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Rebaudioside A Enhances LDL Cholesterol Uptake in HepG2 Cells via Suppression of *HMGCR* Expression

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Abstract

Background: Rebaudioside A is one of the major diterpene glycosides found in Stevia had been reported to possess anti-hyperlipidemic effects. In this study, we explore the potential cholesterol-regulating mechanisms of Rebaudioside A in the human hepatoma (HepG2) cell line in comparison with simvastatin.
Methods: Cells were incubated with Rebaudioside A at several concentrations (0-10 µM) to determine the cytotoxicity by the MTT assay. Cells were treated with selected dosage (1 and 5 µM) in further experiments. Total cellular lipid was extracted by Bligh and Dyer method and subjected to quantitative colorimetric assay. To illustrate the effect of Rebaudioside A on cellular lipid droplets and low-density lipoprotein receptors, treated cells were subjected to immunofluorescence microscopy. Finally, we investigated the expression of experimental gene patterns of cells in response to treatment.
Results: In this study, cytotoxicity of Rebaudioside A was determined at 27.72 µM. Treatment of cells with a higher concentration of Rebaudioside A promotes better hepatocellular cholesterol internalization and ameliorates cholesterol-regulating genes such as *HMGCR*, *LDLR*, and *ACAT2*.
Conclusions: In conclusion, our data demonstrated that Rebaudioside A is capable to regulate cholesterol levels in HepG2 cells. Hence, we proposed that Rebaudioside A offers a potential alternative to statins for atherosclerosis therapy.

Keywords: Rebaudioside A, Anti-hypercholesterolemia, Lipid droplets, Low-density lipoprotein, *HMGCR*.

Introduction

An evidence-based phytotherapy pathway that relies on scientific and clinical data is a crucial approach before the establishment of plant-based derivatives in medical practice (1). *Stevia rebaudiana Berto*ni is an intense, non-nutritive natural sweetener that has been a popular sugar substitute used in various industries as well as phytotherapy (2). Following the first domestication in Japan, large-scale cultivation now exists in other countries to fulfil the economical demands (3). Phytochemical of Stevia leaves revealed the presence of steviol glycosides, and other important secondary metabolites each with various therapeutic potentials (3, 4). To date, there are 11 major steviol glycosides detected in the plant (5).

Rebaudioside A accounted for up to 6.5% of the total dry weight of the leaf is of interest due to its potent sweetness estimated to be 400 times sweeter than sucrose (6). There exist quite a lot of discussion as to therapeutic applications of Rebaudioside A (7-10). Recent investigations

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demonstrated the potential role of Rebaudioside A as a hepatoprotective agent (11, 12). More importantly, it has been shown that an improvement in the lipid profile of experimentally induced diabetic rats can be achieved by oral administration of this diterpenoid glycoside (13) in which further support the notion that Rebaudioside A possesses potential lipid-regulating activity.

Atherosclerosis is a chronic inflammatory disease affecting the intima of medium and large-sized arteries, likely to occur at arterial bifurcations (14, 15). The disease has been recognized as the principal cause of clinically important cardiovascular diseases (CVDs) (16). Pioneering work on atherosclerosis has been carried out earlier by Alexander Iosifovich Ignatowski in 1907 in which he provided evidence of aortic atherosclerotic lesion in experimental rabbits fed with various types of animal proteins (17). Conceptually similar work has also been carried out by Anichkov and Semen Chalatov in 1912 that extends the idea of cholesterol in atherosclerosis development (17). In addition to non-pharmacological approaches, few medications have been developed to intervene with abnormal plasma cholesterol levels (18). A class of cholesterol-modifying medications known as statins is commonly prescribed to hyperlipidemic patients (18). Likewise, considering that reactive oxygen species (ROS) and oxidative stress initiate atherosclerosis which cause coronary heart disease, antioxidants are beneficial to prevent such events (19). Despite the efficiency of statins in lowering cholesterol, many side effects have been discussed (20-22) thus further motivates the need for an alternative to replacing statins. Therefore, in this study, we are interested in examining the unexplored cholesterol-lowering potential of Rebaudioside A in the HepG2 cell line.

Materials and Methods

Cell culture

HepG2 cells (ATCC number: ATCC® HB-8065™) were grown in 90% advanced minimum essential media (AMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% amphotericin-B. All cell culture reagents were from Gibco (USA). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Preparation of Rebaudioside A and Simvastatin

HPLC grade of Rebaudioside A (≥ 96%) and Simvastatin (≥ 97%) were from Sigma Aldrich (USA). Rebaudioside A (RA) was prepared as a stock solution in a sterile dimethyl sulfoxide with a final concentration of 1mM. Simvastatin was activated as mentioned by (23). Both solutions were stored at 4 °C until further analysis.

Cell viability assay

Cells ($1x10^4$ cells per well) were incubated in 96 well-plates for 24 hours. The following day, the culture medium was removed and cells were further incubated with RA (0-10 µM) overnight. Upon 24 hours, the cytotoxic effect was determined by the MTT assay (24). The half-maximal inhibitory concentration (IC_{50}) was determined by graphically plotting the value of cell viability (%) accordingly:

Cell viability (%) = (Mean Treated $OD_{(A570)}$-Mean Background $OD_{(A570)}$) / (Mean Control $OD_{(A570)}$-Mean Background $OD_{(A570)}$) x 100

Cell treatment

For cholesterol quantification and cDNA expression analysis cells ($3x10^5$ cells per well) were seeded in 6 well-plates and for immunofluorescence microscopy ($1x10^3$ cells per glass coverslip) in 4 well-plates. Cells were treated for 24 hours accordingly: 1) Base control (BC) AMEM and FBS only; 2) Negative control (NC) AMEM, FBS, CD lipid concentrate, 50X cholesterol lipid concentrate, and human plasma LDL; 3) Positive control (PC) AMEM, FBS, CD lipid concentrate, 50X cholesterol lipid concentrate, and human plasma LDL with 10 µM simvastatin; 4) Low dosage RA (LRA) AMEM, FBS, CD lipid concentrate, 50X cholesterol lipid concentrate, and human plasma LDL with 1.0 µM RA; and 5) High dosage RA (HRA) AMEM, FBS, CD lipid concentrate, 50X cholesterol lipid concentrate, and human plasma LDL with 5.0 µM RA. For cholesterol quantification and

cDNA analysis, the treatment medium was removed, cells were washed with PBS, scraped off, and collected into a 1.5ml tube. For immunofluorescence imaging, cells were stained, mounted on coverslips, and viewed.

Cholesterol quantification and protein determination

Before analysis, cells were homogenized for 2 minutes at 20% amplitude on ice using an ultrasonic dismembrator (Thermo Fisher Scientific, USA) followed by lipid extraction according to Bligh and Dyer method (25). Total cellular cholesterol (TCC) was quantified by Cholesterol Liquicolor Kit (Human Diagnostic, USA) according to the manufacturer's instruction at 540nm wavelength using a spectrophotometer. TCC was calculated as follow:

Cholesterol concentration (mmol/L)= 5.17 x [Mean Treated $OD_{(A540)}$/ Average Blank $OD_{(A540)}$] / [Average Standard $OD_{(A540)}$/ Average Blank $OD_{(A540)}$]

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Data acquired from protein determination was used to normalize data of cholesterol quantification analysis.

Immunofluorescence microscopy

Qualitative expression of the nucleus (N), lipid droplets (LDs), and low-density lipoprotein receptors (LDLR) were observed by immunofluorescence microscopy. Cells were fixed with 4% paraformaldehyde (Sigma Aldrich, USA) in PBS for 60 minutes at room temperature. The fixative agent was discarded and cells were washed with PBS three times, each for 5 minutes. Afterward, cells were submerged in a concoction of 2% BSA (Amresco, USA) to block non-specific binding and 0.2% Triton X-100 (Sigma Aldrich) to aid cellular permeabilization. Then, cells were incubated with primary antibody against LDLR (1:50, ab175883, Abcam, UK) for 1 hour. Counterstaining was carried out using fluorescently tagged Alexa Fluor® 594 Donkey Anti-Rabbit IgG [H+L] secondary antibody (1:200; Thermo Fisher Scientific, USA), Bodipy 493/503 (1:50; Abcam, USA) and Hoechst 33342 (1:50; Gibco, USA). Finally, the coverslip was mounted onto a glass slide with FluorSave™ Reagent (Sigma Aldrich, USA) and viewed at 200X magnification using an inverted fluorescence microscope (Nikon Eclipse Ti-S, Japan).

RNA isolation and optimization of primers

RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) as mentioned in the manual. The purity and quantity of total cellular RNA were assessed spectrophotometrically using a NanoQuant Plate™ (Tecan, Switzerland). Optimization of experimental primers (IDT, Singapore) in Table 1 with standardized total RNA (100 ng/µl) was carried out by conventional reverse transcriptase-polymerase chain reaction (RT-PCR) using the Access RT-PCR System master mix (Promega, USA) in the Thermoblock 96 thermocycler (Sensoquest, Germany). The resulting PCR products were separated by agarose gel electrophoresis (2% agarose, w/v; at 80V for 40 minutes) containing SYBR® Safe DNA gel stain (Thermo Fisher Scientific, USA) and visualized using the Gel DOC XR+ System Image Analyser (Bio-Rad, USA).

Table 1. The sequences and accession number of experimental primers used for conventional RT-PCR and 2-step RTqPCR.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number
GAPDH	GTCTTCACCACCATGGAGAAG	GCTTCACCACCTTCTTGATGTCATC	NM_0013579432
HMGCR	TTACTCCTTGGTGATGGGAGCTTG	TCCTGTCCACAGGCAATGTAGATG	NM_000859.3
LDLR	CAATGTCTCACCAAGCTCTG	TCTGTCTCGAGGGGTAGCTG	NM_001195799.2
ACAT2	ACACCTCGATCTTGGTCCTGCCATA	GGAATGCAGACAGGGAGTCCT	NM_003578.4

Gene expression analysis by 2-step real-time quantitative PCR (2-step RTPCR)

The expression of experimental genes was analyzed in Applied Biosystem™ StepOnPlus™ qPCR System (Thermo Fisher Scientific, USA) using the GoScript™ Reverse Transcription System (Promega, USA) and GoTaq® qPCR Master Mix (Promega, USA) according to the manufacturer's instructions. In brief, the cycling conditions were 95 °C for 2 minutes followed by 40 cycles of denaturation (95 °C, 3 seconds), annealing (*GAPDH*: 52.0 °C, *HMGCR*: 58.7 °C, *LDLR*: 64.0 °C, and *ACAT2*: 57.7 °C, 15 seconds) and extension (60 °C, 1 minute). *GAPDH* was used as the internal control, and the expression of experimental genes was calculated according to the ΔΔCT method. Data were presented as fold-change to the control.

Statistical analyses

Data were analyzed by One-Way ANOVA test using the GraphPad Prism version 7 for Windows, GraphPad Prism (USA). Results were presented as mean ± standard deviation and *P*-value< 0.05 was considered as statistically significant.

Results

Cell viability assay

HepG2 cells were treated with RA (0-10 µM) for 24 hours and the results were depicted in Figure 1. The trend of the results illustrated that cell viability decreases dose-dependently with RA incubation for 24 hours. Notably, the IC_{50} of RA was found to be at 27.72 µM. In line with the findings from this assay, 1 µM and 5 µM of RA were used to treat cells in the following experiments.

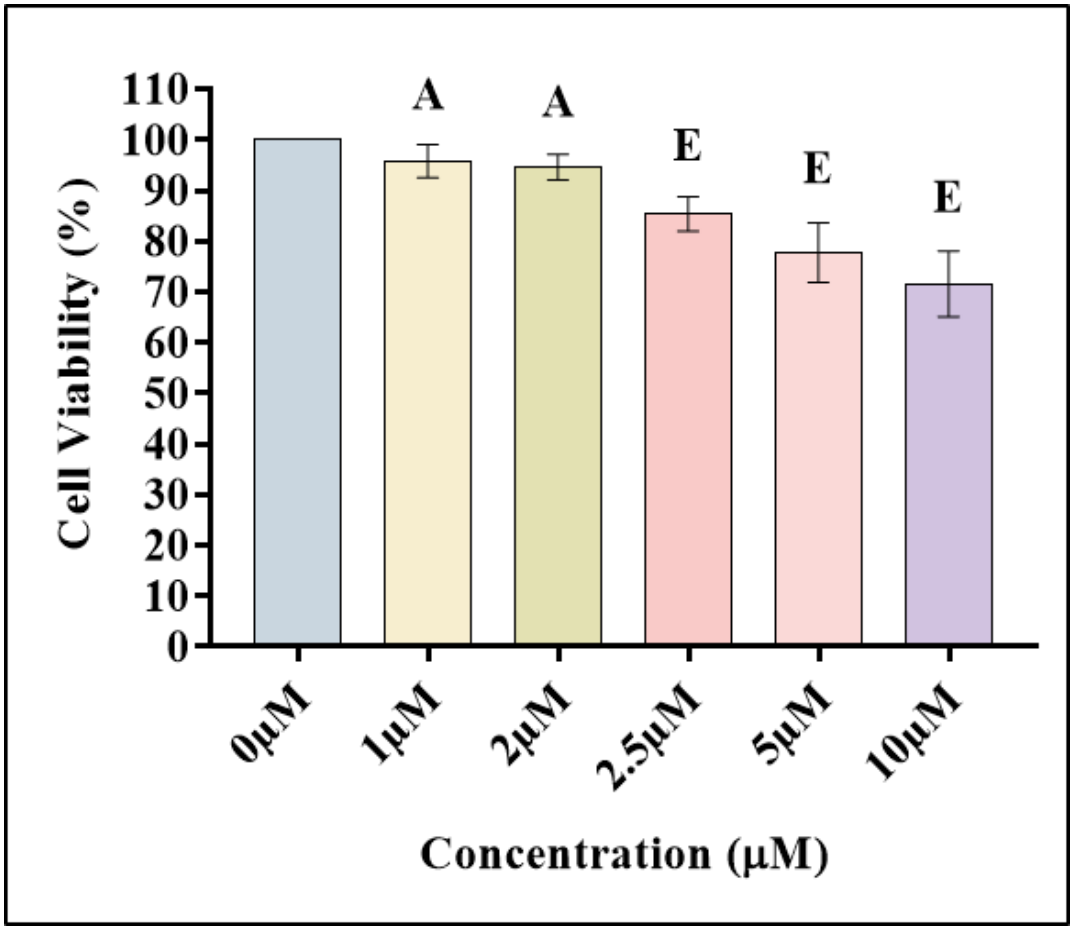

Fig. 1. Cytotoxic of Rebaudioside A on HepG2 cells for 24 hours at 1 µM (95.8±3.271), 2 µM (94.6±2.51), 2.5 µM (85.4±3.362), 5 µM (77.8±5.891) and 10 µM (71.6±6.427) against control group (0 µM). Graph followed by different upper case letter (A-E) represents a significant difference (p< 0.05) treatment wise (n= 5).

Cholesterol quantification and protein determination

Data illustrated in Figure 2 indicated the extracted total cellular cholesterol (TCC) level in different groups of cells was considerably affected by treatments. According to the results, the highest value for TCC was recorded in HRA following PC> LRA> NC, while the lowest was observed in BC. Surprisingly, treatment of cells with LRA has little to no effects on TCC as the result is almost similar to NC.

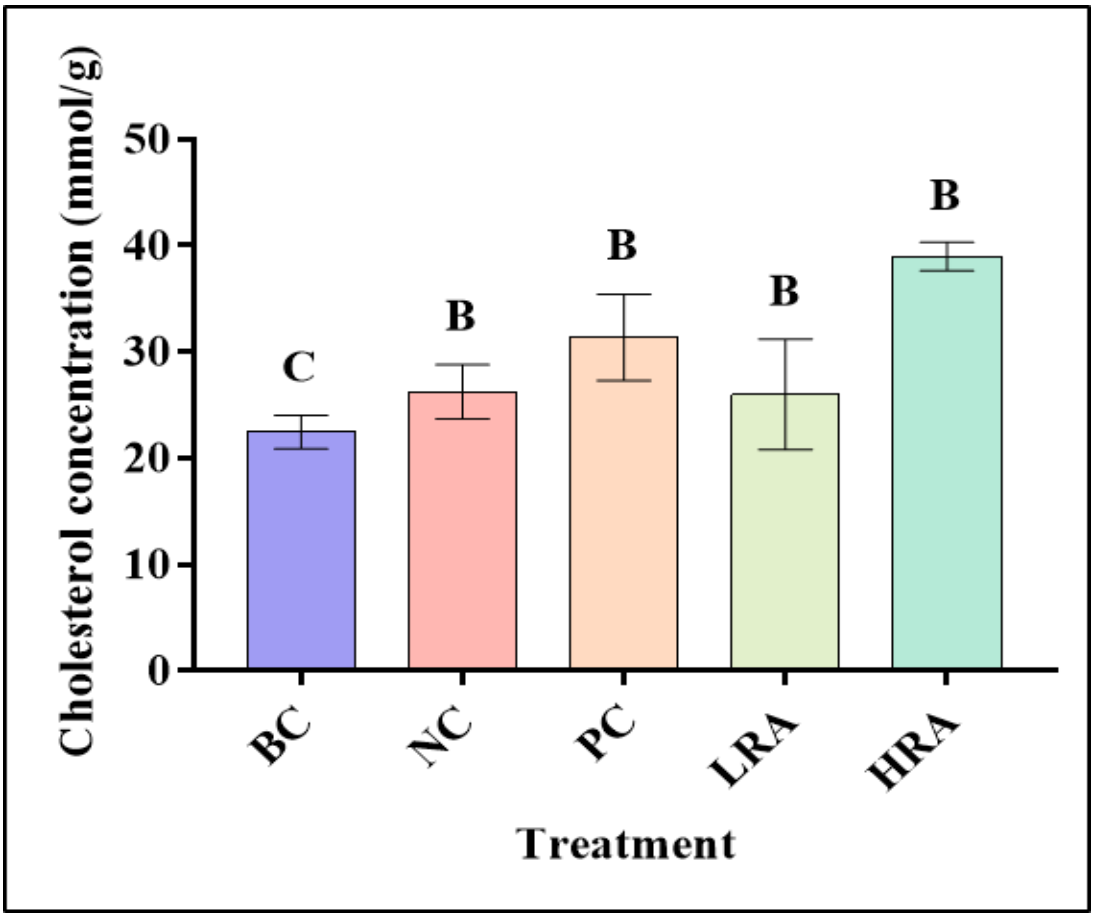

Fig. 2. TCC extracted from treated cells for 24 hours, BC (22.49±1.581), NC (26.2±2.555), PC (31.38±4.067), LRA (25.99±5.177) and HRA (38.98±1.318). Graph followed by different upper-case letter (A-E) represents a significant difference ($p< 0.05$) treatment wise (n= 5).

Immunofluorescence microscopy

To determine the expression of LDLR and LDs, a qualitative assessment by fluorescent staining was employed. The microphotographs depicted in Figure 3 suggest that HRA promoted the up-regulation of LDLR expression on cells' surface causing higher intracellular lipid mobilization and further reserved into LDs. The expression of LDLR was qualitatively prominent in HRA> LRA> PC but absent in both NC and BC. However, the presence or absence of LDLR does not affect LDs formation observed in all groups. It is important to highlight that no strong conclusions could be drawn based on the qualitative assessment of immunofluorescence staining although it reflects the results of the cholesterol quantification assay. Therefore, to ensure the molecular mechanism of RA-inducing cellular uptake of cholesterol is properly understood, gene expression analysis was conducted.

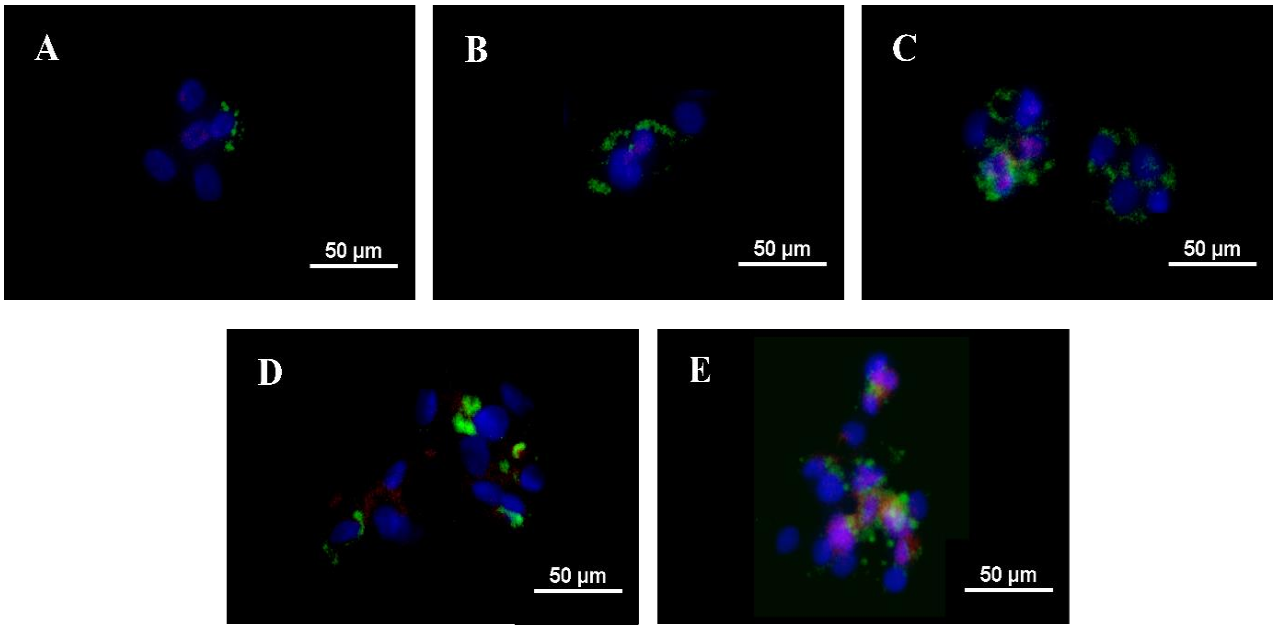

Fig. 3. Qualitative assessment of immunofluorescence microphotographs on treated cells for 24 hours (A; BC, B; NC, C; PC, D; LRA and E; HRA). Fluorescence staining was as follows: blue (nucleus), green (lipid droplets), and red (low-density lipoprotein receptor) with magnification at 200X (n= 4).

Gene expression

The purity of extracted RNA samples was in a range of 2.0 to 2.1 at an absorbance ratio of 260-280 nm wavelengths. The annealing temperature of each experimental and internal control gene was determined by conventional RT-PCR followed and qualitative assessment of bands via agarose gel electrophoresis (*GAPDH*: 490 bp, *HMGCR*: 747 bp, *LDLR*: 258 bp, and *ACAT2*: 117 bp). The optimized annealing temperatures were adapted to cycling conditions for gene expression analysis by 2-step RT-PCR. The expression of experimental genes was illustrated as expression-fold in Figure 4. It was found that HRA significantly down-regulated *HMGCR* expression and at the same time up-regulating *LDLR* and *ACAT2* genes as compared to other treatment groups.

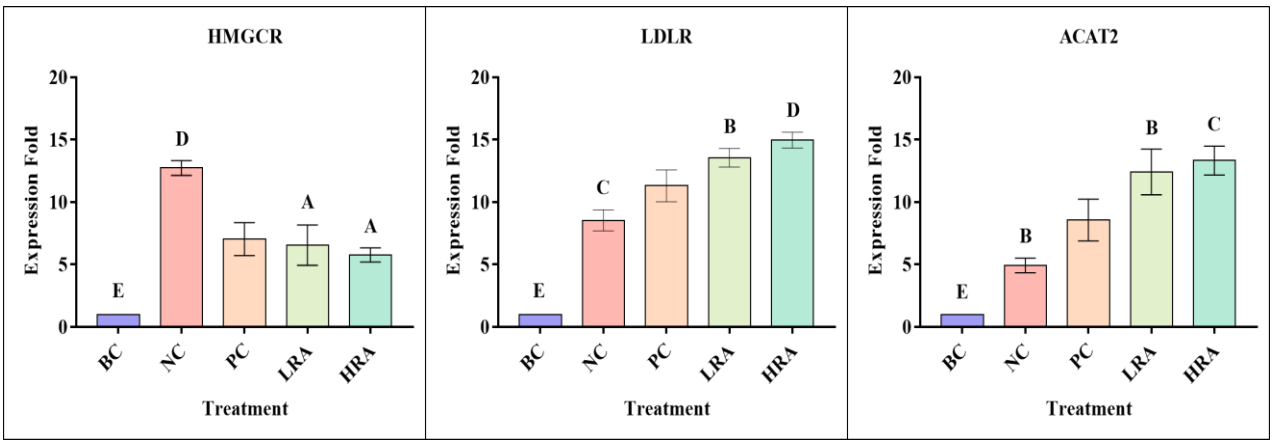

Fig. 4. Regulation of experimental genes involve in cholesterol metabolism for *HMGCR*: BC (1±0), NC (12.74±0.592), PC (7.04±1.316), LRA (6.56±1.613) and HRA (5.776±0.569); *LDLR*: BC (1±0), NC (8.55±0.849), PC (11.30±1.275), LRA (13.56±0.748) and HRA (14.96±0.640); and *ACAT2*: BC (1±0), NC (4.93±0.588), PC (8.57±1.676), LRA (12.43±1.839) and HRA (13.33±1.155). Graph followed by different upper-case letter (A-E) represents a significant difference ($p < 0.05$) treatment wise (n= 3).

Discussion

To our knowledge, this work is novel in describing the anti-hypercholesterolemia activity of RA and the potential molecular regulation of cholesterol levels in HepG2 cells. The cell line was used as an in-vitro model as it expresses multi-differentiated functions of the liver, especially in cholesterol metabolism (26, 27). Available data from this in-vitro study could provide scientific evidence on the application of RA therapeutical outcomes.

We first validated the cytotoxic effects of RA in HepG2 cell lines via MTT assay. The present experiment has illustrated three interesting findings. First, the incubation of cells with 1 µM and 2 µM of RA did not show any significant difference in cell viability compared to untreated cells (0 µM). Second, a decrease in cellular viability with a significant difference was observed as the concentration of RA given to cells increased (2.5-10 µM). Third, cellular proliferation was absent in all treatments which may suggest the anti-proliferative effects of RA. Following the current findings, the toxicity effect of RA is observed to be cell dependant. The higher concentration of RA significantly decreased the cellular viability and proliferation of these cells due to membrane ruptures induced by high osmotic pressure (28).

Next, we examined the effects of RA in regulating intracellular cholesterol mobilization. Although cholesterol is important in maintaining cellular architecture, dynamics, and functions (27, 29) high circulating cholesterol levels are a predisposing factor of atherosclerotic cardiac disease (27, 30, 31). Here we observed the TCC levels in the HRA group are the highest. The analysis presumed that HRA in this study is a potential candidate of cholesterol-lowering substitute but could not be definitively proven by comparing the TCC levels and fluorescence staining entirely. Therefore, to ensure the molecular mechanism is properly understood, gene expression analysis was done.

In this analysis, cells treated with HRA show promising results by significantly altered cholesterol-regulating genes (*HMGCR*, *LDLR*, and *ACAT2*) in comparison to other treatment groups. The first experimental gene observed, *HMGCR* was down-regulated in HRA further suggest that RA is a potential cholesterol-lowering alternative to simvastatin. This gene encodes the rate-limiting enzyme in endogenous cholesterol synthesis, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*) (32). Statins possess a similar structure as *HMGCR* which competitively inhibits the enzyme results in decrease cholesterol levels in cells (33). We postulated that RA exerts similar biochemical and physiological effects as statins. Treatment of cells with HRA also caused an up-regulation of *LDLR* and *ACAT2* gene expression. The mechanisms could be further elaborated by a brief understanding of the negative feedback mechanism evoked by strings of proteins interaction initiated by sterol regulatory element-binding protein (SREBP).

SREBP is a transcriptional factor protein that controls lipids synthesis in cells (34, 35). This protein is encoded by two genes (*SREBF1*: SREBP-1 and *SREBF2*: SREBP-2) that regulate cholesterol synthesis genes (27, 36). Among the existing isoforms, SREBP-2 is expressed ubiquitously and tightly regulated by cellular cholesterol levels (34, 35, 37). SREBP-2 is present in the endoplasmic reticulum (ER) membrane as an inactive precursor bound to SREBP cleavage-activating protein (SCAP) (38). SCAP possesses a cholesterol-sensing domain that responds to intracellular cholesterol depletion causing translocation of SREBP-2 to the Golgi in a vesicle. Here, the Site-1 and -2 proteases are cleaved which release the NH_2-terminal transcription-activation domain of the SREBP-2. The NH_2-terminal enter the nucleus binds to a sterol response element and ameliorates the expression of SREBP-2 targeted genes (34, 37-39).

We also demonstrated that RA decreased the expression of *HMGCR* and its transcription product, the *HMGCR* enzyme. Inhibition of the enzyme results in alleviated intracellular

cholesterol levels which initiate the proteolytic activation of SREBP-2 (33). The activation of SREBP-2 initiates the transcription of the *LDLR* gene results in increased expression of LDLR on the cell surface. *LDLR* gene encodes the genetic information required for LDLR synthesis involved in cellular uptake of circulating LDLC (33, 40). The regulation of LDLC is important in providing balance cholesterol homeostasis necessary to prevent certain cardiovascular diseases development (41). Circulating LDLC binds to LDLR and endocytosed through clathrin-mediated endocytosis. Through chains of enzymatic reactions, LDLR is recycled to the cell surface via an endocytic recycling compartment, whereas non-esterified cholesterol is hydrolyzed from LDLC by lysosomal acid lipase (LAL).

Next, we observed that the expression of the *ACAT2* gene in HRA is significantly up-regulated in parallel to the expression of *LDLR*. *ACAT2* encodes the acyl-CoA:cholesterol acyltransferase (ACAT2), an enzyme that catalyzes the esterification of cholesterol (42). ACAT2 is one of the membrane-bound O-acyltransferase (MBOAT) families that plays important role in neutral lipid synthesis for lipid droplets biogenesis (43). This enzyme is primarily localized to the ER that utilizes non-esterified lipids (*e.g.* fatty acyl CoA and cholesterol) to form cholesterol esters which are reserved in between the leaflets of the ER bilayer and give rise to oil lens as the concentration increases (43, 44). Subsequently, LDs will bud off into the cytosol (43). LDs are highly dynamic intracellular organelles that prevent lipotoxicity and oxidative stress in cells by buffering potentially toxic lipids and proteins accumulation (45). Figure 5 depicted the proposed molecular pathway of RA in regulating the intracellular intake of LDLC.

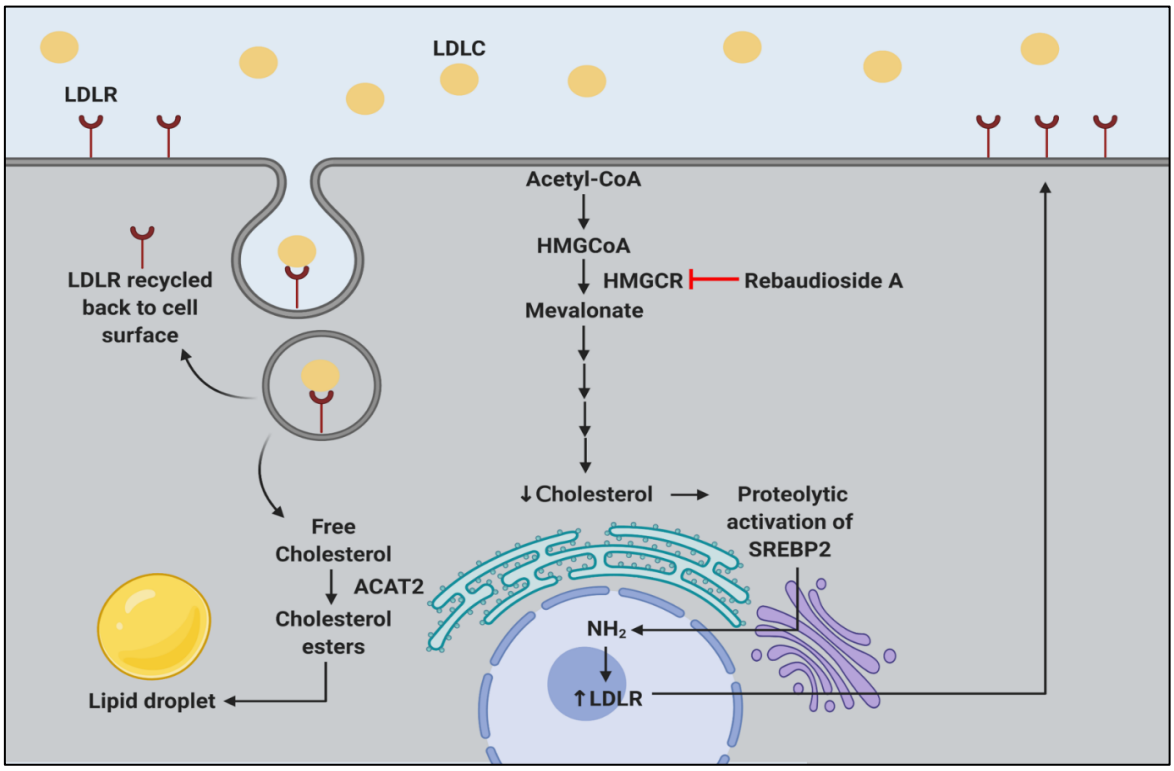

Fig. 5. A proposed molecular pathway of Rebaudioside A in ameliorating the expression of cholesterol-synthesis genes (*HMGCR*, *LDLR* and *ACAT2*). Rebaudioside A competitively inhibits the enzymatic reaction of *HMGCR* causing intracellular cholesterol depletion. Proteolytic activation of SREBP-2 was stimulated as the SCAP/SREBP-2 complex translocated to the Golgi followed by releasing of NH_2-terminal into the nucleus. The consequences of this event lead to *LDLR* gene transcription and more LDLR proteins exist on the cell surface. Circulating LDLC binds to LDLR and internalized via the clathrin-mediated endocytosis. In cells, LDLC segregates from its receptor (recycle to the surface) due to acidic pH and the former was hydrolysed by LAL. The non-esterified cholesterol molecules (free cholesterol) are toxic to cells, thereby esterified by ACAT2 protein into neutral lipids (cholesterol esters) and further stored in lipid droplets (This figure was created with BioRender.com).

In summary, this novel in-vitro study shows that Rebaudioside A is a potential cholesterol-lowering candidate for statins. Considering its natural and non-cytotoxic characteristics, Rebaudioside A possesses few other therapeutical advantages to statins drawback. Despite the success demonstrated, this work is limited by its absence of other cholesterol synthesis genes namely *SREPB-2*, *HMGCS*, and *MVK*. Hence, future research should therefore seek to address this issue by implying these genes as well as other possible approaches such as large-scale multi-omics. Regardless, the findings in this study provide a very useful insight to understand the mechanism of Rebaudioside A in lowering cholesterol levels.

Acknowledgements

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