Research in Pharmaceutical Sciences, June 2019; 14(3): 190-200
Received: September 2018
Accepted: April 2019

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Siamese neem flower extract suppresses cholesterol absorption by interfering NPC1L1 and micellar property *in vitro* and in intestinal Caco-2 cells

Acharaporn Duangjai[1], Atcharaporn Ontawong[1,2], and Chutima Srimaroeng[2,*]

[1]*Division of Physiology, School of Medical Sciences, University of Phayao, Phayao, Thailand.*
[2]*Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.*

Abstract

Siamese neem (*Azadirachta indica A. Juss* var. *siamensis Valeton*) (*A. indica)* leaf extract, a traditional ayurvedic medicine, has been reported to exhibit antipyretic, antibacterial, antidyslipidemic, and antihyperglycemia effects. This study investigated the mechanism of hypocholesterolemic effect of methanolic extract of Siamese neem flowers in *in vitro* studies and in Caco-2 cells. Pancreatic cholesterol esterase and 3-hydroxy 3-methylglutaryl-CoA (HMG-CoA) reductase activities were assessed. Cholesterol micelle formation was prepared for *in vitro* cholesterol physicochemical property analyses, micelle size and solubility, and transport of cholesterol into the Caco-2 cells. The expression of niemann-pick C1 like 1 (NPC1L1), and its major regulator, peroxisome proliferator-activated receptor δ (PPARδ), were determined by western blot and real time polymerase chain reaction, respectively. *A. indica* flower extract inhibited pancreatic cholesterol esterase activity and increased cholesterol micelles size. Uptake of cholesterol into Caco-2 cells was inhibited by *A. indica* flower extract in a dose-dependent manner. In addition, *A. indica* extract inhibited HMG-CoA reductase activity, resulting in low level of intracellular cholesterol accumulation, together with increased cytosolic NPC1L1 protein expression and decreased PPARδ gene expression. In conclusion, *A. indica* flower extract has cholesterol-lowering effects by inhibiting intestinal cholesterol absorption, interfering micellar cholesterol formation, and attenuating cholesterol synthesis. As such, *A. indica* flower extract has potential for developing into nutraceutical product for prevention of hypocholesterolemia.

Keywords: *A. indica*; Cholesterol; HMGR; Micelle; NPC1L1.

INTRODUCTION

Cholesterol homeostasis is maintained by intestinal absorption, endogenous biosynthesis, and removal of cholesterol from the blood circulation (1). Intestinal cholesterol absorption is a multi-step process in which cholesterol is micellized in the lumen, taken up by the enterocytes, and transported to the blood circulation. Interestingly, inhibiting cholesterol absorption is the primary approach to reduce plasma cholesterol levels. Niemann-pick C1-like 1 (NPC1L1) protein is a cholesterol transporter localized on the brush border membrane of enterocytes (2). As previously shown, a lack of NPC1L1 results in reduced plasma cholesterol and low-density lipoprotein (LDL) and improved fatty liver in high-cholesterol diet fed mice (3), while mice fed with cholesterol-restricted diet demonstrated upregulation of intestinal NPC1L1 expression (4). Moreover, hepatocyte nuclear factors 4 α (HNF4α), sterol regulatory element-binding protein 2, and peroxisome proliferator-activated receptor α agonist, fenofibrate, positively activated NPC1L1 transcription in hepatocellular carcinoma (HepG2) cells (5,6). HNF1α also increased NPC1L1 promoter activity and mRNA expression in human hepatocyte derived cellular carcinoma cells (7).

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.258485

*Corresponding author: C. Srimaroeng
Tel: +66-53935362, Fax: +66-53935365
Email: chutima.srimaroeng@cmu.ac.th

On the other hand, activation of PPARδ down regulated intestinal NPC1L1 gene expression in hamsters (8). Since, the liver controls whole body cholesterol homeostasis, this organ plays an important role in *de novo* cholesterol synthesis through a rate limiting step enzyme, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), and through controlling reversed cholesterol transport pathway. At present, ezetimibe and statins are prescribed as lipid-lowering drugs which target NPC1L1 and HMGR, respectively (9,10); and nonresponsive individuals and adverse effects have been reported (11,12).

Natural supplements are gaining attention as alternative treatments for hypercholesterolemia. Several natural plants show potential hypocholesterolemic and anti-atherosclerotic effects. For instance, curcumin inhibits cholesterol uptake by decreasing levels of NPC1L1 protein and mRNA expression in intestinal Caco-2 cells (13). The combination of garlic and ezetimibe decreased cholesterol level, low-density lipoprotein cholesterol levels, liver weight and atherogenic index (14). Moreover, major polyphenols in grape seed (*e.g.*, gallic acid, catechin, and epicatechin) lower cholesterol by inhibiting pancreatic cholesterol esterase activity, binding to bile acids, and suppressing solubility of cholesterol in micelles as shown by delayed cholesterol absorption (15). Recently, bitter melon aqueous extract was shown to decrease intestinal cholesterol absorption via inhibition of pancreatic cholesterol esterase and micelle formation (16), and *Morus indica* (mulberry) inhibited HMGR activity, suggesting it also has a role in reducing cholesterol levels (17).

Azadirachta indica A. Juss var. siamensis Valeton (A. indica) is well-known as neem and belongs to the Meliaceae family. Neem is native to East India and Burma, and grows widely in South East Asia including Thailand (18). Each part of the neem tree is used in traditional ayurvedic medicine and several active compounds have been isolated from different parts including azadirachtin, nimbidin, nimbin, nimbinin, nimbidinin, nimbolide, nimbidic acid, nimbidin, sodium nimbidate (18), quercetin, and ß-sitosterol (19). Neem shows a variety of pharmacological effects such as antipyretic, antiviral, analgesic, antibacterial, contraceptive, hepatoprotective (18), and anti-dyslipidemic (20). In addition, neem's ethanolic leaf extract reduces total cholesterol, LDL, VLDL, and triglyceride in streptozotocin-induced diabetic rats (20,21). However, the mechanisms behind the hypocholesterolemic effect of *A. indica* flower extract remains unclear. The present study investigated the effects of metanolic *A. indica* flower extract on cholesterol absorption and synthesis using intestinal Caco-2 cells and *in vitro* studies. The cholesterol-lowering mechanisms of *A. indica* flower extract were also identified.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified eagle medium (DMEM-F12), fetal bovine serum (FBS), penicillin and streptomycin solution, and trypsin-ethylenediaminetetra acetic acid were purchased from Life Technologies (Eugene, OR, USA). Phosphate buffered saline (PBS) was obtained from Biochrom AG (Berlin, Germany). HMG-CoA reductase assay kit was purchased from Sigma Chemical Co. (St. Louis, MO, USA). (1α,2α(n)-^{3}H) Cholesterol (specific activity, 49 Ci/mmol) was purchased from Perkin-Elmer (Wellesley, MA, USA). NPC1L1 antibody was bought from Novus Biologicals (Littleton, CO, USA). Ezetimibe was purchased from Schering-Plough Research Institute (Kenilworth, NJ, USA). Folin-Ciocalteu reagent, sodium bicarbonate, 1,2-di-O-lauryl-rac-glycero-3-glutaric acid 6'-methylresorufin ester, taurocholic acid sodium salt hydrate, glycodeoxycholic acid, taurodeoxycholic acid, and hydrazine hydrate solution were received from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemical reagents used in this study were obtained from commercial sources.

Methanolic extract of A. indica preparation

A. indica was harvested at Tumbon Maeka, Phayao District Mueang Phayao, Phayao Province, Thailand. The plant was identified by a botanist, and plant specimens were collected into herbarium of the Faculty of Biology, Naresuan University, Phitsanulok, Thailand (voucher specimen No. 003805). Flower part of *A. indica* was collected and dried in a hot air oven at 37 °C.

Dried materials were cut and ground into small pieces. One hundred g of the dried plant material were weighed and extracted twice with 300 mL methanol (95%) by reflux extraction. Subsequently, the extract was filtered and evaporated in a rotavapor apparatus at 55-60 °C. Crude extract was stored at -20°C and dissolved in dimethylsulfoxide (DMSO) prior to use.

Total phenolic content determination

The content of total phenolic compounds in *A. indica* methanolic extract was determined according to the Folin-Ciocalteu method (22). Briefly, crude extract at 2 mg/mL was mixed with Folin-Ciocalteu reagent and Na_2CO_3 solution, and incubated at room temperature for 30 min. Total phenolic content of the mixture solution was measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard phenolic content. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g of dry material.

Pancreatic cholesterol esterase activity determination

Pancreatic cholesterol esterase activity was determined using p-nitrophenyl butyrate as described previously (15). Briefly, *A. indica* extract at 0.01-10 mg/mL was incubated with mixtures containing 5.16 mM taurocholic acid, 0.2 mM p-nitrophenyl butyrate, and 100 mM NaCl at pH 7.0. The reaction was initiated by adding porcine pancreatic cholesterol esterase (1 mg/mL) and incubating for 5 min at 25 °C. Liberated p-nitrophenoxide was determined by measuring absorbance at 405 nm. Inhibition of pancreatic cholesterol esterase activity was compared with control (absence of *A.indica* extract). The data are shown as the half maximal inhibitory concentration (IC_{50}).

Cholesterol micelle size determination

Cholesterol micelles were prepared by addition of 1 µM cholesterol and 50 µM phosphatidylcholine dissolved in chloroform, 2 mM sodium taurocholate dissolved in methanol, and evaporated with N_2 gas which modified from Yamanashi *et al*. (23) and Kirana *et al.* (24). Reconstituted cholesterol micelle with PBS was filtered through 0.22 µm membrane to obtain the similar size range of micelle cholesterol particles and *A. indica* extract was subsequently co-incubated at 37 °C for 3 h. Particle size was measured using a particle size analyzer (Zetasizer Nano, Malvern Instruments, Malvern, UK).

Micellar cholesterol solubility assay

The method to determine the solubility of cholesterol in micelle was modified from Kirana *et al.* (24). Briefly, *A. indica* extract (12.5, 25, 50, 75, and 100 µg/mL) was incubated with micelle solution containing 10 mM cholesterol, 1 mM sodium taurocholate, and 0.6 mM phosphatidylcholine for 3 h at 37 °C. The mixed solution was filtered through a 0.22 µm membrane to eliminate nonmicellar fraction. Cholesterol content in the filtrate was measured, defining the micellar cholesterol solubility.

Cell culture preparation

Human Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells at passages 2 to 22 were maintained in 25 cm^2 flasks in a 95% air, 5% CO_2 atmosphere in the presence of media containing DMEM/F-12, 1.2 g/L $NaHCO_3$, 10% FBS, and 100 unit/mL penicillin-streptomycin solution.

Cell viability assay

Viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT). Briefly, cells were plated at 2.5×10^4 cells/well in a 96-well plate for 24 h. Cells were treated with *A. indica* extract (12.5, 25, 50, 75, or 100 µg/mL) for 3, 24, and 48 h, respectively. Subsequently, MTT solution (5 mg/mL) was added and incubated for 4 h. At the end of experiment, MTT solution was aspirated and the formazan product was eluted from cells by the addition of DMSO for 30 min. Absorbance of dissolved formazan was measured at 595 nm using Synergy™ HT microplate reader (Biotek, VT, USA). Cell viability was calculated according to the following equation:

Cell viability (%) = (absorbance of treated group/absorbance of control group) × 100

Cholesterol uptake study

To determine the effect of *A. indica* extract on cholesterol transport in human intestinal epithelial cells, Caco-2 cells were seeded at a density of 5×10^4 cells/mL into a 24-well plate and were grown for 18-21 days. Differentiated Caco-2 cells were incubated in medium containing 1 μCi/mL of [^{3}H]-cholesterol micelles in the presence or absence of *A. indica* extract at various concentrations (12.5, 25, 50, 75, or 100μg/mL), and 40 μg/mL of ezetimibe, cholesterol absorption inhibitor, for 3 h. At the end of the experiment, cells were washed three times with ice-cold PBS and were lyzed with 1 N sodium hydroxide and neutralized by 1 N hydrochloric acid. Radioactivity was quantified by liquid scintillation spectroscopy (Perkin Elmer, MA, USA). Uptake of cholesterol was calculated as fmole and normalized by mg protein using Bradford protein assay (Bio-Rad, CA, USA).

Subcellular fractionation and western blot analysis

To measure protein expression of cholesterol transporter, NPC1L1, subcellular fractions extracted from Caco-2 cells were prepared using differential centrifugation. Treated cells (with A. indica extract at 100 μg/mL) and control cells (without extract) were lysed using CelLytic MT mammalian tissue lysis/extraction reagent (Sigma-Aldrich Co., MO, USA) containing 1% complete protease inhibitor mixture (Merck, Darmstadt, Germany) according to the manufacturer's protocol. Briefly, the sample was homogenized and centrifuged at 5,000 *g* at 4 ºC for 10 min. Supernatant was designated as whole cell lysate. Half of the supernatant was recentrifuged at 100,000 *g* for 2 h. The supernatant fraction from this step was designated as cytosolic fraction while the pellet was resuspended by the same buffer and used as membrane fraction. Samples were stored at -80 °C prior to use. For western blot analysis, protein concentration was determined, resolved in 4× Laemmli solution, electrophoresed on 10% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (GE Healthcare, WI, USA). Non-specific binding was eliminated by blocking with 5% (w/v) non-fat dry milk in 0.05% Tween® 20 in tris-buffered saline for 1 h. Polyclonal anti-rabbit NPC1L1, monoclonal anti-mouse alkaline phosphatase, or anti-mouse β-actin antibody were incubated at 4 °C overnight. Polyvinylidene difluoride membrane was washed with tris-buffered saline and incubated with horseradish peroxidase-conjugated immunopure secondary goat antirabbit or antimouse IgG (Merck, Darmstadt, Germany) for 1 h. The target protein was detected using Super Signal West Pico Chemi-luminescent substrate (GE Healthcare, WI, USA) and quantitatively analyzed with the Image J program from the Research Services Branch of the National Institute of Mental Health (Bethesda, MD, USA).

Real time polymerase chain reaction analysis

To evaluate the effect of A. indica extract on PPARδ expression, total RNA was extracted and purified from Caco-2 cells. The cells were incubated in the presence (treated cells) or absence (control cells) of *A. indica* extract at 100 μg/mL using TRIzol® reagent (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instruction. The first strand cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad, CA, USA) and real time polymerase chain reaction (RT-PCR) was performed using SYBR RT-PCR master mix (Bioline, London, UK) on ABI 7500 (Life Technologies, NY, USA). Forward and reverse primers were purchased from Macrogen (Seoul, Korea) and used at a final concentration of 0.4 μM. Human PPARδ (forward primer: 5'-GTCACACAACGCTATCCGTTT-3', reverse primer: 5'-AGGCATTGTAGATGTGCTT GG-3') and human GAPDH (forward primer: 5'-AGCCTTC TCCATGGTGGTGAAAC-3', reverse primer: 5'-CGGAGTCAACGGATT TGGTCG-3'). Gene expression was normalized to GAPDH mRNA and reported as relative fold changes. RT-PCR amplification was performed in duplicate for each cDNA.

HMG-CoA reductase inhibitory activity

To determine the effect of *A. indica* extract on cholesterol synthesis, HMG-CoA reductase activity assay kit was used as recommended by the manufacturer (Sigma-Aldrich, MO, USA). The reaction containing A. indica extract at 100 μg/mL, NADPH, and different concentration

of HMG-CoA substrate (400, 800, and 1600 mg/mL) was initiated by addition of the catalytic domain of human recombinant HMG-CoA reductase. Pravastatin (0.25 μM) was used as an inhibitory control for the activity of HMGR. Oxidation of NADPH by the catalytic subunit of HMGR was measured using Synergy™ HT microplate reader (Biotek, VT, USA) at the wavelength of 340 nm at 37 °C with a kinetic program. HMGR activity was expressed as a percentage of inhibition compared with control (absence of test compound) and HMG-CoA at 400 μM.

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test. Statistical analyses were conducted using SPSS statistical software version 23 (IBM Corp., NY, USA). Differences were considered significant at $P < 0.05$.

RESULTS

Percent yield and total phenolic content of metanolic A. indica extract

A. indica flowers were extracted with 95% methanol. The percent yield and total phenolic content of the crude extract were 11.43% (w/w) and 23.73 ± 0.69 mg/L of gallic acid, respectively.

Effect of A. indica extract on pancreatic cholesterol esterase activity, cholesterol micelle size and solubility

As shown in Fig. 1A, *A.indica* extract's potent inhibitory effect on pancreatic cholesterol esterase was dose-dependent with an IC_{50} value of 3.36 ± 1.24 mg/mL. In addition, methanolic *A.indica* extract increased micelle size in a dose dependent manner (Fig. 1B). However, *A. indica* extract at 12.5-100 μg/mL had no effect on cholesterol micelle solubility (Fig. 1C), indicating potential inhibition of cholesterol absorption by *A. indica via* partial modulation of pancreatic cholesterol esterase enzyme and cholesterol micelle size.

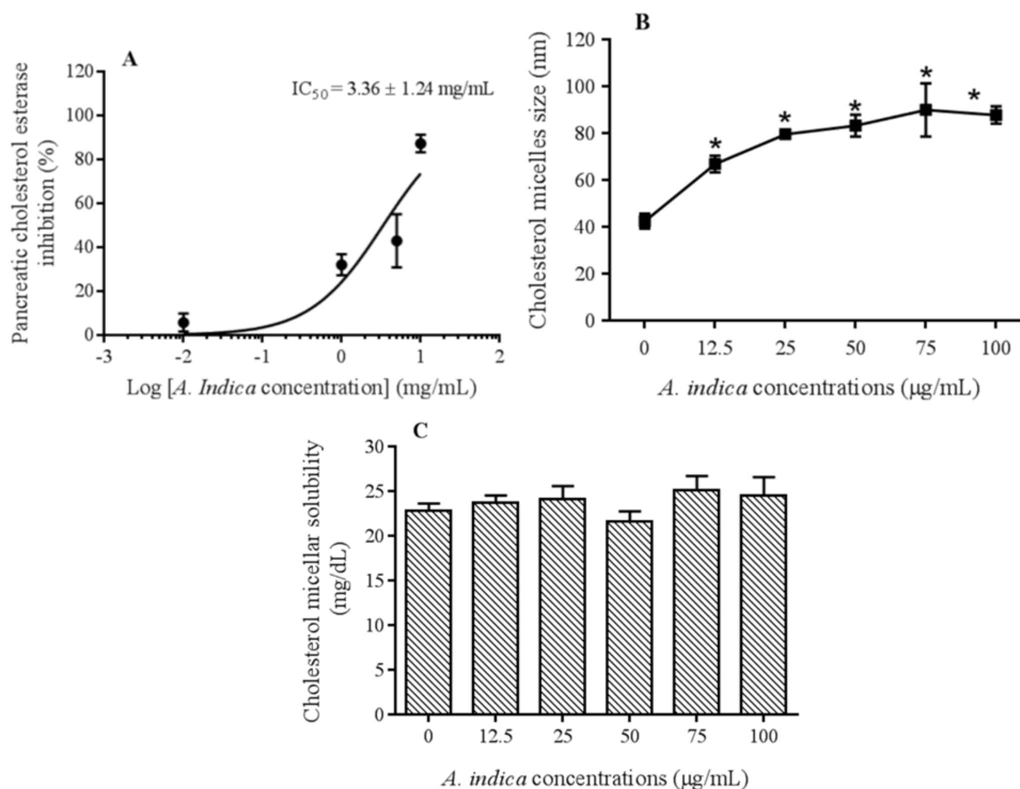

Fig. 1. Effect of *A. indica* extract on physicochemical property of cholesterol micelles on (A) pancreatic cholesterol esterase activity represents as the inhibitory concentration at 50% (IC_{50}) (n = 3), (B) cholesterol micelle particle size, and (C) intermicellar cholesterol levels. Values are represented as mean ± SEM; n = 4; * $P < 0.05$ compared with control. *A. indica, Azadirachta indica*.

Effect of A. indica extract on cholesterol uptake and cell viability in Caco-2 cells

Differentiated Caco-2 cells were incubated with serum-free medium containing 1 µCi/mL of [^{3}H]-cholesterol micelles in the presence or absence of either *A. indica* extract at 12.5, 25, 50, 75, and 100 µg/mL or 40 µg/mL of ezetimibe for 3 h. *A. indica* extract decreased cholesterol uptake in a dose-dependent manner at a maximum inhibition much like ezetimibe (Fig. 2A). Furthermore, *A. indica* extract at 12.5-100 µg/mL did not interfere cell viability at 3, 24, or 48 h, respectively (Fig. 2B-2D), indicating *A. indica* extract inhibited cholesterol absorption without any cytotoxic effect.

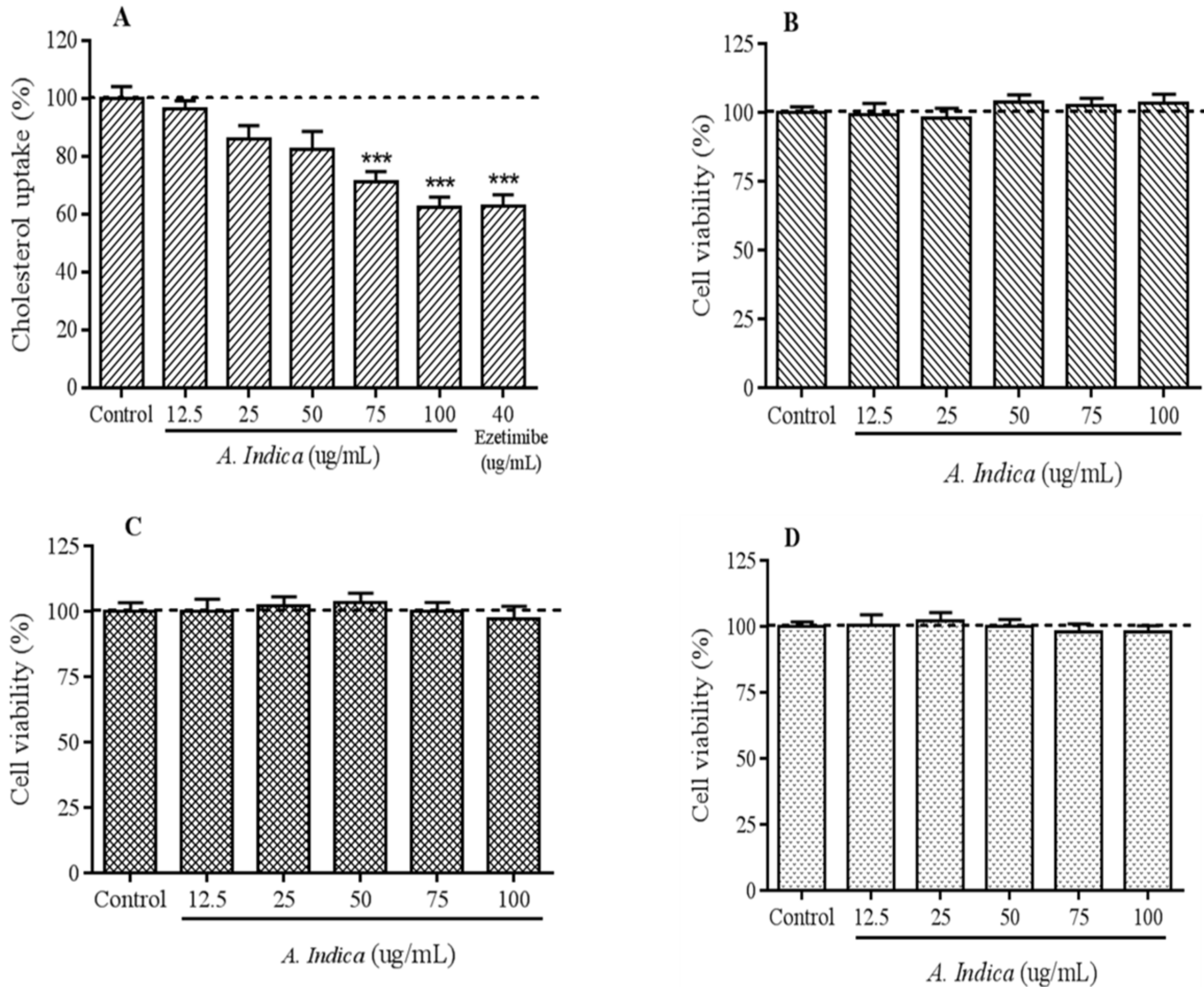

Fig. 2. Effect of *A. indica* extract on cholesterol uptake and cell viability in differentiated Caco-2 cells. (A) Cells were incubated with different concentration of *A. indica* extract at 12.5, 25, 50, 75, and 100 µg/mL or 40 µg/mL of ezetimibe for 3 h at 37 °C. The radioactivity of [3H]-micelle cholesterol was measured and expressed as percent of control. Viability of Caco-2 cells after exposure to either *A. indica* extract or ezetimibe for (B) 3 h, (C) 24 h, and (D) 48 h was determined using MTT assay. Each experiment was performed separately, n = 5. *** Indicates significant differences compared with control ($P < 0.001$). *A. indica, Azadirachta indica*.

Effect of A. indica extract on niemann-pick C1 like 1 expression in Caco-2 cells

To further determine whether *A. indica* extract interferes NPC1L1 expression or trafficking, the maximum and effective dose of *A. indica* on cholesterol absorption inhibition at 100 μg/mL was used to determine cellular protein expression of NPC1L1 using western blot analysis. Membrane and cytosolic NPC1L1 protein expression significantly increased by *A. indica* extract when compared with control cells (Fig. 3). The findings indicated *A. indica* extract may have blocked NPC1L1 trafficking and internalization, consequently interfering with cholesterol absorption.

Effect of A. indica extract on peroxisome proliferator-activated receptor δ in Caco-2 cells

Since PPARδ activation has previously been shown to decrease NPC1L1 mRNA expression in Caco-2 cells (8), the next step was to determine if *A. indica* extract at 100 μg/mL regulates PPARδ. As shown in Fig. 4, *A. indica* extract down-regulated PPARδ mRNA expression, leading to increased membrane and cytosolic NPC1L1 protein expression.

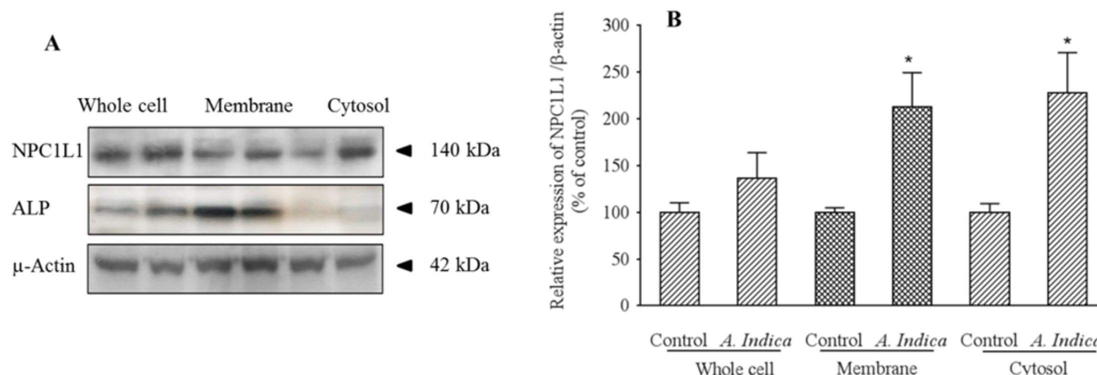

Fig. 3. NPC1L1 expression in 100 ug of whole cell, 50 ug of membrane, and 50 ug of cytosolic fractions extracted from Caco-2 cells. Cells were incubated with 100 ug/mL of A. indica for 3 h. NPC1L1 proteins were detected using anti-NPC1L1 antibody. ALP and anti-β-actin antibodies were also used as apical membrane marker and loading control, respectively. Data are expressed as mean ± SEM, n = 5. (A) A representative blot of NPC1L1 and β-actin protein expressions and (B) indicated quantification of relative NPC1L1/β -actin. * Shows significant differences compared to control, $P < 0.05$. NPC1L1, niemann-pick C1 like 1; A. indica, Azadirachta indica; ALP, anti-alkaline phosphatase.

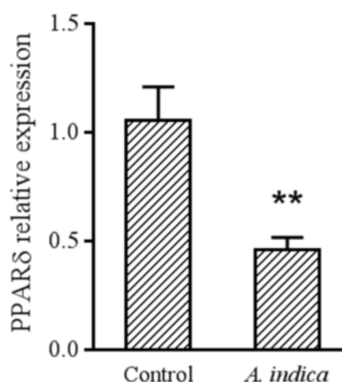

Fig. 4. Effect of *A. indica* extract on PPAR δ mRNA expression in Caco-2 cells. Cells were incubated with 100 μg/mL of *A. indica* extract for 3 h. Total RNAs were extracted from Caco-2 cells and PPAR δ mRNA level was determined using quantitative polymerase chain reaction. Data are expressed as mean ± SEM from 3 separate experiments. ** Indicates significant differences compared to control, $P < 0.01$. *A. indica, Azadirachta indica;* PPAR, peroxisome proliferator-activated receptor.

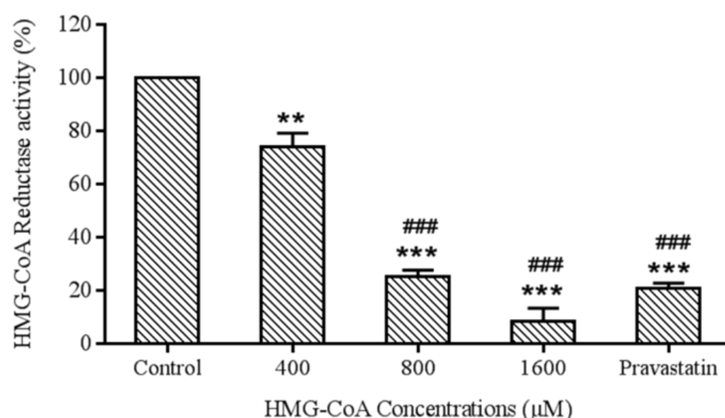

Fig. 5. Effect of *A. indica* extract on HMG-CoA reductase activity using *in vitro* cell-free based assay. *A. indica* extract at 100 µg/mL or pravastatin 0.25 µM was incubated with HMG-CoA at 400, 800, and 1600 mg/mL. Value represented as mean ± SEM, n = 3. **, *** Indicate significant differences compared with control, $P < 0.01$ and $P < 0.001$. ### Shows significant differences in comparison with HMG-CoA at 400 µM, $P < 0.001$. *A. indica, Azadirachta indica;* HMG-CoA, 3-hydroxy 3-methylglutaryl-CoA.

Effect of A.indica extract on HMG-CoA reductase activity

To determine if *A. indica* extract inhibits cholesterol synthesis, HMGR activity was evaluated. NADPH concentration was fixed and HMG-CoA substrate was used in the range of 400-1600 mg/mL in the presence of either *A. indica* extract (0.1 mg/mL) or pravastatin (0.25 µM). Oxidation of NADPH by the catalytic subunit of HMGR was measured. As shown in Fig. 5, *A. indica* extract inhibited HMGR activity in a dose-dependent manner, indicating *A. indica* methanolic extract directly inhibited HMGR activity resulting in decreased endogenous cholesterol synthesis.

DISCUSSION

This study examined the cholesterol-lowering mechanisms of methanolic *A. indica* extract, specifically, on inhibition of cholesterol transport and interference in cholesterol metabolism. Since cholesterol absorption is a multi-step process, factors interfering with digestion, micelle formation, or transport influence intestinal cholesterol absorption (1). Previous studies report anti-dyslipidemic properties of ethanolic *A. indica* leaf extract in streptozotocin-induced diabetic rats (20,21). As pancreatic cholesterol esterase hydrolyzes cholesterol esters into un-esterified cholesterol and free fatty acids, a deficit of this enzyme leads to accumulation of cholesterol esters and triglycerides in cells and tissues (25). Accordingly, inhibiting cholesterol esterase has been suggested as a useful agent to lower cholesterol levels (26). In this study, methanolic *A. indica* extract demonstrated an inhibitory effect against pancreatic cholesterol esterase activity similar to a previous *in vitro* study where polyphenols present in grape seed inhibited pancreatic cholesterol esterase (15). Another study, using phytochemical screening, showed *A. indica* contains secondary metabolites such as alkaloids, glycosides, flavonoids, and polyphenols (27). Moreover, the methanolic *A. indica* seed oil extracts also revealed the presence of saponins, terpenes, tannins and steroids (18,28). Our recent study and other independent studies also revealed that *A. indica* plant contains steroids such as campesterol, beta-sitosterol, and stigmasterol (29-31). Taken together, it is possible the polyphenols in *A. indica* extract enacted an inhibitory effect on pancreatic cholesterol esterase activity, subsequently diminishing lipid digestion and limiting intestinal cholesterol absorption.

Additionally, modulated cholesterol micelle size and solubility interfere with cholesterol transport and inhibition of its absorption. A major constituent of green tea catechins, epigallocatechin-3-gallate, alters the physicochemical properties of lipid

emulsion by increasing particle size, leading to an interference of intestinal lipid absorption (32). Sitosterol, the most common dietary plant sterol, also reduces micellar cholesterol solubility by displacing cholesterol from micelles (33). Similar to *A. indica*, ß-sitosterol at a greater concentration demonstrated a greater reduction of micellar cholesterol solubility (33), and this compound subsequently inhibited intestinal cholesterol absorption (33,34). Thus, *A. indica* extract containing β-sitosterol (19) might reduce cholesterol absorption by disturbing micelle formation.

Previous study reports that cholesterol absorption in NPC1L1-deficient mice has been reduced while ezetimibe treatment does not produce the same effect. This indicates NPC1L1 plays a critical role in intestinal cholesterol absorption (2). Consistently, the present study showed *A. indica* extract reduced cholesterol uptake in a dose-dependent manner similar to ezetimibe (Fig. 2A). Several pieces of evidence exhibited point to involvement of polyphenols in cholesterol absorption. For instance, curcumin and quercetin decreased NPC1L1 protein and mRNA expressions in Caco-2 cells, resulting in reduced cholesterol absorption (34) similar to the way grape seed and red wine, which are rich in polyphenols, inhibit cholesterol uptake in human epithelial cells – HT29, HepG2, and Caco-2 cells (35). Previous studies reported that limonoids, flavonoids, rutin, and quercetin can be isolated from methanolic extract of *A. indica* flowers (36,37). Hence, *A. indica* extract may inhibit cholesterol transport *via* polyphenol activity. This study also further demonstrated that *A. indica* extract up-regulated NPC1L1 protein expression in membrane and cytosolic fractions (Fig. 3) and exhibited a statin-like effect by inhibiting HMGR activity (Fig. 4). Likewise ezetimibe, a cholesterol absorption inhibitor, blocks internalization of NPC1L1 causing retention on plasma membrane (38) while atorvastatin, an HMGR inhibitor, induces total protein expression of NPC1L1 for 33% by likely activating sterol regulatory element-binding protein (SREBP)-2 and HNF4-α in hyperlipidemic patients (39). Hence, *A. indica* may dominantly block NPC1L1/cholesterol internalization, leading to an increase in membrane NPC1L1 expression. Furthermore, it may also induce transcription factors regulated NPC1L1 expression, SREBP-2, and HNF4-α, similar to that of atorvastatin's effect as previously shown, resulting in up-regulation of cytosolic NPC1L1 expression. In addition, activation of PPARδ reduces NPC1L1 mRNA expression in Caco-2 cells (8), and cholesterol levels significantly decrease in PPARδ knock-in mice (40). Together, these data suggest that PPARδ controls cholesterol level partly through up-regulation of NPC1L1. In this study *A. indica* down-regulated PPARδ mRNA expression. Thus, *A. indica* extract may have reduced cholesterol transport independently from PPARδ. Further study could identify the molecules responsible for *A. indica* mediated transcriptional regulation of NPC1L1.

Human HMGR contains three major domains, catalytic, linker, and anchor and the active sites in the catalytic domain reduce HMG-CoA substrate (41). Here, *A. indica* extract exhibits direct inhibition of HMGR activity. Likewise, phenols extracted from grapefruit peels (42) and *Moringa oleifera* leaves (43) inhibit HMGR activity. By binding to HMGR and blocking nicotinamide adenine dinucleotide phosphate ($NADP^+$) binding sites, the latter polyphenols partially occupy active sites, hence inhibiting HMGR activity (44). Thus, *A. indica* extract not only interferes with cholesterol absorption, but also inhibits *de novo* cholesterol synthesis, suggesting *A. indica* extract could be developed as a nutraceutical to lower lipid levels due to its concomitant mechanisms of cholesterol homeostasis.

CONCLUSION

Hypocholesterolemic action of methanolic *A. indica* flower extract appears to involve inhibition of cholesterol absorption and synthesis. Reducing cholesterol uptake, pancreatic cholesterol esterase activity as well as interfering with physiochemical properties of cholesterol micelles and blocking HMGR activity were identified. The data indicate a vital role for *A. indica* in cholesterol-lowering treatment; however, further *in vivo* study

is needed to validate its hypocholesterolemic effects in humans.

ACKNOWLEDGEMENTS

This research was financially supported by the Faculty of Medicine Endowment Fund, Chiang Mai University, Chiang Mai, Thailand (66/2558 to CS and AD) and the NSTDA Chair Professor Grant (the Fourth Grant) of the Crown Property Bureau Foundation and the National Science and Technology Development Agency to Professor Dr. Vatcharin Rukachaisirikul. The authors would like to thank Ms. Sineenart Santidherakul, Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University, Thailand, for technical assistance.

REFERENCES

1. Wang DQ. New concepts of mechanisms of intestinal cholesterol absorption. Ann Hepatol. 2003;2(3):113-121.
2. Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, *et al*. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303(5661):1201-1204.
3. Telford DE, Sutherland BG, Edwards JY, Andrews JD, Barrett PH, Huff MW. The molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin. J Lipid Res. 2007;48(3):699-708.
4. Davis HR Jr, Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, *et al*. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J Bio Chem. 2004;279(32):33586-33592.
5. Iwayanagi Y, Takada T, Suzuki H. HNF4α is a crucial modulator of the cholesterol-dependent regulation of NPC1L1. Pharm Res. 2008;25(5):1134-1141.
6. Iwayanagi Y, Takada T, Tomura F, Yamanashi Y, Terada T, Inui K, *et al*. Human NPC1L1 expression is positively regulated by PPARα. Pharm Res. 2011;28(2):405-412.
7. Pramfalk C, Jiang ZY, Cai Q, Hu H, Zhang SD, Han TQ, *et al*. HNF1α and SREBP2 are important regulators of NPC1L1 in human liver. J Lipid Res. 2010;51(6):1354-1362.
8. Mathur SN, Watt KR, Field FJ. Regulation of intestinal NPC1L1 expression by dietary fish oil and docosahexaenoic acid. J Lipid Res. 2007;48(2):395-404.
9. McKenney JM. Pharmacologic characteristics of statins. Clin Cardio. 2003;26(4 Suppl 3):32-38.
10. Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, *et al*. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci U S A. 2005;102(23):8132-8137.
11. Florentin M, Liberopoulos EN, Elisaf MS. Ezetimibe-associated adverse effects: what the clinician needs to know. Int J Clin Pract. 2008;62(1):88-96.
12. Golomb BA, Evans MA. Statin adverse effects : a review of the literature and evidence for a mitochondrial mechanism. Am J Cardiovasc Drugs. 2008;8(6):373-418.
13. Feng D, Ohlsson L, Duan RD. Curcumin inhibits cholesterol uptake in Caco-2 cells by down-regulation of NPC1L1 expression. Lipids Health Dis. 2010;9(1):40-44.
14. Abbasi Oshaghi E, Mohammadi A, Zynolebadi N, Nabi Moradi M, Ghasemi H, Totonchi A, *et al*. New insight in ezetimibe/garlic combination in hypercholesterolemic mice. Res Pharm Sci. 2012;7(5):S757.
15. Ngamukote S, Mäkynen K, Thilawech T, Adisakwattana S. Cholesterol-lowering activity of the major polyphenols in grape seed. Molecules. 2011;16(6):5054-5061.
16. Su J, Wang H, Ma C, Liu C, Gao C, Nie R, *et al*. Hypocholesterolaemic mechanism of bitter melon aqueous extracts via inhibition of pancreatic cholesterol esterase and reduction of cholesterol micellar solubility. Int J Food Sci Nutr. 2016;67(1):20-28.
17. Reddy Palvai V, Urooj A. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*ex vivo*) by *Morus indica* (Mulberry). Chinese Journal of Biology. 2014;2014. Article ID: 318561.
18. Nishan M, Subramanian P. Pharmacological and non pharmacological activity of Azadirachta indica (Neem) -A review. Int J Biosci. 2014;5:104-112.
19. Mahmoud DA, Hassanein NM, Youssef KA, Abou Zeid MA. Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens. Braz J Microbiol. 2011;42(3):1007-1016.
20. Bisht S, Sisodia S. Anti-hyperglycemic and antidyslipidemic potential of *Azadirachta indica* leaf extract in STZ-Induced diabetes mellitus. J Pharm Sci Res. 2010;2(10):622-627.
21. Chattopadhyay RR, Bandyopadhyay M. Effect of *Azadirachta indica* leaf extract on serum lipid profile changes in normal and streptozotocin induced diabetic rats. Afr J Biomed Res. 2005;8:101-104.
22. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vicult. 1965;16(3):144-158.
23. Yamanashi Y, Takada T, Suzuki H. Niemann-Pick C1-like 1 overexpression facilitates ezetimibe-sensitive cholesterol and β-sitosterol uptake in CaCo-2 cells. J Pharmacol Exp Ther. 2007;320(2):559-564.
24. Kirana C, Rogers PF, Bennett LE, Abeywardena MY, Patten GS. Naturally derived micelles for rapid

in vitro screening of potential cholesterol-lowering bioactives. J Agric Food Chem. 2005;53(11): 4623-4627.
25. Stępień AE, Gonchar M. A simple method for the determination of the cholesterol esterase activity. Acta Biochim Pol. 2013;60(3):401-403.
26. Heidrich JE, Contos LM, Hunsaker LA, Deck LM, Vander Jagt DL. Inhibition of pancreatic cholesterol esterase reduces cholesterol absorption in the hamster. BMC Pharmacol. 2004;4:5. DOI: 10.1186/1471-2210-4-5.
27. Johnson UE, Akwaji PI, Effiong US, Aniedi-Abasi M, Effiom OE. Phytochemical composition, antimicrobial effect of *Azadirachta indica* and carica papaya extracts on fungi isolated from gmelina arborea seedling. Int J Phytopathol. 2014;3(3): 109-115.
28. Muhammad H, Kabiru A, Makun H, Adefolalu F, Fasiku O, Abdullah A. Evaluation of methanolic and ethanolic extracts of *Azadirachta indica* seed oil for hypoglycaemic properties and effects on some biochemical parameters in diabetic mice. Iosr J Pharmacy. 2013;3(3):52-58.
29. Duangjai A, Nuengchamnong N, Lee LH, Goh BH, Saokaew S, Suphrom N. Characterisation of an extract and fractions of Azadirachta indica flower on cholesterol lowering property and intestinal motility. Nat Prod Res. 2017:1-4.
30. Sitasiwi AJ, Isdadiyanto S, Mardiati SM. Effect of ethanolic Neem (*Azadirachta indica*) leaf extract as an herb contraceptive on Hepato-somatic Index of the male mice (Mus musculus). J Phys: Conf Series; 2018. DOI :10.1088/1742-6596/1025/1/012043.
31. Maithani A, Parcha V, Pant G, Dhulia I, Kumar D. *Azadirachta indica* (neem) leaf: a review. J Pharm Res. 2011;4(6):1824-1827.
32. Koo SI, Noh SK. Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. J Nutr Biochem. 2007;18(3):179-183.
33. Jesch ED, Carr TP. Sitosterol reduces micellar cholesterol solubility in model bile. Nutr Res. 2006;26(11):579-584.
34. Nekohashi M, Ogawa M, Ogihara T, Nakazawa K, Kato H, Misaka T, *et al*. Luteolin and quercetin affect the cholesterol absorption mediated by epithelial cholesterol transporter niemann–pick c1-like 1 in caco-2 cells and rats. PloS one. 2014;9(5):e97901.
35. Leifert WR, Abeywardena MY. Grape seed and red wine polyphenol extracts inhibit cellular cholesterol uptake, cell proliferation, and 5-lipoxygenase activity. Nutr Res. 2008;28(12):842-850.
36. Chaisawangwong W, Gritsanapan W. Quality assessment and scavenging activity of *Siamese neem* flower extract. Nat Prod Res. 2013;27(4-5):394-401.
37. Kitdamrongtham W, Ishii K, Ebina K, Zhang J, Ukiya M, Koike K, *et al*. limonoids and flavonoids from the flowers of *Azadirachta indica* var. siamensis, and their melanogenesis-inhibitory and cytotoxic activities. Chem Biodivers. 2014;11(1): 73-84.
38. Xie C, Zhou ZS, Li N, Bian Y, Wang YJ, Wang LJ, *et al*. Ezetimibe blocks the internalization of NPC1L1 and cholesterol in mouse small intestine. J Lipid Res. 2012;53(10):2092-2101.
39. Tremblay AJ, Lamarche B, Lemelin V, Hoos L, Benjannet S, Seidah NG, *et al*. Atorvastatin increases intestinal expression of NPC1L1 in hyperlipidemic men. J Lipid Res. 2011;52(3): 558-565.
40. Gross B, Hennuyer N, Bouchaert E, Rommens C, Grillot D, Mezdour H, *et al*. Generation and characterization of a humanized PPARδ mouse model. Br J Pharmacol. 2011;164(1):192-208.
41. Friesen JA, Rodwell VW. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol. 2004;5(11):248-254.
42. Ademosun AO, Oboh G, Passamonti S, Tramer F, Ziberna L, Boligon AA, *et al*. Phenolics from grapefruit peels inhibit HMG-CoA reductase and angiotensin-I converting enzyme and show antioxidative properties in endothelial EA. Hy 926 cells. Food Sci Hum Wellness. 2015;4(2):80-85.
43. Reddy VP, Ahmed F, Urooj A. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme a (hmg co-a) reductase in liver microsomes by *Moringa Oleifera* L. polyphenols. Int J Pharm Sci Res. 2012;3(8):2510-2516.
44. Islam B, Sharma C, Adem A, Aburawi E, Ojha S. Insight into the mechanism of polyphenols on the activity of HMGR by molecular docking. Drug Des Devel Ther. 2015;9:4943-4951.