{min}_{\text{sample}}$$

Determination of Apo-A1, SRB1, LDLR, HL and LCAT using cell culture

In this study, the ability of TCAE to reduce cholesterol metabolism in human hepatoma cancer cell line (Hep G2) was carried out following the method by Hubert et al. (2001) and Peter et al. (2010) with slight modification. This experiment was carried out after the effective concentration of TCAE had been identified. Five TCAE concentrations (0.625, 1.25, 2.5, 5, and 10 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 1×10^6 cells/well followed by incubation in CO_2 incubator at 37°C for 48 h. The cells were divided into 4 groups: normal control (N) group (Hep G + medium only), untreated (NC) control group (Hep G2+ 10 µl LDL), pravastatin control group (Hep G2 + 10 µl LDL + 10 µM simvasttatin) and treatment groups (Hep G2 + 10 µl LDL + TCAE (10, 5, 2.5, 1.25 and 0.625 mg/ml). The plate was incubated in a 5% of CO_2 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) 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.

Statistical analysis

All data were analyzed using the computer software Statistical Package for Social Sciences (SPSS) version 20.0 and were expressed as mean + standard deviation. Comparisons of group means were done by one-way analysis of variance (ANOVA) with a probability less than 0.05 ($p < 0.05$) taken as indicative of significant difference. The mean value (x) and standard deviation (SD) were calculated for each variable measured. Turkey's pos hoc test was used for multiple group comparison. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Cytotoxicity screening of TCAE

In vitro cytoxicity screening in the present study was exercised to determine the optimum concentration of TCAE, since beside their therapeutic potentials TCAE, may also cause adverse effect. This is to ensure that the concentrations selected will not cause necrosis to the cell which later may disrupt cholesterol metabolism activity of Hep G2; hence, the effects of both TCAE in regulating cholesterol metabolism cannot be monitored.

The uses of Hep G2 in this study is due to its ability to retain normal cholesterol metabolism, that is, normal hepatocytes cell (Hasan et al., 2015; Dashti, 1992; Yanagita et al., 1994) and its ability to express several genes involved in cholesterol homeostasis including LDLR, HMGR, Apo A1, LCAT, and SRB1. Hep G2 cell line was 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).

Result from the present study revealed that, the treatment of TCAE from concentration ranging from 0.002 to 20 mg/ml did not cause 50% cell death to Hep G2 cell population, indicating that the concentrations used did not impede cholesterol metabolism activity of the cell. This data is in accordance with Zulkhairi et al. (2008) who reported *T. crispa* extract with concentrations varying from 50 to 900 µg/ml showed no toxic effect on HUVEC cell, normal cell lines ($p<0.05$) (Figure 1).

It is also found that *T. crispa* extract produced no toxic effect on brine shrimp survival and does not demonstrate any IC_{50} even up to an extreme concentration of 1 g/ml. Similar finding was reported by Tungpradit et al. (2010) who stated that *T. crispa and Tinospora cordifolia* water and methanol extracts had no significant cytotoxicity to HL 60, Hep G2 and MCF-7 cancer cells with the IC_{50} up to 500 µg/ml. Moreover, many previous studies done on *T. crispa* in several experimental animals had reported no evidence of organ damage (Pingale, 2011; Talubmook and Buddhakala, 2013; Abu et al., 2015)

However, our finding was contradicted with Md et al. (2011) who found that chloroform, petroleum ether and methanol extract of *T. cripsa* exhibited very significant cytotoxicity with IC_{50} value of 11.5, 12.6 and 12.0, µg/ml, respectively, in the brine shrimp lethality bioassay. The reason for the differences could be due to different extraction medium used in which most of those chemicals although reported to be useful but they are also very toxic (Pruthu, 2015). The use of water also is in line with recent trends in extraction techniques known as green extraction which largely focused on finding solutions that minimize the use of solvents, while also enabling process of strengthening and a cost-effective production of high quality extracts (Chemat et al., 2012). Meanwhile, it was reported that 10 µM simvastatin caused no cytotoxic effect in the HepG2 cells (Peter et al., 2010).

Effects of TCAE in regulating cholesterol metabolism in HEP G2 cells

Reverse cholesterol transport (RCT) is a pathway by

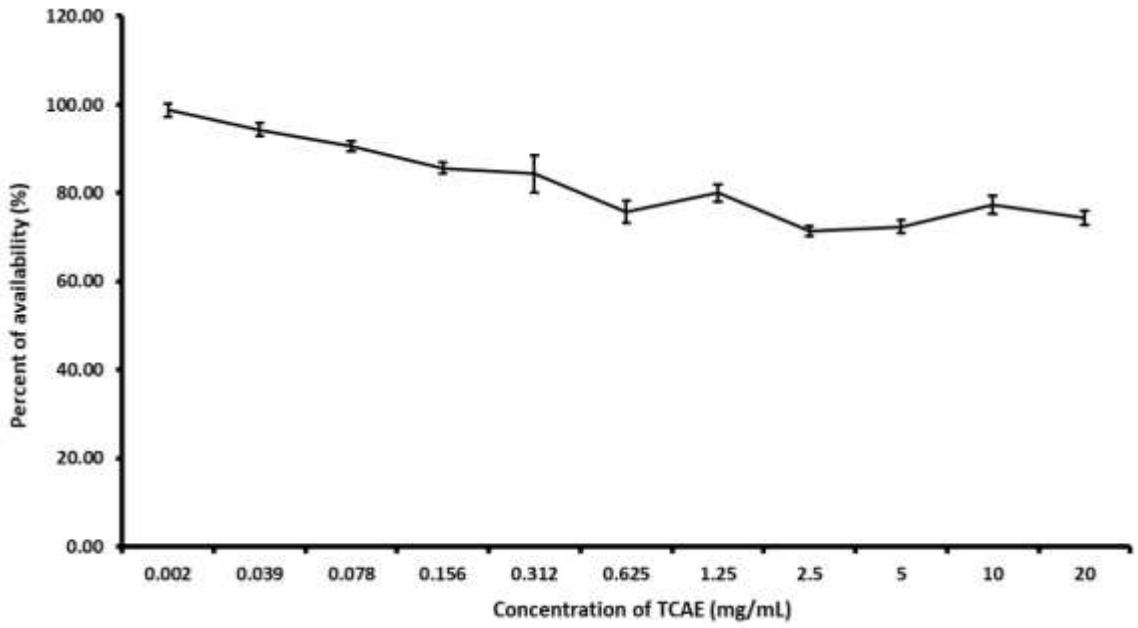

Figure 1. Percentage of viability of Hep G2 cells in 96-well plate against concentration of TCAE. The cells were seeded in a 96-well plate in amount of 1×10^4 cell per well and were nurtured with 100 µl media (RPMI 1640) per well. The cells were treated with different concentration of TCAE and incubated in 5% CO_2 humidified incubator at 37°C for 24 h.

which accumulated cholesterol is transported from the vessel wall to the liver for excretion, thus preventing atherosclerosis. Major constituents of RCT include acceptors such as HDL and Apo A-I, and enzymes such as LCAT, hepatic lipase (HL) and cholesterol ester transfer protein (CETP). A critical part of RCT is cholesterol efflux, in which accumulated cholesterol is removed from macrophages in the sub intima of the vessel wall by ATP-binding membrane cassette transporter A1 (ABCA1) or by other mechanisms, including passive diffusion, scavenger receptor B1 (SR-B1), and collected by HDL and Apo A-I. RCT plays a major role in anti-atherogenesis and modification of these processes may provide new therapeutic approaches to cardiovascular disease (Ohashi et al., 2005).

Apo A1

The results of the Apo A1 are shown in Figure 2. Result showed that TCAE was able to stimulate Hep G2 cell to secrete APO A-1 although it is not comparable to that of simvastatin. The Apo A1 concentration of groups treated with 10, 5, 2.5, 1.25 and 0.625 mg/ml of TCAE were increased by 52.11, 50.72, 37.82, 39.96, and 26.28%, respectively, whereas simvastatin increased Apo A1 levels by up to 101.82%, compared to NC group. Paul et al. (1997) reported that one of the effects of statin drug in reducing cholesterol is stimulating the secretion of the Apo A1 by the Hep G2 cells thus enhancing the reverse transport of circulating cholesterol. Apo A-I is vital in the formation of HDL, in which due to its absence no HDL will be presented in plasma (Hiromitsu et al., 2002).

LCAT

LCAT plays a central role in HDL-mediated transport of excess cholesterol from peripheral tissues for disposal in the liver involved in RCT. During the process, cholesterol is absorbed into HDL particles and esterified with a long chain fatty acid by LCAT before being taken up to the liver and excreted in the bile (Milada and Jiri, 1999; Sander et al., 1989). Kuivenhoven et al. (1997) reported LCAT shortage will trigger to significant reduction in plasma HDL cholesterol concentration, formation of cholesterol-laden cells in various tissues, corneal opacification, premature atherosclerotic cardiovascular disease and progressive renal insufficiency. Thus, any substance that can increase LCAT is considered very beneficial in the treatment of high blood cholesterol.

The results of the LCAT are shown in Figure 3. Result revealed that supplementation of TCAE significantly increased ($p<0.05$) production of LCAT in Hep G2 cells compared to NC group. Interestingly, TCAE at 10 mg/ml exhibited significantly ($p<0.001$) higher concentration of LCAT than simvastatin drug does indicating that TCAE might have strong anti-cholesterol effect by enhancing

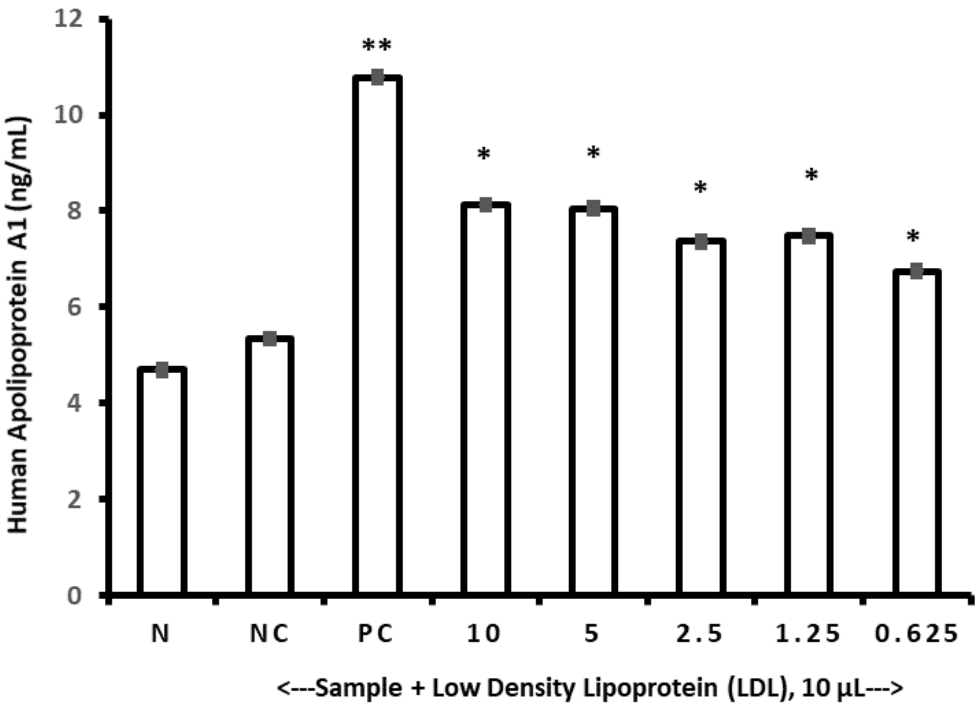

Figure 2. Graph of Human Apo A1 concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. Data expressed as mean ± SD. *Significantly different compared to NC at $p<0.05$. **Significantly different compared to NC at $p<0.01$.

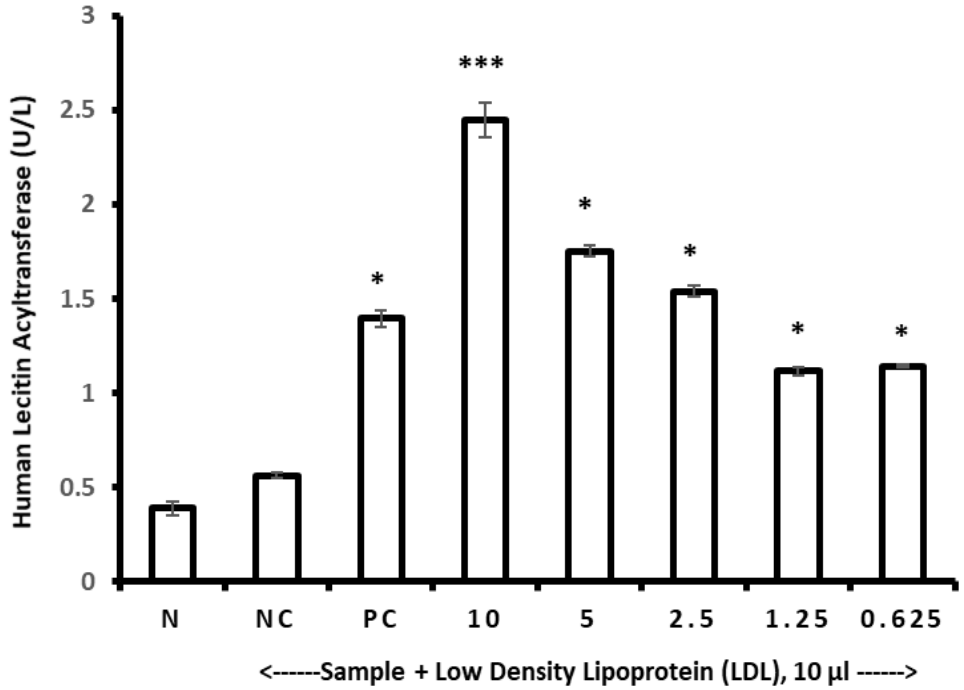

Figure 3. Graph of human LCAT concentration against treatment of samples. The samples are Simvastatin as control and different concentration of TCAE and syringin as mean ± SD. *Significantly different compared to NC at $p<0.05$. **Significantly different compared to NC at $p<0.01$. ***Significantly different compared to NC at $p<0.001$.

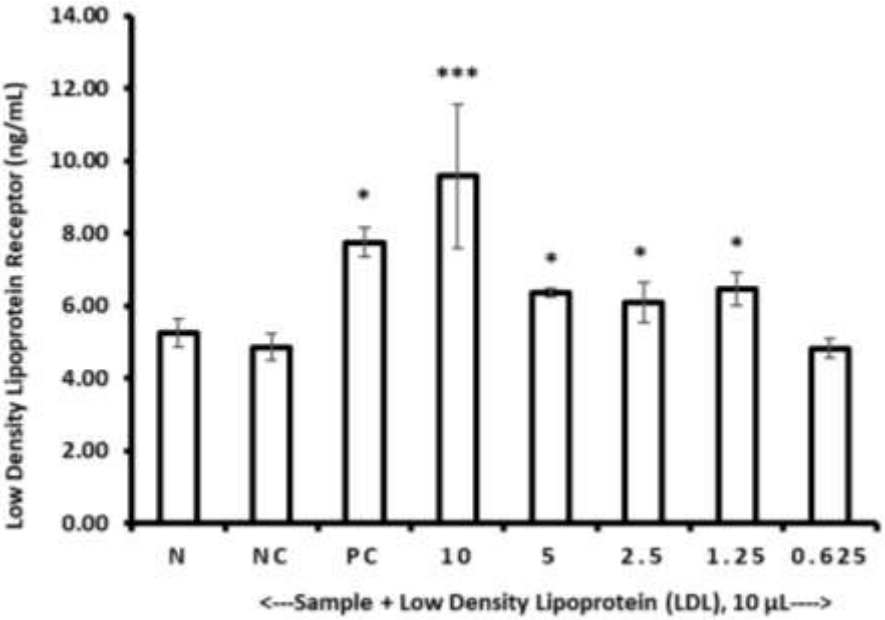

Figure 4. Graph of Human LDLR concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. *Significantly different compared to NC at $p<0.05$. *** Significantly different compared to NC at $p<0.001$.

the RCT pathway. Our finding is in line with Kumar et al. (2013), who reported that lipid metabolism disorders may increase in LDL levels of the alloxan-induced diabetic rats and consequently decrease the plasma levels of LCAT and HDL as well post-heparin lipolytic activity (PHLA). In contrast, treatment with *T. cordifolia* was able to stimulate LCAT enzyme and partially recover the level of HDL in diabetic rats.

LDLR

The results of the LDLR are shown in Figure 4. Results revealed that treatment of TCAE and simvastatin caused the increment of LDLR level in Hep G2 cell. Interestingly, TCAE at concentration of 10.0 mg/ml was able to increase the secretion of LDLR by Hep G2 cells stronger than simvastatin with the increment of 1.97 and 1.59 folds, respectively. LDL, known as bad cholesterol, is cleared from plasma by the action of the LDLR as one of the mechanism involved in RCT (Ghanya et al., 2010). Commercial available anti-cholesterol drugs such as statin and simvastatin increase the expression of LDLR and LDL uptake by liver resulting in low cholesterol level in blood plasma (Yokoyama et al., 2007; Polisecki et al., 2008).

SRB-1

SRB-1, also known as HDL receptor, functions as a key regulator of HDL metabolism (good cholesterol) and as a receiving platform of the triglyceride contents in the liver (Gillard et al., 2017). It facilitates the efflux of cholesterol from cells in peripheral tissues to HDL and mediates the selective uptake of cholesteryl esters from HDL in the liver in the RCT pathway (Van Eck et al., 2003). Therefore, any substance that can increase the concentration of SRB-1 is considered very useful in combating hypercholesterolemia. Result as presented in Figure 5 revealed that, the untreated group which was supplemented with 10 µl LDL only (NC group) exhibited low levels of SRB-1 compared to other treatment groups, indicating that high levels of LDL caused low secretion of SRB-1 in hepatocytes (Atshaves et al., 2009). SRB-1 deficiency could lead to the accumulation of HDL cholesterol within the circulation as a direct consequence of an impaired delivery to the liver, thus affect the availability of cholesterol for excretion into the bile (Van Eck et al., 2003). In contrast, administration of TCAE normalized SRB-1 concentration. Improvement of SRB-1 deficiencies through the introduction of TCAE could enhance reverse cholesterol transport in facilitating the transport of accumulated cholesterol from the plasma into the liver for excretion and thus might prove their abilities to lower the risk of cardiovascular complications.

HL

The graph of HL concentrations against treatment

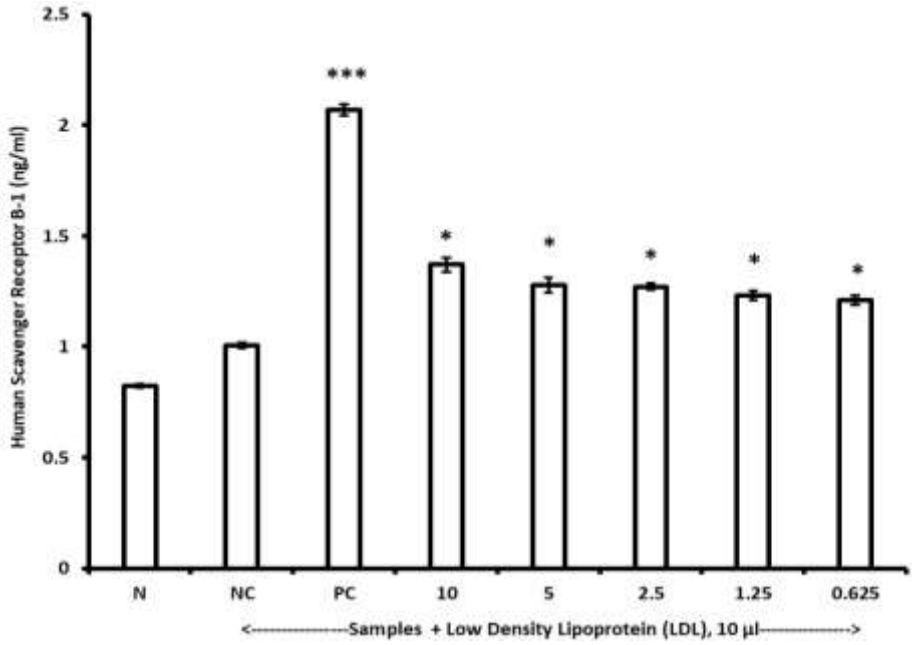

Figure 5. Graph of Human SRB-1 concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. *Significant difference compared to NC at $p<0.05$. ***Significantly different compared to NC at $p<0.0001$.

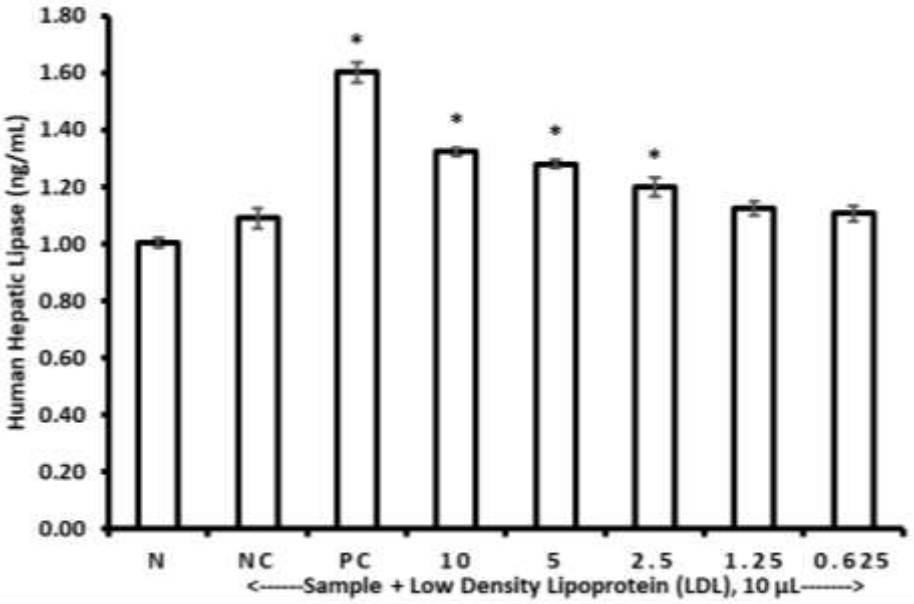

Figure 6. Graph of Human HL concentration against treatment of samples. The samples are Simvastatin as control and different concentration of *T. crispa* aqueous extract. Data expressed as mean ± SD. *Significantly different compared to NC at $p<0.05$.

samples is as shown in Figure 6. Result revealed that, supplementation of TCAE at concentration ranging from 0.625 to 10.0 mg/ml were able to promote Hep G2 cell to significantly increase ($p<0.05$) the HL production when compared with NC group. Interestingly, TCAE at 10.0, 5.0 and 2.5 mg/ml increased HL levels by which the

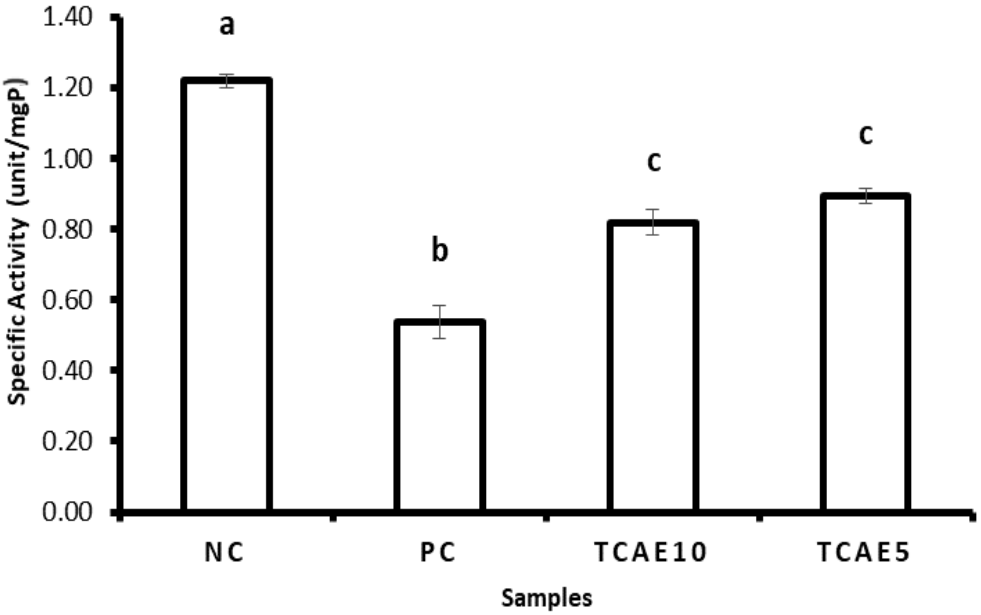

Figure 7. Graph of HMG-CoA Reductase activity against treatment of samples. The samples are Simvastatin as Positive Control (PC) and different concentration (mg/mL) of TCAE. Data expressed as mean ± SD. Data with same uppercase alphabet are not significantly different at $p<0.05$.

increment was comparable to PC group indicating a probable lipid lowering ability of TCAE in the *in vitro* system. The finding was in similar trend with *in vivo* study done by Kumar et al. (2013) who reported that the reduction of hepatic lipases in alloxan induced diabetic-dyslpidemia rats had caused hyper β-lipoproteinemia in which their reactivation by the treatment with *T. cordifolia* had played a significant role in regulation of lipoprotein metabolism back to the normal state. According to Andrés-Blasco et al. (2015), HL deficiency can trigger the increment of triglycerides and cholesterol levels in the blood resulting to the risk of developing atherosclerosis and heart disease. Therefore, high level of HL in the plasma triggered by TCAE can accelerate RCT and enhance catabolism of excess LDL from the liver.

Effects of TCAE on HMGR activity

Two-third of total cholesterol is synthesized endogenously by hepatocytes, whereas one-third of the total cholesterol is derived from diet (Kishor et al., 2007). In the liver, enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGR) plays important role in catalysing the cholesterol biosynthesis as a direct pathway in supplying cholesterol to peripheral cells (Meisel et al., 2011). Thus, it is well accepted that, one of the important approach to treat hypercholesterolemia is by inhibiting the HMGR activity as what simvastatin drug does (Farnier et al., 2012).

Result in Figure 7 showed that supplementation of TCAE at 10 and 5.0 mg/ml significantly reduced ($p<0.05$) HMGR activity in Hep G2 cells in dose dependent manner with the reduction of 32.9 and 26.7%, respectively, when compared with NC group. However, TACE ability to inhibit HMGR activity was found not comparable with that of simvastatin. This indicates that TCAE exhibited moderate inhibition of HMGR activity in Hep G2 cells which might explain the findings from the previous *in vivo* studies (Nagaraja et al., 2008; Zulkhairi et al., 2009). Hence, it is suggested that the anti-hypercholesteroleamic effects of *T. crispa* reported possibly contributed through other pathway such as RCT.

Meanwhile, the group treated with 10 µM simvastatin, showed the highest inhibition (56%) of HMGR activity when compared with NC group. This result is consistent with the study done by Bergstrom et al. (1998), who reported that atorvastatin and simvastatin caused inhibition of HMG-CoA reductase activity in Hep G2 cells. Similar findings was reported by Wilcox et al. (1999) who demonstrated that supplementation of 10 µM simvastatin decreased cellular cholesterol synthesis and CE mass in Hep G2 cell by up to 96% ($P < 0.001$) and 52% ($P < 0.001$), respectively and furthermore these inhibitors have been widely used to lower plasma cholesterol levels. Simvastatin was reported to decrease in plasma lipid levels by two different mechanisms: inhibition of HMG-CoA reductase and depression of *de novo* synthesis of PC via the cytidine diphosphate-choline pathway (Hwang et al., 2017).

Conclusions

Conclusively, the results suggest that TCAE shows strong cholesterol reducing effects demonstrated by a significant increase in molecules levels involved in reverse cholesterol transport (Apo A1, LCAT, LDL-R, SRB-1 and HL) in Hep G2 cells. The efficacy of these activities is appreciably good when compared with standard drug simvastatin. However, TCAE shows moderate effect in controlling mevalonate pathway. It could be suggested, that the pathway of TCAE action in lowering the total cholesterol possibly more on reverse cholesterol transport. However, this study was done on cell culture which has certain limitation and weakness. *In vivo* study needs to be conducted further to comprehend the anti-cholesterol effects of TCAE in the actual biological system. Besides that, it is also important to determine the chemical compounds that are involved in the up-regulation of the cholesterol metabolism.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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