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เรื่อง ขอแจ้งผลรับรองการตีพิมพ์บทความ
เรียน ผู้นิพนธ์ (รศ.ดร.รัตนา อินทรานุปกรณ์)

ตามที่ท่านได้ส่งบทความ เรื่อง INFLUENCE OF EXTRACTION TECHNIQUES AND SOLVENTS ON α -MANGOSTIN AMOUNTS FROM MANGOSTEEN (*Garcinia mangostana* L.) PERICARP ผลของวิธีการสกัดและตัวทำละลายต่อบริมาณสารแอลฟ่าเมงโกรสตินที่สกัดได้จากเปลือกมังคุด เสนอต่อองค์กรน้ำอุตสาหกรรมวารสารไทยไกเซชันนิพน์ คณะเภสัชศาสตร์ มหาวิทยาลัยศิลปากร เพื่อตีพิมพ์ลงในวารสารไทยไกเซชันนิพน์

บันทึกนี้ บทความดังกล่าวได้ผ่านการพิจารณาจากองค์กรน้ำอุตสาหกรรมเป็นที่เรียบร้อยแล้ว จึงขอแจ้งผลการรับรองบทความ เรื่อง INFLUENCE OF EXTRACTION TECHNIQUES AND SOLVENTS ON α -MANGOSTIN AMOUNTS FROM MANGOSTEEN (*Garcinia mangostana* L.) PERICARP ผลของวิธีการสกัดและตัวทำละลายต่อบริมาณสารแอลฟ่าเมงโกรสตินที่สกัดได้จากเปลือกมังคุด ให้ได้รับการตีพิมพ์ลงในวารสารไทยไกเซชันนิพน์ ปีที่ ๑๐ ฉบับที่ ๒ เดือน กรกฎาคม – ธันวาคม ๒๕๕๘ (Volume 10 Number 2 (July - December), 2015 ทั้งนี้หากวารสารฯ ได้ตีพิมพ์แล้วเสร็จจะดำเนินการจัดส่งให้แก่ท่านต่อไป

จึงเรียนมาเพื่อโปรดทราบ

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มหาวิทยาลัยศิลปากร

งานการศึกษาต่อเนื่องทางเภสัชศาสตร์

โทร./โทรสาร ๐-๓๔๗๔-๔๔๖๗๓

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ผลของวิธีการสกัดและตัวทำละลายต่อปริมาณสารแอลฟ่าเมงโกลสตินที่สกัดได้จากเปลือกมังคุด
INFLUENCE OF EXTRACTION TECHNIQUES AND SOLVENTS ON α -MANGOSTIN AMOUNTS FROM MANGOSTEEN (*Garcinia mangostana* L.) PERICARP

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บทคัดย่อ

จากการศึกษาเบรี่ยนเที่ยบวิธีการสกัด 3 แบบ (การใช้เครื่องเขย่า การใช้คลื่นเสียงความถี่สูง และ การสกัดแบบต่อเนื่อง) และตัวทำละลายที่ใช้ในการสกัด 2 ชนิด (เมทานอสและเอทานอล) ที่สามารถสกัดสารสำคัญแอลฟ่าเมงโกลสตินจากเปลือกมังคุดได้สูงสุด พบว่าการสกัดแบบต่อเนื่องด้วยตัวทำละลายเมทานอสและเอทานอลให้ปริมาณแอลฟ่าเมงโกลสตินมากกว่าการสกัดด้วยวิธีอื่น การสกัดแบบต่อเนื่องด้วยตัวทำละลายเมทานอสจะให้ปริมาณแอลฟ่าเมงโกลสตินสูงสุด ในขณะที่การสกัดด้วยคลื่นเสียงความถี่สูงโดยใช้ตัวทำละลายเอทานอลจะให้ปริมาณแอลฟ่าเมงโกลสตินต่ำสุด ดังนั้นการสกัดแบบต่อเนื่องด้วยตัวทำละลายเมทานอสจัดเป็นอีกทางเลือกหนึ่งของวิธีสกัดที่ให้ปริมาณแอลฟ่าเมงโกลสตินสูงจากสารสกัดและผลิตภัณฑ์เปลือกมังคุด นอกจากนี้ยังได้พัฒนาวิธีวิเคราะห์ด้วยเครื่องໂຄมาໂໂກฟาร์มิซึ่งเหลสมรด堪สูง (ເອົ້າພື້ອລົ້າ) ให้ได้ວິທີທີ່ງ່າຍ รวดเร็วและจำเพาะแก่การวิเคราะห์แอลฟ่าเมงโกลสตินในสารสกัดจากเปลือกมังคุดทั้งเชิงคุณภาพและปริมาณ พร้อมทั้งทำการตรวจสอบความถูกต้องของวิธีวิเคราะห์ที่พัฒนาขึ้น พบว่าเป็นวิธีวิเคราะห์ที่ปริมาณสารแอลฟ่าเมงโกลสตินมีความสัมพันธ์เป็นเส้นตรงกับตัวที่วัดได้ อีกทั้งมีความถูกต้องและแม่นยำสำหรับการควบคุมคุณภาพ

คำสำคัญ: แอลฟ่าเมงโกลสติน, สารสกัดเปลือกมังคุด, การตรวจสอบความถูกต้อง, เครื่องเขย่า, การใช้คลื่นเสียงความถี่สูง, การสกัดแบบต่อเนื่อง

Abstract

The efficiency of three different techniques (stirring, ultrasonication and Soxhlet extractions) and two solvents (methanol and ethanol) were intensively evaluated for extraction of α -mangostin in mangosteen (*Garcinia mangostana* L.) pericarps. When compared with the other techniques, Soxhlet extraction showed good results with both methanol and ethanol. The extraction yield of α -mangostin obtained by Soxhlet extraction with methanol was the highest, whereas the lowest yield of α -mangostin obtained by ultrasonication with ethanol was revealed.

Therefore, Soxhlet extraction using methanol as solvent is considered as an alternative technique for obtaining the bioactive α -mangostin with high concentration from mangosteen pericarp extracts and products. Furthermore, the simple, rapid, and specific high performance liquid chromatography (HPLC) method has been established and validated for identification and quantification of α -mangostin from mangosteen pericarp extracts under various extraction conditions. The developed method was found to be satisfactory linearity, precision and accuracy for quality assessment.

Keywords: α -mangostin, mangosteen pericarp extract, method validation, stirring, ultrasonic, Soxhlet extraction

Introducion

The pericarp of the mangosteen fruit, *Garcinia mangostana* L., has a long history of several medicinal purposes for treatment of dysentery, skin infections, urinary disorders, cystitis and gonorrhea¹⁻³. α -Mangostin, a xanthone derivative, is one of the main active ingredients in mangosteen pericarp⁴ that has been found to exhibit a wide range of health promoting properties and pharmacological effects: antioxidant^{2,5,6}, anti-inflammatory⁷, antibacterial activity^{1,8}, anticancer activity^{6,8,9} and immunomodulatory¹⁰. Due to its remarkable medicinal benefits, products containing mangosteen pericarp extracts are now distributed increasingly all over the world and have triggered more and more attention in recent years.

Each of the technique has its own advantages and the choice of extraction technique depends on several factors including sample matrix, operating cost, simplicity of operation, etc. Various solvents

extraction techniques such as soxhlet¹¹⁻¹³, maceration¹⁴⁻¹⁶ and ultrasonication¹⁷, with different solvents (methanol¹¹, ethanol¹², 70% acetone^{18,19}, ethyl acetate¹¹ etc.) have been commonly used for extracting α -mangostin from mangosteen pericarps. However, none of these studies had investigated the effect of various solvents and extraction techniques on the yield of α -mangostin which were conducted on one homogenous sample obtained by grinding mangosteen pericarps procured from a single source. Furthermore, the evaluation of the extraction methods and solvents has not been finalized. In addition, previous studies⁴ showed some drawbacks of quantification of α -mangostin due to long retention period and complicated solvents used.

This study specifically focused on residual sources, mangosteen pericarps, which are the waste parts from consumption and food industry. Not only the method but also the solvent influences the extraction results. Three different

extraction techniques and two solvents for the extraction of main bioactive α -mangostin from mangosteen pericarps were used. The results indicated the efficiency of extraction technique and the solvent for optimal extraction. Furthermore, the development of a fast, simple and quantitative analysis method for the determination of α -mangostin was carried out on mangosteen pericarp extract.

Methods

Plants materials and Chemicals

Ripe Mangosteens (*G. mangostana* L.) were obtained during August-September 2012 from local markets in Samutprakarn province, Thailand. α -Mangostin standard was purchased from Sigma-Aldrich (Singapore). Methanol, ethanol, dichloromethane and *n*-hexane (Merck Company, Germany) used for mangosteen pericarp extraction were of analytical grade solvents. Methanol, acetonitrile, formic acid and orthophosphoric acid for HPLC analysis were obtained from Merck Company (Germany).

Sample preparation

Mangosteens were peeled off to obtain mangosteen pericarps, which were chopped into small pieces and dried at 50°C. The dried mangosteen pericarps were ground into coarse powder and stored in a dry place before the extraction process started.

Comparison of extraction techniques and solvents

The dried mangosteen pericarp powder (5 g) was accurately weighed and extracted by using three different methods: stirring, ultrasonication and soxhlet extraction. Each extraction method was carried out using 150 mL of different single extraction solvent (methanol and ethanol) and extraction time of 1 h. All extractions were carried out in triplicate.

For the stirring and ultrasonication extraction technique, the process was carried out by placing extraction flask on a magnetic stirrer (Heidolph® MR3001, hot plate magnetic stirrer, Germany) and in an ultrasonic bath (Branson® 2510, Ultrasonic Corporation, USA), respectively.

For Soxhlet extraction, the dried plant powder was placed in a thimble inside Soxhlet extraction apparatus, which was fitted with a 250 mL round bottom flask. The extraction was carried out at a boiling point of each extraction solvent approximately 4 cycles/h. The extraction time started after the condensed extractant dripped onto the plant powder.

After extraction, the crude extracts from each technique and solvent were then filtered through filter paper and removed solvent under reduced pressure by using rotary evaporator (Buchi®, R-215, Rotavapor, Switzerland) at 45°C. The residue was

suspended in water to produce an aqueous solution and then partitioned in turn with *n*-hexane and dichloromethane to afford *n*-hexane and dichloromethane extracts, respectively. The dichloromethane extract was found to have α -mangostin and then it was selected for further analysis. After that, the solvent (dichloromethane) was removed with vacuum rotary evaporator under the reduced pressure at 40°C and the residue was dissolved in 10 mL of methanol. The α -mangostin profiles in extracts were quantitatively analysed by a HPLC method. Comparison of extraction techniques and solvents was achieved by comparing HPLC peak areas and the quantitative calibrations were made according to the linear calibration curves of standard.

Optimisation of the chromatographic condition

Before selecting the condition for the optimisation, a number of preliminary trials were conducted with different mixtures and ratios of solvents. Formic acid / orthophosphoric acid, water and acetonitrile mixtures are most often chosen as an eluent^{4,17}. Different concentrations of water and acetonitrile were tested to achieve the best resolution of examined analytes.

High performance liquid chromatography (HPLC) analysis

The mangosteen pericarp extracts from all extraction procedures and two

different extraction solvents were analysed using Finnigan modular LC system which was composed of a Model P4000 dual pump equipped with a Rheodyne 7725i injector linked to a 20 μ L loop and a Model UV 6000 photodiode array detector. A Phenomenex C18 column (250 x 4.6 mm I.D., particle size 10 μ m) was used for chromatographic separations. The chromatographic data obtained by a PC (Professional Component) system, and a software ChromQuest from Thermo Fisher Scientific was used to acquire and process the data. The analysis was conducted at an ambient temperature, flow rate of 1 mL/min with UV detection at 320 nm. Triplicate HPLC analyses of each extract were carried out. The mobile phase consisted of two eluents: water and acetonitrile. Gradient elution was needed for complete separation of the analysis. The most appropriate gradient elution program was maintained at 40% acetonitrile for 5 minutes, then, increased to 90 percent in 5 minutes and held at 90 percent for another 5 minutes. At the end, the system was set to increase acetonitrile from 90 to 100 percent within 1 minute, holding these conditions for 9 minutes and then returned to the original condition. Total run time was 20 minutes.

The standard solution of α -mangostin in methanol was prepared and used as a stock solution for generating a calibration curve. The α -mangostin stock

solutions were then subsequently diluted with methanol to provide a series of working standard solutions in the concentration range of 0.02 – 0.10 mg/mL and analysed in triplicate using the above analytical method. Calibration curve was generated by linear regression based on peak areas. The identification of the separated compounds in mangosteen extracts was assigned by a comparison of retention times, UV spectra and co-chromatogram with authentic standards. Quantification was carried out by an integration of the peak areas using the external standard method. Calibration curve showed good linear relationships. The extracted samples obtained from various extraction procedures were quantitatively analysed one by one. The best extraction solvent and technique was then selected for a validation test.

Validation of the analytical method

Mangosteen pericarp extract obtained by methanol - Soxhlet extraction method described above (sample A) was used for validation study. The method was validated according to the USP38 NF33 <1225>²⁰ for specificity, accuracy, precision and linearity.

Specificity

The specificity was carried out by the analysis of standard α -mangostin and sample A spiked with standard α -mangostin.

The specificity was then evaluated by comparing the retention times of α -mangostin in the chromatogram of the sample A solution with those in the chromatogram of the standard solution. Peak purity was also evaluated by the photodiode array detection.

Accuracy and precision

The accuracy of the method was evaluated by recovery assay at three levels of standard α -mangostin solution (0.03, 0.04 and 0.05 mg/mL) added to the pre-analysed sample A and analysed quantitatively in triplicate by the proposed HPLC method. The average recovery and % relative standard deviation (RSD) were calculated. To assess the precision of the proposed method, six replicates of the sample A were determined on the same day (intra-day precision) and on five consecutive days (inter-day precision).

Linearity

The linearity was determined by using five concentrations of standard α -mangostin solution in the range of 0.02–0.10 mg/mL (n=3). The calibration curve was constructed by plotting the peak area versus the concentration of standard solutions and subjected to the linear least-square regression analysis to calculate the calibration equation and correlation coefficient.

Statistical analysis

All report data were subjected to analyses of variance (ANOVA, $\alpha=0.05$) and Scheffe using a Statistical Package for the Social Sciences software (SPSS version 16 for windows from SPSS Inc., Chicago, Illinois, USA).

Results and Discussion

Identification of α -mangostin in mangosteen pericarp extracts from various extraction techniques and solvents by HPLC

The HPLC chromatogram of mangosteen pericarp extracts from each extraction method and solvent showed similar

xanthone profiles. The representative HPLC chromatograms of the α -mangostin standard and mangosteen extract are presented in figure 1. The optimised HPLC condition was achieved after determination of α -mangostin with different combinations of acetonitrile and water. The use of a Phenomenex C18 column with gradient elution consisted of acetonitrile and water as binary mobile phase, resulted in a good resolution and short analysis time of α -mangostin at the retention time less than 13 minutes. The overall separation was completed within 20 minutes per 1 sample which was considerably more rapid than the previous described method²¹.

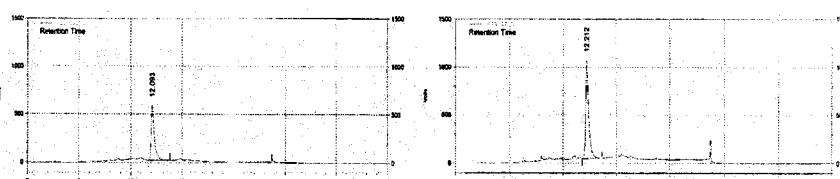


Figure 1 Representative HPLC fingerprints: (a) standard α -mangostin (b) extract A from mangosteen pericarp.

Effect of different extraction methods and solvents on α -mangostin contents

The results from the extraction of mangosteen pericarp using three different techniques (stirring ultrasonication and Soxhlet) and two different solvents (methanol and ethanol) were investigated on the yield of crude extract and α -mangostin content (Table 1). The extractive

values were compared to determine the suitable method and solvent with the highest extraction efficiency.

The results of quantitative analysis revealed that both solvent and extraction method are significant factors affecting α -mangostin contents ($p<0.05$). Table 1 depicted the highest yield of crude extract and α -mangostin obtained by Soxhlet

extraction using methanol as solvent, which showed the amount of 11.57 ± 0.30 % dry weight and 3.74 ± 0.34 mg/g, respectively. Ultrasonic technique with ethanol provided the lowest yield of α -mangostin (0.65 ± 0.14 mg/g) but the medium yield of crude extract (4.29 ± 0.52 %dry weight). The stirring method with ethanol gave the lowest yield of crude extract at 2.27 ± 0.06 %dry weight and low α -mangostin content at 0.89 ± 0.06 mg/g. Soxhlet extraction affording the highest yield of α -mangostin was probably due to the result of repeated fresh solvent contact with the sample many times and allow almost 100% active material recovery. Stirring and ultrasonication were significantly less efficient than the Soxhlet extraction. This may be due to the extraction time of 1 hour which is insufficient for a complete α -mangostin extraction from the mangosteen pericarp and the solvent properties are less relevant for the recoveries. Furthermore, it

was found that ultrasound enhanced the degradation of phenolic compounds by increasing their oxidation²². These results might cause the lower α -mangostin extraction yield. The xanthones in plant extract are more often associated with other molecules like proteins, polysaccharides, terpenes, chlorophyll and inorganic compounds²³. Thus, it requires suitable solvents for the extraction of α -mangostin. Literature data shows that polar solvents such as methanol and ethanol have been commonly used for extraction of α -mangostin from mangosteen pericarp and enabled the process to extract high concentration of α -mangostin^{11,12}. Therefore the impact of both extraction solvents on the assay of α -mangostin from mangosteen pericarp was investigated. Results of the present study indicated that methanol was obviously more powerful for quantitative extraction of α -mangostin than ethanol.

Table 1 Effect of different methods and solvents on yield of crude extract (%dry weight) and α -mangostin contents in mangosteen pericarp

| Method | Solvent | Yield of crude extract* | |
|--------------------|----------|-------------------------|------------------------------|
| | | (%dry weight) | α -Mangostin content* |
| Stirring | Methanol | $3.41 \pm 0.08^{**}$ | $2.52 \pm 0.29^{**}$ |
| | Ethanol | $2.27 \pm 0.06^{**}$ | $0.89 \pm 0.06^{**}$ |
| Ultrasonication | Methanol | $9.43 \pm 0.20^{**}$ | $2.87 \pm 0.34^{**}$ |
| | Ethanol | $4.29 \pm 0.52^{**}$ | $0.65 \pm 0.14^{**}$ |
| Soxhlet extraction | Methanol | $11.57 \pm 0.30^{**}$ | $3.74 \pm 0.34^{**}$ |
| | Ethanol | $7.60 \pm 0.14^{**}$ | $1.69 \pm 0.09^{**}$ |

* expressed as mean \pm standard deviation (S.D) of three analytical replicates (n=3).

**Values indicated significant difference at p<0.05.

HPLC Method validation

In the present study, simple chromatographic condition for separation of α -mangostin from mangosteen pericarp extract using HPLC was optimised. The developed method was found to be very specific for α -mangostin as no other co-eluting peak was detected (Figure 1-b). The peak corresponding to α -mangostin in the sample was confirmed by comparing the spectrum obtained by photodiode array detector, which was completely in agreement with the standard. Furthermore, the method presents a linear response between added concentration and peak area for α -mangostin in the sample; therefore it should be considered specific. The accuracy of the method was evaluated by spiking known amounts of standard α -mangostin into pre-analysed sample A. The recovery at three different levels of α -mangostin was 97.34, 95.50 and 100.73%

at concentration levels of 0.03, 0.04 and 0.05 mg/mL, respectively (Table 2). The obtained results of α -mangostin had shown recoveries between 90-107% within the range of the Association of Official Agricultural Chemists (AOAC) guidelines^{24,25}. The intra-day and inter-day precisions were investigated by determining α -mangostin in sample A six times per day and on five consecutive days, respectively. The precision experiment results were presented as RSD values in Table 2 and indicated lower than 2%, which were considered as a satisfactory precision of the method, complying with the evaluation criterion of the AOAC guidelines^{24,25}. The calibration curve showed linearity within the range of 0.02 - 0.10 mg/mL with regression equation of $Y = 253.29 \times 10^6 + 22,471.97$ that demonstrated the excellent correlation coefficient of 0.9970, as shown in Table 3 and Figure 2.

Table 2 Recovery studies and precision of α -mangostin by the proposed HPLC method

| Quantity in sample (mg/mL) | Accuracy (n=3) | | | | Precision (n=6) | |
|----------------------------|------------------------|---------------|--------------|---------|-------------------|-------------------|
| | Standard added (mg/mL) | Found (mg/mL) | Recovery (%) | RSD (%) | Intra-day RSD (%) | Inter-day RSD (%) |
| | 0.04 | 0.03 | 97.22 | 8.41 | 1.48 | 1.37 |
| | | 0.04 | 95.58 | 6.54 | | |
| | | 0.05 | 100.73 | 12.23 | | |

RSD = relative standard deviation

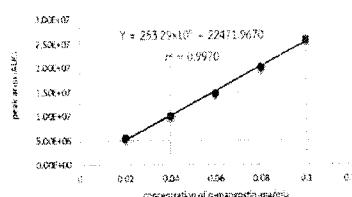


Figure 2 Calibration curve of α -mangostin standard concentration ranging from 0.02 to 0.10 mg/mL.

Table 3 Concentrations and peak areas of standard α -mangostin

| Concentration (mg /mL) | Peak Area | | | Average | RSD | R^2 |
|---------------------------|-----------|---------|---------|---------|--------|-------|
| | n1 | n2 | n3 | | | |
| 0.02 | 5420075 | 5499892 | 5693736 | 5537901 | 0.0254 | 0.997 |
| 0.04 | 9947643 | 9999820 | 9714050 | 9972956 | 0.0026 | |
| 0.06 | 1467233 | 1460732 | 1483562 | 1470509 | 0.0080 | |
| 0.08 | 1993958 | 1998182 | 2026405 | 2006182 | 0.0088 | |
| 0.10 | 2528365 | 2600659 | 2617849 | 2582291 | 0.0184 | |

Conclusion

Screening of extraction power of different solvents and techniques, in terms of the α -mangostin content, clearly illustrated that the Soxhlet extraction using methanol as solvent achieved the highest yield. The developed HPLC method for quantification of α -mangostin was validated and shown reliable, accurate, precise and linear (in the concentration range of 0.02 - 0.1 mg/mL).

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