High-Dose Edible Bird's Nest Extract (EBN) Upregulates *LDL-R* via Suppression of *HMGCR* Gene Expression in HepG2 Cell Lines

(Sarang Burung Walet (EBN) Berdos Tinggi Meningkatkan *LDL-R* melalui Perencatan Gen *HMGCR* dalam Sel HepG2)

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ABSTRACT

Edible bird's nest (EBN) is an ancient food that had been consumed by Chinese people for well-being purposes. Nevertheless, the effect of EBN on cholesterol metabolism is poorly studied. Since the cholesterol is primarily being metabolized in the liver, HepG2 cell lines was selected as the model for in-vitro preliminary assessment. Our EBN extract (EBNE) showed 71% cell viability in HepG2 cell lines, even at the highest dose (1.5 mg/mL). Simultaneously, EBNE was significantly up-regulated the LDL-R *gene expression via suppression of* HMGCR *at the highest concentration. Besides that,* ACAT2 *was up-regulated significantly to allow exogenous cholesterol storage. Expression analysis of these genes were correlated with high immunofluorescence distribution of the* LDL-R *and LDs compared to other treatment groups. Consistent with gene expression and immunofluorescence staining, intrahepatocellular cholesterol concentration was increased significantly at the highest dose of EBNE. The EBNE had significantly improved cholesterol metabolism in the HepG2 cell line via suppression of the* HMGCR *gene and subsequently up-regulated* LDL-R *gene expression at the highest dose. Therefore, this study provides an insight to understand the effect of EBNE in improving cholesterol metabolism.*

Keywords: 3-hydroxy-3-methylglutharaldehyde CoA reductase (HMGCR)*; cholesterol metabolism; edible bird's nest (EBN); HepG2; low-density lipoprotein receptor* (LDL-R)

ABSTRAK

Sarang burung Walet (EBN) merupakan makanan tradisi yang diamalkan oleh masyarakat China bagi tujuan kesihatan. Dalam pada itu, kesan EBN ke atas metabolisme kolesterol masih belum dikaji dengan terperinci sehingga kini. Organ hati memainkan peranan sebagai organ utama di dalam tubuh badan manusia yang melibatkan metabolisme kolesterol terutamanya pada hati. Justeru, sel HepG2 iaitu sel hepatosit hati dipilih sebagai model kajian rintis terhadap peranan EBN pada organ hati secara in-vitro. *Menerusi kajian yang telah dijalankan terhadap ekstrak EBN (EBNE) menemukan bahawa, walaupun dos tersebut paling tinggi (1.5 mg/mL) digunakan, namun kelangsungan sel HepG2 hidup adalah sebanyak 71%. Pada masa yang sama, EBNE telah meningkatkan pengekspresan gen* LDL-R *dengan ketara melalui perencatan gen* HMGCR *pada EBNE berkepekatan tertinggi. Manakala,* ACAT2 *turut jelas sekali meningkat dalam memainkan peranan ia membolehkan penyimpanan kolesterol eksogenus di dalam sel. Analisis ekspresi gen-gen ini berkait dengan taburan imunopendarfluor* LDL-R *dan LD yang tinggi berbanding dengan kumpulan rawatan seumpamanya. Rentetan kajian pengekspresan gen dan pewarnaan imunopendarfluor, didapati kepekatan kolesterol intrahepatosel telah meningkat pada dos EBNE yang tertinggi nyata sekali. EBNE telah menambahbaik metabolisme kolesterol di dalam sel HepG2 melalui perencatan gen* HMGCR *serta meningkatkan pengekspresan gen* LDL-R *pada dos tertinggi. Lantaran itu, kajian ini memberikan pemahaman mengenai kesan EBNE dalam menambahbaikkan metabolisme kolesterol di dalam tubuh badan manusia.*

Kata kunci: 3-hidroksi-3-metilglutharaldehid CoA reduktase (HMGCR)*; metabolisme kolesterol; sarang burung walet (EBN); HepG2; reseptor lipoprotein berketumpatan rendah* (LDL-R)

The use of edible bird's nest (EBN) in traditional Chinese medicine has been documented since the Tang Dynasty (618-907 CE). EBN is used as a natural supplement that possesses various medicinal benefits towards human wellness, including anti-viral, anti-inflammatory, immune modulator, respiratory supplement, anti-aging and metabolic stimulant (Haghani et al. 2016; Marcone 2005). EBN derived from salivary secretion of two sublingual's palaeotropical swiftlets (Looi & Omar 2016); in particular male swiftlets during mating season (Ma & Liu 2012). Most of the Chinese community would consume this tonic food in the form of bird's nest soup and serve as a traditional remedy (Ma & Liu 2012). In general, proximate analysis of EBN from *Aerodramus* genus nest consists of proteins (62.0-63.0%), carbohydrates (25.62-27.26%), ash (2.1%), and lipid (0.14-1.28%) (Yu-Qin et al. 2000). Most of the proteins and carbohydrates found in the form of glycoproteins such as sialic acid, galactosamine and glucosamine, which linked via carbohydrate-peptide linkages (Ma & Liu 2012). In the matrix-rich glycoproteins of EBN, sialic acid is the major essential sugar component in the EBN, which constitute of 9-11% (Colombo et al. 2003; Yida et al. 2015a) and this glycoprotein served as important metabolite in improving cholesterol metabolism (Lindbohm et al. 2000; Millar 2001). Scientifically, the EBN was claimed as a complete food ingredient that can be consumed by the human (Chua et al. 2014).

Low-density lipoprotein cholesterol (LDL-C) or commonly termed as a bad cholesterol can lead to cardiovascular disease (CVD) (Gradinaru et al. 2015). When LDL-C is excessively circulating in the bloodstream, human body will be stimulated to express the LDL receptor (LDL-R) on the plasma membrane particularly hepatocytes for excess LDL-C internalization (Charlton-Menys & Durrington 2007). Liver serves as the major internal organ in metabolizing excess cholesterol in bloodstream (Rogers et al. 2015; Shimano & Sato 2017) Subsequently, degraded LDL-C will be stored in the lipid droplet in form of cholesterol ester for future use under catalyzation of acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2) (Chang et al. 2009; Rogers et al. 2015). For past decade, researchers have been targeting cholesterol rate-limiting enzyme called 3-hydroxy-3-methyglutaryl-coenzyme A reductase (HMGCR) to reduce high cholesterol in the blood (Steinberg 2006). To date, CVD is a disease leading in death worldwide, particularly in developed and developing country (Ladeiras-Lopes et al. 2015). The primary cause of CVD is due to cumulative effect of high cholesterol in the blood, which eventually lead to plaque formation, and this disorder can be termed as atherosclerosis (Chan et al. 2014). High blood cholesterol can be medically managed by consumption of anti-cholesterol drugs (Ladeiras-Lopes et al. 2015).

In recent years, many nutraceutical researchers are promoting homeopathic medicinal approach to reduce cholesterol in dyslipidaemia patients. Most of the natural product is started from the ethnic beliefs that have been claimed to have a medicinal effect. Same goes to EBN that have been consumed traditionally for metabolic stimulant by the royal family of China. Based on Yida et al. (2015a), EBN could reduce blood cholesterol in an insulin resistance animal model. Nevertheless, they were not further elaborated on by which the EBN is improving cholesterol. Therefore, this article reports the biological functions of EBNE on the cholesterol metabolism in HepG2 cells, as a preliminary study. This study will provide a better understanding on the potential role of EBN as an alternative food to reduce blood cholesterol in future.

EBNE PREPARATION AND EXTRACTION

Edible bird's nest was purchased from a certified manufacturer in a raw form before being soaked, and cleaned manually in the PBS (Phosphate Buffer Solution). Cleaned EBNs were pooled together and air dried in 100% ventilated incubator at 30 °C for 24 h. Dried EBN was ground into a small granule using table top grinder before the extraction procedure. 1.0 g of dried EBN was soaked in 15 mL PBS and vortexed for 30 s. Soaked EBN were stewed in a pre-warmed water bath at 70 °C for 5 h, to get the EBN extract (EBNE). EBNE was centrifuged for 10 min at 4,400 rpm. Supernatant was collected for protein precipitation via cold acetone technique. Finally, protein pellet was dissolved in 1.0 mL chilled PBS (4.0 °C) by re-suspending with micropipette and vortexed. Final product was subjected for protein determination using BCA assay kits (Thermo Fisher Scientific, USA).

HepG2 CELL MAINTENANCE AND TREATMENT

HepG2 was purchased from American Type Culture Collection (ATCC) and maintained in Advanced Minimum Essential Medium (AMEM) supplemented with 10% FBS (Foetal Bovine Serum) as suggested from the manufacturer. Additional 1% Penicillin-Streptomycin and Fungizone (2.5 µg/mL) was added to avoid secondary infection. Cells were maintained in T-25 cell culture flask under humidified incubator at 37.0 °C with 5% CO_2 until 90% confluent before sub-cultured into different plate. Media were changed regularly for every two days while in maintenance phase. For treatment protocol, 1×10^6 cells were cultured in 6-wells plate sized 3.0 cm in diameter for 24 h before treatment was started. HepG2 cells were then cultured in AMEM and 1% FBS with or without exogenous cholesterol (1:250) and LDL-C (10 µg/mL), in the presence or absence of EBNE that already quantified at different concentrations (0.5, 1.0 and 1.5 mg/mL) for

24 h. Treatment regime consists of three controls which include baseline control (BC-media only), negative control (NC-media with exogenous cholesterol and LDL-C) and positive control (PC-media with exogenous cholesterol, LDL-C and anti-cholesterol drug (Simvastatin-4.60 μg/mL)).

HepG2 CELL VIABILITY ASSAY

Cells were seeded in 96-wells microplate at 3×10^4 cells per well and treated with different concentrations of EBNEs (0.2, 0.5, 0.8, 1.0 and 1.5 mg/mL). After incubation for 24 h, treatment media was removed and replaced with new media containing AMEM and 1% FBS before 10 μL of MTT reagent (5 mg/mL) was added into the wells. Cells were incubated again for 4 h before discarding old media containing MTT reagent and replaced with 100 μL of DMSO. Cell viability curve was established and IC$_{50}$ was determined.

LDL-R, *ACAT2* AND *HMGCR* mRNA EXPRESSION

Cell pellets were subjected for RNA extraction using Qiagen RNeasy® Mini Kit. RNA quality was determined using NanoQuant plate™ (Tecan, Switzerland) via Infinite M200Pro Spectrophotometer (Tecan, Switzerland). Total extracted RNA was standardized into 100 ng/μL before subjected for primers (Table 1) optimization in the Thermoblock 96 thermocycler (Sensoquest, Germany) using Access RT-PCR System master mix (Promega, USA), and electrophoretically validated in 1.5% agarose gel. Qualitative bands observation was visualized using Gel Doc XR+ System Image Analyser (Bio-Rad, USA) before optimized annealing temperature was determined. RNA was amplified using GoTaq® qPCR master mix (Promega, USA) according optimized *LDL-R*, *ACAT2*, *HMGCR,* and *GADPH* (housekeeping gene). At first, gene amplification was started with reverse transcription at 45 °C for 45 min, followed by initial denaturation at 95 °C for 5 min. Both processes were done for one cycle only. Denaturation at 95 °C for 1 min, followed by annealing at 64 °C (*LDL-R*), 59 °C (*ACAT2*), 50 °C (*HMGCR*) and 64 °C (*GAPDH*) for 1 min, and extension at 72 °C for 20 s were performed for 40 cycles. Final extension was setup at 72 °C for 7 min, followed by incubation at 4 °C were applied to all genes. The product size for *LDL-R*, *ACAT2*, *HMGCR,* and *GAPDH* are 369, 70, 210, and 453 base pair (bp), respectively. The $\Delta\Delta C_T$ quantification was used to determine the expression of target genes using *GADPH*; as an internal control gene via CFX-96 Touch™ Real-Time PCR Detection System (BioRad, USA). Target gene mRNA level was expressed in relative-fold to the baseline control using the same system.

TABLE 1. Description of analysed genes in the *in-vitro* experimental study

Gene Name	Description	Forward (F) and Reverse (R) Primer Sequence (5'-3')	Accession Number
LDL-R	Low-density lipoprotein receptor; cellular membrane-bound protein receptor for internalization/ endocytosis of low-density lipoprotein into the cellular cytosol	(F) CCCCGCAGATCAACCCCCACTC (R) AGACCCCCAGGCAAAGGAAGACGA	NM000527.3
ACAT2	Acyl-coenzyme A: cholesterol acyltransferase; integral membrane-bound enzyme of rough endoplasmic reticulum which plays role in catalyzation of cholesteryl ester formation	(F) GCAAGTCCCTGCTTGATGAGC (R) CCAGCGATGAACATGTGGTAGAT	NM003578.3
HMGCR	3-hydroxy-3-methylglutharyl-coenzyme A reductase; rate-limiting enzyme in cholesterol biosynthesis in the mevalonate pathway	(F) GGGACCAACCTACTACCTC (R) GTCGAAGATCAATTTACAA	BC033692

| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase; endogenous gene control for gene normalization | (F) GACCACAGTCCATGCCATCAC
(R) TCCACCACCCTGTTGCTGTAG | NM002046.3 |

HepG2 IMMUNOFLUORESCENCE STAINING

For immunofluorescence staining, 1.0×10^3 HepG2 cells in 50 µL of media were cultured on top of round-cover slips inside 6-wells plate for 1 h in the CO_2 incubator. Post cells attachment, media containing treatments were added in the well up to 2.0 mL and kept for 24-h. Old media was removed and replaced with 4% paraformaldehyde (PA) for fixation. PA was removed and cells were washed three times with 2.0 mL PBS for five minutes in each washing steps. Cells fixation continued for overnight in 4 °C with 1% PA. Cells were washed again with PBS in the same manner before permeabilization of cells with 0.2% triton-X 100 and subsequently soaked in a blocking solution of 2% BSA in PBS. Cover slips were incubated with 1:50 primary antibody (LDL-R) (ab52818, Abcam, UK) and secondary antibody (Alexa Fluor® 594 Donkey Anti-Rabbit IgG [H+L]). Lipid immunofluorescence staining (Bodipy 493/503) was used to stain the lipid droplets and DAPI staining was used for nuclear staining. Cells were viewed with Nikon Eclipse Ti-S fluoroscopy (Nikon, Japan).

HepG2 INTRAHEPATOCELLULAR CHOLESTEROL QUANTIFICATION

Lipid extraction was performed on the treated and control cells by acidified Bligh and Dryer method. Cells were collected into the respective glass tubes with 0.5 mL PBS and homogenized with a TissueRuptor and Disposable Probes (Qiagen, Germany). About 100 µL of cell suspension were reserved for protein determination via BCA assay, and 500 µg of protein in 1.0 mL PBS was used for lipid extraction. Chloroform containing lipid extracts were evaporated with nitrogen gas flow in a pre-heated water bath at 55 °C. Cholesterol quantification was performed with a cholesterol assay kit from Cholesterol Liquicolor (Human Diagnostics Worldwide, Germany). Cholesterol concentration was determined via absorbent at 540 nm wavelength using Infinite M200Pro Spectrophotometer (Tecan, Switzerland).

STATISTICAL ANALYSIS

Data were expressed in mean and standard deviation (SD) before data screening and normalization were conducted via normality test. Independent t-test was used for data analyses by comparing groups at p-value <0.05 to show significance differences between corresponding data.

RESULTS AND DISCUSSION

HepG2 CELL VIABILITY

HepG2 cell lines were selected in this study, as the cholesterol metabolism is mainly occurred in the liver (Rogers et al. 2015; Shimano & Sato 2017). Although the HepG2 is a human liver cancer cell lines, the usage of this cell is the most established and reported *in-vitro* model in assessing cholesterol metabolism in previous studies (Al-Naqeb et al. 2010; Chong et al. 2011; Leng et al. 2018; Nielsen et al. 2013; Zhao et al. 2016). Figure 1 shows the effect of different EBNE concentrations (0.2, 0.5, 0.8, 1.0 and 1.5 mg/mL) on the HepG2 cell viability post 24-h treatment. The cell viability for the respective concentration was 97.46 ± 1.52, 90.01 ± 2.62, 84.53 ± 2.04, 77.56 ± 0.99 and 70.94 ± 1.52 (n=9). Therapeutic dosage of simvastatin (4.3 µg/mL) showed insignificant cell mortality (106.29 ± 3.92). The 50% inhibitory concentration (IC_{50}) was unable to determine, as the cell line appeared viable more than 50% at the highest concentration (1.5 mg/mL). HepG2 was appeared to be viable at the highest concentration with 71% cell viability, and at 80% cell viability was calculated at 0.93 mg/mL. Since the HepG2 cell viability at 1.5 mg/mL was 71%, the cell viability test was not conducted at the higher concentration, and this justification is in line with previous study that mentioned, a good cell viability for *in-vitro* study must be maintained more than 70% (Al-Naqeb et al. 2010). Meanwhile, 1.0 mg/mL of EBNE was 78%, which was correlated with previous data that shows the cell viability of HepG2 at 1.0 mg/mL of EBNE, can be up to 80% (Yida et al. 2014). This finding is validated with recent study that shows EBN can serve as antioxidant mediator through transcriptional regulation of hepatocytes antioxidant gene in *in-vitro* and *in-vivo* model (Yida et al. 2015b, 2014), subsequently maintained the integrity and viability of the cell. This statement is supported with *in vivo* result that have shown the EBNE was not causing pathological changes in mice, and finally classified as category 5 or unclassified according to the GHC based on

OECD guidelines (Haghani et al. 2016). On the other type of cell line such as MDCK, IC$_{50}$ of EBNE can be ranging between 2.9-4.5 mg/mL (Haghani et al. 2017), which can be considered high for a natural product origin.
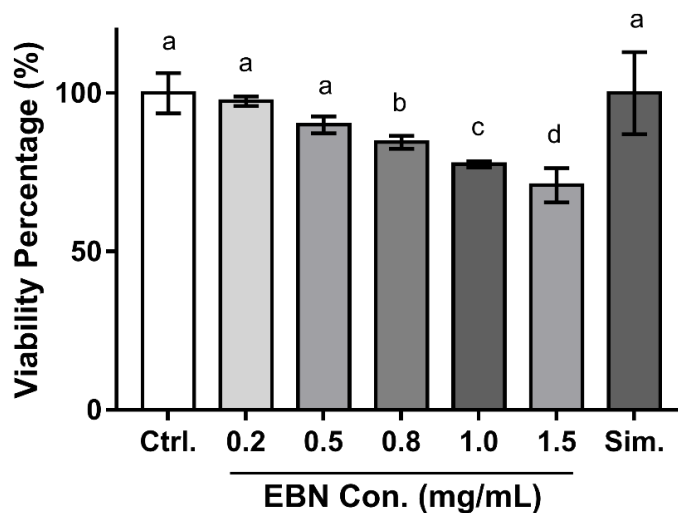

FIGURE 1. Bar chart of the cell viability for HepG2 against EBN extract for 24 h. Graph was plotted at control, different concentration of EBN extract (0.2, 0.5, 0.8, 1.0 and 1.5 mg/mL) and Simvastatin (4.3 µg/mL). Data with a different letter indicates significant differences when compared with control. Data were presented in means ± standard deviation (SD). Data with a different letter indicates significantly differences (n=9)

LDL-R, *ACAT* AND *HMGCR* GENE EXPRESSION

Gene expression is an initial point for the biochemical pathway regulation at the cellular level. Therefore, to investigate the effect of EBNE on cholesterol metabolism, quantitative PCR was done to measure the fold expression of several genes that are involved in cholesterol metabolism. Figure 2 shows the expression of gene of interest (GOI) *LDL-R* via gel electrophoresis in each control (baseline (BC), negative (NC) and positive (PC) control) and EBNE treatments (0.5, 1.0 and 1.5 mg/mL). Qualitatively, all samples were expressing the *LDL-R* gene (369 bp). The intensity of the LDL-R band appeared in a dose-dependent manner; with 1.5 mg/mL of EBNE showed the most intensified band among the EBNE treatments.
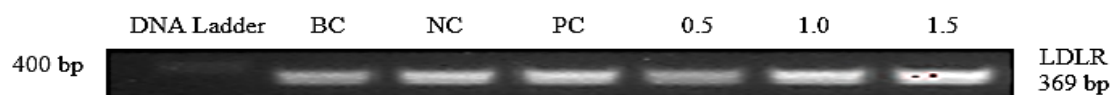

FIGURE 2. The *LDR-R* gene expression in controls and EBNE treatments. Baseline control (BC) containing only media; negative control (NC) containing media, exogenous cholesterol and LDL-C; positive control (PC) containing media with exogenous cholesterol, LDL-C and Simvastatin; 0.5, 1.0 and 1.5, respectively represents concentration of EBNE (0.5, 1.0 and 1.5 mg/mL) with exogenous cholesterol and LDL-C

Figure 3 shows the fold expression of selected GOI (*LDL-R*, *ACAT2*, and *HMGCR*). All data were normally distributed and fold expression was expressed in mean (n=9) and standard deviation (SD). As presumed, the PC group was showing the highest fold expression (49.64 ± 8.26) compared to the BC. Meanwhile, the NC group demonstrated the lowest fold expression (3.24 ± 0.85). Negative control was used in this study to observe how the cell will react and survive in a high cholesterol environment without any treatment, and to show that normal human liver cell will survive and upregulates *LDL-R* expression significantly as a response to high cholesterol condition (Charlton-Menys & Durrington 2007), but not as efficient as cells supplemented with EBNE. In general, EBNE (0.5, 1.0 and 1.5 mg/mL) upregulates *LDL-R* expression significantly compared to the baseline control in a dose-dependent manner (3.30 ± 0.78, 6.18 ± 2.57 and 7.41 ± 3.04).

For *ACAT2*, the PC showed the highest fold of gene expression (16.68 ± 0.25), and similar trend was demonstrated in the EBNE group (0.5, 1.0 and 1.5 mg/mL) with positive dose dependent manner (2.05 ± 0.16, 3.78 ± 0.17 and 5.33 ± 0.16). This finding was corresponded with the expression of *LDL-R*. As the LDL-C had been internalized into the cell cytoplasm together with LDL-R inside the clathrin-coated endocytic vesicle, contained-LDL-C will undergo acidic hydrolysis to produce cholesterol and amino acid, before fused with endoplasmic reticulum (ER), and cholesterol will be hydrolysed by ACAT2 and causing increment of cholesteryl ester production in between of ER's phospholipid bilayer

(Chang et al. 2009; Martin & Parton 2006; Rogers et al. 2015). This accumulation will lead to budding of new independent organelle such as lipid droplet that associated with specific protein on the monolayer phospholipid (Martin & Parton 2006).

HMGCR is a rate-limiting enzyme in cholesterol biosynthesis via mevalonate pathway (Shimano & Sato 2017), and inhibition of this enzyme gain attention from researchers for the past decades in reducing high cholesterol in dyslipidaemic patient (Steinberg 2006). Previously, Vanillin Rich Fraction (VRF) was detected to downregulate *HMGCR* expression and upregulate *LDL-R* significantly in a dose dependent manner (Al-Naqeb et al. 2010). In general, EBNE (0.5, 1.0 and 1.5 mg/mL) downregulated *HMGCR* expression in negative dose dependent manner (0.88 ± 0.09, 0.73 ± 0.29 and 0.69 ± 0.16). Nevertheless, only the highest concentration (1.5 mg/mL) showed significant gene suppression at 31% compared to BC. Therefore, we suggest that the effective dosage for the EBNE to give an anti-cholesterol effect should be at 1.5 mg/mL. This finding is consistent with our laboratory collaborator, that had found serum paraoxonase/lactonase 3 (PON-3) protein in our EBNE, which serves as prodrug of HMGCR inhibitor (Unpublished data). Insignificant finding in 0.5 and 1.0 mg/mL EBNE in the present study could be due to insufficient amount of PON-3 protein in our extract to cause the anti-cholesterol effect. As expected, the Simvastatin was significantly suppressed the *HMGCR* gene at 65% and this finding was in line with the study by Charlton-Menys and Durrington (2007) on the effect of statin drugs.
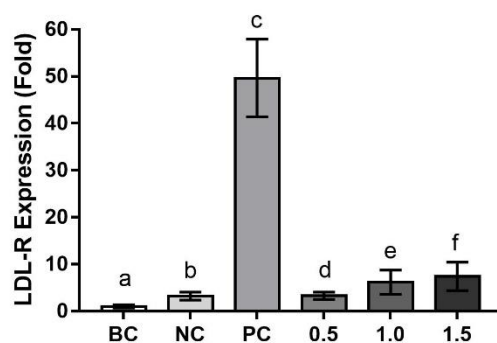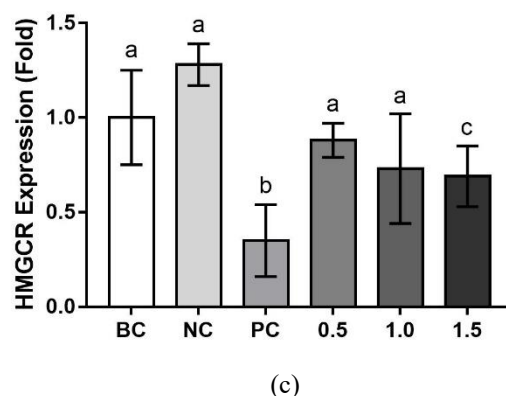

(a)
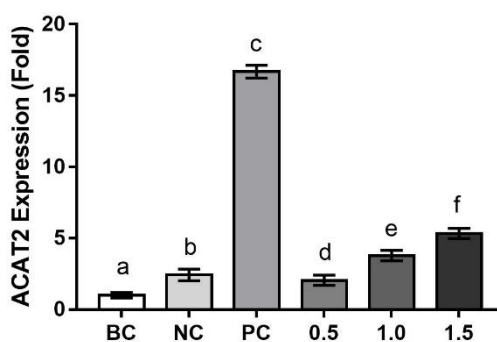
(b)

FIGURE 3. Bar chart of gene expression for (a) *LDL-R*, (b) *ACAT2,* and (c) *HMGCR* in HepG2 cell lines against controls (BC; baseline control, NC; negative control, PC; positive control) and EBNE treatment groups (0.5, 1.0 and 1.5 mg/mL). Data were presented in means ± standard deviation (SD). Data with a different letter indicates significantly differences (n=9)

HepG2 IMMUNOFLUORESCENCE STAINING

In order to visualize the LDL-R protein expression and the final product of internalized LDL in the lipid droplets (LDs), LDL-R, and LDs were enhanced with immunofluorescence-labelled antibody. In the immunofluorescence staining imaging, LDL-R, LDs, and nucleus were stained with red, green and blue, respectively (Figure 4). As the treatment concentration (EBNE) increased, the distribution of LDL-R fluorescence (red) was gradually increased on the cell lines, which indicated the abundant expression of the LDL-R protein on the membrane plasma. This finding was consistent with our expression analysis (Figure 3) and also validated the effect of EBNE in increasing the LDL-R protein expression in HepG2. For the controls, fluorescence distribution of the LDL-R in BC was observed in the absent of LDL-R expression. This could be explained as the media only containing AMEM, and absent of exogenous stimulus (cholesterol) that must be presence to initiate the LDL-R protein expression (Shimano & Sato 2017).

Conversely, positive control that contained media, exogenous stimulus, and Simvastatin showed the highest LDL-R distribution, as the Simvastatin increased the LDL-R protein expression upon the treatment (Chan et al. 2014). In correlation with high LDL-R fluorescence distribution, the LDs was gradually increased in size as the EBNE concentration increased, which means more exogenous cholesterol and LDL-C was internalized by the LDL-R, and stored in the LDs. This data was also consistent with the expression of *ACAT2* (Figure 3). As the nature of hepatocytes to contain LDs in the cytoplasm (Rogers et al. 2015), baseline control was observed to have a tiny and smaller LDs compared to positive control which contained larger LDs; as Simvastatin upregulates LDL-R and increases storage of cholesterol particularly LDL-C (Charlton-Menys & Durrington 2007).
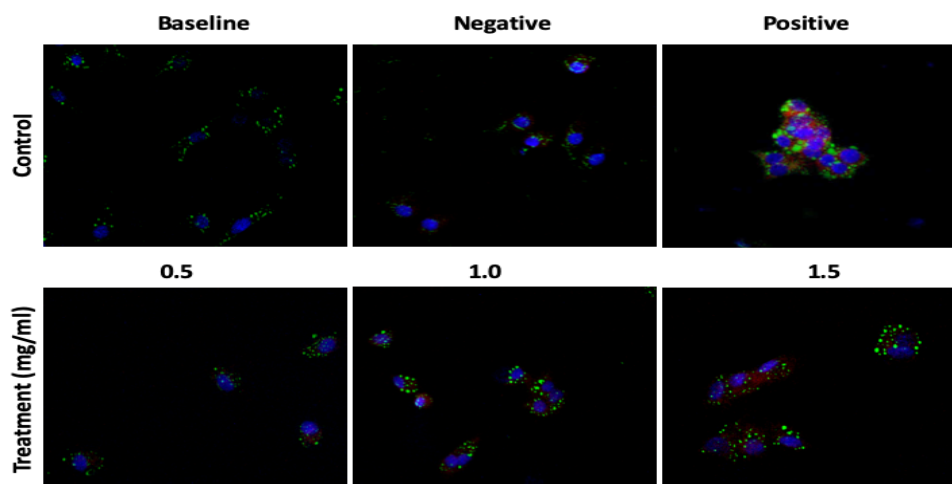

FIGURE 4. Microphotographs of immunofluorescence (IF) staining of LDL-R (red), LDs (green) and nucleus (blue) in controls (BC; baseline control, NC; negative control, PC; positive control) and EBNE (0.5, 1.0 and 1.5 mg/mL). Magnification 200X

INTRAHEPATOCELLULAR CHOLESTEROL CONCENTRATION OF HepG2

Calorimetric assay (ELISA) was performed to quantify the cytosol cholesterol. Figure 5 shows cholesterol concentration of controls (BC, NC, and PC) and treated cells at different dosage of EBNE (0.5, 1.0 and 1.5 mg/mL). All data were significantly increased compared to the BC. As expected, PC group that treated with anti-cholesterol drug (Simvastatin) was recorded as the highest cholesterol concentration (129.0 ± 7.0), and this finding is consistent with the pharmacodynamic of statin drug group in lowering exogenous cholesterol (Steinberg 2006), and stored in the lipid droplets (Chang et al. 2009). Conversely, BC recorded the lowest cholesterol concentration (7.0 ± 2.0), and this result was predicted earlier as there is pre-existing cholesterol in the hepatocytes that made up the plasma membrane of cell and organelles, and the lipid droplets (Gartner 2017). In the NC, the cholesterol concentration (12.0 ± 1.0) was slightly increased compared to the BC, as the normal physiological function of the hepatocytes will increase internalization of excess cholesterol for lowering the exogenous cholesterol in order to maintain the homeostatic condition (Charlton-Menys & Durrington 2007). Nevertheless, the uptake might be less due to limited *LDL-R* expression on the outer layer of plasma membrane. For the EBNE treated cells, cholesterol concentrations were expressed in a dose-dependent manner (15.0 ± 3.0, 32.0 ± 7.0, and 96.0 ± 2.0). These results were parallel with our *ACAT2* expression (Figure 3), which indicate that the EBNE able to increase internalization of excessive exogenous cholesterol, and may contribute to the cholesterol storage in the lipid droplets (Rogers et al. 2015). Besides that, this finding also supported by previous study that suggests the present of sialic acid in the EBN (Yida et al. 2015a) is imperative in preventing oxidation of exogenous LDL-C and in preserving the conformation of this lipoprotein which lead to the consistent internalization of LDL-C into the cytosol (Lindbohm et al. 2000; Millar 2001). From the data, the negative control showed insignificant difference with 0.5 mg/mL of EBNE. This finding suggests that, to have an effect for internalization of excessive exogenous cholesterol for storage, the EBNE dose must be higher than 0.5 mg/mL and this will be the benchmark for further *in-vivo* study.
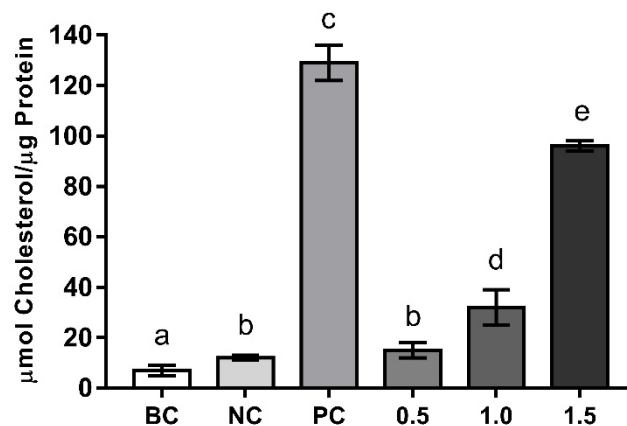

FIGURE 5. Bar chart of gene expression for (a) *LDL-R*, (b) *ACAT2,* and (c) *HMGCR* in HepG2 cell lines against controls (BC; baseline control, NC; negative control, PC; positive control) and EBNE treatment groups (0.5, 1.0 and 1.5 mg/mL). Data were presented in means ± standard deviation (SD). Data with a different letter indicates significantly differences (n=9)

Based on the results, we propose a schematic diagram (Figure 6) on by which the EBNE improved the cholesterol metabolism. Upon supplementation of EBNE, suppression of the *HMGCR* gene that acts as cholesterol rate-limiting enzyme will lead to the intracellular cholesterol deprivation. As the internal regulatory responses, the *LDL-R* gene will be upregulated to restore the homeostatic cholesterol level inside the cell. The LDL-R will allow the internalization of excess exogenous cholesterol into the cytoplasm, particularly the LDL-C. This LDL-C will be transported to the endoplasmic reticulum for further storage inside the new lipid droplets (LDs), under catalyzation of ACAT2 enzyme. These newly biosynthesis LDs will be utilized for further cellular physiology.
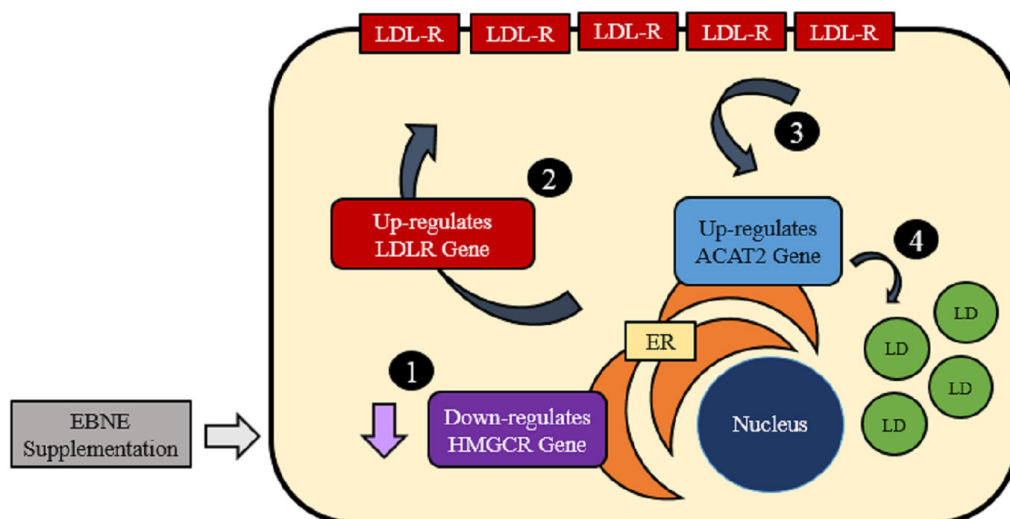
FIGURE 6. A proposed schematic pathway for Edible Bird's Nest Extract (EBNE) supplementation in reducing cholesterol on the human liver cell lines (HepG2). EBNE supplementation causes down-regulation of cholesterol rate-limiting enzymes gene expression (*HMGCR*; 3-hydroxy-3-methyglutharyl-coenzyme A reductase) on the endoplasmic reticulum (ER) (1), and resulting in deprivation of intra-hepatocellular cholesterol concentration in the cytosol. As a homeostatic negative feedback mechanism, LDL-R (low-density lipoprotein receptors) gene expression is up-regulated (2) to restore back cholesterol deficiency in the cytosol, via internalization of exogenous LDL-C. Internalized-LDL-C will be transported in the lysosome and metabolized via acidic hydrolysis to form hydrolysed free cholesterol and some amino acids. Hydrolysed free cholesterol is directly transported to the endoplasmic reticulum for storage under catalyzation of ACAT2 (acyl-coenzyme A: cholesteryl acyltransferase 2) gene that is also up-regulated (3) for the hydrolysed cholesterol to be stored in the form of cholesteryl esters. Accumulation of cholesteryl esters in the intramembrane of endoplasmic reticulum will lead to newly synthesis of lipid droplets budding and will be released into the hepatic cytosol (4)

CONCLUSION

In conclusion, EBNE could upregulate *LDL-R* and *ACAT2* expressions in a positive dose-dependent manner, and suppressed *HMGCR* expression significantly at the highest concentration. These resulted in formation of abundant lipid droplet from numerous expression of LDL-R protein on the plasma membrane, and dramatically increased the cholesterol concentration upon EBNE supplementation in a dose-dependent manner. This finding provides an insight in elucidating the effect of EBNE on the cardiovascular health in animal model.

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