Research Article

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PRELIMINARY QUALITY CONTROL OF THAI TRADITIONAL MEDICINE FORMULA "THOR-RA-NEE-SAN-THA-KAT"

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ABTRACT

The present study aimed to define preliminary quality control standards for Thor-ra-nee-san-tha-kat (TSK), a Thai traditional medicine indicated for the treatment of muscle pain caused by abdominal rigidity and for severe constipation. TSK is comprised of black pepper seeds (*Piper nigrum*, 60% w/w), Aloe vera sap (12.5% w/w) and 24 other ingredients. Organoleptic, microscopic, phytochemical and physicochemical properties, and levels of heavy metal and microbial contamination were determined according to the methods of the Thai Herbal Pharmacopoeia. TSK is a brown, bitter tasting powder with a pungent odor. Microscopic examination revealed mesocarp filled with yellow oil glands, beaker cells of endocarp and stone cells from *P. nigrum*. Phytochemical analysis revealed the presence of alkaloids, anthraquinones and polyphenolics. Heavy metal analysis indicated the presence of arsenic, cadmium and lead within the allowable range limits.

Key words: Thai traditional medicine, black pepper, *Piper nigrum*, preliminary quality control

INTRODUCTION

Thai traditional medicine is the compilation of Buddhist principles, cultural medicinal practices, and traditional philosophies passed down from generation to generation. Historically, the originator of Thai traditional medicine is an Indian doctor, Jivaka Komarabhacca[1-3]. The majority of Thai traditional dispensatories integrate Ayurvedic herbal medicines, Chinese herbal medicines and Thai medicinal plants[4-6]. Several Thai traditional medicines have been included in the list of herbal medicinal products as essential medicines since 1999. In 2016, there were 50 Thai traditional dispensatories and 24 products developed from herbs listed in the National List of Essential Medicines (NLEM)[7].

The present study examines Thor-ra-nee-san-tha-kat (TSK), a Thai traditional formula used since ancient times and included in the list of herbal medicinal products in 2016. The formula is composed of twenty-six plants; the main components are black pepper; *Piper nigrum* L. (60 % w/w) and *Aloe vera* Burm.f. (12% w/w) [Table 1]. In Thai traditional medicine, black pepper is regarded as a warming tonic herb, and is used to stimulate appetite, enhance digestion, and ease digestive disorders such as flatulence, diarrhea, and indigestion[8-10]. Aloe is used internally to treat constipation, coughs, ulcers, headaches, arthritis, and immune-system deficiency[11-12]. TSK is classified as a musculoskeletal drug for the cure of the muscle pain caused by abdominal rigidity and severe constipation. The dosage forms of TSK are as a 500mg capsule, as a 500mg tablet and as a bolus8. Administration is by the oral route, once a day, before breakfast or at bedtime. Currently, there are no guidelines for the quality control of TSK products and guidelines are only available for 5 out of 26 of the raw materials listed in the TSK formulation in the Thai Herbal Pharmacopoeia. The objectives of this study are to determine preliminary standards for TSK using powder microscopic characterization, phytochemical screening and thin layer chromatography (TLC) fingerprint profiling for the detection of standard compounds from black pepper (piperine), *Aloe vera* (aloin) and *Garcinia hanburyi* Hook. f. (gambogic acid).

MATERIAL AND METHODS

Collection and identification of plant materials

Authentic samples of the herb ingredients in TSK were procured in the Northeastern part of Thailand. The identity and authenticity of the raw materials were confirmed by organoleptic and powder microscopy and comparison with characters mentioned in the Thai, Indian and Chinese Herbal Pharmacopoeia[13-15]. A voucher specimen of each ingredient was deposited in the museum of the Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. *Aloe vera* sap was purchased from African Aloe (Uniondale, South Africa) to ensure sap with high laxative action, as indicated in traditional preparations. The Thai Herbal Pharmacopoeia TSK was prepared as per the method and ratios described in Thai Herbal Pharmacopoeia, 2016. Ingredient powders were passed through a #30 sieve and mixed thoroughly to obtain a homogeneous blend. Solvents and other chemicals used in the phytochemical, physicochemical and TLC studies were of analytical grade and purchased from Merck (Darmstadt, Germany) and S.D. Fine Chemicals (Mumbai, India). Piperine (purity 97%), aloin (purity 98%) and gambogic acid (purity 98 %) were purchased from Sigma-Aldrich (St Louis, USA).

Pharmacognostic study

Organoleptic parameters (color, odor, taste, morphology) of TSK powder was analyzed and recorded as follows. A small quantity of TSK powder was dissolved with distilled water and a few drops were spread on a glass slide, covered with a cover slip and excessive water was removed with filter paper. Microscopic evaluation of TSK powder was done with and without staining

and studied under a Carl-Zeiss Trinocular microscope. For preliminary phytochemical studies, 5g of TSK powder was successively extracted with ethanol and water. The extracts were concentrated by distillation under reduced pressure. Aqueous and ethanol extracts of TSK samples were analyzed for the presence of alkaloids, glycosides, flavonoids, tannins and sterols[16-19].

Physicochemical evaluations

Physicochemical characteristics of the TSK sample were analyzed by quantitative analysis for total ash, water-soluble ash, acid-insoluble ash, water soluble extractives, alcohol-soluble extractives, loss on drying, as recommended by the Thai Herbal Pharmacopoeia 2016 (p.576-579). TSK samples were analyzed for presence of lead (Pb), arsenic (As) and cadmium (Cd) by atomic absorption spectroscopy (model AA 240, Varian, The Netherlands) [20-23].

Microbial testing

Microbial testing involved determination of total bacterial count and total fungal count. Each of the microbial experiments was performed in a sterilized laminar air flow chamber. The sample stock solution was prepared by taking 10 mg of TSK powder and making up the volume to 100 ml with Soybean Casein Digest broth. One ml of the stock solution was used as the test sample for all microbial tests. For total bacterial count, the test and control samples were incubated on Soybean Casein Digest Agar plates at 35˚C for 2-3 days. For total fungal count, the samples were incubated on Sabouraud Dextrose Agar (SDA) plates at 25˚C for 5-7 days[24-25]. Colonies of bacteria and fungi were counted and contamination was assessed according to Thai Herbal Pharmacopoeia (THP) (p. 588-620) and ANNEX III ASEAN guidelines on limits of contamination for traditional medicines and health supplements 2014.

TLC Chromatogram study of TSK formula

A chromatogram study of TSK samples for identification of chemical markers was performed by TLC densitometry. Silica gel 60 F254 TLC plates with aluminum sheet support (0.2 mm thickness) (E. Merck) were used. A 100 µL glass syringe (Hamilton) and Camag Linomat V spotting device was used (Camag, Muttenz, Switzerland). The developing chamber was a Camag glass trough chamber (20 × 10 cm) previously saturated with mobile phase vapor for 30 min. Stock solutions of 1000 µg/ml of piperine, aloin and gambogic acids were prepared in methanol[26-27]. TSK samples were prepared in the concentration of 1 mg/ml. Various mobile phase systems using various solvents (toluene, ethyl acetate, formic acid, methanol and water) were used for the visualization of the selected chemical markers in TSK extract.

RESULTS AND DISCUSSION

The original preparation of TSK formula is to mix the powder of 26 raw materials and perform a decoction. The present dosage form of TSK formula is available in hospitals as 500 mg capsules containing the powder of the 26 herbs as listed in Table 1. This study is the first to collect authentic samples of all 26 herbs with voucher specimens. As shown in Table 1, 19 herbs of the TSK dispensary have Sanskrit names which reflect the major influence of Ayurvedic medicine. *Atractylodes lancea* (thumb.) DC and *Rheum officinale* Baillon are Chinese herbal medicines and the remaining five herbs are local Thai medicinal plants.

The organoleptic characters of the TSK formula is tabulated in Table 2. The preliminary phytochemical screening for various functional groups is tabulated in Table 3. The heavy metal content, physicochemical parameters and microbial counts of TSK formula is tabulated in Tables 4, 5 and 6, respectively.

The TSK powder was a brown-colored powder, with a bitter taste, smooth texture and the pungent odor of black pepper mixed with various aromatic and spicy herbs. Since the TSK formula consists of 26 herbs, the identification of all organelles from the herbs by microscopic analysis is impractical. Therefore, the microscopy study was concentrated on the components with the highest proportion by weight. In addition, there are several components in the TSK formula that are derived from sap and resin, such as *Aloe vera* sap, camphor and resin from *Garcinia hanburyi* and *Ferula assa-foetida*. All of these constituents have acellular structure, and therefore there are no specific organelles that can be observed. Microscopic characterization revealed the diversity of organelles as shown in Figure 1 (A-N). The most abundant organelles were from *Piper nigrum* L. including epicarp, mesocarp, beaker cells of endocarp and stone cells, which corresponds to black pepper making up the highest proportion of TSK (60 % by weight). Mesocarp of *Piper nigrum* L. was filled with yellow oil glands (Figure 1: A) and this is one of the distinct organelles found in the TSK formula. Other abundant organelles were epicarp, and fragment fibres and crystals from *Phyllanthus emblica* L. There were numerous organelles found in the powder at levels much lower than those from *Piper nigrum* L. and *Phyllanthus emblica* L.

As seen in Table 3, the preliminary phytochemical screening of hot aqueous and ethanolic extracts of TSK indicated the presence of alkaloids, flavonoids, steroids, tannins and anthraquinones. The presence of alkaloids and anthraquinones corresponded to the chemical markers piperine and aloin from black pepper and aloe, respectively. Heavy metal contents of TSK samples were found to be within permissible limits (Table 4). The results of microbial content study showed the absence of *Escherichia coli*, *Salmonella* spp, *Staphylococcus* spp, and *Clostridium* spp. However, *Enterobacteria* spp were found within the acceptable limit (Table 5). Thin layer chromatograms of the ethanol extract of TSK under short wavelength (254 nm) and long wavelength (365 nm) UV light are presented in Figure 2. A mixture of toluene: ethyl acetate: formic acid (5:4:1) was found to be the best mobile phase for the separation of piperine and gambogic acid in the extract. However, there was no separation of the aloin standard with the toluene: ethyl acetate: formic acid 5:4:1 mobile phase. Piperine, and gambogic acid are the main chemicals from *Piper nigrum* L., and *Garcinia hanburyi* Hook.f. in the TSK formula. Piperine is the most suitable chemical marker for the TSK formula because it is from the major ingredient and remains stable in the product. Although black pepper is included in several traditional medicines available in Thailand (such as Trikatu), there are only a few formula that contain black pepper as the main ingredient (more than 50%). Furthermore, TSK formula is the only traditional medicine product that contains black pepper and *Garcinia hanburyi* Hook. f. The detection of piperine and gambogic acid can therefore be considered as the distinctive characteristic of the TSK formula. Suitable chemical markers from *Aloe vera*, the second major ingredient in TSK, will need to be identified to complete this quality control test for TSK. Overall, the microscopic study and the TLC chromatogram of TSK showed that the majority of organelles and chemical markers in TSK came from black pepper, which makes up 60% of the formula by weight.

Table 1: Ingredients of Thor-ra-nee-san-tha-kat

Scientific name	Thai name	Sanskrit name	Part used	Part
Piper nigrum L.	Prik Thai	Maricha	Seed	60
Aloe vera (L.) Burm.f.	Ya Dam	Kumair	Sap (Leaf)	12.5
Terminalia chebula Retz.	Samor Thai	Harithaki	Fruit	3.75
Ferula assa-foetida L.	Ma Ha Hing	Hingu	Resin	3.75
Cinnamomum camphora (L.) Presl.	Ga La Boon	Kapur	Waxy solid	3.75
Garcinia hanburyi Hook.f.	Rong Thong	-	Sap (Stem)	2.5
Iresine herbstii Hook.f.	Pak Paew Dang	-	Aerial	1.25
Phyllanthus emblica L.	Ma Kham Pom	Amla	Fruit	1.25
Alocasia indica Schott.	Kra Dard Khao	Alooka	Tuber	0.625
Alocasia indica var.*metallica* Schott.	Kra Dard Dang	-	Tuber	0.625
Amomum testaceum Ridl.	Kra Wan	-	Fruit	0.625
Amomum xanthioides Wall.	Raew	-	Fruit	0.625
Amorphophallus campanulatus- Blume ex. Decne.	Bok	Arsaghna	Tuber	0.625
Atractylodes lancea (thumb.) DC.	Kot Ka Mao	-	Rhizome	0.625
Cuminum cyninum L.	Tien Khao	Jeeraka	Seed	0.625
Dioscorea hispida Dennst.	Gloy	Hastyaluka	Tuber	0.625
Glycyrrhiza glabra L.	Cha Em Thed	Yashti-madhu	Root	0.625
Gloriosa superba L.	Dong Dueng	Kalihari	Tuber	0.625
Myristica fragrans Houtt.	Luke Chan	Jatiphala	Seed	0.625
Myristica fragrans Houtt.	Dok Chan		Aerial	0.625
Nigella sativa L.	Tien Dam	Kalonji	Seed	0.625
Plumbago indica L.	Chet Ta moon	Chitrak	Root	0.625
Rheum officinale Baillon.	Kot Nam Tao	-	Rhizome	0.625
Saussurea lappa Clarke.	Kot Kra Doke	Kushtha	Root	0.625
Syzygium aromaticum (L.) Merr. & Perry.	Kan Plu	Lavanga	Flower bud	0.625
Zingiber officinale Roscoe.	Khing	Sunthi	Rhizome	0.625

Table 2: Organoleptic properties of Thor-ra-nee-san-tha-kat

Parameters	Powder
color	Dark brown
taste	Bitter and spicy
odor	Pungent smell
dosage form	Powder

Table 3: Phytochemical Screening of Thor-ra-nee-san-tha-kat

Chemical test (reagent)	Aqueous extract	Ethanol extract
Alkaloid		
Dragendorff's reagent test	+	+
Wagner's reagent Test	+	+
Mayer's reagent test	+	+
Hager's reagent test	+	+
Tannic acid test	+	+
Anthraquinone (Modified Borntrager test)	+	+
Flavonoids (Shinoda test)	+	+
Tannins (Folin–Denis reagent)	+	+
Saponin (Foam test)	-	-
Cardiac Glycoside (Keller-Killiani test)	-	-
Phytosterol (Liebermann's – Burchard's test)	+	+

+ = present; - = absent

Table 4: Heavy metal contents of Thor-ra-nee-san-tha-kat

Test parameters	Result	limit	Analytical method
Arsenic (As)	1.3 ppm	< 4 ppm	Inductively coupled plasma atomic emission spectroscopy (ICP-AES)
Cadmium (Cd)	Absence	< 0.3 ppm	
Lead (Pb)	4.4 ppm	< 10 ppm	

Table 5: Physicochemical parameters of Thor-ra-nee-san-tha-kat

parameters	Pharmacopoeia standard	Result	Limit	Analytical method
Powder characteristics	Not available	Pass	Fine brown powder with unique pungent odor filled in white capsule	-
Weight variation	Not available	Pass	+/-10% (Capsule contained 500 mg powder)	-
Foreign matter	Not available	Absence	< 2%	-
Level of pesticide contamination	Not available	In the safety range	Analysis of the residue of anti-choline esterase pesticides	Colorimetric cholinesterase inhibitor assay
Loss on drying at 105 ° C	Not available	8%	< 10%	-
Total ash	Not available	4.20%	< 9%	-
Acid insoluble ash	Not available	0.18%	< 3%	-
Alcohol soluble extractive	Not available	26.02%	> 13%	-
water soluble extractive	Not available	21.37%	> 11%	-

Table 6 Microbial counts of Thor-ra-nee-san-tha-kat

Test parameters	THP	observation	Analytical method
Total viable aerobic count (bacteria)	$\leq 5.0\times10^5$/g	< 250 per g	Thai herbal Pharmacopoeia 2016 and WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues
Total viable aerobic count (fungi)	$\leq 5.0\times10^5$/g	< 10 per g	
Enterobacter spp.	$< 10^3$	< 10 per g	
Escherichia coli	Absence	Absence	
Staphylococcus spp.	Absence	Absence	
Salmonella spp.	Absence	Absence	
Clostridium spp.	Absence	Absence	

* Thai herbal Pharmacopoeia (THP)

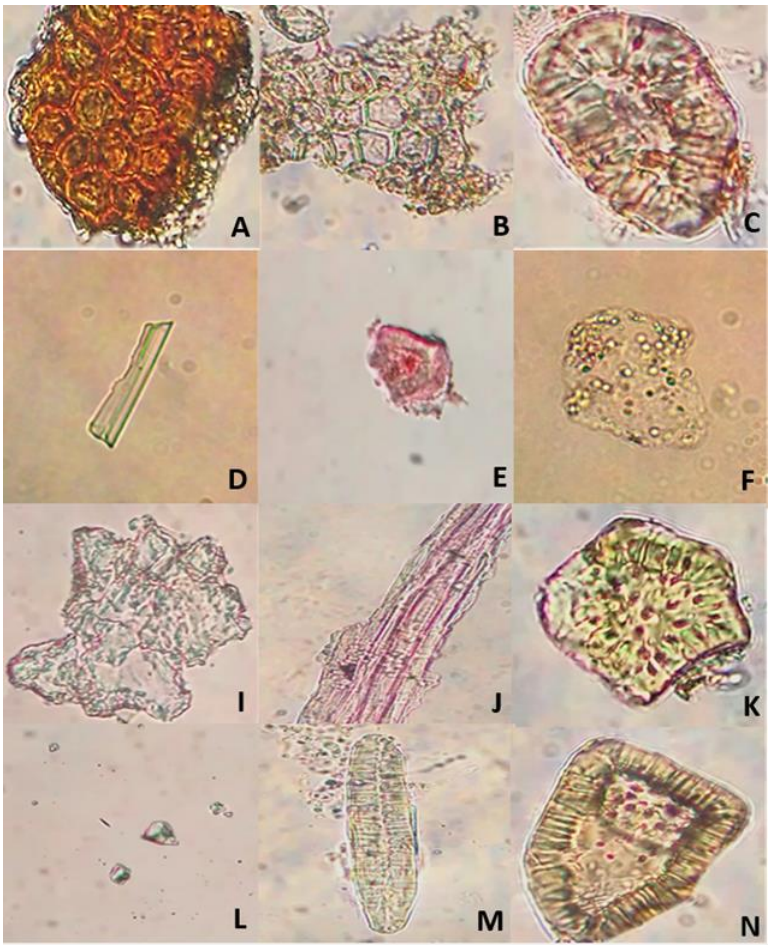
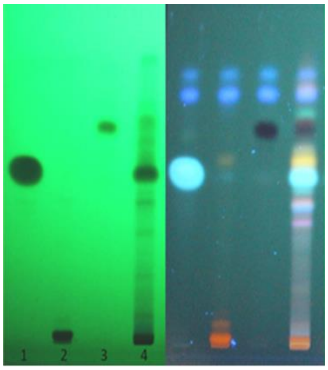


Figure 1: Microscopic analysis of Thor-ra-nee-san-tha-kat

A. Epicarp in surface view, B. Polygonal cells of mesocarp, C. Stone cells, D. Monoclinic crystals, E. Beaker cells of endocarp, F. Starch grains, (A-F: organelles from black pepper, *Piper nigrum*), I. Epicarp in surface view, J. Fragment of Fibres, K. Sclereid, L. Prismatic crystals, M. Fibrous sclereids, (I-M: organelles from *Phyllanthus emblica* L.), N. Sclerenchyma of endocarp (*Terminalia chebula* Retz.)



A **B**

Figure 2: TLC chromatogram of Thor-ra-nee-san-tha-kat

Lane 1 Standard piperine, Lane 2 Standard aloin, Lane 3 Standard gambogic acid, Lane 4 TSK extract, A and B Mobile phase toluene: ethyl acetate: formic acid 5:4:1, (piperine R_f = 0.53, gambogic acid R_f = 0.7), A visualization under UV light at 254 nm, B visualization under UV light at 365 nm

CONCLUSION

The present work was carried out for the standardization of TSK formula. The in-house formulation was studied for various physicochemical parameters as well as phytochemical screening and TLC analysis. The organelles that were ubiquitously distributed in TSK powders were from black pepper, *Terminalia chebula* Retz and *Phyllanthus emblica* L. TLC chromatography has been performed for the detection of piperine and gambogic acid from TSK product. The overall quality-control parameters and the developed TLC methods may be considered as a tool for assistance for scientific organizations and manufacturers in developing standards. Hence quality control of TSK formula is established in the given standard conditions and this study outcome may be considered as a reference standard for future scientific studies.

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