Original Article

Chemical and Biological Stability under Forced Degradation and Accelerated Storage Conditions of the Anti-Acne Formulation of Pra-Sa-Mang-Khud Ethanolic Extract

Kalyarut Phumlek[1], Arunporn Itharat, Ph.D.[2*], Ubonwan Saesiw[1], Weerachai Pipatrattanaseree, Ph.D.[3], Krit Piwngam Ph.D.[2], Papawee Sookdee[4], Neal M. Davies, Ph.D., R.Ph.[5]

Abstract

Introduction: Pra-Sa-Mang-Khud (PSM) formula is the mixture of *Garcinia mangostana* pericarp and Ha-Rak remedy developed for acne treatment. However, the chemical and biological stability of PSM extract is necessary for an optimal manufacturing process. Therefore, this study was aimed to investigate the stability of PSM ethanolic extract under forced degradation and accelerated storage conditions.

Methods: Thermal degradation, moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, and oxidation were performed in forced degradation studies. In addition, PSM extract was kept in accelerated storage conditions at 40 ± 2ºC with 75 ± 5% RH for 6 months. Finally, PSM extracts under forced degradation and accelerated storage conditions were evaluated for α-mangostin and pectolinarigenin contents by high performance liquid chromatography and antibacterial activity against *C. acnes.*

Results: PSM extract under acid hydrolysis and oxidation conditions had significantly degraded α-mangostin content and reduced anti-*C. acnes* activity. In contrast, the treatments with high temperature, moisture hydrolysis, and alkaline hydrolysis increased α-mangostin content. For accelerated storage conditions, α-mangostin content, pectolinaligenin content, and anti-*C. acnes* activity were shown to be stable in all samples.

Conclusions: Avoiding preparation in an acidic environment is essential for optimal PSM extract efficacy. Moreover, an antioxidant agent that prevents degradation is necessary. Both the hot and cold processes can be effectively used to prepare the product containing PSM extract. PSM extract can have a tentative shelf-life of two years due to its demonstrated stability under accelerated storage conditions.

Keywords: Forced degradation, Accelerated storage conditions, Pra-Sa-Mang-Khud, α-Mangostin, Pectolinaligenin

Received: 20 May 2022 **Revised: 28 June 2022** **Accepted: 30 June 2022**

[1] Student of Doctor of Philosophy Program in Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[3] Regional Medical Science Center 12 Songkhla, Department of Medical Sciences, Songkhla, Thailand
[4] Department of Applied Thai Traditional Medicine, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, Thailand
[5] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada
***Corresponding author:** Arunporn Itharat, Ph.D., Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
Email: iarunporn@yahoo.com

Introduction

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidance suggests that stability testing is required to understand how the quality of a drug substance or product changes over time under a variety of environmental conditions.[1] Forced degradation or stress testing is the study on degradation of new drug material and product under conditions more severe than accelerated conditions to monitor extract stability in the formulation.[2] Accelerated stability testing is a method for determining shelf-life and storage stability.[1] These data can be used in the manufacturing process to evaluate degrading products and estimate product stability.[3]

Pra-Sa-Mang-Khud (PSM) formula consists of 50% w/w of *G. mangostana* (GM) pericarp mixed with 50% w/w of Ha-Rak (HR) remedy that was developed for acne treatment. In Folk medicine, the pericarp of GM, mangosteen rind, has an astringent taste and has been used for wound healing, eczema, and skin infections.[4] In addition, previous studies had shown that GM had a strong antimicrobial effect against *Cutibacterium acnes* and *Staphylococcus epidermidis*.[5-7]

Listed as a Thai traditional medicine (TTM) in the Thai National List of Essential Medicines, HR remedy consists of five herbal roots: *Capparis micracantha, Clerodendrum petasites, Ficus racemosa, Harrisonia perforata,* and *Tiliacora triandra* and has been used as an antipyretic drug.[8] Additionally, HR has been used to treat fever with skin rash in the Takkasila scripture.[9] The main cause of fever with skin rash in TTM is the increase of Pit-ta (fire) which parallels mechanistically the flare ups that occur in acne. The bitter and cold taste of HR affects the level of Pit-ta (fire). Moreover, research on the biological activities of HR have demonstrated that the HR ethanolic extract has significant antimicrobial activity.[10]

PSM formula has the potential for development as an anti-acne product. However, the chemical and biological stability of the extract is a major concern since it impacts the drug's efficacy.[1] Therefore, this study was aimed to evaluate the stability of Pra-Sa-Mang-Khud ethanolic extract under forced degradation and accelerated storage conditions in terms of active chemical constituents α-mangostin and pectolinarigenin (Figure 1) using HPLC and anti-*C. acnes* activity.

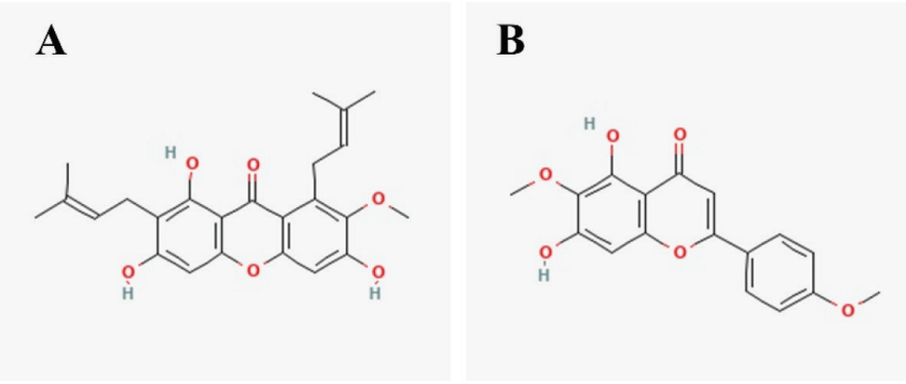

Figure 1 Chemical structures of α-mangostin (A) and pectolinarigenin (B).

Methods

Plant Materials

The ingredients of PSM consist of six herbs, namely *G. mangostana* (SKP 214 09 13 01), *C. micracantha* (SKP 391 03 13 01), *C. petasites* (SKP 202 03 09 01), *F. racemosa* (SKP 117 06 18 01), *H. perforata* (SKP 178 08 16 01), and *T. triandra* (SKP 114 20 20 01). The ripe fruits of GM were collected from Chanthaburi, Thailand. Other plant constituents were collected from Chachoengsao, Thailand.

Preparation of PSM Extract

The five plant roots were sliced into small pieces. The pericarps of GM were separated from fruits. Plant materials were washed and dried in a hot air oven at 50°C for 3 days. These materials were ground and homogeneously combined at the ratio of 1:1:1:1:1:5. The dried powdered plant material (400 g) was macerated with 95% ethanol (2,000 ml) for 72 hours and then evaporated to dryness using a rotary evaporator (Rotavapor R-205, Germany). The maceration was repeated twice, and the dried extracts were combined.

Forced Degradation Studies

Thermal degradation, moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, and oxidation were performed following the recommendation of the ICH guideline.[1] In addition, the samples were analyzed for α-mangostin and pectolinarigenin contents by HPLC and investigated for their antibacterial activities.

Thermal Degradation

A 50 mg of extract was weighed into a glass tube and heated at 80ºC for 3 hours, then allowed to cool to room temperature.

Moisture Hydrolysis

A 50 mg of extract was weighed into a glass tube, followed by 60 µl of deionized water was added and heated to 80ºC for 3 hours, then allowed to cool to room temperature.

Acid Hydrolysis

A 50 mg of extract was weighed into a glass tube, followed by 60 µl of 3N hydrochloric acid was added and heated to 80ºC for 3 hours. The sample was allowed to cool to room temperature and neutralized with sodium hydroxide.

Alkaline Hydrolysis

A 50 mg of extract was weighed into a glass tube, followed by 60 µl of 3N of sodium hydroxide was added and heated to 80ºC for 3 hours. The sample was allowed to cool to room temperature and neutralized with hydrochloric acid.

Oxidation

A 50 mg of extract was weighed into a glass tube, followed by 60 µl of 30% hydrogen peroxide was added and heated to 80ºC for 3 hours, then allowed to cool to room temperature.

Accelerated Storage Conditions

The crude extracts were packed into grass vials with caps and stored at 40 ± 2°C with 75 ± 5% RH for 6 months according to the ICH guideline.[1] The extracts were prepared in triplicate and taken on days 0, 15, 30, 60, 90, 120, 150, and 180 to analyze chemical content by HPLC and investigated for antibacterial activity.

Quantitative Analysis of Chemical Contents Using HPLC

The content of α-mangostin and pectolinarigenin in PSM ethanolic extract was analyzed by HPLC according to Sakpakdeejaroen, Juckmeta, and Itharat, 2014[11] with some modifications. The HPLC system (Agilent® 1200) is equipped with a photodiode array detector. The HPLC column was a C18 column (Agilent®, 4.6 x 250 mm, 5 microns). A sample volume of 10 µl was injected into the HPLC system. The gradient elution was performed as follow: 0.1% phosphoric acid in water (A): acetonitrile (B) 95:5 at 0 min; 5:95 at 30 min; 95:5 at 35 min; at a flow rate of 1.0 ml/min. α-Mangostin and pectolinarigenin were determined at 316 nm and 331 nm, respectively.

Antibacterial Activity

A microtiter plate-based assay was used to evaluate MIC following Pan-In et al., 2015[12] with some minor modifications. *C. acnes* (DMST 14916) was cultured on brain heart infusion (BHI) for 72 hours under anaerobic conditions using GasPak systems (AnaeroPack®-MicroAero, Japan). The test materials were dissolved in dimethyl sulfoxide (DMSO) and 2-fold serially diluted with medium to obtain final concentrations ranging from 128 to 1 µg/ml of PSM ethanolic extract, 16 to 0.125 µg/ml of α-mangostin, and 1,000 to 7.8125 µg/ml of pectolinarigenin. Clindamycin (Sigma-Aldrich, USA) was used as the positive control. The broth culture containing *C. acnes* was added into each well at a ratio of 1:1. The *C. acnes* plates were incubated at 37°C for 72 hours. Then 10 µl of resazurin was added into each well. The lowest concentration that still demonstrated blue color was the MIC value. An aliquot from the wells that inhibited the growth of tested strains was applied onto BHI agar. The MBC value was calculated by the lowest concentration that showed no growth of the tested strain.

Statistical Analysis

Data were presented as mean ± standard deviation (SD) of triplicate independent experiments using statistical software. Differences were compared with the control group and considered statistically significant for *P*-values lower than .05.

Results

PSM Extract

The quality of plant materials; *C. micracantha, C. petasites, F. racemosa, H. Perforata, T. triandra,* GM, HR, and PSM were evaluated before extraction. The percentage loss on drying ranged from 6.52% to 8.84%. The total ash and acid insoluble ash were between 3.14% to 7.77% and 0.02% to 1.90%, respectively. The contents of As, Cd, and Pd were from 0.0077 ppm to 0.0739 ppm. All plant materials passed the quality standard according to requirements in the Thai herbal pharmacopoeia. After maceration the yield of PSM extract was found to be 15.28% w/w. PSM extract showed a significant anti-*C. acnes* activity with MIC value of 4 µg/ml. α-Mangostin was a major active compound of GM, and pectolinarigenin was a marker of HR. Chromatograms and spectrums were shown in Figure 2.

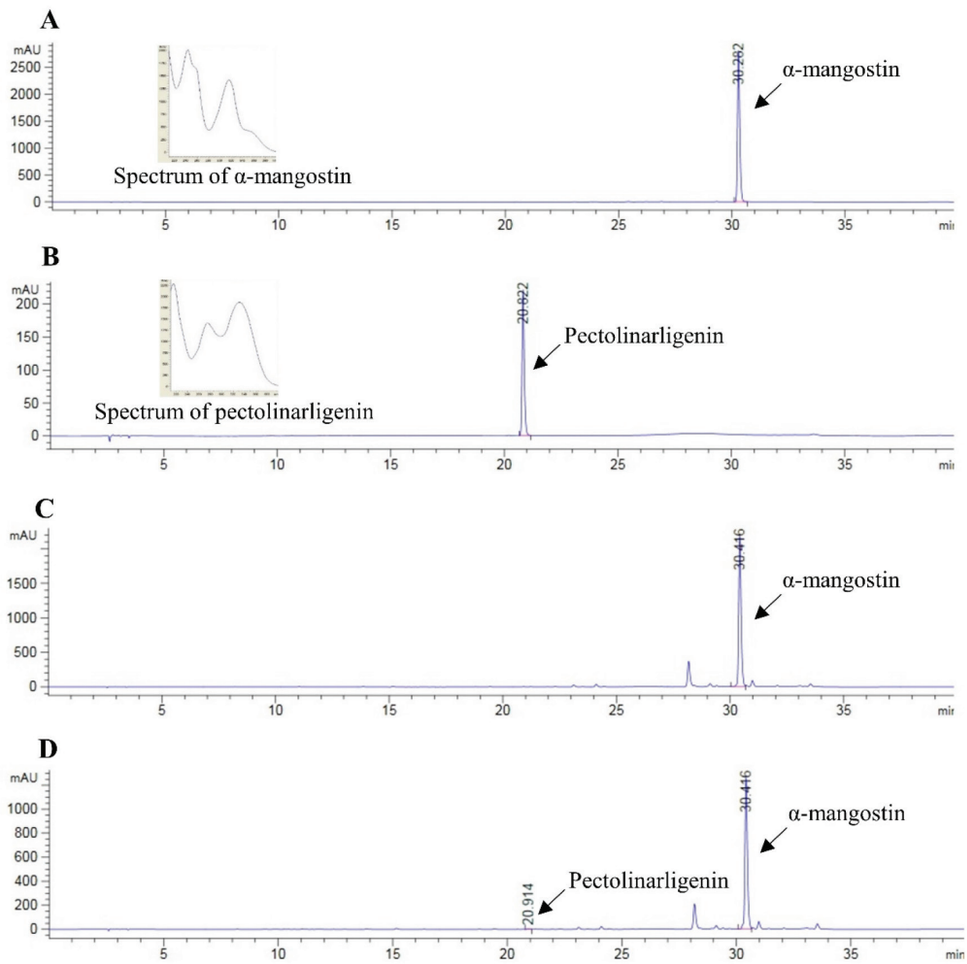

Figure 2 (A) HPLC Chromatogram of α-mangostin (concentration 400 µg/ml) at 316 nm, (B) Chromatogram of pectolinarigenin (concentration 25 µg/ml) at 331 nm, and chromatogram of PSM extract (concentration 2.5 mg/ml) at 316 nm (C) and 331 nm (D).

The Stability of PSM Extract Under Forced Degradation Studies

PSM extract under acid hydrolysis and oxidation had a degraded α-mangostin content from 130.12 ± 4.77 mg/g to 22.05 ± 0.27 mg/g and from 130.12 ± 4.77 mg/g to 66.87 ± 0.22 mg/g, respectively, which resulted in its reduced anti-*C. acnes* activity (from MIC value of 4 µg/ml to 16 µg/ml

in acid hydrolysis and from 4 µg/ml to 32 µg/ml in oxidation). The effect of high temperature, moisture hydrolysis, and alkaline hydrolysis did not degrade α-mangostin content and anti-*C. acnes* activity. On the contrary, the content of α-mangostin under heating, moisture, and alkaline hydrolysis resulted in a significant increase in the PSM extract.

Pectolinarigenin content was significantly decreased after the forced degradation studies. However, the percentage of remaining pectolinarigenin content was more than 90% under thermolysis, moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, and oxidation (Table 1 and Figure 3).

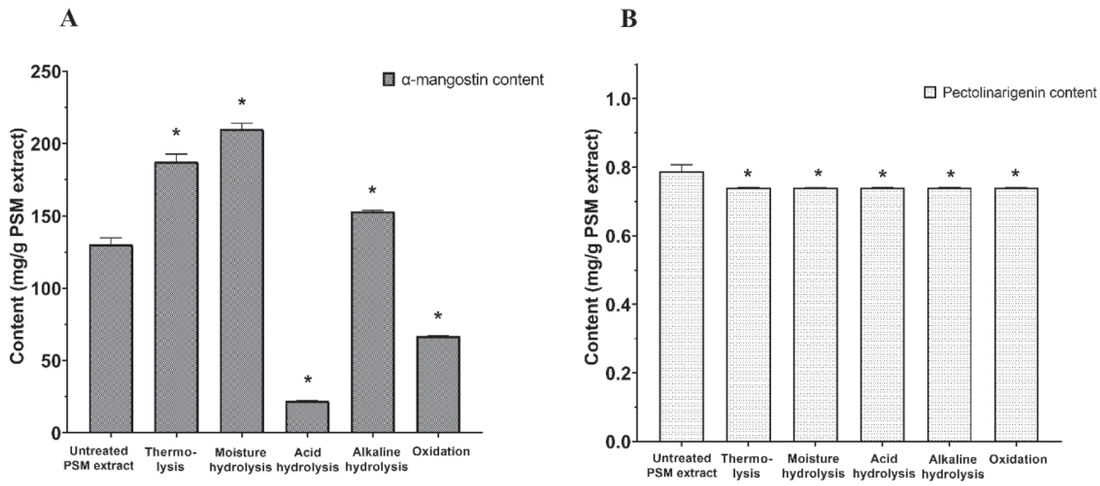

Figure 3 (A) α-Mangostin and (B) pectolinarigenin contents of PSM extract treated under forced degradation conditions (**P*-value < .05).

The Stability of PSM Extract Under Accelerated Storage Conditions

α-Mangostin content significantly increased on day 120 from 130.12 ± 4.77 mg/g to 175.21 ± 7.13 mg/g and slightly increased with no significant difference on days 15, 60, 90, 150, and 180. In contrast, pectolinarigenin content had a very slight but significantly decrease. Although pectolinaligenin content was decreased after accelerated storage conditions, however, the anti-*C. acnes* activity was unchanged in all samples when compared with PSM day 0, this could be due to constant α-mangostin content in PSM extract (Table 2 and Figure 4).

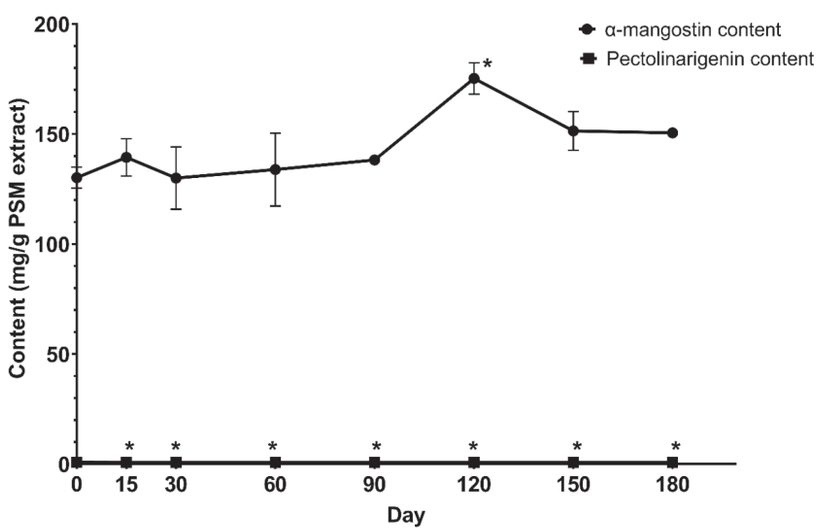

Figure 4 α-Mangostin and pectolinarigenin contents of PSM extract after stability test at 40 ± 2°C with 75 ± 5% RH for 6 months (**P* -value < .05).

Table 1 α-Mangostin and pectolinarigenin contents and anti-*C. acnes* of PSM extract-treated under forced degradation studies

Samples	α-Mangostin content (mean ± SD)		Pectolinarigenin content (mean ± SD)		Anti-*C. acnes* activity	
	Content (mg/g)	%Remaining	Content (mg/g)	%Remaining	MIC (μg/ml)	MBC (μg/ml)
PSM extract	130.12 ± 4.77	100.00	0.7887 ± 0.0187	100.00	4	8
PSM treated under forced degradation conditions						
Thermolysis	187.35 ± 5.37*	143.98	0.7406 ± 0.0004*	93.91	8	16
Moisture hydrolysis	209.91 ± 4.21*	161.31	0.7403 ± 0.0002*	93.87	8	16
Acid hydrolysis	22.05 ± 0.27*	16.95	0.7396 ± 0.0014*	93.78	16	32
Alkaline hydrolysis	152.98 ± 0.83*	17.56	0.7398 ± 0.0011*	93.81	8	16
Oxidation	66.87 ± 0.22*	51.39	0.7400 ± 0.0006*	93.83	32	64
α-Mangostin	-	-	-	-	1	4
Pectolinarigenin	-	-	-	-	250	500
Clindamycin	-	-	-	-	0.031	0.063

*Significant difference at *P*-value < .05 compared with untreated PSM extract

Table 2 α-Mangostin and pectolinarigenin contents and anti-*C. acnes* of PSM extract after stability test at 40 ± 2°C with 75 ± 5% RH for 6 months

Samples	α-Mangostin content (mean ± SD)		Pectolinarigenin content (mean ± SD)		Anti-*C. acnes* activity	
	Content (mg/g)	%Remaining	Content (mg/g)	%Remaining	MIC (μg/ml)	MBC (μg/ml)
PSM Day 0	130.12 ± 4.77	100.00	0.7887 ± 0.0187	100.00	4	8
PSM Day 15	139.36 ± 8.44	107.10	0.7403 ± 0.0005*	93.86	4	8
PSM Day 30	129.95 ± 14.16	99.86	0.7402 ± 0.0006*	93.85	4	8
PSM Day 60	133.79 ± 16.59	102.82	0.7401 ± 0.0005*	93.84	8	16
PSM Day 90	138.18 ± 1.30	106.19	0.7392 ± 0.0004*	93.73	8	16
PSM Day 120	175.21 ± 7.13*	134.65	0.7401 ± 0.0003*	93.84	8	16
PSM Day 150	151.35 ± 8.84	116.31	0.7400 ± 0.0002*	93.84	8	16
PSM Day 180	150.50 ± 1.53	115.66	0.7400 ± 0.0004*	93.82	8	16

* Significant difference at *P*-value < .05 compared with PSM extract day 0

Discussion

At present, many commercial products containing GM are marketed as anti-acne treatments. GM has proven to possess strong antibacterial activity against acne-causing bacteria.[5-7] The major constituent from the pericarp of GM was α-mangostin, which has antibacterial activity,[6,13] antioxidant, anticancer, anti-inflammatory, antiallergy, analgesic, antifungal, and antiviral properties.[13] Furthermore, Pectolinarigenin and O-methylalloptaeroxylin, the main chemical compounds in HR remedy, showed a strong anti-inflammatory by production inhibition of NO with IC_{50} values of 7.15 μg/ml and 14.16 μg/ml, respectively.[14] Therefore, PSM developed from GM and HR mixture was considered as a potential candidate for acne treatment with dual mechanisms of action i.e. antibacterial and anti-inflammatory.

The results of this study indicated that PSM extract was unstable under acid hydrolysis and oxidation conditions due to the degradation of α-mangostin content and anti-*C. acnes* activity. Interestingly, the content of α-mangostin under heating, moisture, and alkaline hydrolysis significantly increased in the PSM extract. The previous study found that the rise of α-mangostin content in the fresh mangosteen pericarp juice was related to increased heating temperature up to 65°C.[15] This research supported the effect of heating temperature on α-mangostin content. α-Mangostin, β-mangostin, γ-mangostin, gartanin, and garcinone that have a close chemical structure were determined in GM. The increased temperature may have caused shifting of alkyl functional groups onto the structure of α-mangostin, resulting in an increase in α-mangostin content.[16] Although pectolinarigenin content was significantly decreased under forced degradation studies, the remaining percentage of 90% was undegraded.[17] From the results, PSM extract should avoid intense acidic conditions during preparation, and an antioxidant agent should be considered to prevent degradation. The hot or cold process can be used in product preparation owing to the stability of α-mangostin content and antimicrobial activity after thermolysis. Moreover, it can be concluded that α-mangostin is the most suitable chemical marker of PSM extract for anti-*C. acnes* activity due to the exhibited relationship between α-mangostin and antibacterial activity. For 6 months of accelerated storage conditions, α-mangostin content increased on days 15, 60, 90, 120, 150, and 180. This can be explained for two reasons. The first reason is the effect of humidity or moisture under force degradation which showed in Table 1. It was found that α-mangostin can be increased in high moisture. Thus, α-mangostin can be also increased in accelerated storage conditions which had high moisture content when compared with day 0. The second reason is the effect of temperature. The rise of α-mangostin content was related to increased heating temperature.[15] Therefore, PSM extract which was kept at 40 ± 2°C with 75 ± 5% RH had the potential to increase α-mangostin content more than PSM extract on day 0, which was kept at -20°C until used and thawed at the room temperature when used. The results of accelerated storage conditions indicated that PSM extract showed chemical content stability and anti-*C. acnes* activity. These findings suggested that PSM extract can have a shelf-life of two years.

This study supports the further preparation and development of anti-acne products containing PSM extract for clinical use. As inflammation is an important etiological aspect of acne, a further study on the anti-inflammatory stability in PSM extract is required to investigate its anti-inflammatory activity under forced degradation and accelerated storage conditions.

Acknowledgements

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

All authors report no conflicts of interest relevant to this article.

References

1. Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research. *ICH guidelines, Q1A (R2): Stability Testing of New Drug Substances and Products (revision 2)*. U.S. food & drug administration. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q1ar2-stability-testing-new-drug-substances-and-products. Published 2003. Accessed April 5, 2022.

2. Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-A review. *J Pharm Anal.* 2014;4:159-165.
3. Maheswaran R. FDA Perspectives: Scientific Considerations of Forced Degradation Studies in ANDA Submissions. *Pharm Technol.* 2012;36:73-80.
4. Intusaitrakul C. *Thai herbal medicines used in primary health care.* Bangkok: Duabgjamol publishing; 2008.
5. Pothitirat W, Chomnawang MT, Gritsanapan W. Anti-acne-inducing bacterial activity of mangosteen fruit rind extracts. *Med Princ Pract.* 2010;19(4):281-286.
6. Pothitirat W, Chomnawang MT, Supabphol R, Gritsanapan W. Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. *Fitoterapia.* 2009;80(7): 442-447.
7. Chomnawang MT, Surassmo S, Nukoolkarn VS, Gritsanapan W. Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. *J Ethnopharmacol.* 2005;101 (1-3): 330-333.
8. National drug system development committee. *National List of Essential Medicines.* Bangkok: The agricultural cooperative federation of Thailand; 2013.
9. Ayuraved-Wittayarai Foundation. *Thai traditional medicine textbook (pâet sàat sŏng krór).* Bangkok: See Thai press; 1998.
10. Nuaeissara S, Kondo S, Itharat A. Antimicrobial Activity of the Extracts from Benchalokawichian Remedy and Its Components. *J Med Assoc Thai.* 2011;94(S7):172-177.
11. Sakpakdeejaroen I, Juckmeta T, Itharat A. Development and validation of RP-HPLC method to determine anti-allergic compound in Thai traditional remedy called Benjalokawichien. *J Med Assoc Thai.* 2014;97(8):76-80.
12. Pan-In P, Wongsomboon A, Kokpol C, Chaichanawongsaroj N, Wanichwecharungruang S. Depositing α-mangostin nanoparticles to sebaceous gland area for acne treatment. *J Pharmacol Sci.* 2015;129(4):226-232.
13. Ibrahim MY, Hashim NM, Mariod AA, Mohan S, Abdulla MA, Abdelwahab SI, Arbab IAα-Mangostin from *Garcinia mangostana* Linn: An updated review of its pharmacological properties. *Arab J Chem.* 2016;9(3):317-329.
14. Juckmeta T, Pipatrattanaseree W, Jaidee W, et al., Cytotoxicity to Five Cancer Cell Lines of the Respiratory Tract System and Anti-inflammatory Activity of Thai Traditional Remedy. *Nat Prod Commun.* 2019;14(5):1-6.
15. Mulia K, Hasanah F, Krisanti EA. The effect of heating temperature on cytotoxicity and α-mangostin yield: Mangosteen pericarp juice and mangosteen extract. *IOP Conf Ser: Mater Sci Eng.* 2018;316:1-7.
16. Pedraza CJ, Cardenas RN, Orozco IM, Perez RJM. Medicinal properties of mangosteen (*Garcinia mangostana*). *Food Chem Toxicol.* 2008;46(10):3227-3239.
17. Deshpande MM. Analytical, Bioanalytical, Stability-Indicating Methods: Key Part of Regulatory Submissions. In: Srivastva AN, ed. *Analytical Chemistry - Advancement, Perspectives and Applications.* IntechOpen; 2021.