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Original Article

Development and validation of a high-performance thin layer chromatography method for the simultaneous quantitation of α- and γ-mangostins in Thai stingless bee propolis

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ABSTRACT

Stingless bees (Apoidea) are widely distributed and commercially cultivated in artificial hives in fruit gardens. Their propolis are commonly used in traditional medicine to treat various diseases (*e.g.*, abscesses, inflammations, and toothaches) and as a constituent of numerous health products. Thus, this study aimed to (i) develop and validate a high-performance thin layer chromatography method for the quantitation of major active constituents (α- and γ-mangostins) in propolis produced by five stingless bee species (*Tetragonula fuscobalteata* Cameron, *T. laeviceps* Smith, *T. pagdeni* Schwarz, *Lepidotrigona terminata* Smith, and *L. ventralis* Smith) cultivated in Thai mangosteen orchards and (ii) determine an optimal extraction solvent. Separation was performed on a silica gel 60 F_{254} plate using toluene/ethyl acetate/formic acid (8:2:0.1, v/v/v) as a mobile phase, and the developed method was validated to assure its linearity, precision, accuracy, and limits of detection/quantitation. Propolis extract from *T. fuscobalteata* exhibited the highest mangostin content, and acetone was shown to be more a more effective extraction solvent than dichloromethane, ethanol, or methanol. Thus, the simplicity and reliability of the developed method make it well suited for the routine analysis (*e.g.*, for quality control) of commercial products containing stingless bee propolis.

Introduction

Stingless bees, a large tribe (Meliponini) of the bee family (Apoidea), are represented by ~500 species and 64 genera (*e.g.*, Melipona, Tetragonisca, and Plebeia in Latin America and Lepidotrigona, Tetrigona, and Tetragonula in tropical Asia) found in subtropical or tropical regions (Nordin et al., 2018) and produce a range of products (beehive pollen, honey, propolis etc.) with valuable nutritional and medicinal properties. In particular, stingless bee propolis is valued for its antioxidant, antiproliferative, and antimicrobial activities (da Cunha et al., 2013; Dutra et al., 2014) and exhibits broadly variable chemical composition and biological activity reflecting the diversity of plant populations and bee species (Bankova, 2005; Silici and Kutluca, 2005; Kustiawan et al., 2014).

In Thailand, stingless bees are represented by ten genera and 32 species, among which Lepidotrigona and Tetragonula are the ones most widely cultured and harvested in gardens (Boongird, 2011; Chuttong et al., 2016). The propolis of these stingless bees is traditionally used to treat diabetes, inflammations, and skin diseases (Umthong et al., 2011; Choudhari et al., 2012), and the corresponding products are marketed as creams, ointments, sprays, shampoos, and mouthwash. In terms of chemical composition, Thai stingless bee propolis from mangosteen orchards has been reported to contain prenylated xanthones, namely α-, β-, and γ-mangostins, mangostanin, 8-deoxygartanin, gartanin, and garcinone B (Sanpa et al., 2015; Vongsak et al., 2015; Ishizu et al., 2018). Among them, α- and γ-mangostins were identified as major constituents and believed to play a vital role in determining the antibacterial, anti-diabetic, antiproliferative, and antioxidant activities of propolis extracts (Sanpa et al., 2015; Vongsak et al., 2015, 2016; Kongkiatpaiboon et al., 2016; Ishizu et al., 2018). These highly interesting compounds are usually found in plants of the Clusiaceae family (*e.g.*, mangosteens) and exhibit a wide range of activities, *e.g.*, anti-tumor, anti-microbial, anti-inflammatory, and anti-obesity ones (Chang and Yang, 2012; Ibrahim et al., 2016).

Although several methods of quantifying α- and γ-mangostins in plant extracts are available, they cannot be practically applied to propolis extracts in view of their complex composition (Pothitirat

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and Gritsanapan, 2008; Michel et al., 2012), which necessitates the development of more reliable techniques. High-performance thin layer chromatography (HPTLC) is well suited for qualitative and quantitative analyses of natural product extracts because of its simplicity, few requirements, and low cost (Pedan et al., 2018). Additionally, this technique provides useful information on the complex matrix of propolis and can be used to evaluate its botanical origin based on the proportion of the main plant material (Guzelmeric et al., 2018; Shawky and Ibrahim, 2018). Additionally, extraction of natural products with dissimilar solvents also displayed variable results (Kongkiatpaiboon et al., 2018). Therefore, we herein employ HPTLC-densitometry for the simultaneous analysis of propolis from five stingless bee species (*Tetragonula fuscobalteata* Cameron, *T. laeviceps* Smith, *T. pagdeni* Schwarz, *Lepidotrigona terminata* Smith, and *L. ventralis* Smith) harvested in a mangosteen orchard, aiming to (1) develop and validate an HPTLC technique for the simultaneous determination of α- and γ-mangostins in Thai stingless bee propolis, (2) evaluate the content of these compounds in different propolis types collected in the same mangosteen orchard, and (3) compare the effects of extraction solvent on the results of quantitative analysis of these compounds.

Materials and methods

Materials and chemicals

Propolis from five stingless bee species (*Tetragonula pagdeni* Schwarz, *T. laeviceps* Smith, *T. fuscobalteata* Cameron, *Lepidotrigona terminata* Smith, and *L. ventralis* Smith) was collected in December 2016 from an apiary in the same mangosteen orchard (Makham district, Chanthaburi province, Thailand). Stingless bees were identified by Dr. Chama Inson (Department of Entomology, Faculty of Agriculture, Kasetsart University) and deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand (specimens 121601–121605, respectively). Standard α- and γ-mangostins (>98%) were purchased from Chengdu Biopurify Phytochemicals Ltd., Sichuan, China. All reagents were of analytical grade.

Sample extraction

Different stingless bee species propolis (10 g) was cleaned, cut into small pieces, extracted with 50 vol% aqueous ethanol, and sonicated in methanol (200 ml) at 40 °C for 30 min. The obtained suspension was centrifuged at 4200 × *g* for 5 min at 20 °C, and the supernatant was kept, while the pellet was re-extracted as described above. The supernatants were combined and concentrated in a rotary evaporator, and the resulting crude residue was stored in the refrigerator at 0–4 °C.

Standard solution preparation

For stock solution (1 mg/ml) preparation, α- and γ-mangostin reference standards were accurately weighed and dissolved in methanol in a volumetric flask. Working standard solutions were prepared by diluting stock solutions with methanol to achieve final concentrations of 100 μg/ml (α-mangostin) and 50 μg/ml (γ-mangostin).

Instrumentation and chromatographic conditions

HPTLC chromatography was performed on Al sheets coated with silica gel 60 F_{254} (20 cm × 10 cm, Cat. No. 1.05548.0001, Merck, Germany). Sample and standard solutions were spotted on the HPTLC plate using a Linomat V automatic sample spotter (Camag, Switzerland) equipped with a 100-μl syringe. Samples were applied as 7-mm-wide bands (=14 tracks per plate) located 10 mm away from the lower edge of the plate. The application rate was held constant at 150 nl/s. The plate was developed in a twin trough chamber (Camag, Switzerland) up to a distance of 8 cm using toluene/ethyl acetate/formic acid (8:2:0.1, v/v/v) as a mobile phase. After development, the plate was dried with cold air from a hair dryer for 5 min and scanned at 254 nm in absorbance mode. Densitometric scanning was performed using a TLC scanner 3 (Camag, Switzerland) and WinCAT software. Slit dimensions of 6.0 × 0.45 mm and a scanning speed of 10 mm/s were employed.

Method validation

The developed method was validated according to the International Conference on Harmonization guideline (ICH, 1996/2005) in terms of linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

Linearity

α- and γ-mangostin working standard solutions (0.5–3.5 μl) were applied to the HPTLC plate to afford concentrations of 100–700 ng/band (α-mangostin) and 50–350 ng/band (γ-mangostin). Calibration curves were obtained by plotting the absorbance peak area vs. amount of the standard.

Precision

The measurement of repeatability and intermediate precision was done by the analysis of six replicates of sample solution at 100% concentration of α-mangostin and γ-mangostin. The repeatability was determined by analyzing six times within 1 day, while the intermediate precision was examined for three consecutive days by the proposed method. All precisions were expressed as relative standard deviation percentages (%RSD).

Accuracy

Accuracy was evaluated by recovery determination. The recoveries of α- and γ-mangostins were determined for samples spiked with three different standard concentration levels (~50, 100, and 150% of the determined propolis extract content). For each concentration, recovery determination was performed in triplicate, and recovery (%) was calculated as 100% × (amount found − original amount)/amount spiked.

LOD and LOQ

LOD and LOQ were determined based on the standard deviation of y-intercepts of regression lines (SD) and the slope of the calibration curve (*S*) of the sample in the range of LOD, LOQ according to the formula: LOD = 3.3(SD/*S*) and LOQ = 10(SD/*S*).

Robustness

The robustness of the developed method was determined by varying chromatographic conditions at α- and γ-mangostin loadings of 300 and 150 ng/band, respectively. The composition of the toluene/ethyl acetate/formic acid mobile phase was slightly changed in the range of ±5% as 8:2:0.1, 8.4:2:0.1, and 7.6:2:0.1, v/v/v. The time between plate spotting and development was varied in the range of 5, 15, and 30 min, and the time between plate development and densitometric scanning was varied in the same range. The %RSD of α- and γ-mangostin reference standard amounts were calculated for all robustness variations.

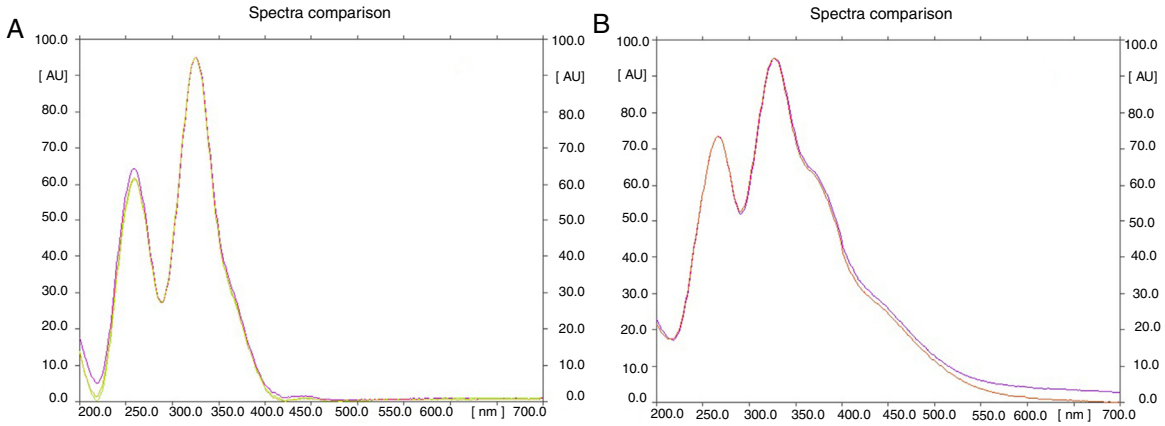

Fig. 1. Overlayed UV spectra (200–700 nm). A. α-Mangostin reference standard and propolis extract; B. γ-mangostin reference standard and propolis extract.

Quantification of α- and γ-mangostins in propolis extract

Accurately weighed propolis extract samples (100 mg) were dissolved in methanol and sonicated for 30 min, and the dispersion volume was then adjusted to 10 ml in a volumetric flask. Each sample solution was filtered through a 0.45-μm nylon membrane filter and applied onto the HPTLC plate at a loading of 2 μl/band. The sample with the highest major compound content was used for further experiments. All analyses were performed in triplicate.

Extraction solvent optimization

Propolis from *T. fuscobalteata* was extracted by 30-min sonication in five different solvents (50 vol% aqueous ethanol, 95 vol% aqueous ethanol, methanol, acetone, and dichloromethane) at 40 °C, and the contents of α- and γ-mangostins were determined by the validated HPTLC method. Additionally, each propolis extract sample (100 mg) was accurately weighed and separately extracted by 30-min sonication in methanol (10 ml). Sample solutions were applied onto the HPTLC plate at a loading of 2 μl/band and analyzed in triplicate. The solvent yielding the highest content of major compound in the propolis extract was concluded to be best suited for extraction.

Statistical analysis

The average contents of α- and γ-mangostins in propolis extract were statistically investigated using one-way analysis of variance (ANOVA) with least significant difference by SPSS for Windows® 16.0. The results were reported as mean ± SD ($n = 3$). A statistical probability (p value) of less than 0.05 indicated a statistically significant difference between groups.

Results and discussion

An 8:2:0.1 (v/v/v) toluene/ethyl acetate/formic acid system afforded the best separation and resolution of α- and γ-mangostins, with respective R_f values determined as 0.37 ± 0.01 and 0.20 ± 0.01. The identities of α- and γ-mangostins were confirmed by overlaying the absorption spectra of samples with those of reference compounds (Fig. 1). The specificity of the analyzed peaks was checked at three different peak positions, *i.e.*, start, apex, and end. The proposed HPTLC method showed acceptable validation parameters (Table 1). In particular, α- and γ-mangostin calibration curves were linear in the ranges of 100–700 and 50–350 ng/band, respectively. The corresponding correlation coefficients were equal or greater than 0.995, which confirmed the good linearity of the developed method. Repeatability and intermediate precision were determined as <5%. The LOD/LOQ of α- and γ-mangostins were calculated as 28.71/87.01 and 14.26/43.21 ng/band, respectively, while the respective average recoveries equaled 102.2 and 100.3%. Thus, the developed technique was concluded to exhibit good precision and accuracy. Considering robustness studies, %RSD was found to be less than 5% for all variations (Table 2). In addition, this validated method also had priority over the previous HPLC technique owing to its lower-cost, and more simplicity in analysis (Kongkiatpaiboon et al., 2016).

Propolis extract produced by *Tetragonula* bees exhibited higher α- and γ-mangostin contents than that produced by *Lepidotrigona* bees. Specifically, the highest contents of α- and γ-mangostin

Table 1
Validation parameters of the proposed HPTLC method.

Parameter	α-Mangostin	γ-Mangostin
Linearity range	100–700 ng/band	50–350 ng/band
Regression equation ($n = 5$)	$Y = 9.4466X + 1754.1$	$Y = 12.356X - 31.769$
Correlation coefficient (r^2)	0.9956	0.9986
Repeatability, % RSD	1.83%	2.49%
Intermediate precision, %RSD	3.53%	2.97%
LOD	28.71 ng/band	14.26 ng/band
LOQ	87.01 ng/band	43.21 ng/band
% Recovery	98.2 ± 1.2% to 102.2 ± 1.6%	97.1 ± 1.4% to 102.3 ± 1.9%

X, concentration of α-mangostin and γ-mangostin in ng/ml; Y, peak area.

Table 2
Robustness of α- and γ-mangostin analysis.

Optimization condition	α-Mangostin (%RSD)	γ-Mangostin (%RSD)
Mobile phase composition (toluene/ethyl acetate/formic acid; 8:2:0.1, 8.4:2:0.1, 7.6:2:0.1, v/v/v)	3.37	2.97
Time from spotting to chromatography (5, 15, 30 min)	1.89	1.98
Time from chromatography to scanning (5, 15, 30 min)	0.90	0.57

Table 3
Contents of α- and γ-mangostins in propolis extract determined by the validated HPTLC method.

Sample	Content of major compound[a] (wt%)	
	α-Mangostin	γ-Mangostin
Tetragonula fuscobalteata Cameron	2.77 ± 0.01[a]	1.95 ± 0.09[a]
T. laeviceps Smith	1.13 ± 0.14[b]	0.40 ± 0.07[b]
T. pagdeni Schwarz	1.94 ± 0.13[c]	0.43 ± 0.04[b]
Lepidotrigona terminata Smith	0.35 ± 0.03[d]	0.18 ± 0.01[c]
L. ventralis Smith	0.80 ± 0.02[e]	0.13 ± 0.02[d]

Dissimilar superscript letters in the same column indicate significantly different results at $p < 0.05$.
[a] Expressed as mean ± SD ($n = 3$).

Table 4
Contents of α- and γ-mangostin in *Tetragonula fuscobalteata* propolis determined by the validated HPTLC method using different extraction solvents.

Solvent extraction	Content of major compound[a] (% w/w)	
	α-Mangostin	γ-Mangostin
Acetone	6.67 ± 0.40[a]	3.39 ± 0.32[a]
Dichloromethane	5.22 ± 0.01[b]	2.39 ± 0.08[b]
50 vol% aq. ethanol	2.77 ± 0.01[c]	1.95 ± 0.09[c]
95 vol% aq. ethanol	4.20 ± 0.03[d]	2.42 ± 0.08[b]
Methanol	4.67 ± 0.03[e]	2.76 ± 0.04[d]

Dissimilar superscript letters in the same column indicate significantly different results at $p < 0.05$.
[a] Expressed as mean ± SD ($n = 3$).

were observed in the case of *T. fuscobalteata* (2.77 and 1.95 wt%, respectively), while the lowest contents were observed for *L. terminata* (Table 3). HPTLC chromatograms of standard α-mangostin, γ-mangostin, and propolis samples are shown in Fig. 2. For extraction solvent optimization, the most mangostin-rich propolis (from *T. fuscobalteata*) was extracted by five different solvents, and acetone was identified as the most effective one (Table 4, Fig. 3). Thus, acetone should be selected as an extraction solvent for real-life product analysis.

The chemical composition of propolis is directly related to that of bud exudates collected by bees from plant sources as well as to climate and geographic origin. For instance, propolis produced in tropical and subtropical regions has a chemical composition different from that of European propolis, which contains typical "poplar bud" phenolics, flavonoid aglycones, and phenolic acids and their esters (Bankova, 2005; Silici and Kutluca, 2005; Kustiawan et al., 2014). In a previous study, liquid chromatography–mass spectrometry analysis of stingless bee propolis from a mangosteen orchard indicated the presence of α- and γ-mangostin peaks similar to those observed for the resin of mangosteen fruit (Ishizu et al., 2018). Herein, all propolis samples were produced by five different stingless bee species in the same mangosteen orchard. The presence of *Garcinia mangostana* basic compounds such as α- and γ-mangostins confirmed that *G. mangostana* was the main source of our propolis samples. Moreover, the chemical composition of propolis also depended on bee species, in agreement with the results of a previous study, wherein propolis collected by three different races of honeybees in the same apiary was shown to exhibit different chemical composition and major compound concentrations by gas chromatography-mass spectrometry (Silici and Kutluca, 2005).

In the eastern part of Thailand, various stingless bee species are commercially cultivated in artificial hives in fruit gardens for crop pollination and honey harvesting, with *Tetragonula* being the main genus widely occurring in almost all Thailand provinces (Boongird, 2011). In 1980, the Thai government recognized the importance of beekeeping to the national economy and has subsequently actively supported agricultural apiarists, as reflected by the establishment of the Agricultural Extension and Development Center (Beekeeping) for promoting and educating farmers on beekeeping to produce honey and other bee products (Seanbualuang, 2012). According to the results of this study, *T. fuscobalteata* is suited for rearing in bee farms to acquire high-quality propolis products, and acetone is the best extraction solvent. Thus, under the conditions of proper quality control and standardization, propolis from mangosteen orchards can be globally marketed as a component of functional foods and nutraceutical/cosmetic products.

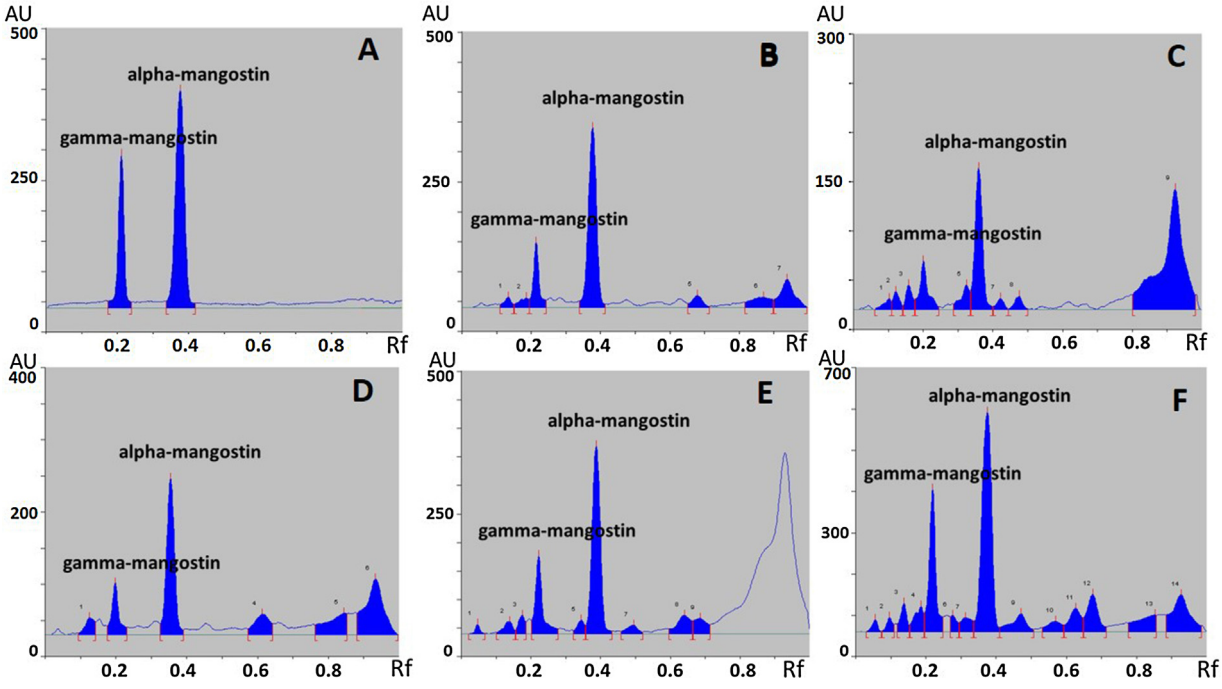

Fig. 2. HPTLC chromatograms (recorded at 254 nm). A. α- and γ-mangostin reference standards; B. *Tetragonula pagdeni* propolis extract; C. *Lepidotrigona terminata* propolis extract; D. *L. ventralis* propolis extract; E. *T. laeviceps* propolis extract; F. *T. fuscobalteata* propolis extract.

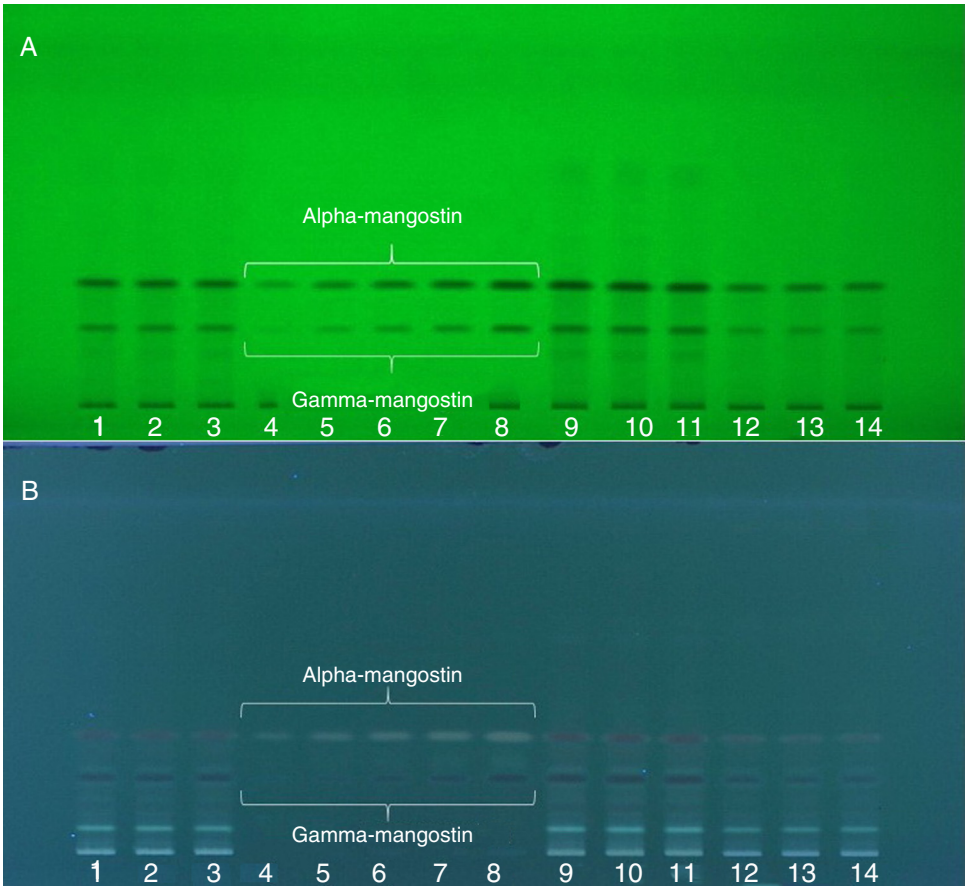

Fig. 3. HPTLC chromatogram of *T. fuscobalteata* propolis extracts using different extraction solvents (silica gel F_{254}, toluene:ethyl acetate:formic acid 8:2:0.1 v/v/v); track 1–3 = 95 vol% aq. ethanol extract, track 4–8 = alpha-mangostin and gamma-mangostin, track 9–11 = acetone extract, track 12–14 = 50 vol% aq. ethanol extract, (A) detected under ultraviolet (UV) 254 nm, (B) detected under UV 366 nm.

Conclusion

Herein, we developed a simple, fast, accurate, and precise HPTLC method for the simultaneous quantitation of α- and γ-mangostins in mangosteen propolis and identified the extraction solvent and bee species affording the best yield of these constituents. Several validation parameters were determined according to the International Conference on Harmonization guideline, and the proposed method was concluded to be well suited for the quality assessment of propolis to ensure the efficacy and safety of related raw materials and nutraceutical products.

Authors' contributions

SC's contribution included HPTLC analysis, analyzing the results, and preparing the paper. BV's contribution included collecting samples, designing and performing laboratory work, analyzing the results, and preparing the paper. The authors have read the final manuscript and approved of the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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