Original Article

Quality Control of Raw Plant Materials and Stability Testing under Accelerated Storage Conditions of Kheaw-Hom Remedy Extract

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Abstract

Introduction: Kheaw-Hom remedy (KH), a Thai traditional antipyretic medicine, is included in the National List of Essential Medicine. Currently, there is no scientific report on its standard requirements for quality control and stability testing.

Objectives: To determine the quality control and stability testing of KH on anti-inflammatory activity and its bioactive marker

Methods: Quality control methods (loss on drying, total ash, acid-insoluble ash, extractive values, and heavy metals) of KH and raw plant materials were performed according to Thai Herbal Pharmacopoeia (THP). The stability testing of the ethanolic extract of KH (KHE) was stored under 40 ± 2 ºC and 75 ± 5% relative humidity for 180 days. Anti-inflammatory activity on LPS-induced nitric oxide (NO) production in RAW264.7 macrophage cells were evaluated. Ethyl *p*-methoxycinnamate (EPMC), a bioactive marker, was analyzed using high performance liquid chromatography (HPLC).

Results: KH showed loss on drying, total ash, acid-insoluble ash, ethanol soluble extractive and water-soluble extractive values of 8.66 ± 0.47%, 6.17 ± 0.06%, 1.14 ± 0.07%, 10.62 ± 0.12%, and 13.78 ± 0.54%, respectively. Eighteen plant materials met the requirements of THP. The anti-inflammatory activity on nitric oxide inhibition and EPMC content of KHE on day 180 exhibited no significant difference when compared with day 0.

Conclusions: This study is the first report on quality control and stability testing of KH. All KH and its plant components conformed to the standard requirements of THP. KHE could be stored at room temperature for two years.

Keywords: Quality control, Stability testing, Accelerated storage conditions, Kheaw-Hom

Volume 24, Issue 1, Page 49-61
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Received: 29 June 2023 **Revised: 15 December 2023** **Accepted: 11 January 2024**

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Introduction

Kheaw-Hom remedy (KH) is a Thai traditional antipyretic medicine, which has been officially published in the National List of Essential Medicine (NLEM).[1] Folk doctors and Thai traditional practitioners in hospitals have long used this remedy to treat fever with rash in children with illnesses, such as measles, chickenpox, and aphthous ulcers.[2-4] The remedy has a cool and bitter taste which serves dual purposes, as it can be ingested and also applied externally to reduce body temperature during fever.[5]

KH is composed of eighteen medicinal plants, i.e., *Pogostemon cablin* (Blanco) Benth. (Phim-Sen), *Limnophila rugosa* (Roth) Merr. (Phak-Kra-Chom), *Cordyline fruticosa* (L.) A.Chev. (Red leaf, Mak-Phu), *Cordyline fruticosa* (L.) A.Chev. (Green leaf, Mak-Mia), *Eupatorium fortunei* Turcz. (San-Phra-Hom), *Vetiveria zizanioides* (L.) Nash ex Small (Faek-Hom), *Kaempferia galanga* L. (Proh-Hom), *Myristica fragrans* Houtt. (Chan-Thet), *Dracaena cochinchinensis* (Lour.) S.C.Chen (Chan-Daeng), *Angiopteris evecta* (G.Forst.) Hoffm. (Wan-Kip-Rat), *Globba variabilis* Ridl. (Wan-Ron-Thong), *Tacca chantrieri* André (Nae-Ra-Phu-Sri), *Sophora exigua* Craib (Phit-Sa-Nat), *Cyathea gigantea* (Wall. ex Hook.) Holttum (Ma-Had-Sa-Dam), *Mimusops elengi* L. (Phi-Kun), *Mesua ferrea* L. (Bun-Nak), *Mammea siamensis* T. Anderson (Sa-Ra-Phi) and *Nelumbo nucifera* Gaertn. (Bua-Luang).[1] Each medicinal plant composition is added at an equivalent ratio. Previous studies on KH have demonstrated its various beneficial properties, including anti-inflammatory activity, antioxidant activity, antimicrobial activity, and antimalarial activity.[6-9] Currently, there is no established monograph on the quality control of KH in Thai Herbal Preparation Pharmacopoeia (THPP) and there is a lack of scientific reports on stability testing.[10] Recently, the standardized monographs are only available for 5 out of the 18 raw plant materials listed in this remedy in the Thai Herbal Pharmacopoeia (THP) i.e., *K. galanga*, *D. cochinchinensis*, *M. elengi*, *M. ferrea*, *N. nucifera*.[11]

Quality control and stability testing are very important since it impacts the efficacy and shelf life of a medicine product.[12,13] Therefore, the objectives of this study were to evaluate the quality control of raw plant materials and stability testing of the ethanolic extract of KH (KHE) under accelerated storage conditions: in terms of the inhibitory effect on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophage cells and the quantitation of the bioactive marker. These results are essential for establishing precise specifications to ensure the consistent quality and shelf life of KH.

Methods

Chemicals and Reagents

Acetonitrile, chloroform, dimethyl sulfoxide (DMSO), hydrochloric acid (HCl), methanol, were purchased from RCI Labscan (Bangkok, Thailand). Commercial grade 95% ethanol was purchased from C.M.J. Anchor Co., Thailand. Fetal bovine serum (FBS), penicillin-streptomycin, trypan blue, 0.5% trypsin-EDTA, Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin were purchased from Gibco BRL Life Technologies (NY, USA). Phosphate buffer saline (PBS) was purchased from Amreso 27 (OH, USA). Lipopolysaccharide (LPS, Serotype: *Escherichia coli* O55:B5), 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl -2H-tetrazolium bromide (MTT), *N*-(1-naphthy) ethylenediamine dihydrochloride, sulfanilamide, and phosphoric acid were purchased from Sigma-Aldrich (MO, USA).

Plant materials

KH consists of eighteen medicinal plants. Each plant was purchased from Charoensuk Osot Pharmacy, Nakhon Pathom, Thailand. The identification of plant materials was confirmed by comparison with authentic voucher specimens deposited at the Herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla Province, Thailand. The plant material details are shown in Table 1.

Table 1 List of plant materials of Kheaw-Hom remedy

Scientific name	Family name	Voucher specimen number	Thai name	Part used	Source	Proportion (%w/w)
Angiopteris evecta (G.Forst.) Hoffm.	Marattiaceae	SKP 110-1 01 05 01	Wan-Kip-Rat	Rhizome	Sukhothai	5.56
Cordyline fruticosa (L.) A.Chev.	Asparagaceae	SKP 005 03 06 01	Mak-Phu	Green leaf	Phetchaburi	5.56
Cordyline fruticosa (L.) A.Chev.	Asparagaceae	SKP 005 03 06 01	Mak-Mia	Red Leaf	Phetchaburi	5.56
Cyathea gigantea (Wall. ex Hook.) Holttum	Cyatheaceae	SKP 059 03 07 01	Ma-Had-Sa-Dam	Rhizome	Suratthani	5.56
Dracaena cochinchinensis (Lour.) S.C.Chen	Asparagaceae	SKP 065 04 12 01	Chan-Daeng	Wood	Nakhon Ratchasima	5.56
Eupatorium fortunei Turcz.	Compositae	SKP 051 05 19 01	San-Phra-Hom	Leaf	Ratchaburi	5.56
Globba variabilis Ridl.	Zingiberaceae	SKP 206 07 13 01	Wan-Ron-Thong	Rhizome	Nakhon Pathom	5.56
Kaempferia galanga L.	Zingiberaceae	SKP 206 11 07 01	Proh-Hom	Rhizome	Ratchaburi	5.56
Limnophila rugosa (Roth) Merr.	Plantaginaceae	SKP 177 12 18 01	Phak-Kra-Chom	Leaf	Nakhon Pathom	5.56
Mammea siamensis T. Anderson	Calophyllaceae	SKP 083 13 19 01	Sa-Ra-Phi	Flower	Ang Thong	5.56
Mesua ferrea L.	Calophyllaceae	SKP 083 13 06 01	Bun-Nak	Flower	Phetchabun	5.56
Mimusops elengi L.	Sapotaceae	SKP 171 13 05 01	Phi-Kun	Flower	Nakhon Pathom	5.56
Myristica fragrans Houtt.	Myristicaceae	SKP 121 13 06 01	Chan-Thet	Wood	Australia	5.56
Nelumbo nucifera Gaertn.	Nelumbonaceae	SKP 125 14 14 01	Bua-Luang	Stamen	Nakhon Sawan	5.56
Pogostemon cablin (Blanco) Benth.	Lamiaceae	SKP 095 16 03 01	Phim-Sen	Leaf	Phetchaburi	5.56
Sophora exigua Craib.	Leguminosae	SKP 072 19 05 01	Phit-Sa-Nat	Rhizome	Sukhothai	5.56
Tacca chantrieri André.	Taccaceae	SKP 189 20 03 01	Nae-Ra-Phu-Sri	Rhizome	Nakhon Ratchasima	5.56
Vetiveria zizanioides (L.) Nash ex Small	Poaceae	SKP 081 22 26 01	Faek-Hom	Root	Nakhon Ratchasima	5.56

Quality control of raw plant materials

Quality control methods include loss on drying, extractive values, total ash, acid-insoluble ash, and heavy metals. These methods were performed following the THP guideline and the quality control methods for herbal materials by World Health Organization (WHO).[8,14]

Loss on drying[14]

Loss on drying or moisture content was analyzed using an electric moisture analyzer (Scaltec instrument, Germany). The sample powder (2 g) was placed on moisture analyzer at 105 °C. The weight of dried sample was displayed. The loss on drying value was calculated using the following equation:

$$\% \text{ Loss on drying} = \frac{\text{Weight of sample before dry (g)} - \text{Weight of sample after dry (g)}}{\text{Weight of sample before dry (g)}} \times 100$$

Total ash[11]

The sample powder (2 g) was weighed in a crucible and burned in a muffle furnace (Nabertherm, Germany) at 450 °C for 9 hours. After cooling down in a desiccator, the crucible was burned in a muffle furnace at 450 °C for 5 hours and put in a desiccator until cool down. This process was repeated until the weight was constant. The percentage of total ash was calculated using the following equation:

$$\% \text{ Total ash} = \frac{\text{Stable weight after burning (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

Acid-insoluble ash[11]

The total ash was boiled in 25 ml of 10% HCl for 5 minutes. The insoluble matter was collected on a Whatman ashless filter paper No. 42 and washed with deionized water until the filtrate is neutral. The filter paper was put in the crucible and burned in a muffle furnace at 500 °C for 9 hours. The crucible was cooled in a desiccator and weighed. The procedure was repeated until the weight was constant. The percentage of acid-insoluble ash was calculated using the following equation:

$$\% \text{ Acid-insoluble ash} = \frac{\text{Stable weight after burning (g)}}{\text{Weight of beginning sample (g)}} \times 100$$

Extractive values[11]

Extractive values include ethanol-soluble extractive value and water-soluble extractive value. The sample powder (5 g) was macerated with 100 ml of 95% ethanol in an Erlenmeyer flask for 24 hours, shaking frequently during the first 6 hours and then allowing it to stand for 18 hours. The extract was filtered rapidly. The filtrate (20 mL) was transferred to an evaporating dish and evaporated to dryness at 105 °C until the weight was constant. The water-soluble extractive value method is similar to the above method but uses 0.25% chloroform in water instead of ethanol. The percentage of ethanol and water-soluble extractive values were calculated using the following equation:

$$\% \text{ Ethanol or water-soluble extractive} = \frac{\text{Weight of the extract (g)}}{\text{Weight of dried powder (g)}} \times 100$$

Heavy metals[11]

The sample was prepared using the wet digestion method following THP. The contents of arsenic (As), cadmium (Cd), and lead (Pb) were determined using Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS) (Hitachi®, Model Z-8200 Series) with the modified methods.[15,16] The concentration of As, Cd, and Pb was diluted in the ranges of 5-80 ppb, 0.25-4.00 ppb, and 10-80 ppb, respectively with correlation coefficients (R^2) greater than 0.995.

Stability testing under accelerated storage conditions[13]

Stability testing was performed according to the International Conference on Harmonization (ICH) Q1A (R2) guideline.[13] KHE was contained in amber glass bottles with lids and stored in a stability chamber (Termaks, Norway) under accelerated storage conditions of 40 ± 2 ºC and 75 ± 5% relative humidity (RH) for 180 days. KHE inhibitory effect on LPS-induced NO production in RAW264.7 macrophage cells and bioactive marker stability was determined using high performance liquid chromatography (HPLC) on days 0, 15, 30, 60, 90, 120, 150, and 180, respectively.

Determination of NO production in LPS-induced RAW 264.7 cells[17]

Cell culture

RAW 264.7 (ATCC® TIB-71™), murine macrophage cells, were purchased from American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium containing 10% FBS, 10,000 units/ml of penicillin, and 10,000 µg/mL of streptomycin at 37 °C, 5% CO_2 and 95% relative humidity (RH). All experiments were approved by Institute Biosafety Committee of Thammasat University (Number 067/2018) and performed under biosafety level 2.

Determination of NO production using Griess reagent

RAW 264.7 cells ($1x10^5$ cells/well) were seeded into a sterile 96-well plate and incubated at 37 °C, 5% CO_2, and 95% RH for 24 hours. The old medium was replaced with fresh medium, some without LPS (100 µL/well) and some containing 10 ng/ml LPS (100 µL/well), together with the test samples at various concentrations (100 µL/well). Medium containing 0.2% DMSO without any sample served as a negative control. Prednisolone was used as a positive control. After incubation for 24 hours, 100 µL of supernatant was transferred to another sterile 96-well plate, followed by 100 µL of Griess reagent. The NO production was determined by measuring the accumulation of nitrite which interacted with the Griess reagent. The optical density (OD) was measured using a microplate reader at 570 nm. The percentage of inhibition was calculated using the following equation and IC_{50} values were calculated using a Prism program.

$$\% \text{ Inhibition} = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

Where, $OD_{control}$ = mean of $OD_{control\ (+LPS)}$ - mean of $OD_{control\ (-LPS)}$

OD_{sample} = mean of $OD_{sample\ (+LPS)}$ - mean of $OD_{sample\ (-LPS)}$

Determination of cytotoxicity using MTT assay

Viable cells were determined using the MTT assay as described by Makchuchit.[17] The method used was the same as above. MTT solution (10 µL, 5 mg/ml in PBS) was added into the well, which was without LPS, and incubated at 37 °C in 5% CO_2 for 2 hours. The supernatant was removed and 100 µL of isopropanol contained 0.04 M HCl was added to dissolve the formazan production produced in the cells. The OD was measured using the microplate reader at 570 nm. The percentage of toxicity was calculated using the equation given below and performed in triplicate. If the percentage of toxicity was greater than 30%, the test samples were considered to be toxic.

$$\% \text{ Toxicity} = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

Where; mean of $OD_{control}$ = mean of $OD_{control\ (-LPS)}$

mean of OD_{sample} = mean of $OD_{sample\ (-LPS)}$

Determination of bioactive marker using HPLC

Ethyl *p*-methoxycinnamate (EPMC), a bioactive marker of KHE, was determined using HPLC system, with diode array detector and automatic injector. A reversed-phase column, Agilent® Eclipse XDB-C18 (size 4.6 x 250 mm, 5µm) with guard column was used as a stationary phase. The mobile phase was composed of water : acetonitrile starting from 95:5 v/v to 90:10 v/v in total run time 60 minutes. The pump flow rate was set at 1 ml/min. Diode array detector was set at 320 nm. KHE (10 mg) was dissolved in 1 ml of methanol and filtered through a 0.45 µm nylon filter. EPMC was determined within the concentrations of 1-400 µg/mL. A volume of 10 µL was injected into the HPLC column and EPMC was separated under the above chromatographic condition. The peak area of EPMC was calculated against the standard curve using the linear least-squares regression equation.

Statistical analysis

All experiments were performed in triplicate. The quality control results are expressed as the mean ± standard deviation (SD). The stability testing results are expressed as mean ± standard error of mean (SEM). IC_{50} values were calculated by Prism program. The difference between each group was analyzed using one-way of variance (ANOVA). *P*-value less than 0.05 was considered significant.

Results

Quality control of raw plant materials

The results of quality control are shown in Table 2. The percentage loss on drying of KH was 8.66 ± 0.47%. Among all the samples tested, *A. evecta* demonstrated the highest percentage of loss on drying (9.93 ± 0.27%), while *D. cochinchinensis* showed the lowest percentage (5.98 ± 0.33%).

The percentage of total ash of KH was 6.17 ± 0.06%. *V. zizanioides* showed the highest percentage of total ash (10.94 ± 0.57%), whereas *A. evecta* showed the lowest percentage (3.15 ± 0.11%). The percentage of acid-insoluble ash of KH was 1.14 ± 0.07%. Among all the samples tested, *V. zizanioides* demonstrated the highest percentage of acid-insoluble ash (2.23 ± 0.05%), whereas *D. cochinchinensis* showed the lowest percentage (0.32 ± 0.03%).

The percentage of ethanol and water-soluble extractive values of KH were 10.62 ± 0.12 and 13.78 ± 0.54%, respectively. *D. cochinchinensis* showed the highest percentage of ethanol-soluble extractive value (25.25 ± 1.04%), whereas *N. nucifera* demonstrated the lowest (1.22 ± 0.02%). The highest water-soluble extractive value was found in *V. zizanioides* (56.49 ± 0.13%), whereas *M. fragrans* showed the lowest (1.69 ± 0.06%).

The results revealed that KH met the requirements of THP. The heavy metals of KH and its plant ingredients are shown in Table 3. Arsenic (As), cadmium (Cd), and lead (Pb) contents of KH were 0.01 ± 0.00 ppm, 0.01 ± 0.00 ppm, and 0.02 ± 0.00 ppm, respectively. The amounts of heavy metals of eighteen plant materials in this study met the standard criteria.

Table 2 The results of quality control of KH and eighteen plant materials

Sample	% Loss on drying		% Total ash		%Acid insoluble ash		% Extractive values			
							Ethanol-soluble		Water-soluble	
	Mean ± SD	Limit	Mean ± SD	Limit	Mean ± SD	Limit	Mean ± SD	Limit	Mean ± SD	Limit
Angiopteris evecta	9.93 ± 0.27	≤ 10	3.15 ± 0.11	≤ 10	0.46 ± 0.01	≤ 2	2.08 ± 0.08	-	14.43 ± 0.33	-
Cordyline fruticosa (Green leaf)	7.11 ± 0.70	≤ 10	7.68 ± 0.22	≤ 10	0.59 ± 0.04	≤ 2	5.24 ± 0.28	-	17.96 ± 0.76	-
Cordyline fruticosa (Red leaf)	7.17 ± 0.66	≤ 10	7.99 ± 0.46	≤ 10	0.53 ± 0.12	≤ 2	5.71 ± 1.12	-	17.42 ± 0.58	-
Cyathea gigantea	7.63 ± 0.12	≤ 10	3.37 ± 0.33	≤ 10	0.57 ± 0.23	≤ 2	2.89 ± 0.31	-	6.72 ± 0.35	-
Dracaena cochinchinensis	5.98 ± 0.33	≤ 8*	0.95 ± 0.32	≤ 1*	0.32 ± 0.03	≤ 2	25.25 ± 1.04	≥ 12*	3.04 ± 0.23	≥ 1*
Eupatorium fortunei	6.39 ± 0.18	≤ 10	5.63 ± 0.52	≤ 10	0.67 ± 0.08	≤ 2	3.36 ± 0.17	-	18.48 ± 0.85	-
Globba variabilis	8.85 ± 0.64	≤ 10	7.42 ± 0.16	≤ 10	1.10 ± 0.02	≤ 2	4.84 ± 1.08	-	11.34 ± 0.94	-
Kaempferia galanga	6.67 ± 0.23	≤ 11*	5.52 ± 0.14	≤ 8*	1.32 ± 0.04	≤ 2*	3.45 ± 1.21	≥ 2.5*	14.61 ± 0.55	≥ 14*
Limnophila rugosa	6.39 ± 0.18	≤ 10	9.82 ± 0.71	≤ 10	0.83 ± 0.21	≤ 2	7.62 ± 0.70	-	15.55 ± 1.12	-
Mammea siamensis	7.74 ± 0.17	≤ 10	7.98 ± 0.32	≤ 10	0.43 ± 0.02	≤ 2	2.84 ± 0.17	-	25.39 ± 1.27	-
Mesua ferrea	8.57 ± 0.59	≤ 11*	4.91 ± 0.21	≤ 5*	1.49 ± 0.34	≤ 1.5*	5.66 ± 0.10	≥ 4.5*	8.24 ± 0.49	≥ 2.5*
Mimusops elengi	8.59 ± 0.36	≤ 16*	5.97 ± 0.19	≤ 7*	1.29 ± 0.30	≤ 3*	8.23 ± 1.31	≥ 8*	10.30 ± 0.27	-
Myristica fragrans	6.10 ± 0.22	≤ 10	8.58 ± 0.26	≤ 10	1.78 ± 0.02	≤ 2	1.39 ± 0.09	-	1.69 ± 0.06	-
Nelumbo nucifera	8.69 ± 0.34	≤ 12*	5.01 ± 0.63	≤ 6*	0.69 ± 0.31	≤ 1*	1.22 ± 0.02	-	10.62 ± 0.49	≥ 10.5*
Pogostemon cablin	9.55 ± 0.39	≤ 10	9.52 ± 0.11	≤ 10	1.18 ± 0.14	≤ 2	3.37 ± 0.29	-	13.13 ± 0.37	-
Sophora exigua	6.35 ± 0.32	≤ 10	4.60 ± 0.06	≤ 10	1.07 ± 0.08	≤ 2	13.52 ± 0.58	-	15.78 ± 0.65	-
Tacca chantrieri	7.31 ± 0.98	≤ 10	4.89 ± 0.07	≤ 10	0.82 ± 0.08	≤ 2	5.61 ± 0.84	-	17.55 ± 0.23	-
Vetiveria zizanioides	8.84 ± 0.74	≤ 10	10.94 ± 0.57	≤ 10	2.23 ± 0.05	≤ 2	2.36 ± 0.21	-	56.49 ± 0.13	-
Kheaw-Hom remedy	**8.66 ± 0.47**	**-**	**6.17 ± 0.06**	**-**	**1.14 ± 0.07**	**-**	**10.62 ± 0.12**	**-**	**13.78 ± 0.54**	**-**

Note: * indicated the standard value of THP 2022
- indicated not reported

Table 3 The results of heavy metals of KH and eighteen plant materials

Sample	Heavy metal (ppm)		
	As	**Cd**	**Pb**
Angiopteris evecta	0.14 ± 0.01	0.03 ± 0.00	ND
Cordyline fruticosa (Green leaf)	0.18 ± 0.07	0.03 ± 0.02	ND
Cordyline fruticosa (Red leaf)	0.10 ± 0.04	0.01 ± 0.00	ND
Cyathea gigantea	0.16 ± 0.04	0.02 ± 0.01	ND
Dracaena cochinchinensis	0.13 ± 0.03	0.04 ± 0.05	ND
Eupatorium fortunei	0.13 ± 0.05	0.20 ± 0.01	ND
Globba variabilis	0.11 ± 0.06	0.05 ± 0.06	ND
Kaempferia galanga	0.03 ± 0.00	0.16 ± 0.00	ND
Limnophila rugosa	0.09 ± 0.02	0.03 ± 0.02	ND
Mammea siamensis	0.19 ± 0.06	0.04 ± 0.06	ND
Mesua ferrea	0.17 ± 0.04	0.00 ± 0.00	ND
Mimusops elengi	0.17 ± 0.00	0.11 ± 0.01	ND
Myristica fragrans	0.09 ± 0.02	0.00 ± 0.00	ND
Nelumbo nucifera	0.13 ± 0.05	0.02 ± 0.02	ND
Pogostemon cablin	0.15 ± 0.06	0.00 ± 0.00	ND
Sophora exigua	0.11 ± 0.03	0.11 ± 0.06	ND
Tacca chantrieri	0.12 ± 0.10	0.12 ± 0.03	ND
Vetiveria zizanioides	0.19 ± 0.03	0.03 ± 0.01	ND
Kheaw-Hom	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Limit*	**≤ 4**	**≤ 0.3**	**≤ 10**

Note: * indicated the standard value of THP 2022
ND = not detected

Stability testing under accelerated conditions

The stability testing results of KHE under accelerated conditions at day 0, 15, 30, 60, 90, 120, 150, and 180 on inhibitory effects of NO production and EPMC content are shown in Table 4. The inhibitory effect on NO production and EPMC content of KHE from day 15 until day 180 did not show a significant difference when compared with day 0 ($p > 0.05$). The chromatograms on the stability of EPMC content are shown in Figure 1.

Table 4 Stability of KHE on inhibitory effects of NO production and EPMC content during 180-day storage under accelerated conditions at 40 ± 2 °C and 75 ± 5% RH

Samples	IC_{50} of NO production (μg/mL)	EPMC content (mg/g of extract)	% Remaining of EPMC
Day 0	34.79 ± 0.90	16.53 ± 0.15	100.00
Day 15	36.63 ± 2.85	16.50 ± 2.93	99.77
Day 30	37.33 ± 3.55	15.97 ± 1.91	96.60
Day 60	32.86 ± 3.19	16.22 ± 1.65	98.13
Day 90	33.44 ± 2.68	15.94 ± 2.73	96.41
Day 120	36.97 ± 2.51	16.30 ± 0.81	98.61
Day 150	38.91 ± 2.01	16.91 ± 0.08	102.26
Day 180	39.09 ± 1.37	15.02 ± 2.21	90.87

The data were analyzed using one-way ANOVA followed by Dunnett's Multiple Comparison Test.
*Significantly differences ($p < 0.05$) compared with day 0

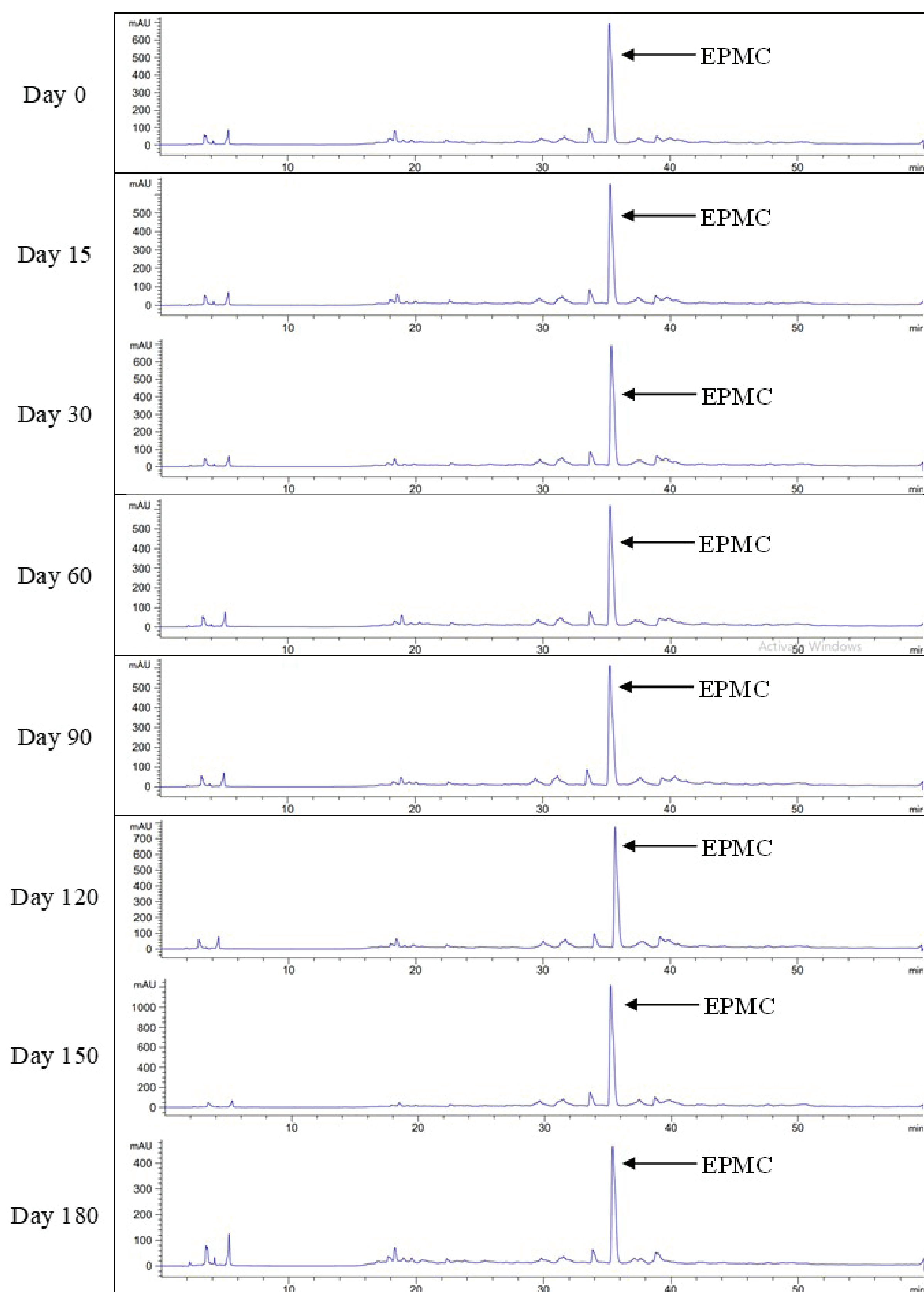

Figure 1 HPLC chromatogram on the stability of EPMC content in KHE during 180-day storage under accelerated conditions at 40 ± 2 °C and 75 ± 5% RH

Discussion

KH, a Thai traditional antipyretic remedy for children, has been included in the NLEM. However, according to the THPP, there are four monographs of antipyretic remedies, namely Chan-Tha-Li-La, Ha-Rak, Pra-Sa-Chan-Daeng, and Pra-Sa-Pro-Yai remedy.[10] Unfortunately, there is currently a lack of available data on the quality control of KH within the THPP. In this study, the quality control and stability of KH were evaluated following the standard guidelines to support the establishment of a quality specification for ensuring and controlling the quality of KH.

The quality controls of KH and its eighteen plant materials were conducted in accordance with the THP and WHO guidelines including loss on drying, extractive values, total ash, acid-insoluble ash, and heavy metals.[11,14] Loss on drying is one of the most important for quality control because an excess of water in plant materials will encourage microbial growth, the presence of fungi or insects, and the deterioration of active compounds following hydrolysis. Total ash includes both physiological ash, which is from the plant tissue itself, and non-physiological ash, which is from extraneous matter (sand and soil) adhering to the plant surface. Acid-insoluble ash is the amount of silica, especially sand and siliceous earth, that is a residue derived after boiling the total ash with diluted acid and burning the remaining insoluble matter. Extractive values are used to determine the quantity of active constituents extracted with solvents of plant materials because no suitable chemical or biological assay exists.[14]

The standard requirements on quality control are only available for 5 out of 18 of the raw plant materials listed of this remedy in THP i.e., *K. galanga*, *D. cochinchinensis*, *M. elengi*, *M. ferrea*, and *N. nucifera*.[11] Although there is no specific requirement for KH in THPP, the general requirements of loss on drying, total ash, and acid-insoluble ash for the herbal plants should be less than 10%, 10%, and 2%, respectively.[18] The results revealed that KH and all plant materials met the standard criteria except *V. zizanioides*. The total ash and acid-insoluble ash values of *V. zizanioides* were higher than the general standard values. These results were similar to the previous study which reported that the total ash and acid-insoluble ash of *V. zizanioides* roots were 10.63 ± 3.68% and 9.10 ± 3.41%, respectively.[19] A possible explanation for the high values of total ash and acid-insoluble ash is contamination from sand or small amounts of gravel in the root part of the plant.

The amounts of heavy metals which are As, Cd, and Pb depends on the location, the soil quality, or the water quality during cultivation. THP suggested that the maximum amounts of the heavy metals, based on the acceptable daily intake values, are as follows: As 4 ppm, Cd 0.3 ppm and Pb 10 ppm.[11] The results revealed that the amounts of heavy metals of KH and all plant materials in this study met the standard criteria of THP.

Stability testing was performed following the ICH guideline to provide evidence related to shelf life and recommended storage conditions of the drug.[13] Previous studies reported that KHE and EPMC exhibited high anti-inflammatory activities by inhibition of NO and prostaglandin E_2 (PGE_2) production. Similarly, KHE also exhibited high EPMC content. These results correlated with its bioactivity.[20] Therefore, EPMC was used as bioactive marker of KHE. The results indicated that KHE was stable under accelerated storage conditions until day 180 without losing the NO production inhibitory activity and EPMC content. Thus, KHE could be stored at room temperature for at least 2 years. However, a long-term storage stability study of KHE should be conducted to confirm the results from accelerated storage conditions testing.

This study is the first report on the quality control and stability testing of KH. The information on quality control and stability of KH can serve as a valuable dataset for the establishment of a reference standard for KH remedy and become a reference standard for future scientific studies and manufacturing to ensure the quality and shelf life of KH.

Financial support

This work was supported by the Thailand Science Research and Innovation Fundamental Fund (TUFF28/2565) and Center of Excellence on Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University.

Compliance with Ethics Requirements

The experiments were approved by the Institute Biosafety Committee of Thammasat University (Number 014/2561) and performed under biosafety level 2.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgments

The authors would like to thank the Thailand Science Research and Innovation Fundamental Fund and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University for funding and providing equipment and facilities for this research.

Author Contributions

Kanmanee Sukkasem, a Ph.D. degree student, carried out all experiments, performed the data analyses, and drafted the manuscript. **Arunporn Itharat**, the supervisor of Kanmanee Sukkasem, contributed to submitting all research grants, designed the study, evaluated the results, and approved this manuscript. **Pakakrong Thongdeeying**, **Weerachai Pipatrattanaseree, Sunita Makchuchit** provided technical guidance for the experiments. **Chonthicha Kongkwamcharoen** contributed to the implementation of the experiment. **Neal M. Davies** edited the manuscript.

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