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THE CHOLESTEROL ESTERASE INHIBITION AND TOTAL PHENOLIC CONTENT OF AQUEOUS EXTRACT OF TRIPHALA AND MODIFIED TRIPHALA FORMULAS

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Abstract: Hypercholesterolemia is the important public health problems. Triphala is traditional medicinal formula that is recognized to demonstrate the antioxidative activity and the blood cholesterol reducing property. It composes of fruits of three herbal plants, *Terminalia bellirica*, *Terminalia chebula* and *Phyllanthus emblica*. The objective of this study is to investigate the effect of aqueous extract of Triphala and modified Triphala formulas on cholesterol esterase inhibition and total phenolic content. Each formula was extracted in duplicate called extraction 1 and extraction 2. The results indicated that modified Triphala formula 2 (MT.F.2), which contained *T. bellirica*, *T. chebula* and *P. emblica* in 12:8:4 weight ratio, expressed the highest total phenolic content at 514.4±5.1 µg GAE/mg plant extract for extraction 1 and 516.3±22.9 µg GAE/mg plant extract for extraction 2, respectively. The cholesterol esterase (CEase) was inhibited only 4-11% by aqueous extract of Triphala and modified Triphala formulas at concentration of 2.0 mg/ml. The MT.F.2 manifested the highest percent inhibitory values of 8.4±0.4 for extraction 1 and 10.6±0.5 for extraction 2, respectively. In conclusion, total phenolic content involve in the proportion of *T. bellirica* in formula. On the contrary, the inhibition of CEase seemed not related to the amount of each herbal plant in formula.

Key Words: Triphala, total phenolic content, cholesterol esterase, enzyme inhibition

บทคัดย่อ: ภาวะคอเลสเตอรอลในเลือดสูงเป็นปัญหาสุขภาพที่สำคัญ ตำรับตรีผลาเป็นสูตรสมุนไพรโบราณที่มีฤทธิ์ต้านอนุมูลอิสระและคุณสมบัติในการลดระดับคอเลสเตอรอลในเลือด ตำรับตรีผลาประกอบไปด้วยผลของสมุนไพร 3 ชนิด คือ สมอพิเภก สมอไทย และมะขามป้อม วัตถุประสงค์ในการทำวิจัยครั้งนี้ เพื่อทดสอบผลของสารสกัดชั้นน้ำของตำรับตรีผลาและตำรับตรีผลาที่เปลี่ยนแปลงสูตรต่อการยับยั้งการทำงานของเอนไซม์คอเลสเตอรอล เอสเทอเรส และปริมาณสารประกอบฟีนอลรวม แต่ละสูตรจะสกัด 2 ครั้ง เรียกว่า การสกัดครั้งที่ 1 และการสกัดครั้งที่ 2 ผลการทดลองแสดงให้เห็นว่าตำรับตรีผลาที่ปรับปรุงสูตรที่ 2 (MT.F.2) ซึ่งประกอบไปด้วย สมอพิเภก สมอไทย และมะขามป้อม ในอัตราส่วนต่อน้ำหนักเป็น 12:8:4 จะให้ปริมาณสารประกอบฟีนอลรวมสูงที่สุด โดยการสกัดครั้งที่ 1 มีค่าเทียบเท่ากับกรดแกลลิกที่ 514.4±5.1 ไมโครกรัมต่อมิลลิกรัมของสารสกัด และการสกัดครั้งที่ 2 มีค่าเท่ากับ 516.3±22.9 ไมโครกรัมต่อมิลลิกรัมของสารสกัด ตามลำดับ สารสกัดชั้นน้ำของตำรับตรีผลาและตำรับตรีผลาที่ปรับปรุงสูตรที่ความเข้มข้น 2.0 มิลลิกรัมต่อมิลลิลิตร สามารถยับยั้งการทำงานของเอนไซม์คอเลสเตอรอล เอสเทอเรสได้เพียง 4-11 เปอร์เซ็นต์เท่านั้น โดยตำรับตรีผลาที่ปรับปรุงสูตรที่ 2 ให้ค่าการยับยั้งการทำงานของเอนไซม์คอเลสเตอรอล เอสเทอเรสสูงสุดที่ 8.4±0.4 และ 10.6±0.5 เปอร์เซ็นต์ตามลำดับ กล่าวโดยสรุปปริมาณสารประกอบฟีนอลรวมเกี่ยวข้องกับสัดส่วนของสมอพิเภกในสูตรตำรับ ซึ่งต่างจากการยับยั้งการทำงานของเอนไซม์คอเลสเตอรอล เอสเทอเรส ที่ไม่มีความสัมพันธ์กับปริมาณของพืชสมุนไพรแต่ละชนิดในสูตรตำรับ

คำสำคัญ: ตรีผลา ปริมาณสารประกอบฟีนอลรวม เอนไซม์คอเลสเตอรอล เอสเทอเรส การยับยั้งเอนไซม์

INTRODUCTION

Triphala is a traditional Ayurvedic and Thai medicines for balancing body elements and detoxification. It has commonly been used to relief headache and dyspepsia, improve blood circulation, control blood pressure, blood sugar and blood cholesterol (Sabu and Kuttan, 2002). Triphala consists of equal amount of fruits of three medicinal plants, *Terminalia bellirica* (Gaertn.) Roxb. (Sa Mor Phi Phek), *Terminalia chebula* Retz. (Sa Mor Thai) and *Phyllanthus emblica* L. (Ma Kham Pom). Nevertheless, the proportion of each plant in Triphala has been modified for widely used in many specific treatments followed Ayurvedic medicines including Pitta formula is used for relief the effect of fire element, Wata formula is used for relief the effect of wind element, and Semha formula is used for relief the effect of water element on body in summer. Scientific evidences have demonstrated that Triphala formula composes of a various groups of secondary metabolites, especially phenolic/polyphenolic compounds and it shows very high antioxidative activity by DPPH, hydroxyl radicals, superoxide radicals, $ABTS^{\cdot-}$ tests (Naik *et al*., 2005; Naik *et al*., 2006)

Hypercholesterolemia is a main cause of cardiovascular disease which is one of the important public health problems. Previous studies indicate that increasing of total cholesterol, especially the low density lipoprotein cholesterol is a major factor of cardiovascular disease (Castelli, 1984; Weststrate and Meijer, 1998; Elahi *et al*., 2009). Several mechanisms have been proposed to regulate cholesterol metabolism. The inhibition of enzymes that control cholesterol absorption and transportation is considered as a good target for reducing blood cholesterol.

Cholesterol esterase (CEase, EC 3.1.1.13) is the one of crucial enzyme in cholesterol metabolism. CEase stimulates the hydrolysis of cholesterol ester, by liberated free cholesterol from dietary cholesterol ester. It plays the important role in the regulation of cholesterol metabolism by extending cholesterol intestinal absorption and transportation to enterocytes (Howles *et al*., 1996; Heidrich *et al*., 2004). Many cardiovascular drugs are studied the mechanism of CEase inhibition. The results indicate that CEase is inhibited by these drugs both *in vitro* and *in vivo* studies (Pioruńska-Stolzmann and Pioruńska-Mikołajczak, 2001; Chiou *et al*., 2006).

Presently, besides the synthetic drugs, to reduce lipid and cholesterol level in blood circulation system, the traditional medicines are used as a good alternative treatment. In the present study, we investigate the effect of aqueous extract of Triphala and modified Triphala formulas on CEase inhibition and total phenolic content. These results might provide more information about blood cholesterol reduction.

MATERIALS AND METHODS

Materials

CEase from porcine pancreas (Lot.No.26745), Folin–Ciocalteu solution, gallic acid, orlistat, *p*-nitrophenyl butyrate (*p*-NPB), and taurocholic acid sodium salt hydrate were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide (DMSO) purchased from Carlo Erba Reagent (USA). Other chemicals were analytical grade obtained from Univar (USA).

Plant preparation and extraction

The whole dried fruits of *T. chebula*, *T. bellirica*, and *P. emblica* were ground to powder, then, prepared each formula/herbal plant according to Table 1 in the total weight of 8.4 g. After that, the powders were boiled in 840 ml hot water for 15 min, filtered through Whatman no.1 paper, and the filtrate was dried by lyophilizer. The aqueous extracts were

stored in glass bottle, kept in desiccator and protect from light until use. The aqueous extract of each sample was prepared in duplicate sets.

Determination of total phenolic content

Total phenolic content was analyzed according to Folin–Ciocalteu method (Singleton *et al*, 1999). The aqueous extracts were prepared in distilled water at the concentration of 0.2 mg/ml, and 20 µl was mixed carefully with 100 µl of 10% (v/v) Folin–Ciocalteu solution in 96-well plate. After mixing for 6 min, 80 µl of 7.5% (w/v) sodium carbonate was added, and the reaction mixed was allowed to stand in dark for 60 min at room temperature. The absorbance was monitored at 760 nm using microplate reader (Bio-Rad, USA). Total phenolic content was calculated using the linear equation of gallic acid (12.5-200 µg/ml). The results were expressed as µg of gallic acid equivalents per milligram plant extract (µg GAE/mg plant extract). All samples were made in triplicate.

Table 1. The proportion of herbal plants in Triphala and modified Triphala formulas: T.F. = Triphala formula; MT.F. = modified Triphala formula

Formula/herbal plant	Proportion of each herbal plant			Note
	T. chebula	*T. bellirica*	*P. emblica*	
T.F. 1	8	8	8	The national core drug medications
MT.F. 2	8	12	4	Pitta formula
MT.F. 3	12	4	8	Wata formula
MT.F. 4	4	8	12	Semha formula
T. chebula	1	-	-	
T. bellirica	-	1	-	
P. emblica	-	-	1	

Inhibition of pancreatic cholesterol esterase activity

CEase enzyme inhibitory activity was evaluated in the presence of bile salt and *p*-NPB. The aqueous extracts were prepared at 2.0 mg/ml with 10% DMSO. The final concentration of DMSO in reaction was 2%. According to Adisakwattana (2012) method with some modifications, reaction mixed was prepared by mixing 1 volume of 1.0 mM taurocholic acid sodium salt, with 1 volume of 1.0 mM *p*-NPB in 100 mM sodium phosphate buffer, pH 7.0 and with 1 volume of 500 mM sodium chloride. The stock solution of porcine CEase (3.2 µg/ml) was dissolved in 100 mM sodium phosphate buffer, pH 7.0. The reaction was started by adding 50 µl of each aqueous extract solution and CEase into 96-well plate, and incubated at 25ºC for 5 min. After, 150 µl of reaction mixed was added and continuously incubated at 25ºC for 5 min. The absorption values were determined by measuring the optical density at 405 nm. The % inhibition was calculated by comparison between the OD_{405} of reaction in presence and absence of the inhibitor. Orlistat was used as a positive control. All measurements were made in triplicate.

Statistical analysis

Results were carried out in triplicate and expressed as mean values ± standard deviation. Two sets of extraction were compared by independent samples T-test. One-way analysis of variance (ANOVA) and Tukey's multiple comparison post hoc test were used for analyzes between formulas or each herbal plant. Significant differences were determined at $p< 0.05$.

RESULTS AND DISCUSSION

The total phenolic contents of all aqueous extracts presented very high values in a range of 300 to 600 µg GAE/mg plant extract (Figure 1). Earlier studies showed the same results of total phenolic content in aqueous extract of Triphala about 400 µg GAE/mg extract (Russell *et al.*, 2011). The total phenolic contents between two extractions were not significantly different; however, MT.F.2 showed the highest total phenolic content among four formulas with significantly different ($p< 0.05$) at 514.4±5.1 µg GAE/mg plant extract for extraction 1 and 516.3±22.9 µg GAE/mg plant extract for extraction 2, respectively. Noticeable, the amount of total phenolic content in each formula was high to low content relative to the proportion of *T. bellirica* in each formula. Moreover, total phenolic content of MT.F.2 was lower than herbal plant, *T. bellirica*, inconsiderable value. It implies formulas composed of high proportion of *T. bellirica* may relate to high total phenolic content. These results demonstrated that amount of total phenolic contents in formulas related to the proportion of *T. bellirica* and it displayed equal total phenolic values in both extractions.

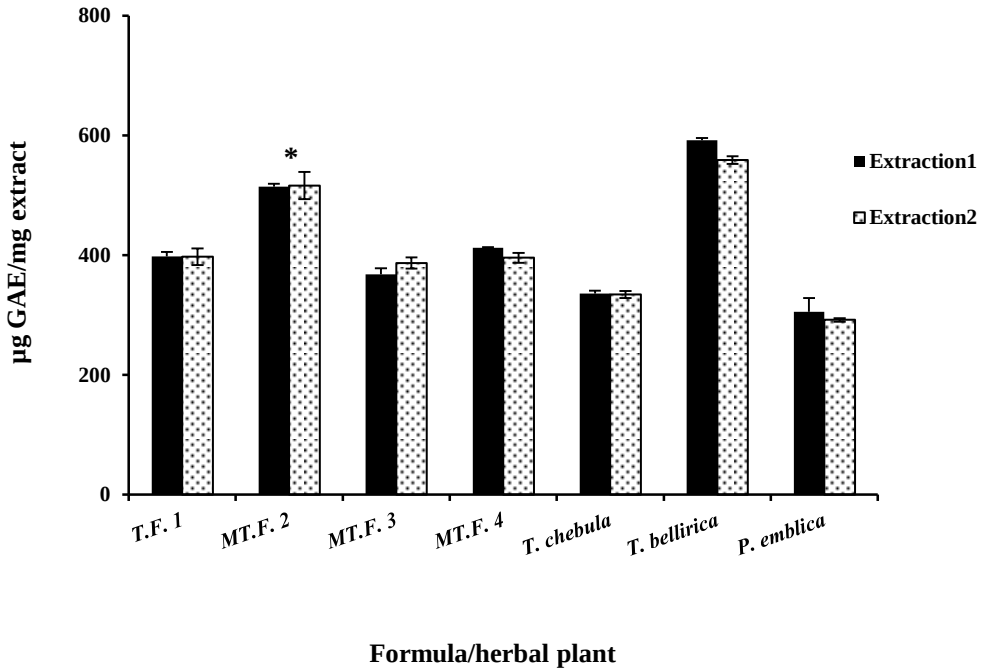


Figure 1. Total phenolic contents of aqueous extracts of Triphala, modified Triphala formulas and herbal plants. * significantly difference between four formulas at $p<0.05$.

All herbal formulas showed less CEase inhibition (Table 2) at concentration of 2.0 mg/ml, while the activity of enzyme was strongly suppressed by the positive control (orlistat). The CEase inhibition of two extractions was significantly different ($p< 0.05$). The extraction 2

showed higher CEase inhibitory values than extraction 1 might indicate that herbal powder sampling for extraction 1 and extraction 2 were non-homogenous mixture. Besides, the active compound for CEase inhibition might be partly extracted in boiling water or was not completely extracted within 15 min. Among Triphala formulas the MT.F.2 expressed the highest CEase inhibition with 8.4±0.4 % for extraction 1 and 10.6±0.5 % for extraction 2, respectively. Its CEase enzyme inhibition was significantly different from other formulas ($p<$ 0.05). Nevertheless the enzyme inhibition of MT.F.2 was moderately 9 times lower than that of orlistat (0.2 µg/ml). For each individual plant, the *P. emblica* demonstrated the highest CEase inhibition, but *T. bellirica* was lowest CEase inhibition. The MT.F2 which contained the lowest proportion of *P. emblica* showed significantly different CEase inhibition higher than MT.F.4. These results may suggest that the proportion of each herbal plant in Triphala and modified Triphala formulas did not relate to the inhibition of CEase.

Table 2. The inhibition of CEase in aqueous extracts of Triphala, modified Triphala formulas and herbal plants at concentration of 2.0 mg/ml

Formula/herbal plant	% Inhibition	
	Extraction 1	Extraction 2
T.F. 1	5.7 ± 0.3	8.2 ± 0.2
MT.F. 2	8.4 ± 0.4 *	10.6 ± 0.5 *
MT.F. 3	6.5 ± 0.4	8.6 ± 0.2
MT.F. 4	6.8 ± 0.5	9.6 ± 0.2
T. chebula	9.0 ± 0.8	11.0 ± 0.3
T. bellirica	4.7 ± 0.8	7.2 ± 0.6
P. emblica	24.5 ± 0.1 #	31.6 ± 0.2 #
Orlistat (0.2 µg/ml)	84.1 ± 0.5	

* significantly difference between four formulas at $p<0.05$.
# significantly difference between each individual plant at $p<0.05$.

CONCLUSION

Triphala and modified Triphala formulas showed relatively low CEase inhibition (less than 10%) at concentration of 2.0 mg/ml. All formulas demonstrated the total phenolic contents higher than 350 µg GAE/mg plant extract. The MT.F.2 exhibited the highest value of total phenolic content and CEase inhibition. Total phenolic content may relate to the proportion of *T. bellirica* in formula. Meanwhile, the inhibition of CEase seemed not related to the amount of each herbal plant in formula and total phenolic content directly. To understand the mechanism of triphala on CEase inhibition, the future study is further investigated.

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