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ORIGINAL ARTICLE

In vitro 3-Hydroxy-3-methylglutaryl-coenzyme: A Reductase Inhibition Assay of Triphala Ayurvedic Formulation

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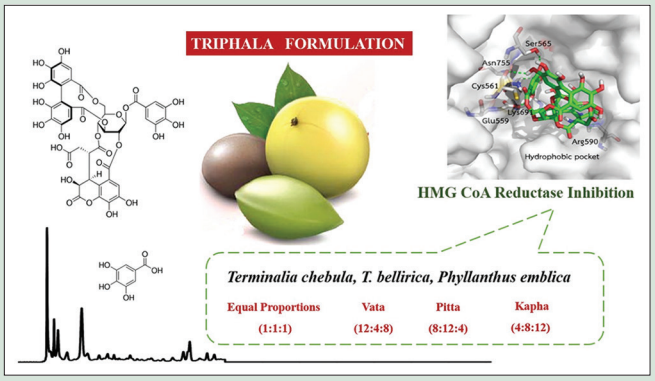
ABSTRACT

Background: Triphala, the Ayurvedic herbal formulation composed of *Terminalia chebula* Retz. (Combretaceae), *Terminalia bellirica* Roxb. (Combretaceae), and *Phyllanthus emblica* L. (Euphorbiaceae) fruits. It has been reported the cholesterol-lowering effect that the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was proposed as a key mechanism of action. Since, triphala formulations in equal proportion (1:1:1) and different ratios of its three fruit constituents (vata, pitta, and kapha) have been prescribed by the traditional practitioners due to the patient's body conditions. The biological activities of each formulation are needed to evaluate. **Objectives:** The objective of the study was to investigate phytochemicals, HMG-CoA reductase inhibitory effect, and HMG-CoA reductase molecular modeling of triphala extracts. **Materials and Methods:** Four triphala extracts were prepared by decoction and determined the contents of gallic acid, ellagic acid, chebulagic acid, and chebulinic acid as markers using high-pressure liquid chromatography analysis. The *in vitro* HMG CoA reductase assay was performed based on ultraviolet spectrophotometry, and molecular modeling was simulated using Autodock 1.5.6 to characterize the binding energy, ligand efficacy, and H-bond interaction. **Results:** All extracts contained gallic acid and chebulagic acid in the high contents, whereas ellagic acid and chebulinic acid were found in a small amount. The enzyme assay revealed pitta extract (at 10 μg/mL) was the most potent enzyme inhibition of 58.4% ± 0.40% ($P \leq 0.05$). Moreover, the modeling results indicated that these four markers can interact the enzyme with different configurations and binding affinities. **Conclusion:** Pitta extract appeared to be a potent HMG-CoA reductase inhibitor. It was a potential natural product as an alternative treatment for hypercholesterolemia.

Key words: Chebulagic acid, gallic acid, kapha, pitta, vata

SUMMARY

- Triphala composed of *Terminalia chebula*, *Terminalia bellirica,* and *Phyllanthus emblica* fruits. This formulation has been prepared in equal proportion and different ratios of its three fruit constituents; vata, pitta, and kapha. Pitta extract exhibited the most potent enzyme inhibitor on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase. High-pressure liquid chromatography analysis indicated that gallic acid and chebulagic acid were major components. Both compounds can interact the enzyme-active sites with different configurations and binding affinities. The HMG CoA reductase inhibitory action of pitta extract suggests its potential as an alternative treatment for hypercholesterolemia.



Abbreviations Used: HMG-CoA: 3-Hydroxy-3-methylglutaryl-coenzyme A; HPLC: High-pressure liquid chromatography; UV: Ultraviolet; PDB: Protein data bank; ADT: AutoDock Tools; LGA: Lamarckian genetic algorithm; GA: Genetic algorithm; RMSD: Root-mean-square deviations.

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INTRODUCTION

Atherosclerosis is a primary cause of cardiovascular diseases. Its major clinical manifestations are ischemic heart disease, ischemic stroke, and peripheral arterial disease.[1] Hypercholesterolemia and elevated low-density lipoprotein cholesterol concentration are mainly responsible for atherosclerosis development.[2] The reduction of plasma cholesterol levels has been considered a target of atherosclerosis management. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is a rate-limiting enzyme in cholesterol biosynthesis that converts HMG-CoA into mevalonic acid at the beginning of the pathway.[3] The enzyme-active binding site composed of HMG-binding pocket and shallow groove for bulky hydrophobic group with crucial two amino acids; Glu559 and Ser565.[4] Inhibition of enzyme activity leads to control cholesterol biosynthesis and lower cholesterol levels. Statins are the most effective HMG-CoA reductase inhibitors that act by competitively binding to the catalytic domain of the enzyme. This class of drugs provides significant clinical benefits in dyslipidemia patients. In a long-term treatment, statins can reduce morbidity and mortality associated with coronary heart diseases.[5] However, the efficacy of statins has been maligned by adverse events involving skeletal muscle.[6] About 10% of patients in the United States discontinued statin therapy due

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to their subjective discomforts.[7] In this regard, natural products have been explored as a source of the alter HMG-CoA reductase inhibitors. Medicine herbs including *Basella alba* L. (Basellaceae), *Amaranthus viridis* L. (Amaranthaceae), and *Ficus palmata* Forsk (Moraceae) were reported the HMG-CoA reductase inhibition property.[8-11]

Triphala is a well-recognized Ayurvedic medicinal formulation. It has been widely used in traditional treatment for various ailments.[12] This herbal formulation consisted of the dried fruits of three plant species, *Terminalia chebula* Retz. (Combretaceae), *T. bellirica* Roxb, and *Phyllanthus emblica* L. (Euphorbiaceae). Phenolics and tannins such as gallic acid, chebulagic acid, and chebulinic acid are the major phytochemicals of triphala.[13,14] In Ayurvedic medicine, triphala is classified as a tridoshic rasayana as it promotes balancing and rejuvenating effects on the three humors; vata (air), pitta (heat), and kapha (water) in the human body.[12] Each fruit constituent of triphala alleviated three humors; vata by *T. chebula*, pitta by *P. emblica,* and kapha by *T. bellirica.* Triphala is normally formulated with equal proportions (1:1:1) of the three fruit constituents which have been used as internal cleansing and detoxifying agents.[15] However, different ratios of the three fruit constituents of triphala have been prescribed by the traditional practitioners due to the patient's body conditions or cause of diseases. Vata is responsible for driving force in the body, pitta is accountable for metabolism, while kapha attends power and resistance.

Scientific studies have shown that triphala (1:1:1) formula has been reported as the potential lipid-lowering effect in the *in vivo* experiment and clinical trials.[16-18] The HMG-CoA reductase inhibitory action has been proposed a mechanism of the hypolipidemic property of triphala. The present study investigated phytochemicals and the inhibitory enzyme action of HMG-CoA reductase of triphala aqueous extracts in the variated ratios of its three fruit constituents. Molecular modeling was also performed to characterize the binding between HMG-CoA reductase enzyme and the phytochemicals of triphala formulas.

MATERIALS AND METHODS

Plant materials and extract preparation

Dry fruits of *T. chebula*, *T. bellerica,* and *P. emblica* were purchased from the traditional drug store in Muang district, Maha Sarakham, Thailand, in April 2019. All plant materials were authenticated through the macroscopic characters as previously described.[19] All voucher specimens were deposited at the Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, Thailand. Each fruit material was removed seed, ground to powder, and sieved through mesh no. 14.

For extract preparation, four formulations of triphala were prepared with equal proportions and the variated ratio of the three fruit constituents of triphala (vata, pitta, and kapha) following Table 1 in the total weight of fine powder fruit materials 15 g. Then, the powders of each triphala formulation were well mixed and boiled with 1 L of distilled water for 1 h. The liquid part was converted to powder by a freeze dryer (ScanVac, Denmark). The extract of each triphala formulation was prepared in triplicated, and the obtained extracts were stored in closed containers, protected from light until used.

Phytochemical determination by high-pressure liquid chromatography analysis

The obtained extracts were high-pressure liquid chromatography (HPLC) quantitative analyzed for gallic acid, ellagic acid, chebulagic acid, and chebulinic acid followed the slightly modified from the previous study.[20] All standard substances (Sigma Aldrich Co., St. Louis, MO, USA) were dissolved in methanol (1 mg/mL) as stock solutions. The desired concentrations of the standard were subsequently diluted in methanol before used. The extracts were dissolved in methanol at a concentration of 1 mg/mL. Chromatographic separation was performed on Shimadzu SCL-10A VP equipped with LC-10 AD binary pumps and SPD-M20A photodiode array detector (Kyoto, Japan). Class-VP version 6.1 was applied for data collection and data processing. Eclipse XDB-C_{18} column (250 mm × 4.6 mm, 5 μm) was used for separation at ambient temperature. The HPLC mobile phase consisted of 0.05% trifluoroacetic acid (A) in water and acetonitrile (B) with a flow rate of 1 mL/min. The mobile phase was filtered through 0.45 μ membrane filter and degassed. HPLC analysis was carried out using a gradient elution in 0–2 min, 5% B; 2–4 min, 5–10% B; 4–12 min, 10–15% B; 12–26 min, 15–30% B; 26–30 min, 30–100% B; 30–35 min, 100% B; 35–40 min, 100–5% B; and 40–45 min, 5% B. Injection volume was kept at 20 μL and was introduced into the HPLC system in triplicate. The absorbance was detected at 270 nm, and the contents of phytochemicals were calculated from calibration curves of standard.

Table 1: Proportions of the individual fruit constituents of triphala formulations

Formulations	Proportions of the individual fruit constituents		
	Terminalia chebula	*Terminalia bellirica*	*Phyllanthus emblica*
Vata	12	4	8
Pitta	8	12	4
Kapha	4	8	12
Triphala (1:1:1)	8	8	8

In vitro 3-hydroxy-3-methylglutaryl-coenzyme A reductase enzyme assay

The inhibition of HMG-CoA reductase was determined based on Ultraviolet spectrophotometry. The enzyme assay kit was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The reaction mixture contained nicotinamide adenine dinucleotide phosphate (400 μM) and HMG-CoA (400 μM) in 200 μL final volume of potassium phosphate buffer (100 mM, pH 7.4). The reaction was initiated by added 2 μL HMG-CoA reductase (0.5–0.75 mg/mL) and incubated at 37°C for 10 min with/without (control) 2 μL sample solution. Pravastatin (Sigma-Aldrich Co., St Louis, MO, USA) was used as a positive inhibitor. The absorbance of the reaction mixture was measured at 340 nm. The percentage of enzyme inhibition was calculated as follows

$$\% \text{ enzyme inhibition} = \frac{\left(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}\right)}{\text{Absorbance}_{\text{control}}} \text{ x } 100$$

3-hydroxy-3-methylglutaryl-coenzyme A reductase molecular modeling

Template preparation

The HMG-CoA reductase template was prepared from a crystal structure of human HMG-CoA reductase bound to the inhibitor atorvastatin (protein data bank [PDB] entry code: 1HWK), downloaded from RCSB Protein Data Bank (https://www.rcsb.org/structure/1HWK). The bound crystal was solved by X-ray crystallography techniques with a resolution of 2.22 Å. Bound atorvastatin and all water molecules were removed, all polar hydrogen parameters were added, and non-polar hydrogens of 1HWK were merged after adding Gasteiger Huckel charges by AutoDock Tools 1.5.6 (ADT 1.5.6). Then, types of atoms were assigned, and grid representation of 1HWK was prepared by AutoGrid4.3.

Ligand preparation

The three-dimensional (3D) structures of crystal ligands, gallic acid, ellagic acid, chebulagic acid, and chebulinic acid were sketched and optimized with ChemBioDraw Ultra 13.0 and ChemBio3D Ultra 13.0 using MM2 method. The ligands were prepared with ADT 1.5.6 as follows: Gasteiger charges were assigned, non-polar hydrogens were merged, aromatic carbons were identified, and the rigid root and rotatable bonds were defined.

Docking procedure

The docking experiments were performed with AutoDock 4.2. The prepared ligands were docked to the template using a Lamarckian genetic algorithm for the ligand conformational search. Other docking parameters were set as follows: number of genetic algorithm runs: 100, population size: 150, maximum number of evaluation: 15,000,000, maximum number of generation: 27000. Final docked conformations were clustered using a tolerance of 2 Å root-mean-square deviations (RMSD). Each cluster consisted of conformers that had similar 3D structures (RMSD < 2 Å). The orientation with the lowest docking energy in the cluster of highest number of member was considered the most stable conformation.

RESULTS

Phytochemical contents of the triphala extracts

The phytoconstituents of triphala formulation; gallic acid, ellagic acid, chebulagic acid, and chebulinic acid were quantified as the chemical markers of vata, pitta, kapha, and triphala (1:1:1) extracts. The HPLC results revealed that all extracts contained gallic acid and chebulagic acid in the high contents comparing with the contents of ellagic acid and chebulinic acid [Table 2]. Their gallic acid contents were in the range of 19.71–26.87 mg per g of the extract, while the chebulagic acid amounts were found as 23.26–36.67 mg per g of extract. To consider the phytochemical components of four extracts, chebulagic acid and chebulinic acid contents of vata extract were significantly different compared to the other extracts, whereas pitta extract revealed the statistics significantly in gallic acid content ($P \leq 0.05$).

3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitory effect of the triphala extracts

Vata, pitta, kapha, and triphala (1:1:1) extracts were investigated their inhibition activity on HMG-CoA reductase at the concentration of 10 µg/mL. Results showed the extract of triphala (1:1:1) exhibited the enzyme inhibition lower than 50% [Figure 1]. Vata and kapha extracts performed the moderate inhibitory action on enzyme activity as 52% approximately. Pitta extract performed the inhibition of 58.4% ± 0.40% that stronger inhibitory ability than triphala (1:1:1) extract ($P \leq 0.05$). The positive HMG-CoA reductase inhibitor, pravastatin (at concentration 5 µg/mL) showed the potent enzyme inhibition of 97.3% ± 4.08% that was significantly higher than all extracts of triphala formulations ($P \leq 0.05$).

Results are expressed as mean ± S. D. Statistical comparison was performed using one-way analysis of variance. The symbol *represents statistically significant comparing with pravastatin and# represents statistical significance compared with triphala (1:1:1) extract ($P \leq 0.05$).

3-hydroxy-3-methylglutaryl-coenzyme A reductase molecular modeling

Gallic acid, ellagic acid, chebulagic acid, and chebulinic acid were used as ligands and docked into the active site of HMG-CoA reductase. Binding affinity was characterized by binding energy (ΔG), ligand efficacy, and the number of hydrogen bond with interacted residues as shown in Table 3.

The active binding site of HMG-CoA reductase composed of HMG-binding pocket and shallow groove for bulky hydrophobic group. Based on the docking results, all phytochemical compounds were located in the hydrophobic pocket of HMG-CoA reductase. Mostly hydroxyl group (-OH) and carboxyl group (-COOH) of compounds interacted with amino acid residues in the HMG-binding pocket, while the bulky phenolic groups were positioned in the hydrophobic pocket, especially in chebulinic acid and chebulagic acid [Figure 2]. Ellagic acid and gallic acid demonstrated good binding energy (ΔG = −6.23 and −6.54 Kcal/mol, respectively) and the lowest number of hydrogen bonds (6 and 5 respectively). Conversely, chebulinic acid and chebulagic acid showed the highest binding energy (ΔG = −3.86 and −1.14 Kcal/mol, respectively) and the highest number of hydrogen bonds (11 and 7, respectively).

DISCUSSION

The basic principle of ayurvedic medicine considers the balance between the natural elements and the tridoshas of the human body to promote wellness and life longevity.[21] Ayurvedic herbals are divided

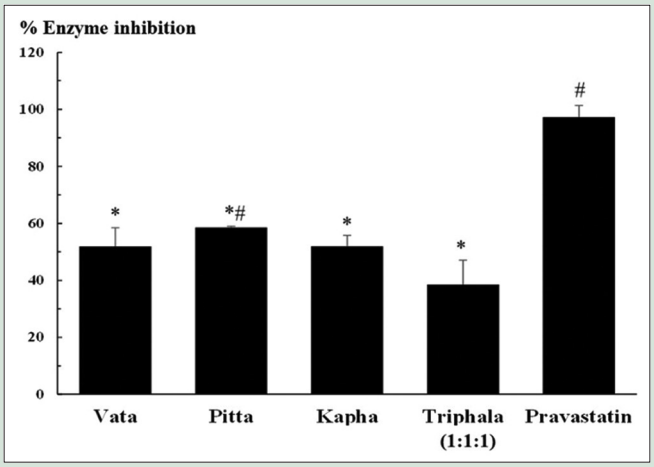

Figure 1: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibition of triphala extracts

Table 2: Phytochemical contents of the triphala extracts

Formulations	Phytochemical contents (mg/g of extract)			
	Gallic acid	Ellagic acid	Chebulagic acid	Chebulinic acid
Vata	26.62±0.994[a]	8.99±0.310	23.26±0.231[a]	1.11±0.007[a]
Pitta	19.71±3.316[b]	8.73±0.794	33.48±2.841[b]	1.98±0.203[b]
Kapha	31.01±1.191[a]	7.43±0.506	36.67±0.731[b]	2.02±0.111[b]
Triphala (1:1:1)	26.87±2.423[a]	7.90±0.380	33.96±1.020[b]	1.80±0.032[b]

Results are expressed as mean±SD with n=3. The difference among means was tested using one-way analysis of variance followed by Duncan multiple range test. P≤0.05 was considered statistically significant. [a,b]Different letters in the same column indicate significance (P≤0.05). SD: Standard deviation

Table 3: Results of docking experiment of -hydroxy-3-methylglutaryl-coenzyme reductase inhibitors and four phytochemical compounds of triphala with the catalytic portion of human 3-hydroxy-3-methylglutaryl-coenzyme reductase

Compound	ΔG (Kcal/mol)	LE*	H-bond	
			Number	Interacted residues
Atorvastatin	−12.09	−0.29	8	Glu559, Ser565, Ser684, Lys691, Lys692, Lys735, Ala751, Asn755
Gallic acid	−6.54	−0.55	5	Arg590, Ser684, Lys691, Lys735, Asn755
Ellagic acid	−6.23	−0.28	6	Ser684, Lys691, Lys692, Lys735, Ala751, Asn755
Chebulagic acid	−1.14	−0.02	7	Glu559, Cys561, Ser565, Arg590, Lys691, Asn755
Chebulinic acid	−3.86	−0.06	11	Glu559, Gly560, Ser565, Ser661, Lys735, Ala751, Asn755

ΔG: Gibbs free energy (Kcal/mol); * Ligand efficacy: ΔG/N, *n* is the number of non-hydrogen atom

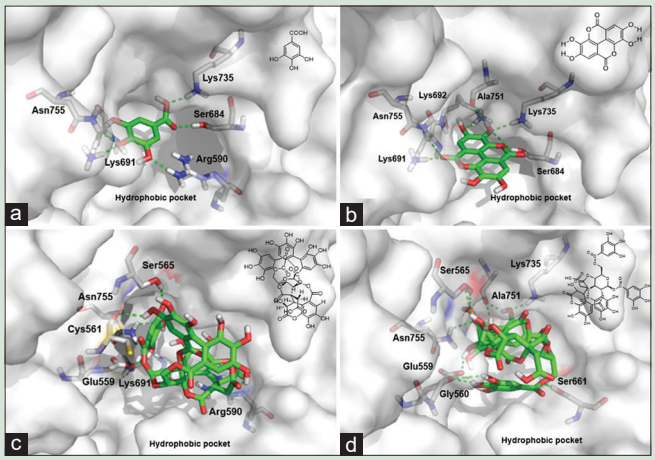

Figure 2: Binding modes of (a) gallic acid, (b) ellagic acid, (c) chebulagic acid, and (d) chebulinic acid in the binding site of HMG-CoA reductase derived from bound crystal (PDB: 1HWK). Hydrogen bonds are displayed as the green dash. The figures were generated using PyMol software

into three classes based on material origin. Among this, the herbal formulation has received increasing global attention in recent years.[22] The combination of several medicinal herbs can be explored to enhance extra therapeutic effectiveness and minimize any potential side effects of treatment. Triphala is an ancient ayurvedic herbal formulation that has been reported to possess a wide range of biological activities. Since the previous study established scientific evidence regarding the cholesterol-lowering properties of triphala and the reduction of HMG-CoA reductase activity was proposed as a key mechanism of the hypolipidemic effect.[16] The present study examined the *in vitro* HMG-CoA reductase inhibitory action of triphala extracts in the variated ratios of its three fruit constituents; vata, pitta, and kapha and triphala (1:1:1). The results demonstrated that pitta extract was the most potent enzyme inhibitor with the estimated IC_{50} lower than 10 μg/mL. Several herbal extracts have been shown their HMG-CoA reductase inhibition. At concentration, 250 μg/mL, *A. viridis,* and *B. alba* leaf extracts showed 71 and 74% inhibition, respectively.[9,10] The inhibitory effect of pitta extract is supposed higher than these extracts, which were reported as the potent inhibitors of HMG-CoA reductase. Moreover, pitta is responsible for enzymes of the body that performing various metabolic activities. It generally relates to the digestive enzyme, bile, internal fire, endocrine, and digestive system.[21] The enzyme inhibitory effect of pitta extract reveals the correlation with tridosha concept in Ayurveda.

Terminalia bellerica is a majority fruit constituent of pitta extract. So far the scientific information of *T. bellerica* on HMG-CoA reductase action has been not established. Only polyphenolics have been reported to inhibit this enzyme activity.[23] In the current study, pitta extract contained gallic acid and chebulagic acid as major components, whereas ellagic acid and chebulinic acid were found in small amount. Our HMG-CoA reductase docking results indicated the binding energy of gallic acid, and ellagic acid was less than chebulagic acid and chebulinic acid because of the small structure. However, their orientation was fit in the pocket due to the rigid structure of four fused rings and no steric hindrance between ligand and active binding site. The good fit between ligand and target led to strong hydrophobic interactions. In comparison, atorvastatin (HMG-CoA inhibitor) was nicely bound to the hydrophobic pocket of HMG-CoA reductase through hydrophobic interaction, and the binding was stabilized by eight hydrogen bonds. The oxygen atom of carbonyl group forms one hydrogen bond with the hydrogen atom of hydroxyl backbone of Ser565, and a proposed catalytic mechanism suggests the residues Glu559 participate directly in the reduction of substrate HMG-CoA.(4) The structure of chebulagic acid and chebulinic acid were located into the hydrophobic pocket with high number of hydrogen bonds and interacted with crucial amino acid, Glu559 and Ser565, similarly to the binding mode of atorvastatin, nevertheless, the binding strength was not as good as that from atorvastatin.

CONCLUSION

Among four triphala extracts in the variated ratios of its three fruit constituents, pitta extract appears to be a potent HMG-CoA reductase inhibitor. Although the phytoconstituents of pitta extract, gallic acid, ellagic acid, chebulagic acid, and chebulinic acid are not structurally similar to statins. Molecular modeling study suggests that these active compounds can interact the enzyme with different configurations and binding affinities. Further investigation of active compounds and a mixture of active compound in different ratio on HMG-CoA reductase activity should be performed to confirm the potential of pitta extract as an alternative treatment for hypercholesterolemia.

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Conflicts of interest

There are no conflicts of interest.

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