นิพนธ์ต้นฉบับ ▪ Original Article

สมุนไพรที่เป็นส่วนประกอบของพิกัดนวโกฐ 4 ชนิดควบคุมเมแทบอลิซึมของโคเลสเตอรอลโดยผ่านการแสดงออกของยีน *LDLR, HMGCR, SR-BI* และ *ApoA-1*

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Four Main Components of Phikud Navakot Promote Cholesterol Metabolism Through *LDLR, HMGCR, SR-BI* and *ApoA-1* Genes

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หลักการและวัตถุประสงค์: ยาหอมนวโกฐ (Ya-Hom Navakot; NY) ประกอบด้วยสมุนไพร 54 ชนิด เป็นตำรับยาไทยที่ใช้กันมานานนับศตวรรษ เพื่อใช้แก้ลม และบรรเทาอาการวิงเวียน สมุนไพรที่เป็นส่วนประกอบหลักของยาหอมนวโกฐ หรือที่เรียกว่าพิกัดนวโกฐ (Phikud Navakot;PN) มีอยู่ 9 ชนิด ซึ่งมีสรรพคุณช่วยควบคุมสภาวะสมดุลของโคเลสเตอรอลผ่านการแสดงออกของยีน *LDLR* และ *HMGCR* ได้ดีกว่ายาหอมนวโกฐ โครงการศึกษานี้ จึงต้องการศึกษาว่าฤทธิ์ลดโคเลสเตอรอลของพิกัดนวโกฐ เกิดจากสมุนไพรเดี่ยวที่เป็นส่วนประกอบหลักของยาหอมนวโกฐ ชนิดใด เพื่อเป็นแนวทางในการนำเอาสมุนไพรในพิกัดนวโกฐไปใช้เป็นทางเลือกในการลดโคเลสเตอรอลต่อไป

วิธีการศึกษา: ฤทธิ์ลดไขมันของสารสกัดแอลกอฮอล์จากสมุนไพรเดี่ยวที่เป็นส่วนประกอบของพิกัดนวโกฐทั้ง 9 ชนิด จะทำการศึกษาจากการแสดงออกของยีน *LDLR, HMGCR, SR-BI* และ *ApoA-1* ในเซลล์ HepG2 โดยใช้วิธี quantitative real-time PCR (qRT-PCR)

ผลการศึกษา: สมุนไพรเดี่ยวที่เป็นส่วนประกอบของพิกัดนวโกฐทั้ง 9 ชนิด มีฤทธิ์ยับยั้งการแสดงออกของยีน *HMGCR* เมื่อเปรียบเทียบกับยาซิมวาสแตติน นอกจากนี้ สารสกัดจากโกฐสอ (*Angelica dahurica*; AD), โกฐเขมา (*Atractylodes lancea*;

Background and Objectives: Ya-Hom Navakot (NY), a combination of fifty-four Thai medicinal herbs, has been used as a traditional medicine for decades especially when dizziness and fainting. Phikud Navakot (PN), nine selected herbal remedies from those components in NY, regulates *HMGCR* and *LDLR* genes leading to enhance cholesterol homeostasis. The cholesterol-lowering effect of PN is found to be more potent than that observed in NY. Hence, the objective of this study was to assess the cholesterol-lowering effect of all nine individual herbal extracts of PN which might be used as an alternative treatment for hypercholesterolemia.

Methods: Lipid lowering effect of the ethanolic extract of all nine individual herbal extracts of PN was examined focusing on expression of the genes encoding *LDLR, HMGCR, SRBI* and *ApoA-1* in HepG2 cells by quantitative real-time PCR (qRT-PCR).

Results: The ethanolic extracts from all nine individual herbs of PN were found to downregulate

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AL) โกฐชฎามังสี (*Nardostachys jatamansi*; NJ) มีฤทธิ์กระตุ้นการแสดงออกของยีน *LDLR* และ *SR-BI* ส่วนสารสกัดจากโกฐจุฬาลัมพา (*Artemisia pallens*; AP) มีฤทธิ์กระตุ้นการแสดงออกของยีน *ApoA-I*

สรุป: ฤทธิ์ลดโคเลสเตอรอลของพิกัดนวโกฐ เป็นผลมาจากสมุนไพร 4 ชนิดที่เป็นส่วนประกอบของพิกัดนวโกฐ ซึ่งเกี่ยวข้องกับการลดโคเลสเตอรอล การรักษาภาวะสมดุลของโคเลสเตอรอลหรือการเพิ่มการสลายโคเลสเตอรอลโดยผ่านการควบคุมการแสดงออกของยีน *HMGCR, LDLR* และ *ApoA-1* ตามลำดับ จึงอาจกล่าวได้ว่า สมุนไพรในพิกัดนวโกฐ น่าจะเป็นอีกหนึ่งทางเลือกที่ใช้ในการลดโคเลสเตอรอลต่อไปในอนาคต

expression of the HMGCR gene comparing with the effect of simvastatin. The extracts of Kot Soa (*Angelica dahurica*; AD), Kot Khamao (*Atractylodes lancea*; AL), and Kot Jatamansri (*Nardostachys jatamansi*; NJ) could additionally upregulate the LDLR and SRB1 genes. Kot Chulalumpa (*Artemisia pallens*; AP) increased the expression of the ApoA1 gene.

Conclusions: Cholesterol-lowering effect of PN was attributable to the four ingredients of PN which possessed high capability to decrease cholesterol production, maintain cholesterol balance, and promote cholesterol clearance via regulation of the HMGCR, LDLR, and ApoAI genes, respectively. Hence, PN might be an alternative tool to reduce cholesterol level in the future.

Keywords: Hypercholesterolemia, Phikud Navakot, *LDLR*, *HMGCR*, *SR-BI*, *ApoA-I*

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Introduction

Hypercholesterolemia is a major risk factor in cardiovascular disease (CVD) progression which is more prevalent globally. In Thailand, CVD is reported to be the leading cause of mortality during 2007-2014[1, 2]. HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), a key enzyme in the mevalonate pathway, is characterized as a rate-limiting step in cholesterol biosynthesis[3]. Hence, it is the primary target in cholesterol-lowering drug therapy and was regulated at the transcription, translation, post-translational modification and degradation levels[3, 4]. Low-density lipoprotein receptor (LDLR), a cell surface transmembrane protein is a key receptor in maintaining cholesterol homeostasis 5. Furthermore, atherothrombosis with high levels of LDL-C and low levels of high-density lipoprotein-cholesterol (HDL-C) was shown as a major contributor for hypercholesterolemia-induced CVD[2]. *HDL-C*, a key mediator of a reverse cholesterol transport (RCT) involving in atheroprotection is mainly composed of apolipoprotein AI (*ApoA-1*)[5]. It is responsible for the removal of excess cholesterol from extrahepatic tissues back to the liver for biliary secretion or re-utilization via a scavenger receptor class B type I (SR-BI)[5]. Statin, a potent inhibitor of HMGCR, is one of the most popular therapeutic agents to treat atherogenic dyslipidemia[6]. Statin treatment was suggested to reduce plasma LDL-C levels by an upregulation of LDLR mRNA and elevate plasma HDL-C levels by an upregulation of *ApoA-1* mRNA[6]. However, statin treatment was discovered to have many adverse effects[7]. Seeking for an alternative dyslipidemic agent is, hence, a requirement. Ya-Hom Navakot (NY), a Thai polyherbal formula established from the Thai wisdom is widely used as a remedy for an anti-dizziness, antiflatulent, cardiotonic and improvement of blood circulation among the Thai people[7]. Additionally, it has been included in Thailand's list of Herbal Medicinal Products. NY is composed of 54 herbs. Its major ingredient or Phikud Navakot (PN) is a mixture of nine herbs in equal weight ratios including the roots of Kot Soa (*Angelica dahurica*; AD), Kot Chiang (*Angelica sinensis*; AS), and Kot Kradook (*Saussurea costus*; SC); the rhizomes of Kot Khamao (*Atractylodes lancea*; AL), Kot Huabua (*Ligusticum chuanxiong*; LC), and Kot Kanprao (*Picrorhiza kurrooa*; PK); the roots and rhizomes of Kot Jatamansi (*Nardostachys jatamansi*; NJ); the aerial parts of Kot Chulalumpa (*Artemisia pallens*; AP) and the galls of Kot Pungpla (*Terminalia chebula*; TC).

It has been reported that the hydroethanolic extract of PN is effective in improving vascular reactivity and composes of antioxidant properties[8-10]. PN is relatively safe as there was no observed treatment-related mortality in both acute and subchronic toxicity studies in rats[11]. The hydroethanolic extract of PN also preserved the

integrity and osmotic ability of red blood cells-induced oxidative stress via its antioxidative property[12]. In addition, NY and PN were found to upregulate *LDLR* gene expression but downregulate expression of *HMGCR* gene[13]. However, which composition of PN plays a major role in hypocholesterolaemic activity involving in LDL-C and HDL-C metabolisms is still unclear. Therefore, this study aimed to investigate the lipid lowering effect of the ethanolic extracts from nine medicinal plants of PN in comparison with Simvastatin drug on expression of the genes encoding LDLR, HMGCR, SRBI, *ApoA-1* which involve in lipoprotein metabolism in HepG2 cells.

Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, glutamine, non-essential amino acids and sodium pyruvate were purchased from GIBCO Laboratories (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Prolabo (Paris, France). Simvastatin (Zocor®) was from Berlin Pharmaceutical Co. Ltd. (Bangkok, Thailand). GENEzol™ reagent used for RNA extraction was obtained from Geneaid (New Taipei, Taiwan). Reagents for first-strand cDNA synthesis were available at ThermoFischer Scientific (Waltham, MA, USA). FastStart Essential DNA Green Master Kit used in quantitative real-time PCR (qRT-PCR) was from Roche (Mannheim, Germany). MTT [3-(4, 5 di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents were of analytical grade with the highest quality.

Plant materials and preparation of PN extracts

All 54 herbs, which are the ingredients of Ya-Hom Navakot (NY) polyherbal formulation including nine herbs which are the core composition of Phikud Navakot (PN) were purchased, examined and prepared by Dr. Sanya Hokputsa, the Research and Development Institute, Government Pharmaceutical Organization as previously described[13]. All plant mixtures were extracted by 50% ethanol (NYEF, PNEF). As a control, Plant materials excluding PN were prepared before extraction with 50% ethanol (NBEF) as described earlier. Stock solutions of all extracts (200 mg/ml) were dissolved in 100% DMSO (Dimethyl sulfoxide) before the extracts were aliquoted and kept at -20oC until use.

Cell culture

HepG2 cell line (ATCC HB-8065) was cultured in DMEM, supplemented with 10% FBS, antibiotics (100 units/ml of penicillin and 100 µg/mL of streptomysin), 1 mM sodium pyruvate, 2 mM glutamine and 0.1 mM non-essential amino acids (DMEM complete medium) at 37oC in a humidified atmosphere under 5% CO2 and 98% relative humidity[13].

Assay of the cytotoxicity of the hydroethanolic extracts from herbal plants on HepG2 cell survival

Cytotoxic effect of the ethanolic extracts from Phikud Navakot (PNEF), Ya-Hom Navakot (NYEF), Ya-Hom Navakot excluding PN (NBEF) and nine major herbal ingredients of PN were assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay modified from the previous study 14. Briefly, HepG2 cells (1×104 cells) were plated overnight in the 96-multiwell plates in DMEM complete medium. The cells were then individually treated with various concentrations (0.01, 0.05, 0.1, 0.5, and 1 mg/ml) of PNEF, NYEF, NBEF, nine individual herbs of PN (APEF, ADEF, ALEF, NJEF, ASEF, LCEF, PKEF, SCEF and TCEF) and simvastatin for 24 hrs. After incubation, the medium was aspirated. A solution of MTT was added before pre-incubation for 2 h. The medium was then replaced with DMSO to dissolve insoluble formazan. The amount of insoluble formazan was determined by measuring the absorbance at 570 nm using a microplate reader (Biorad, USA). Cytotoxicity of all herbal extracts was calculated as percentage of cell viability from the following equation from three independent experiments. Half maximal inhibitory concentration (IC50) was determined from the graph plotting between % cell viability and concentration of the herbal extracts.

Effect of PNEF, NYEF, NBEF and 9 major components of PN on expression of the genes involving in the LDL-C and HDL-C metabolisms

Lipid-lowering effect of all herbal extracts on

$$\% \text{ Cell viability} = \frac{(O.D_{570} \text{ of the test condition} - O.D_{570} \text{ of the blank}) \times 100}{(O.D_{570} \text{ of the standard control} - O.D_{570} \text{ of the blank})}$$

expression of *LDLR*, *HMGCR*, *ApoA-1* and *SRB1* genes was assessed by qRT-PCR. HepG2 cells were individually treated with DMEM complete medium containing 1 mg/ml of PNEF, NYEF and NBEF or various concentrations (0.01, 0.05, 0.1, 0.5, 1 mg/ml) of the 9 major ingredients of PN for 24 h. The cells were cultured in DMEM complete medium or DMEM complete medium containing 1 mg/ml of simvastatin as a negative and positive controls, respectively.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from untreated or herbal extract-treated HepG2 cells using Trizol reagent (Invitrogen, USA), following the manufacturer's instruction 15. The isolated RNA was used as a template to synthesize first strand cDNA using a RevertAid reverse transcriptase (ThermoFisher Scientific, U.S.A) according to the manufacturer's instruction. Briefly, 500 ng of the extracted RNA was added with oligo(dT)^{18} primer and the volume adjusted with DEPC water to 12.5 µl. Following incubation at 65°C for 5 min and another 5 min on ice, a reverse transcription step was performed in a final volume of 20 µl by the addition of 4 µl 5X reaction buffer, 0.5 µl RNase inhibitor (20 units), 2 µL of dNTP mix (1 mM final concentration), and 1 µL RevertAid reverse transcriptase (200 units). The reaction was incubated at 42°C for 60 minutes prior to termination by heating up to 70°C for 10 minutes. The synthesized cDNA was stored at -20°C until use.

Quantitative RT-PCR was performed using the FastStart Essential DNA Green Master Kit (Roche Mannheim, Germany). Following the manufacturer's instructions, each amplification reaction (total volume 10 µl) comprised of 0.5 µl of cDNA, 5 µl of 2X FastStart Essential DNA Green Master Mix, 0.2 µl of 10 µM both forward and reverse primers, 4.3 µl of PCR grade water. Amplification curves were detected by a Stratagene Mx3005P QPCR (Agilent Technologies, USA).

All specific primers for amplification the *LDLR, HMGCR, SRBI, APOA-1* and *GAPDH* genes designed by OLIGO 7 primer analysis software and the thermal cycle conditions were as previously described[16]. The Ct data was normalized using *GAPDH*. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method[16]. Expression of the genes were calculated as fold expression in relation to simvastatin.

Statistical analysis

Relative changes in expression of *LDLR*, *HMGCR*, *ApoA-1* and *SRBI* genes in relation to simvastatin were reported as means ± SD from 3 independent experiments and compared by an unpaired Student's t-test. In addition, one way analysis of variance (ANOVA), followed by Turkey's post hoc mean comparison were used to analyse the data. Statistical significance was considered by $p< 0.05$ with 95% confidence interval. All statistical analyses were performed by SPSS software package version 18.0 (IBM, USA).

Results

Cytotoxic effect of the hydroethanolic extracts from herbal plants on HepG2 cells

Cytotoxic effect of PNEF, NYEF, NBEF, ADEF, ALEF, APEF, ASEF, LCEF, NJEF, PKEF, SCEF, TCEF and simvastatin on HepG2 cell survival was assessed. The effect of PKEF on HepG2 cell survival was shown as an example of the cytotoxic assessment from all herbal extracts (Figure 1). It was found that NYEF, NBEF, PNEF, ADEF, ALEF, APEF, ASEF, LCEF, PKEF, TCEF and simvastatin were not toxic to HepG2 cells (IC50 ≥1 mg/ml). However, NJEF and SCEF exhibited more cytotoxic effect against HepG2 cells (IC50 = 0.39 and 0.5 mg/ml, respectively). Hence, the optimum concentrations of most individual herbal extracts and simvastatin used to study expression of the genes involving in LDL-C and HDL-C metabolisms were 1 mg/ml except NJEF and SCEF of which the optimum concentrations were 0.1 mg/ml.

Effect of PNEF, NYEF and NBEF on expression of the genes involving in the LDL-C and HDL-C metabolisms

The effect of PNEF, NYEF and NBEF on expression levels of *LDLR*, *HMGCR*, *APOA-1* and *SRBI* genes was determined in comparison with simvastatin. HepG2 cells were individually treated with 1 mg/ml of simvastatin, PNEF, NYEF and NBEF for 24 h. Expression levels of the target genes were quantified by qRT-PCR and the graphs were plotted between mean fold expression of the target gene in relation to simvastatin and herbal extracts (Figure 2).

Surprisingly, PNEF was demonstrated to upregulate expression of the *LDLR* and *SRB1* genes higher than NYEF and NBEF (Figure 2). Upregulation levels of the *LDLR* and *SRB1* genes by PNEF were significantly higher than simvastatin (6.65 ± 0.55 vs 1 ± 0.01 and 9.58 ± 0.66 vs 1± 0.01 folds, respectively, $p<0.05$). Additionally, PNEF could significantly upregulate expression of the *LDLR* (6.65 ± 0.55 vs 4.05 ± 0.08 folds respectively, $p<0.05$) and *SRB1* (9.58 ± 0.66 vs

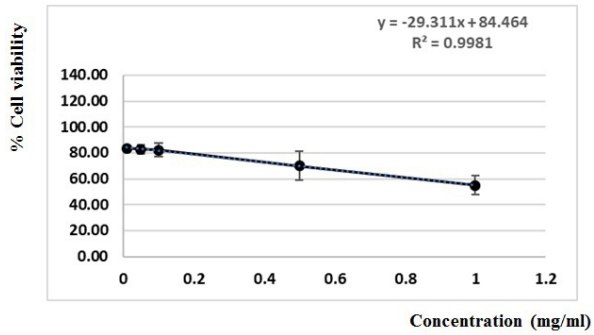

Figure 1 Assessment of the cytotoxic effect of PKEF on HepG2 cell viability:
Effect of the ethanolic extract from *Picrorhiza kurrooa* Royle ex Benth. (PKEF) on viability of HepG2 cells assessed by MTT assay. Cells were treated with various concentrations of PKEF (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) for 24 hrs. The results were expressed as mean ± SD from three independent experiments.

8.02 ± 0.17 folds, p<0.05) genes more than NYEF.

Furthermore, PNEF and NYEF were shown to downregulate the *HMGCR* gene expression more than simvastatin (-1.21 ± 0.22 vs 1 ± 0.01 and -0.95 ± 0.04 vs 1 ± 0.01, folds, respectively). On the contrary, neither PNEF or NYEF could affect upregulation of the *ApoA-1* gene.

NBEF did not upregulate expression of the LDLR or *ApoA-1* genes or downregulate expression of the *HMGCR* gene. NBEF was found to upregulate the *SRB1* gene expression more than simvastatin (7.88 ± 0.21 vs 1 ± 0.01 folds), however, the level of its expression was significantly less than PNEF and NYEF. It was,

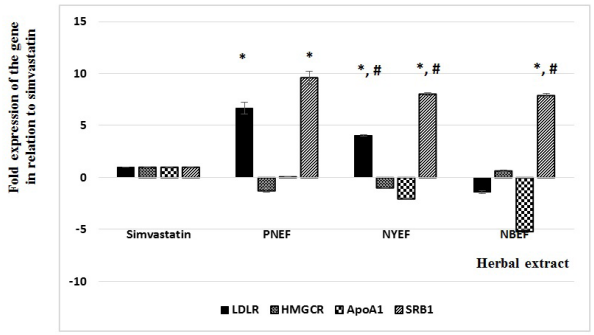

Figure 2 Effect of all herbal extracts on *LDLR, HMGCR, ApoA-1* and *SRB1* genes: Effect of the ethanolic extracts of Phikud Navakot (PNEF), Ya-Hom Navakot (NYEF) and Ya-Hom Navakot excluding PN (NBEF) on expression of the genes encoding *LDLR, HMGCR, ApoA-1* and *SRB1* in HepG2 cells assessed by qRT-PCR. The cells were treated with 1 mg/ml of simvastatin, PNEF, NYEF and NBEF for 24 hrs. The results were expressed as mean ± SD from three independent experiments. A statistically significance was set up at * p < 0.05 compared with simvastatin and # p < 0.05 compared between PNEF and NYEF.

hence, subjected for further investigation to elucidate, which of the main herbal composition of PN plays a role in upregulation or downregulation of the target genes.

Effect of the hydroethanolic extracts from the nine major herbal components of PN on expression of the genes involving in the LDL-C and HDL-C metabolisms

To elucidate which of the 9 major herbal ingredients of PN affects expression of the genes involving in *LDLC* and *HDL-C* syntheses, HepG2 cells were individually treated with 1 mg/ml of simvastatin or various concentrations (0.01, 0.05, 0.1, 0.5, 1 mg/ml) of APEF, ADEF, ALEF, ASEF, LCEF, NJEF, PKEF, SCEF and TCEF for 24 h. Expression levels of the target genes were quantified by qRT-PCR. Dose dependent effects of the ethanolic extract from individual herbal components of PN on expression of *LDLR*, *HMGCR*, *SRB1* and *ApoA-1* genes in comparison with simvastatin were demonstrated (Figures 3A-6A, respectively). Conclusively, expression levels of all 4 target genes in hepG2 cells treated with optimum concentrations of the herbal extracts i.e 1 mg/ml of simvastatin, APEF, ADEF, ALEF, ASEF, LCEF, PKEF and TCEF or 0.1 mg/ml of NJEF and SCEF were also included (Figures 3B-6B, respectively).

It was revealed that ADEF, ALEF and NJEF could significantly upregulate expression of the *LDLR* gene more than simvastatin (8.15 ± 0.09, 6.87 ± 0.45 and 8.83 ± 0.62 folds vs 1 ± 0.01, respectively, p<0.5) (Figure 3B). Interestingly, all herbal extracts were found to downregulate expression of *HMGCR* gene more than simvastatin (Figure 4A, B). Effect of the herbal extracts on expression of *ApoA-1* gene was also assessed (Figure 5A, B). It was shown that 0.5 and 1 mg/ml of APEF could significantly upregulate expression of the *ApoA-1* gene more than simvastatin (6.28 ± 0.01 vs 1 ± 0.01 and 11.44 ± 0.01 vs 1 ± 0.01 folds, p<0.05) (Figure 5A).

Additionally, effect of the herbal extracts on expression of *SRB1* gene was determined (Figure 6A, B). Interestingly, ADEF, ALEF and NJEF were found to significantly upregulate expression of the *SRB1* gene more than simvastatin (47.66 ± 1.92, 45.99 ± 3.65 and 20.8 ± 2.12 folds vs 1 ± 0.01, respectively, p<0.05) (Figure 6B). Effect of the herbal extracts on expression of the genes encoding *LDLR*, *HMGCR, ApoA-1* and *SRB1* was concluded in Table 1.

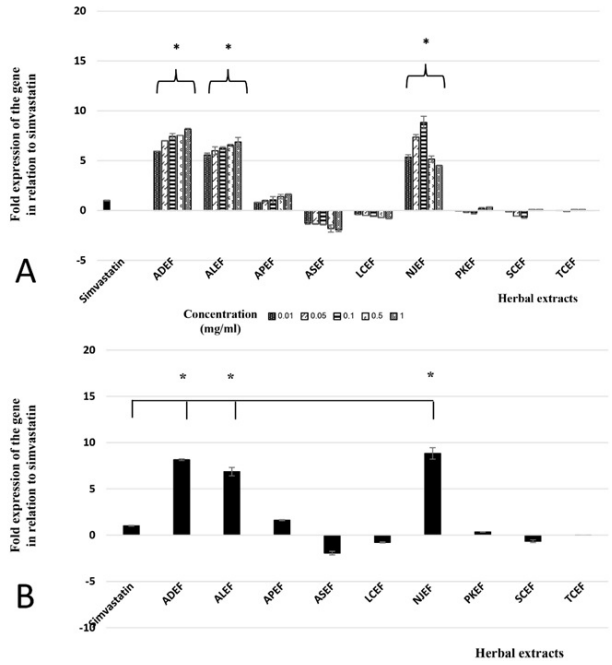

Figure 3 Effect of all herbal extracts on expression of *LDLR* gene: Effect of the ethanolic extract from 9 individual herbs of Phikud Navakot (PN) and simvastatin on expression of the *LDLR* gene in HepG2 cells assessed by qRT-PCR.
(A) The cells were treated with 1 mg/ml of simvastatin or various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of herbal extracts (APEF, ADEF, ALEF, ASEF, LCEF, NJEF, PKEF, SCEF and TCEF) for 24 hrs. (B) Conclusive data from A. The cells were individually treated with 1 mg/ml of simvastatin, 1 mg/ml of APEF, ADEF, ALEF, ASEF, LCEF, PKEF and TCEF or 0.1 mg/ml of NJEF and SCEF for 24 hrs. The results were expressed as mean of fold expression of the gene in relation to simvastatin ± SD from three independent experiments. A statistically significance was set up at * p-value < 0.05 compared with simvastatin.

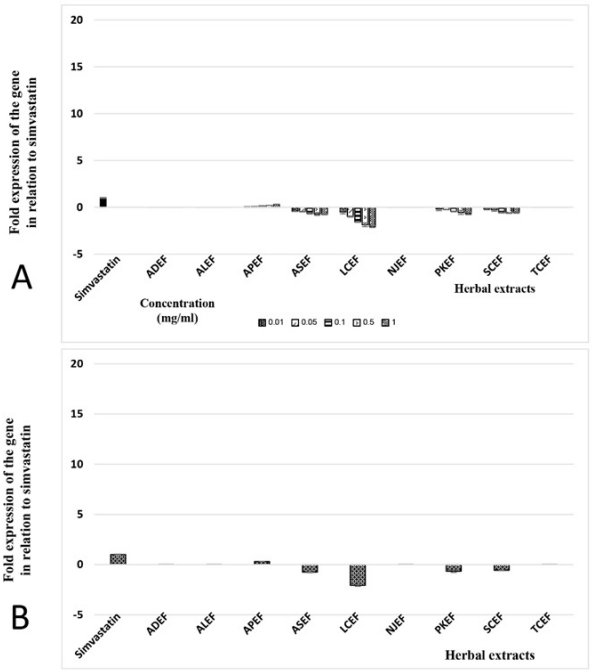

Figure 4 Effect of all herbal extracts on expression of *HMGCR* gene:
Effect of the ethanolic extract from 9 individual herbs of Phikud Navakot (PN) and simvastatin on expression of the *HMGCR* gene in HepG2 cells assessed by qRT-PCR. (A) The cells were treated with 1 mg/ml of simvastatin or various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of herbal extracts (APEF, ADEF, ALEF, ASEF, LCEF, NJEF, PKEF, SCEF and TCEF) for 24 hrs. (B) Conclusive data from A. The cells were individually treated with 1 mg/ml of simvastatin, 1 mg/ml of APEF, ADEF, ALEF, ASEF, LCEF, PKEF and TCEF or 0.1 mg/ml of NJEF and SCEF for 24 hrs. The results were expressed as mean of fold expression of the gene in relation to simvastatin ± SD from three independent experiments. A statistically significance was set up at * p-value < 0.05 compared with simvastatin.

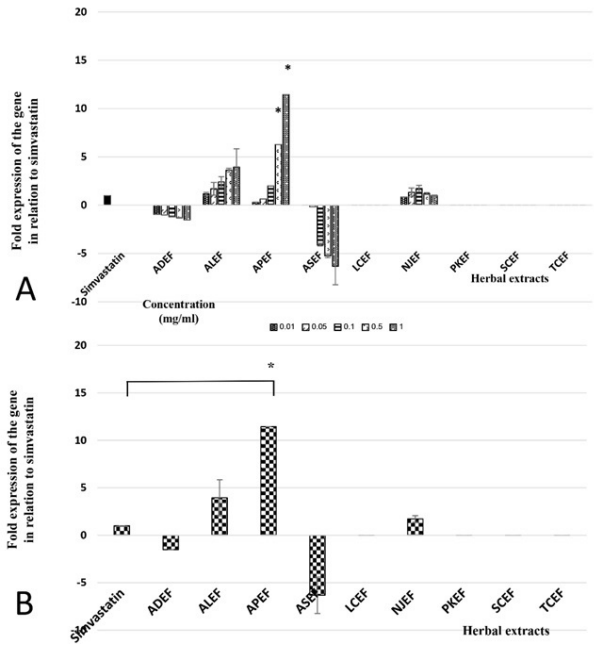

Figure 5 Effect of all herbal extracts on expression of *ApoA-1* gene:
Effect of the ethanolic extract from 9 individual herbs of Phikud Navakot (PN) and simvastatin on expression of the *ApoA-1* gene in HepG2 cells assessed by qRT-PCR. (A) The cells were treated with 1 mg/ml of simvastatin or various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of herbal extracts (APEF, ADEF, ALEF, ASEF, LCEF, NJEF, PKEF, SCEF and TCEF) for 24 hrs. (B) Conclusive data from A. The cells were individually treated with 1 mg/ml of simvastatin, 1 mg/ml of APEF, ADEF, ALEF, ASEF, LCEF, PKEF and TCEF or 0.1 mg/ml of NJEF and SCEF for 24 hrs. The results were expressed as mean of fold expression of the gene in relation to simvastatin ± SD from three independent experiments. A statistically significance was set up at * p-value < 0.05 compared with simvastatin.

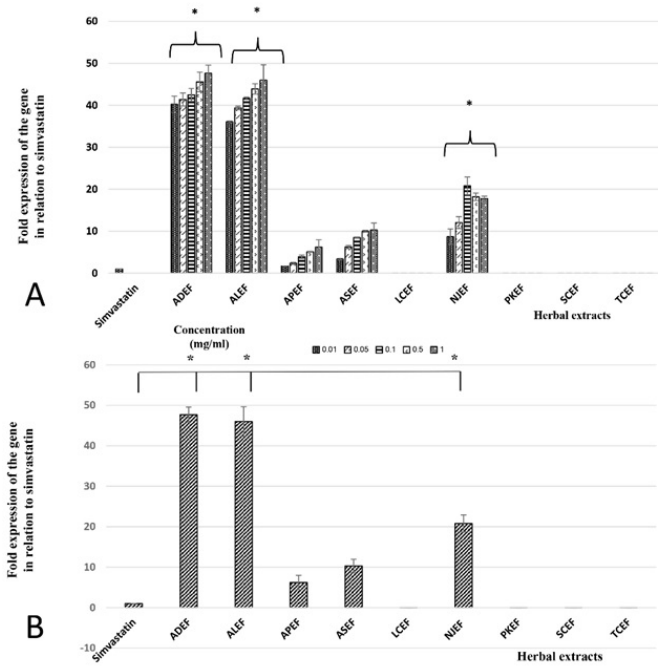

Figure 6 Effect of all herbal extracts on expression of *SRB1* gene: Effect of the ethanolic extract from 9 individual herbs of Phikud Navakot (PN) and simvastatin on expression of the *SRB1* gene in HepG2 cells assessed by qRT-PCR.
(A) The cells were treated with 1 mg/ml of simvastatin or various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of herbal extracts (APEF, ADEF, ALEF, ASEF, LCEF, NJEF, PKEF, SCEF and TCEF) for 24 hrs. (B) Conclusive data from A. The cells were individually treated with 1 mg/ml of simvastatin, 1 mg/ml of APEF, ADEF, ALEF, ASEF, LCEF, PKEF and TCEF or 0.1 mg/ml of NJEF and SCEF for 24 hrs. The results were expressed as mean of fold expression of the gene in relation to simvastatin ± SD from three independent experiments. A statistically significance was set up at * p< 0.05 compared with simvastatin.

Table 1 Effect of the herbal extracts at the optimum concentrations on expression of the genes encoding *LDLR*, *HMGCR*, *ApoA-1* and *SRB1*

Herbal extract	Effect of the herbal extracts on the genes encoding			
	LDLR	HMGCR	Apo A1	SR-B1
PNEF	↑	↓	-	↑
NYEF	↑	↓	-	↑
NBEF	-	NA	-	↑
ADEF	↑	↓	-	↑
ALEF	↑	↓	NS	↑
APEF	NS	↓	↑	NS
ASEF	-	↓	-	NS
LCEF	-	↓		-
NJEF	↑	↓	NS	↑
PKEF	NS	↓	-	-
SCEF	-	↓	-	-
TCEF	-	↓	-	-

↑ ; significant upregulation of the gene
↓ ; significant downregulation of the gene
-; No effect on upregulation of the gene
NS ; expression of the gene was not significantly different compared to simvastatin

Discussion

Previously, our colleagues found that PN showed many eagerness pharmacological properties especially cardioprotective effect in rats [9, 10]. Additionally, it was observed that PNEF and NYEF could upregulate *LDLR* gene but downregulate *HMGCR* gene more than presented in NBEF[13]. These results referred that PN is a major component in NY representing a crucial effect on hypocholesterolaemic activities. However, PN composes of a number of active ingredients, therefore, their achievement and purity have to be considered. Hence, our study was performed to specify which of the nine major herbal components of PN could play roles in upregulation or downregulation of the *LDLR*, *HMGCR*, *SRBI* and *ApoA-1* genes.

Apart from five herbal compositions of PN, four of them had some additional desired hypocholesterolaemic effects (Table 1). The present study revealed that Kot Soa (AD), Kot Khamao (AL) and Kot Jatamansri (NJ) could significantly upregulate expression of *LDLR* and *SRBI* genes in comparison to simvastatin (Figures 3A, B and 6A, B). Additionally, only Kot Chulalumpa (AP) could significantly upregulate the *ApoA-1* gene expression when compared to the other ingredients of PN (Figure 5A, B). Other components of PN, Kot Chiang (AS), Kot Huabua (LC), Kot Kanprao (PK), Kot Kradook (SC) and Kot Pungpla (TC), did not show the regulative activity on *LDLR*, *ApoA-1* and *SRB1* genes. However, downregulation of

the *HMGCR* gene was exhibited in all herbal extracts (Figure 4A, B).

Although the individual pharmacological activities of each active ingredient in PN have been well documented for decades, hypocholesterolaemic effect in relation to *HMGCR*, *LDLR*, *ApoA-1* and *SRB1* genes remains unclear. Kot Soa (*Angelica dahurica*; AD) has been widely used in Chinese, Korean including Thai traditional remedies. Many pharmacological effects from AD root extracts and its active components e.g. furocoumarins, imperatorin, isoimperatorin were observed. Methanolic extract from AD root showed a vasorelaxant activity in isolated rat aortic rings[17]. Moreover, AD could reduce white-fat weight in high-fat-diet hyperlipidaemic mice, decrease total cholesterol and triglyceride concentrations in the livers of both high-fat-diet and Triton WR1339 induced hyperlipidemic mice, and enhance the total hepatic lipase activities[18]. Recently, ethanolic extract of AD was found to improve impaired wound healing by activating angiogenesis in diabetes[19]. Furocoumarins were shown to exhibit strong hepatoprotective activities in HepG2 cells [20] and possess the potential activities in regulating transcriptional activation function of nuclear receptor RXR α [21]. Administration of imperatorin, another active component of AD, was reported to have antihypertensive, antioxidant and vascular remodeling effects [22, 23].

Various pharmaceutical effects of Kot Khamao (*Atractylodes lancea*; AL) including their active constituents were found to exhibit anticancer, anti-inflammatory, antimicrobial and antipyretic activities, as well as activities on central nervous, cardiovascular, and gastrointestinal systems in in vitro, ex vivo, and in animal models[24]. AL crude extracts showed anti-platelet activity in collagen-induced platelet aggregation in rabbit platelets[25]. Furthermore, ethanolic extract of AL rhizome displayed significant lipase inhibition and antiobesity effect in a high-fat diet-induced obesity mice model[26].

Kot Chulalumpa (*Artemisia pallens*; AP)-another important herb for medicinal use was found to establish antioxidant activity[27], anti-inflammatory[28], anticarcinogenic[29] antihyperglycemic and antihypertensive effects[30]. Kot Jatamansri (*Nardostachys jatamansi*; NJ) is another herb generally used in multiple medicinal formulations. It has been known to have several activities including hepatoprotective, cardioprotective, anti-inflammatory, anticancer effects[31-33]. Interestingly, NJ was established to alleviate hyperglycemia by improving insulin s ensitivity and inhibiting gluconeogenesis in the liver[34]. Moreover, NJ also exhibited protective and hypolipidaemic effects against doxorubicin induced myocardial injury in rats[35].

Regarding to the previous literatures, obviously, each component of PN has its own ability to have broad spectrum of pharmacological effects e.g. cardioprotective, hepatotonic, hypoglycaemic and especially hypolipidaemic activities. Comparing to NY and PN, each nine herbal plant has its own ability to promote cholesterol metabolism in several pathways majority on *HMGCR* gene regulation and inferiority on *LDLR*, *SRB1* and *ApoA-1* genes, respectively. Therefore, the cholesterol lowering activity of *ApoA-1* gene in either NY or PN was absent reflecting to the proportional effect. Regarding to our result especially hypocholesterolaemic capacity among NY, PN and each of the 9 active ingredients in PN, it might possess a cholesterol-lowering effect by following mechanisms. Firstly, an upregulation of the *LDLR* gene suggests an increase in the uptake of LDL-C, thereby enhancing LDL-C catabolism. Secondly, an upregulation of *SRB1* gene that could increase the hepatic clearance of plasma HDL-C levels, thus HDL-cholesterol ester (CE) from the vessel wall was transported to the liver for excretion into the bile, bile acid and steroidogenic tissue for hormone production. Thirdly, a downregulation of the *HMGCR* gene refers to the inhibitory effect of cholesterol synthesis. Moreover, the presence of upregulation of *ApoA-1* gene suggests the involvement in the synthesis of HDL-C particles.

Conclusion

It was demonstrated in the present study that the cholesterol lowering effect of PN might be responsible from its active ingredients. The regulatory effect of *HMGCR* gene was predominately presented in all component of PN. Three from nine main components, Kot Soa (AD), Kot Khamao (AL) and Kot Jatamansri (NJ) were found to upregulate the *LDLR* and *SRB1* genes. Additionally, only Kot Chulalumpa (AP) was found to significantly upregulate the *ApoA-1* gene expression suggesting that this effect might be diminished when mixing with other main components of PN. This finding might shed some lights on development of an alternative hypocholesterolaemic agent using PN and its individual herb depended on

their activities.

Declaration of interest statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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