Original article

The Stability of Sahastara Remedy Ethanolic Extract Used for Application in Clinical Study

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Abstract

Introduction: The Sahastara remedy is a Thai traditional medicine which has long been used for muscle and joint pain treatment. It has been reported to be anti-inflammatory drug, so it was developed in the form of ethanolic extract capsule. Thus, stability of the extract capsule should be studied to obtain data for clinical study. This study was to investigate the stability of nitric oxide production inhibition activity and piperine content of Sahastara remedy ethanolic extract capsules.

Method: The Sahastara remedy was extracted with 95% ethanol. The extract was filled in capsule No.0 (sized 500 mg) to contain 100 mg of Sahastara remedy ethanolic extract. The capsules were kept in accelerated conditions (40 C ± 75% Rh) for 180 days. Capsules were sampling on 15, 30, 60, 90, 120, 150 and 180 days. The nitric oxide production inhibition activity was determined to assess the biological activity of Sahastara remedy ethanolic extract capsules. The piperine content was determined by high performance liquid chromatography to assess chemical content in Sahastara remedy ethanolic extract capsules. The results at different time intervals were compared with baseline (Day 0).

Results: IC_{50} of nitric oxide production inhibition of capsule was 2.56 ± 0.81 µg/mL at baseline (Day 0). There is no significant difference at each time interval when compared to baseline data with $p > 0.05$. Piperine content was 24.35 ± 0.21 mg/capsule at baseline (Day 0). The piperine content at each time interval also showed no significant difference when compared to baseline.

Discussion and Conclusion: The Sahastara remedy ethanolic extract capsules was stable at room temperature because its biological activity and chemical content did not change when it was kept in accelerated condition. It is assumed that the Sahastara remedy ethanolic extract capsules could be stored at room temperature for at least 2 years without losing the nitric oxide production inhibitory activity and piperine content. It is thus appropriate to be used in clinical study.

Key words: Sahastara remedy, Stability, Piperine, Nitric oxide inhibition, Anti-inflammation

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Introduction

The Sahastara (SHT) remedy is a Thai traditional medicine which has long been used for muscle and joint pain treatment for longer than 50 years. It is also in the Thai National List of Essential Medicine. It contains 21 medicinal plants. The main ingredients of SHT remedy was piper species which has piperine as major compound[1], so piperine was chosen as marker for this study. In previous study, SHT ethanolic extract showed good biological activity which related to traditional use. It showed high anti-inflammatory effect by inhibiting nitric oxide (NO) production and prostaglandin E_2 production release having IC_{50} = 2.81 and 16.97 µg/mL, respectively[2]. The SHT remedy in powder form also showed equal efficacy to diclofenac in osteoarthritis knee treatment. Thus, it has potential to be developed as modern drug in extract form. The clinical study is a necessary part for phytopharmaceutical development. Thus, the stability at accelerated condition of the drug is necessary for clinical trial study and comparing with modern drugs have to be studied. The stability testing under accelerated condition (40 °C 75% relative humidity, for 6 months or 180 days) was performed which implies that drug would be stable for at least 2 years. These stability data of drug can be used in clinical trial study. However, there is not yet any report on stability of SHT remedy ethanolic extract capsule for clinical study. This study investigated the stability of SHT remedy ethanolic extract capsules under accelerated condition by evaluation its anti-inflammatory activity through NO production inhibition activity and piperine content.

Method

Plants preparation

The Sahastara (SHT) remedy was prepared according to National List of Essential Medicines (NLEM) 2011. Plant ingredients of SHT remedy were collected from several parts of Thailand or purchased from drug stores. The medicinal plants were identified and given specimen voucher numbers (Table 1). These herbs were thin sliced, washed and dried by hot air oven. After that, all medicinal plants were coarsely ground to use for extraction.

Table 1 Medicinal plants in Sahastara remedy formulation (1,000 g.)

Thai name	Scientific name	Voucher specimen	Part used	Weight(g)
Prik-Thai	*Piper nigrum* Linn.	SKP146161401	Fruit	240
Jet-Ta-Mul-Plerng-Dang	*Plumbago indica* Linn.	SKP148160901	Root	224
Sa-mhor-thai	*Terminalia chebula* Retz.	SKP049200301	Fruit	104
Dee-plee	*Piper retrofractum* Vahl.	SKP146160301	Fruit	96
Tong-Tank	*Baliospermum montanum* Muell.A.	SKP121021301	Root	80
Wan-Nam	*Acorus calamus* Linn.	SKP015010301	Rhizome	88
Has-sa-khun-tade	*Kleinhovia hospita* Linn.	SKP183110801	Root	48
Ka-ra-boon	*Cinnamomum camphora* Linn.	SKP096030301	-	14
Dok-Chan	*Myristica fragrans* Houtt.	SKP121130601	Aril of seed	13
Luk-Chan	*Myristica fragrans* Houtt.	SKP121130601	Seed	12
Tien-Dang	*Lepidium sativum* Linn.	SKP057121901	Seed	11
Tien-Ta-Tuk-Ka-Tan	*Anethum graveolens* Linn.	SKP199010701	Fruit	10
Ma-Ha-Hing	*Ferula assafoetida* Linn.	SKP199060101	Resin	10

Table 1 Medicinal plants in Sahastara remedy formulation (1,000 g.)

Thai name	Scientific name	Voucher specimen	Part used	Weight(g)
Tien-Sut-Ta-But	*Pimpinella anisum* Linn.	SKP199160101	Fruit	9
Tien-Khao	*Cuminum cyminum* Linn.	SKP199030301	Fruit	8
Jing-Jor	*Merremia vitifolia* *(Burm.f.) Hallier f.*	SKP054132201	Root	8
Tien-Dum	*Nigella sativa* Linn.	SKP160141901	Seed	7
Kote-Kag-Kra	*Anacyclus pyrethrum* (L.) DC.	SKP051011601	Root	6
Kote-Ka-Mao	*Atractylodes lancea* *(Thunb) DC.*	SKP051011201	Rhizome	5
Kote-Kan-Prao	*Picrorhiza kurroa* Benth.	SKP177161101	Root	4
Kote-Pung-Pla	*Terminalia chebula* Retz. (*gall*)	SKP019200301	Gall	3

Sahastara remedy extraction

The ground plants of SHT remedy were mixed according to the proportions in NLEM. This SHT remedy powder were macerated with 95% ethanol for 3 days, then filtered, and the filtrate was evaporated by rotary evaporator. The residue of SHT remedy was further macerated for 2 more times. All extracts were combined and calculated for percent yield.

Encapsulations

The SHT remedy ethanolic extract capsules was prepared by center of Excellence in Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University. The SHT remedy ethanolic extract 100 mg and excipients were encapsulated in capsule No.0 (500 mg). The quality control of capsules were performed including loss on drying, weight variation, contamination test and disintegration time following Thai Herbal Pharmacopoeia standard[3, 4.]

Anti-inflammatory activity by Nitric oxide production inhibition (Griess's reagent assay)

The NO production inhibition assay was performed to determine the anti-inflammatory activity of SHT remedy ethanolic extract capsules after finishing the stability test. All samples in capsules were extracted with 95% ethanol before testing. The method was slightly modified from previous report of Makchuchit et.al., 2017[5]. Briefly, the murine macrophage cell RAW264.7 was cultured in RPMI 1640 medium, supplemented with 0.1% sodium bicarbonate and 2mM glutamine, penicillin G (100 units/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (FBS). 100 µl of suspended cell was seeded into 96 well-plate (1×10^5 cells/well) and incubated at 37 °C under 5% CO_2 for 24 hours. Then, the medium was replaced with LPS 100 µl/well (except control). The sample extract was dissolved with DMSO and diluted to different concentrations (10, 1, 0.1, 0.01, 0.001 µg/mL) with medium and 100 µL/well were added to 96 well-plate. After being incubated for 24 hours at 37 °C under CO_2, 100 µL/well of supernatant was removed to another 96 wells-plate where 100 µl/well Griess reagent was added and OD was detected at 570 nm. 10 µl/well of MTT was added to another 96-well plate and incubated at 37 °C under CO_2 for 2 hours. After that, supernatant was removed and 0.04M HCL in isopropanol was added 100 µL/well. Then, OD was measured at 570 nm. The OD values were used to calculate for % inhibition (Griess reagent) and % survival (MTT).

Piperine determination by HPLC

Piperine determination was performed to measure piperine content in SHT remedy ethanolic extract capsules. The study was performed using HPLC (Agilent, LC1200) with Photodiode array detector (DAD) and autosampler. Analytic column was Luna C18 (2) (Phenomenex), 250 x 4.6 mm, 5 µm, Guard cartridge (C18), 4 x 3.0 mm (Phenomenex) with guard holder. The analytic condition was slightly modified from previous report of Sakpakdeejaroen and Itharat, 2013[1]. Injection volume is 10 µl at room temperature with flow rate of 1 mL/min. Detection was performed at 340 nm for 70 min runtime. The mobile phase, water and acetonitrile (ACN), using gradient elution (Table 2) was filtered through 0.45 µm Millipore filter. A stock solution of piperine standard was prepared, 1.0 mg of piperine standard was weighed accurately and dissolved in 1 mL ACN. The stock solution of piperine standard was diluted to five different concentrations (400, 200, 100, 50 and 25 µg/mL) with ACN, and 1 mL of each concentration was prepared. Five concentrations of piperine standard were injected to produce the standard curve. The regression equation and co-efficient of correlation (r^2) of piperine standard was performed. 10 mg of SHT extract was weighed accurately and dissolved in 1 mL ACN. The sample was injected under the same conditions as piperine. The piperine content in sample extract was calculated from the regression equation of piperine standard.

Table 2 The gradient solvent elution and time setting for piperine content analysis

Time	Water	Acetonitrile
0	60	40
30	50	50
50	5	95
60	0	100
65	60	40
70	60	40

Stability test in accelerated condition

The stability test was taken place under accelerated conditions (40 °c and 75% RH) in stability incubator (Termaks-KB8400, Norway). The SHT remedy extract capsules were stored for 8 different duration times, namely 0, 15, 30, 60, 90, 120, 150 and 180 days. Then, the NO production inhibition assay and determination of piperine by HPLC were performed on SHT remedy extract capsules at each time interval and compared with Day 0.

Data analysis

The data of anti-inflammatory activity results were carried out in triplicate. IC_{50} was calculated by prism programed. Values of different parameters were represented as the mean ± SEM.The pair-t test was used to compare value change from baseline, $p < 0.05$ indicated a significant difference.

Piperine content data were carried out in triplicate. The value represents as mean ± SD and percentage of piperine content. The pair-t test was used to compare value change from baseline (Day 0), $p < 0.05$ indicated as significant difference.

Results

The SHT remedy extract was brown in color and sticky. The percentage yield (w/w) is 9.80%. SHT capsules meet the quality control requirements. (Table 3)

Table 3 The quality standards of SHT remedy ethanolic extract capsules[3, 4]

Quality control testing	Result	Standard value	Summary
Moisture test (Loss on drying)	8.12%	< 10 %	Pass
Bacterial contamination	< 10 CFU/g	< 5 x 105 CFU/g	Pass
Yeast & Mold contamination	10 CFU/g	< 5 x 103 CFU/g	Pass
Disintegration time	14.01 minutes	< 30 minutes	Pass
Weight variation: average weight*	534 mg	> 462.5 mg	Pass

* Weight difference between capsules < or = 5% of average weight.

The Stability of NO production inhibition activity

IC_{50} of NO production inhibition test was 2.56 ± 0.81 µg/mL at baseline (Day 0). It showed surprisingly that SHT remedy extract has more potent NO production inhibition activity at the end of storage at Day 180 with IC_{50} of 1.11 ± 0.59. However, there is no significant difference when compared between baseline data and value of each different time points with $p > 0.05$ (Table 4). Therefore, it can be concluded that SHT extract capsule is stable without changing anti-inflammatory activity when stored at room temperature for 2 years[6].

Table 4 The NO inhibition testing in stability study by accelerated condition

Sample (n = 3)	IC_{50} of NO production inhibition (µg/mL)*	P - value**
Day 0	2.56 ± 0.81	-
Day 15	1.88 ± 1.01	0.525
Day 30	4.86 ± 1.69	0.213
Day 60	6.13 ± 2.11	0.141
Day 90	5.79 ± 1.75	0.77
Day 120	4.43 ± 1.71	0.289
Day 150	2.03 ± 0.74	0.406
Day 180	1.11 ± 0.59	0.398

* Data represent mean ± SEM of triplicate

** Statistical analysis; Paired t-test which compared with Day 0, Significant difference ($p < 0.05$)

The Stability of piperine content

The percentage of piperine content at each time point was not less than 90% when compared with baseline at Day 0 (Figure 1). It could be assumed that the SHT remedy ethanolic extract capsules could be stored in room temperature for 2 years without losing of piperine content under acceleration stability ICH harmonised tripartite guided line method[6].

Piperine content should not be less than 190 mg/g.extract (19 mg/capsules) at every time point (Table 5). This was a requirement from previous report of SHT remedy powdered drug for treating OA knee patients. This result confirmed that SHT remedy has acceptable piperine content value to be used in clinical study for 2 years.

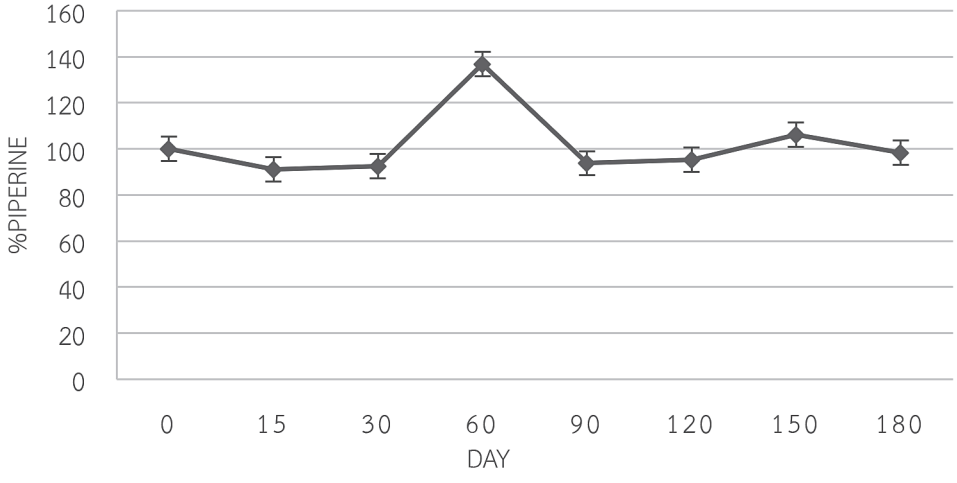

Figure 1 The percentage of piperine in SHT remedy ethanolic extract capsules

Table 5 The piperine content of SHT remedy ethanolic extract capsules

Sample (n = 3)	Piperine content (mg/capsule)	% piperine
Day 0	24.35 ± 0.21	100 ± 0.00
Day 15	22.20 ± 0.21	91.19 ± 0.01
Day 30	22.50 ± 0.14	92.41 ± 0.32
Day 60	33.30 ± 0.23	136.79 ± 0.63
Day 90	22.83 ± 0.27	93.77 ± 0.33
Day 120	23.18 ± 3.83	95.17 ± 14.77
Day 150	25.84 ± 0.08	106.14 ± 1.56
Day 180	23.93 ± 3.91	98.27 ± 16.93

Data represent mean ± SD of triplicate

Discussion and Conclusion

The SHT remedy ethanolic extract capsules contain 100 mg maximum in each capsule where SHT ethanolic extract was sticky and moist. However, the SHT remedy ethanolic extract capsules which contain 100 mg per capsule were passed all the requirement of standard control[3, 4]. In addition, 100 mg SHT remedy ethanolic extract capsule was related to efficacious dose usage in OA knee patients which calculated from percent yield of SHT remedy extract (approximately 10%)[7].

The SHT remedy ethanolic extract capsules show good anti-inflammation activity as NO production inhibition. There is no sample show IC_{50} more than 30 µg/mL. This study related to previous study which suggested that SHT remedy ethanolic extract should has IC_{50} of NO inhibition not higher than 30 µg/mL[8]. It also showed good efficacy to treat OA knee patients with the same criteria of NO production inhibition tested[7]. It is assumed that not only SHT remedy ethanolic extract capsules could be stored at room temperature for at least 2 years, yet it also has good anti-inflammatory effect during such period.

The SHT remedy ethanolic extract capsules have piperine content between 222.0 and 333.3 mg/g. extract. The highest piperine content was on Day 60. The increasing of piperine might reflect the transformation of certain compound to piperine in SHT remedy extract such as chavicine[9]. Although, the piperine content of SHT remedy ethanolic extract capsule on Day 60 was higher than others day, the NO production inhibition activity was not significantly changed. The piperine content in SHT remedy ethanolic extract capsules were not less than 190 mg/g. extract or 19 mg/capsule. This related to previous study of SHT remedy powder drug which SHT remedy extract contain piperine equivalent to 19 mg/capsule must be used in treating OA knee patients[7]. It could be assumed that SHT remedy ethanolic extract could be stored at room temperature for 2 years with no difference of piperine content from the effective dose.

The SHT remedy ethanolic extract capsules are stable regarding both anti-inflammatory activity by NO production inhibition and piperine content when it was kept under accelerated condition. It can be concluded that SHT capsules can be stored at room temperature for at least 2 years without losing biological activity and chemical content. The SHT remedy ethanolic extract capsules are appropriate drug to be used in clinical study. These data are standard information of SHT remedy ethanolic extract capsule to be used in clinical application in the further study.

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Potential conflicts of interest

None.

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บทคัดย่อ

การศึกษาความคงตัวของยาแคปซูลสารสกัดตำรับสหัศธาราเพื่อใช้ในงานวิจัยทางคลินิก

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ผู้ให้ติดต่อ: รองศาสตราจารย์ ดร. อรุณพร อิฐรัตน์ สถานการแพทย์แผนไทยประยุกต์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ และศูนย์ความเป็นเลิศทางการวิจัยแพทย์แผนไทย คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ **อีเมล:** iarunporn@yahoo.com

บทนำ: ยาตำรับสหัศธาราเป็นตำรับยาที่ใช้ในการรักษาอาการปวดของกล้ามเนื้อกระดูกและข้อในทางการแพทย์แผนไทยมาเป็นเวลานาน และอยู่ในบัญชียาหลักแห่งชาติของประเทศไทย จากการศึกษาก่อนหน้าพบว่ายาสหัศธารามีฤทธิ์ในการต้านการอักเสบที่ดี และมีคุณสมบัติที่จะพัฒนาให้อยู่ในรูปของสารสกัดได้ จึงมีความจำเป็นที่จะต้องศึกษาความคงตัวของแคปซูลสารสกัดสหัศธาราเพื่อเป็นข้อมูลสำหรับการวิจัยทางคลินิกต่อไป เพื่อศึกษาความคงตัวของยาแคปซูลสารสกัดสหัศธาราโดยวัดฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่งไนตริกออกไซด์และปริมาณสารสำคัญ piperine ของยาตำรับนี้

วิธีการศึกษา: สกัดยาตำรับสหัศธาราด้วย 95% เอทานอล และสารสกัดสหัศธาราขนาด ๑๐๐ มิลลิกรัม และสารช่วยอื่นๆ ถูกบรรจุในแคปซูลเบอร์ ๐ (ขนาด ๕๐๐ มิลลิกรัม) ศึกษาความคงตัวของยาแคปซูลสารสกัดสหัศธาราในสภาวะเร่งที่อุณหภูมิ ๔๐ องศาเซลเซียส ความชื้นสัมพัทธ์ 75% เป็นระยะเวลา ๑๘๐ วันจากนั้นยาแคปซูลจะถูกสุ่มเก็บในวันที่ ๑๕, ๓๐, ๖๐, ๙๐, ๑๒๐, ๑๕๐ และ ๑๘๐ และทดสอบฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่งไนตริกออกไซด์และหาปริมารสาร piperine ด้วยวิธี HPLC ผลที่ได้จะถูกนำไปเปรียบเทียบความแตกต่างรายคู่กับค่าพื้นฐานที่วันที่ ๐

ผลการศึกษา: ยาแคปซูลสารสกัดสหัศธารามีฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่งไนตริกออกไซด์โดยมีค่า IC_{50} = ๒.๕๖ ± ๐.๘๑ ไมโครกรัม/มิลลิลิตร ในวันที่ ๐ เมื่อเปรียบเทียบความแตกต่างรายคู่ระหว่างระยะเวลาต่างๆ กับวันที่ ๐ พบว่า ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) ในขณะที่ปริมาณสาร piperine มีค่าเท่ากับ ๒๔.๓๕ มิลลิกรัม/แคปซูล (๒๔๓.๕ มิลลิกรัม/กรัมสารสกัด) ในวันที่ ๐ และเมื่อเปรียบเทียบความแตกต่างของปริมาณ piperine รายคู่ระหว่างระยะเวลาต่างๆ กับวันที่ ๐ พบว่าปริมาณสาร piperine ไม่มีความแตกต่างอย่างมีนัยสำคัญ

วิจารณ์ และสรุปผลการศึกษา: ยาแคปซูลสารสกัดสหัศธารามีความคงตัวทั้งฤทธิ์ทางชีวภาพและปริมาณสารเคมี เมื่อเก็บในสภาวะเร่งแสดงว่ายาแคปซูลนี้มีความคงตัวอย่างน้อย ๒ ปี เมื่อเก็บไว้ที่อุณหภูมิห้อง โดยไม่สูญเสียฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่งไนตริกออกไซด์และปริมาณสารสำคัญ piperine จึงสามารถนำไปใช้ในงานวิจัยทางคลินิกได้

คำสำคัญ: ยาตำรับสหัศธารา, ความคงตัว, สารไพเพอรีน, ฤทธิ์ยับยั้งการหลั่งไนตริกออกไซด์, ต้านการอักเสบ