Stevia: limiting cholesterol synthesis in Hep-G2 cells

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Abstract. As of today, no literature has been reported on the efficacy of stevia on lipid regulations conducted *in vitro*. Thus, the current study was focusing on the potential of *Stevia rebaudiana bertonii* as an anti-hypercholesterolemia substitute in limiting the *de novo* cholesterol synthesis in Hep-G2 cell line. The cytotoxicity and lipid internalization effects of stevia on Hep-G2 cells were assessed quantitatively and qualitatively in this study. As evaluated by MTT assay, commercialized stevia (0.5-20.0 mg/ml) and stevioside (1.0-10 µM) inhibited Hep-G2 cells viability in a dose-dependent manner for 24 hours. IC₅₀ was detected at 8.68 mg/ml (commercialized stevia) and 10.91 µM (stevioside). From the assay, suitable concentrations were chosen to study the effect of stevia on cholesterol internalization in Hep-G2 cells supplemented with exogenous lipids. Cholesterol quantification assay revealed that high concentration commercialized stevia and stevioside promoted significant cholesterol internalized in Hep-G2 cells as compared to simvastatin. Finally, immunofluorescent microscopy assessment was done to qualitatively observe the formation of lipid droplets and low-density lipoprotein receptor in relation to total cholesterol extracted. The microphotographs of immunofluorescent microscopy were in parallel to results obtained from the cholesterol quantification assay which further revealed the effect of stevia as a potential anti-hypercholesterolemia agent.

Keywords: anti-hypercholesterolemia, commercialized stevia, lipid droplets, low-density lipoprotein receptor, *Stevia rebaudiana bertonii*, stevioside

INTRODUCTION

Plant-derived medicines are considered as an alternative to currently available medicine due to their remarkable potential in terms of pharmacodynamics and pharmacokinetics activities (Lee, 2017). The applications of plant-based medicine have been documented in Egyptian medicine, traditional Chinese medicine, and Indian Ayurveda medicine (Atanasov *et al.*, 2015). Previous *in-vitro* studies have proven that plant-based medicine derived from *Fragaria ×*

ananassa (strawberry), *Alpinia galangal* (galangal), *Phyllanthus emblica* (Indian gooseberry), and *Ficus carica* (common fig) exert anti-hyperlipidemic property to alleviate both cholesterol and triglycerides serum levels (Atyabi *et al.*, 2015; Forbes-Hernández *et al.*, 2017; Tirawanchai *et al.*, 2019). Apart from that, plant-derived components provide antioxidant property capable to neutralize free radicals preventing oxidative damage in the body (Shukla *et al.*, 2012). These

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findings have given rise to vast discoveries leading to drug innovation with improved efficiency, lesser side effects to consumers and being commercialized as herbal medicine. Recently, stevia extracts have gained attention in the medical field as a result of its extensiveness in health benefits.

Stevia rebaudiana bertonii, generally known as Stevia is a small perennial herbaceous plant famously utilized as a sugar substitute due to its non-toxic and non-caloric characteristics (Kaur *et al.*, 2014; Sharma *et al.*, 2017; Saniah and Samsiah, 2012). Up to date, there are approximately 230 variations of stevia species available but only *S. rebaudiana* and *S. phlebophylla* are producing glycosides (Ullah *et al.*, 2019). Stevia is native to South America and belongs to the *Asteraceae* family grows in sandy soils (Siddique *et al.*, 2012; Shukla *et al.*, 2009). The leaves are loaded with flavonoids, phytochemicals, and terpenes that provide immunological protection to the plant against diseases (Jayaraman *et al.*, 2008). Phytochemicals of interest known as steviol glycosides that made up of stevioside, rebaudioside (A, B, C, D, E, and F), and dulcoside presented in the plant at a different amount (Salazar *et al.*, 2018). The two major bioactive components of steviol glycosides; Stevioside and rebaudioside A were found at 5-10% and 2-4%, respectively that are 200-400 times sweeter than the regular sucrose (Rizwan *et al.*, 2018; Rizzo *et al.*, 2013). Due to their potent sweetness, the extracts are being incorporated in food and beverages to replace sucrose as well as dietary supplements in several countries (Shukla *et al.*, 2009).

Stevia was also reported to play a role as anti-oxidant, renal protective, anti-tumorigenic, anti-inflammatory, reduced atherosclerosis, and promote wound healing (Shivanna *et al.*, 2013; Chen *et al.*, 2018; Greeraert *et al.*, 2010; Das, 2013). Substitution of normal sucrose with stevia is highly recommended for Type-2 diabetes as it does not induce glycemic response due to the inability of humans to metabolize ingested steviol glycosides (Ullah *et al.*, 2019; Geuns *et al.*, 2007). Prolonged consumption has shown no serious effects, which prove that stevia is non-toxic, non-carcinogenic and non-genotoxic to mammals (Koyama *et al.*, 2003). Although the benefits of stevia extracts have been discussed at length, no

reports are available on the regulation of stevia as an anti-hypercholesterolemia agent at the cellular level.

Hyperlipidemia is associated with the elevation of low-density lipoprotein and reduction in circulating high-density lipoprotein (Yang *et al.*, 2019). Hyperlipidemia is a progressive predisposing risk factor to atherosclerosis and coronary heart disease (Maejima *et al.*, 2004). Statin, a HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors are the most commonly prescribed medicine to patients experiencing hypercholesterolemia (Atyabi *et al.*, 2015). A structurally diverse group of statins are available in the market such as atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin to control plasma cholesterol and triglycerides levels (Mashayekhi *et al.*, 2011; Ramkumar *et al.*, 2016). The inhibition of plasma cholesterol levels by HMGCR is well understood.

In the present study, stevia extracts were used to observe the efficiency and efficacy in regulating cholesterol synthesis conducted *in-vitro*. Hep-G2 cells were the most suitable *in-vitro* model for the study because it exhibits a variety of cellular responses to different drugs and involves in cholesterol metabolism (Bao *et al.*, 2012; Levy *et al.*, 2007).

MATERIALS AND METHODS

Chemicals and reagents

Human hepatocellular carcinoma (HepG2) cells were purchased from American Type Culture Collection (Rockville, MD, USA). Advanced Minimum Essential Medium (AMEM), 0.25% Trypsin-EDTA (1X), Fetal Bovine Serum (FBS), Amphotericin-B (200 µg/ml), penicillin-streptomycin (10,000 units/ml and 10,000 µg/ml), chemically defined (CD) lipid concentrate, low-density lipoprotein (LDL) from human plasma, phosphate-buffered saline (PBS), and Hoechst 33342 were purchased from Gibco, USA. Pierce Bicinchoninic Acid (BCA) protein assay kit was acquired from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), HPLC grade Simvastatin (≥97%) and Stevioside hydrate (≥98%), paraformaldehyde powder (95%),

Thiazol Blue Tetrazolium Bromide (98%), FluorSave Reagent, and Triton X-100 were obtained from Sigma Aldrich, USA. Commercialized stevia extract in powdered form with 70% of total glycoside was obtained from Hangzhou Wanray Import & Export CO., LTD. (China). HPLC grade chloroform, methanol, and hydrochloric acid were purchased from RCI Labscan, Australia. Primary antibody (Rb pAb to LDL Receptor, ab175883), secondary antibody (donkey anti-rabbit IgG, Alexa Fluor 594), and Bodipy 493/503 were purchased from Abcam, UK. Cholesterol Liquicolor was from Human Diagnostic, USA. Bovine serum albumin (BSA) was acquired from Amresco, USA.

Cell culture

Hep-G2 cell line was thawed and maintained in a 75 cm² culture flask with 90% AMEM, supplemented with 10% FBS, 1% penicillin-streptomycin and 1% Amphotericin-B in a humidified incubator at 37°C in 5% CO₂. The media was changed every 3 days and passaged every 7 days. Cells were subjected to treatment when the confluency has reached 80-85%.

Stock preparation of commercialized stevia, stevioside and simvastatin

Commercialized stevia was prepared as a stock solution (100 mg/ml) in AMEM and vortexed to dissolve the powder completely. The stock solution was syringe filtered using a 0.22 µm sterile membrane filter to remove contaminants. Stevioside was dissolved in a sterile DMSO as a stock solution (1 mg/ml) and aliquoted into glass vials. Simvastatin was activated in ethanol (100%) followed by 0.1 M sodium hydroxide. Subsequently, the solution was incubated at 50°C for 2 hours on a digital dry bath heater and the pH was adjusted to 7.0 with hydrochloric acid.

Cytotoxicity determination of commercialized stevia and stevioside on Hep-G2 cells

Cells were propagated in a 96-well microplate at a density of 1x10⁴ cells per well and allowed to grow overnight. Hep-G2 cells were supplemented with commercialized stevia (0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 mg/ml) and stevioside (1.0, 2.0, 2.5, 5.0 and 10.0 µM) for 24 hours. The following day, supplemented media was removed and cells were

further incubated with MTT reagent (5 mg/ml) for 3 hours at 37°C in 5% CO₂. DMSO was added into each well and the plate was placed on an orbital shaker for 15 minutes to dissolve the formazan crystals formed. Absorbance was measured at 570 nm using a spectrophotometer (Infinite® M200 Pro Tecan, Switzerland). The mean absorbance of commercialized stevia and stevioside treated cells was compared to the mean absorbance of untreated cells (AMEM only). The half-maximal inhibitory concentration (IC₅₀) was determined as follows:

$$(IC_{50}) = \frac{\text{Average } A_{570nm} \text{ Treated Group}}{\text{Average } A_{570nm} \text{ Control Group}} \times 100\%$$

From the calculation, concentration exhibiting 30% less cytotoxic effects (<70% viability) towards the cells were used in cholesterol quantification assay and immunofluorescence microscopy to further validate the effectiveness of commercialized stevia and stevioside on the internalization of cholesterol in Hep-G2 cells.

Quantification of cholesterol mobilized into HepG2 Cells

To quantify the amount of cholesterol uptake by Hep-G2 cells supplemented with commercialized stevia and stevioside, lipid extraction according to Bligh and Dyer method was adapted (Bligh and Dyer, 1959). Hep-G2 cells at passage 20 were seeded in a 6-wells culture plate with 0.3x10⁶ cells suspended in 2 ml media per well and incubated overnight in 5% CO₂ at 37°C.

The complete growth media was discarded and replaced with supplemented media prepared accordingly: a) **baseline control**; AMEM and FBS, b) **negative control**; AMEM, FBS, and CD lipid concentrate c) **positive control**; AMEM, FBS, CD lipid concentrate, and 4 µM simvastatin, d) **low-concentration commercialized stevia**; AMEM, FBS, CD lipid concentrate, and 0.5 mg/ml commercialized stevia, e) **high-concentration commercialized stevia**; AMEM, FBS, CD lipid concentrate, and 5.0 mg/ml commercialized stevia, f) **low-concentration stevioside**; AMEM, FBS, CD lipid concentrate, and 1.0 µM stevioside, and g) **high-concentration stevioside**; AMEM, FBS, CD lipid concentrate, and 5.0 µM stevioside.

After incubation for 24 hours, Hep-G2 cells were washed twice with sterile, cold PBS to inhibit any further metabolism in cells. The cells were then mechanically detached using a cell scraper, rinsed with 1ml sterile PBS and collected in a 5ml centrifuge tube. Hep-G2 cells were homogenized using Sonic Dismembrator (Thermo Fisher Scientific, USA) for 10 seconds at 20% amplitude on ice. Then, 800 µl of homogenized cells were transferred to a glass tube and subjected to lipid extraction. Cholesterol quantification was determined by cholesterol assay kit according to the manufacturer's instruction and absorbance was taken at 540 nm wavelength using a spectrophotometer. The absorbance was calculated as follow:

$$\text{Cholesterol concentration (mmol/L)} = 5.17 \times \left[\frac{(\text{Average Treated Group } A_{540\text{nm}} / \text{Average Blank } A_{540\text{nm}})}{[(\text{Average Standard } A_{540\text{nm}} / \text{Average Blank } A_{540\text{nm}})]} \right]$$

Assessment of lipid droplets (LDs) and Low-density lipoprotein receptor (LDL-r) in Hep-G2 cells

For immunofluorescence microscopy, cells were cultured on a round-glass coverslip placed in 4-wells cell culture plate at 1×10^3 cells in 2 ml of complete growth media per well. Cells were allowed to grow overnight in 5% CO₂ at 37°C. The following day, cells were supplemented in the same manner as in lipid quantification and incubated for another 24 hours.

After incubation, Hep-G2 cells were fixed with 4% paraformaldehyde in PBS and rinsed three times using cold PBS. Cells were blocked and permeabilized with 2% BSA and 0.2% Triton X-100 prepared in PBS. Subsequently, cells were rinsed three times with PBS for five minutes in each washing and incubated with primary antibody (1:50) for 1 hour. Hep-G2 cells were further incubated with secondary antibody (1:200) and counterstained with Bodipy 493/503 (1:50) for lipid droplets and Hoechst 33342 (1:50) for nucleus. Coverslips were mounted on a glass slide using Fluorsave and observed using an inverted fluorescence microscope (Nikon Eclipse Ti-S, Japan).

Data analysis

To determine the IC₅₀ of commercialized stevia and stevioside, mean absorbance values were used to plot the cytotoxicity curve. For the cholesterol quantification assay, One-way ANOVA was used for data analysis by comparing groups against positive control (PC). P-value <0.05 is considered as significant differences. The values were reported as the mean and standard deviation. Data analysis was performed using the GraphPad Prism version 7 for Windows, GraphPad Prism (USA).

RESULTS

Cytotoxic effect of commercialized stevia and stevioside on Hep-G2 Cells

The assay yielded various results between controlled cells against cells supplemented with either commercialized stevia or stevioside. In Figure 1 (A-B), both commercialized stevia and stevioside markedly inhibited the proliferation rate of Hep-G2 cells in a dose-dependent manner. The IC₅₀ of commercialized stevia on Hep-G2 cells was observed at 8.68mg/ml and stevioside at 10.91 µM. A suitable range of concentration was established from the assay: commercialized stevia (0.5 mg/ml and 5.0mg/ml) and stevioside (1.0 µM and 5.0 µM) as more than 70% of cells were alive at these concentrations. These concentrations were further supplemented to Hep-G2 cells in cholesterol assay quantification and immunofluorescence microscopy evaluation.

Internalization of cholesterol in Hep-G2 cells

Cholesterol quantification assay was performed by supplementing Hep-G2 cells with commercialized stevia (0.5 mg/ml and 5.0 mg/ml) and stevioside (1.0 µM, and 5.0 µM) determined from the previous MT_T assay. Figure 2 shows the cholesterol concentration of controls (**BC**, **NC**, and **PC**) and supplemented cells with 0.5 mg/ml commercialized stevia (**low CS**), 5.0 mg/ml commercialized stevia (**high CS**), 1.0 µM stevioside (**low STV**), and 5.0 µM stevioside (**high STV**) were normalized with protein concentration quantified by the BCA protein determination assay earlier. Data were compared to positive control (PC). In general, BC showed

the least amount of total cholesterol stored in cells followed by NC, Low CS, PC, Low STV, High CS, and High STV. Cells supplemented with high CS (5.0 mg/ml) and high STV (5.0 μ M) promotes cholesterol mobilization with significant results as compared to PC.

Immunofluorescence (IF) imaging

Immunofluorescence signals for nuclei, lipid droplets (LDs), and low-density lipoprotein receptor (LDLr) were obtained by fluorescence microscopy. These signals were detected accordingly: nuclei (blue), LDs (green), and LDLr

(red). In Figure 3, presence of LDs (green) and LDLr (red) were expressed at different degrees between samples. Generally, the exact visualization of these structures remains elusive in regard to their distribution. However, the expression of these signals qualitatively in parallel with the total cholesterol quantified from the assay conducted earlier. In general, cells supplemented with high concentration of stevioside (5.0 μ M) showed a significant fluorescent intensity of LDs and LDLr as compared to all controls.

Figure 1. Viability profiles of Hep-G2 cells supplemented with **(A)** commercialized stevia and **(B)** stevioside followed by 24 hours incubation at different concentrations. The viability of the control was 100% viable cells (n=6).

Figure 2. Total cholesterol concentration (mmol/g) of BC (21.45±0.46), NC (22.86±0.79), PC (30.76±2.95), Low CS (27.99±1.33), High CS (37.98±1.53), Low STV (31.79±2.14), and High STV (40.07±0.19). (n=3).

Figure 3. Presence of nuclei, lipid droplets (LDs), and low-density lipoprotein (LDL-r) on Hep-G2 cells post 24 hours incubation with (A) baseline control, (B) negative control, (C) positive control, (D) commercialized stevia 0.5 mg/ml, (E) commercialized stevia 5.0 mg/ml, (F) stevioside 1.0µM, and (G) stevioside 5.0 µM were observed at 20X magnification using an inverted immunofluorescence microscopy (scale bar: 20 µm). Nuclei, LDs, and LDL-r were detected by blue (Hoechst 33342), green (BODIPY 493/503) and red (Alexa Fluor 594) fluoresced structures, accordingly.

DISCUSSION

Globally, cardiovascular disease (CVD) contributes up to 17 million mortality cases per year caused by the alteration of normal lipoprotein metabolism (Blesso *et al.*, 2018;

Forbes-Hernández *et al.*, 2017). The altered lipoprotein metabolism involving low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides (TG) is termed as dyslipidemia (Kolovou *et al.*, 2005). Elevation of plasma cholesterol specifically LDL coupled with a decrease in HDL are the predisposing factors to

atherosclerosis and other cardiovascular-related diseases (Yang *et al.*, 2019). Atherosclerosis is a pathological condition whereby the normal structure of the arterial intima becomes asymmetrically narrowed as a result of atherosclerotic plaques formation (Wang *et al.*, 2017). The condition could affect either large or medium-sized arteries and the clinical signs are undetected until thrombosis occurred, which leads to other complications (Abdolmaleki *et al.*, 2019). Precursor attributed to atherosclerosis is the deterioration of the endothelial lining and oxidization of progressively accumulated LDL particles along the arterial walls (Moriya, 2019).

Currently, statin therapy is effective in lowering LDL-cholesterol (20-50%), as well as triglyceride (10-20%) and increasing serum HDL-cholesterol levels (5-10%) (Ramkumar *et al.*, 2016). Pharmacodynamic of statin involves the inhibition of *de novo* cholesterol synthesis by competitively blocking HMG-CoA reductase. This rate-limiting enzyme catalyzes the conversion of HMG-CoA to mevalonate, the initial step in cholesterol production (Tirawanchai *et al.*, 2019). Apart from suppressing hepatic cholesterol synthesis, statins aid in the up-regulation of hepatocellular LDL-r resulting in scaling down the LDL-cholesterol level (Funatsu *et al.*, 2001).

Although it is well known that statins are competent in alleviating atherogenic lipoprotein plasma level (20-50%) and triglycerides (10-20%), potential adverse effects are unavoidable (Mullen *et al.*, 2010; Ramkumar *et al.*, 2016). Documented reports on patients experiencing dose-dependent adverse effects of statins such as myositis, myalgia, persistent muscle effects, rhabdomyolysis, headache, liver failure, and gastrointestinal related problems (Golomb *et al.*, 2008; Mashayekhi *et al.*, 2011). Together with adverse effects varying from rare to life-threatening, the cost-effectiveness on types of statins is also a concern (Morrison and Glassberg, 2003).

This study focuses on the effects of stevia as a cholesterol-lowering agent on Hep-G2 cell line supplemented with exogenous lipids. To be developed as an alternative medicine, determination on the half-maximal inhibitory concentration (IC_{50}) is important as it provides valuable information pertaining to the cytotoxic

effects of a supplement on the targeted cell. These effects range from the destruction of the structural integrity of cells to altering their metabolic activities (Aslantürk, 2017). To achieve this objective, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was conducted. MTT is a reliable, reproducible, and economical method used to determine the viability of cells concerning their metabolic activities (Sharma *et al.*, 2019). Hep-G2 cells were supplemented with different concentrations of commercialized stevia and stevioside for 24 hours to assess the immediate response of cells (acute toxicity).

As shown in Figure 1 (A), the IC_{50} of commercialized stevia was determined at 8.68 mg/ml. From the results, supplements that inhibit cell viability less than 30% were chosen further studies. Previous study has reported a significant inhibition of Hep-G2 cell growth observed when the concentration of extracted stevia is at 12.5 mg/ml (Jayaraman *et al.*, 2008). The slight difference of IC_{50} could be due to the presence of steviol glycosides percentages in the commercialized product and the solvent used. According to the guidelines by FDA and EFSA, stevia extracts must contain at least 95% of steviol glycosides (Rizwan *et al.*, 2018). In this study, the content of steviol glycosides in commercialized stevia was 70% while the remaining was suspected to be adulterant which could be a reason to low IC_{50} determined from the current study. Such adulterant was not disclosed by the supplier or the material safety data sheet.

As for stevioside, a significant cytotoxic effect was determined at 10.91 μ M on Hep-G2 cells. Our current findings is in agreement with the IC_{50} of stevioside supplemented to MCF-7 cell line was found to be in a range between 10-20 μ M (Paul *et al.*, 2012). Observation of 24 hours supplementation of stevioside (5 μ M) on HT-29 colon cancer cells significantly inhibited the viability of cells (Ren *et al.*, 2017). However, a higher concentration of stevioside (>200 μ M) is required to induce cytotoxicity in HCT 116 cell and CCD18 Co cells (Sharif *et al.*, 2017). Thus, we can conclude that the resistance of cell lines towards a supplement is different due to several factors. These factors are: 1) the molecular size of the tested compound, 2) genetic variation and composition of the targeted cell, 3) alteration of

the cells internal environment, and 4) the mechanisms of the targeted cells (Sharif *et al.*, 2017). From our findings, 1.0 μM and 5.0 μM of stevioside were used in further experiments as more than 70% of cells were viable at these concentrations.

Aforementioned, commercialized stevia (0.5 mg/ml and 5.0 mg/ml) and stevioside (1.0 μM and 5.0 μM) were supplemented to cells in cholesterol quantification assay. Simvastatin, a HMG-CoA reductase inhibitors served as a positive control in this study was supplemented to cells at 10 μM due to its IC_{50} on Hep-G2 cells (Janine *et al.*, 2012). Inhibition of *de novo* cholesterol synthesis in sample-treated simvastatin is well understood. We hypothesized that a similar effect has taken place in Hep-G2 cells supplemented with commercialized stevia and stevioside. Total cellular cholesterol concentrations were measured to determine the effectiveness of these two supplements in inhibiting the pathway of interest.

Apart from the high proliferation rate (Maria *et al.*, 2015), Hep-G2 cell line was used in this study as majority of cholesterol metabolism takes place in the liver in particularly the hepatocytes. Cholesterol quantification assay revealed that cells supplemented with stevioside (5 μM) treated cells has the highest cholesterol concentration (mmol/g) followed by commercialized stevia (5 mg/ml). It is important to emphasize that no study has been conducted to evaluate the efficacy of stevia as a hypolipidemic agent in *in-vitro*. Most findings were reported on the *in-vivo* model with similar results as in the current study in which stevia helps to improve the serum lipid level. The lipid profile in stevioside-supplemented rats was improved as the total cholesterol, triglyceride, LDL-C, and VLDL lowered with an increment of HDL-C level (Elnaga *et al.*, 2016). To further support the findings, evaluation of the oral intake of stevia extracts caused a reduction of serum cholesterol (240.5 mg/dl to 229.55 mg/dl) in humans (Sharma *et al.*, 2017).

Among the control, simvastatin at 10 μM (PC) was proven to be effective in lipid internalization into cells. Surprisingly, cholesterol assay on BC sample yielded 21.45 mmol/g of cholesterol despite the absence of exogenous lipids/cholesterol in media. However, this could be explained as total cholesterol extracted from

BC was contributed by albumin-bound cholesterol found in the FBS (Okonkowski *et al.*, 2007). To further confirm the results from cholesterol quantification assay, Hep-G2 cells supplemented in the same manner were subjected for immunofluorescence microscopy.

Figure 4. Proposed pathway of stevia in the inhibition of the mevalonate pathway. Stevia has potential anti-hypercholesterolemia property as it inhibits the 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGCR). 1) LDL binds to LDL-r located on the cell surface. 2) LDL and LDL-r are then internalized into the hepatocyte in the clathrin-coated vesicle. 3&4) LDL-r and clathrin-coated vesicles are dissociated from the LDL and recycled back to the cell surface by endosome. 5) Lysosome degraded cholesteryl ester (CE) and triglycerides (TG) to amino acid and cholesterol. 5) Stevia prevents the conversion of HMG-CoA to mevalonate and lower the endogenous cholesterol synthesis by 6) promoting the storage of cholesterol in the form of lipid droplets (LDs).

Qualitative observation on IF microphotographs off supplemented cells revealed that high concentration of commercialized stevia and stevioside regulates the formation of LDLr (red fluorescent) as well as LDs (green fluorescent). LDs are organelles involved in excessive neutral lipid (lipid ester core surrounded by phospholipid monolayer) storage of cells (Guo *et al.*, 2009; Suzuki *et al.*, 2011). Meanwhile, LDLr is a receptor for low-density lipoprotein (LDL) localized on cell surface. Briefly, we hypothesized that these supplementations up-regulate the 3-hydroxy-

3methylglutarate-CoA reductase (HMGCR) at the molecular level. The up-regulation of HMGCR will eventually inhibit the mevalonate pathway in the *de novo* cholesterol synthesis. The inhibition of this enzyme is an effective indicator of lowering circulating cholesterol as it is the main target of commonly used in lipid-lowering medication. Figure 4 depicted the proposed pathway of stevia in regulating cholesterol *de novo* synthesis.

CONCLUSION

In conclusion, it is well known that statins are currently prescribed as anti-hypercholesterolemia drugs as they are highly effective in regulating blood cholesterol levels. However, considering the multiple rare to life-threatening adverse effects, alternative way to replace statins is important. The present study revealed that stevia has the potential to be applied as a cholesterol-lowering drug. However, additional studies are required to further verify how stevia extracts exert anti-hypercholesterolemia property at the cellular and molecular level.

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