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Method Development and Selection of Verbascoside-Rich *Acanthus Species* and their Isolation Using Centrifugal Partition Chromatography

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ABSTRACT

Verbascoside possesses antioxidant, anti-inflammatory, anti-cancer, neuroprotective, and anti-depressant activities. This compound can be found in plants from the Acanthaceae family. However, the production of this compound varies significantly, depending on several factors. This study aims to develop an HPLC method for verbascoside quantification in *Acanthus* species, its isolation from verbascoside-Rich plant is also optimized to obtain the high purity verbascoside. *Acanthus ebracteatus* and *Acanthus ilicifolius* were extracted by sonication. The extracts were subjected to HPLC-UV analysis. It revealed varying verbascoside content between *A. ebracteatus* and *A. ilicifolius* across 22 different locations, with *A. ebracteatus* from Samut Songkhram exhibiting the highest content (0.18% w/w), and *A. ebracteatus* from Samut Sakhon exhibiting the lowest content (0.01% w/w) during March to August collection periods. Chromatographic analysis indicated differing resolutions, with the best resolution (5.79) observed in *A. ebracteatus* from Muang Nakhon Si Thammarat despite low verbascoside content (0.05% w/w), while the runner-up was *A. ebracteatus* from Samut Songkhram (5.50). Thus, verbascoside was isolated from *A. ebracteatus* from Samut Songkhram using Centrifugal Partition Chromatography (CPC). It successfully isolated verbascoside within 2 hours 15 minutes, with some loss observed during the process. Mass spectrometry confirmed verbascoside presence using a reference standard. The study found that 11 mg of verbascoside with a purity of 79% was isolated from 1 g of extract of *A. ebracteatus*. This research underscores the effectiveness of plant material selection and CPC in obtaining high amounts of verbascoside from natural sources, suggesting avenues for further enhancement in isolation techniques to maximize yield.

Keywords: Acanthus ebracteatus, Acanthus ilicifolius, Verbascoside, Chromatography.

Introduction

Verbascoside (also known as acteoside) is a phenylethanoid glycoside found in numerous plant families such as Acanthaceae, Oleaceae, Scrophulariaceae, and Verbenaceae (Figure 1).[1-4] It exhibits notable antioxidant, anti-inflammatory, and wound healing properties, making it valuable in therapeutic cosmetology.[1,5] Additionally, verbascoside shows promise as a lead compound for anti-cancer agents due to its antiproliferative effects against various types of cancer.[6] Furthermore, it displays neuroprotective and anti-depressant effects, suggesting potential applications in the treatment of neurodegenerative diseases and depression.[7,8]

In Thailand, verbascoside is present in *Acanthus ebracteatus* Vahl and *Acanthus ilicifolius* L. (Figure 2), plants that are traditionally used as herbal remedies for promoting longevity.[1,9] Leveraging the potential of these plants, they can serve as alternative sources for isolating verbascoside.

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However, the production of secondary metabolites in plants varies significantly depending on factors such as geographical location, season, and plant maturity. Therefore, the identification, and isolation of verbascoside-rich plant is crucial for optimizing the isolation process. In this study, the plants (*Acanthus ebracteatus* Vahl and *Acanthus ilicifolius* L) were sourced from diverse locations, and the verbascoside content was quantified using High Performance Liquid Chromatogarphy – Ultra violet UV detection (HPLC-UV).

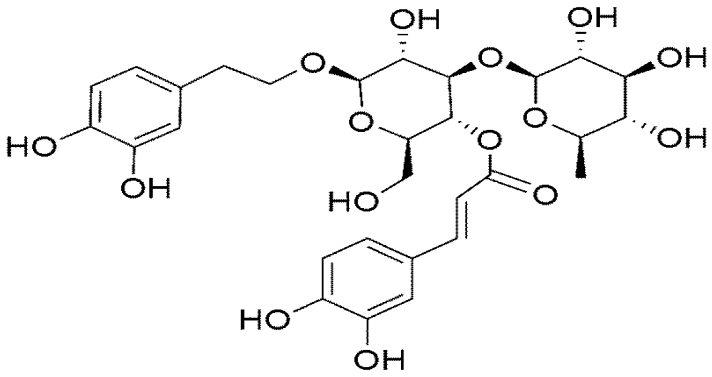

Figure 1: The chemical structure of verbascoside

Centrifugal Partition Chromatography (CPC) represents a silica-free isolation technique scalable from laboratory to industrial settings. While CPC has previously demonstrated effectiveness in isolating verbascoside from *Buddleja globosa* Hope,[10] it is imperative to acknowledge that different plant species contain varying secondary metabolites. Thus, this study introduces an alternative approach aimed

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at achieving high-yield isolation of verbascoside from *A. ebracteatus* or *A. ilicifolius*. The isolated verbascoside holds potential as a versatile ingredient for various applications.

The objective of this study was to identify and isolate verbascoside from verbascoside-rich *A. ebracteatus* or *A. ilicifolius* using Centrifugal Partition Chromatography.

Figure 2: Flowering *A. ebracteatus* (A) and *A. ilicifolius* (B).

Materials and Methods

Collection and identification of plant materials

A. ebracteatus, which grows naturally near rivers or the sea in Central, East, and South Thailand, was collected from March to June, 2019, from twelve different locations, which include; Bang Kachao and Phra Pathom Chedi (Samut Prakan), Samut Songkhram, Samut Sakhon, Nakhon Pathom, Chachoengsao, Chon Buri, Trat, Chumphon, and three areas in Nakhon Si Thammarat (Muang, Pak Phanang, and Khanom) (Figure 3). The GPS coordinates are shown in Table 1.

A. ilicifolius, which grows naturally near the sea in Central, East, and South Thailand, was collected from March to August, 2019, from ten locations, which include; Muang and Pak Phanang (Nakhon Si Thammarat), Surat Thani, Trang, Krabi, Ranong, Samut Sakhon, and three areas in Phang Nga (Muang, Khura Buri, and Thai Mueang) (Figure 3). The GPS coordinates are shown in Table 1.

All samples were identified by a botanist in the Department of Biology, Faculty of Science, Silpakorn University, and assigned voucher specimen number #10100 for *A. ebracteatus* and #10101 for *A. ilicifolius*. The plant materials were washed to remove contaminants and dried at 60°C in a hot air oven (OVD HS-169, Thailand) for 24 hours. The whole plant samples were pulverized in a blender and stored at 30ºC, protected from light.

Table 1: GPS coordinates of locations of plants collection

A. ebracteatus	**Latitude, Longitude**	***A. ilicifolius***	**Latitude, Longitude**
Bang Kachao	13.697542, 100.565276	Samut Sakhon	13.519744, 100.367936
Phra Pathom Chedi	13.568513, 100.525777	Ranong	9.873675, 98.546096
Samut Sakhon	13.497666, 100.309398	Muang Phang Nga	8.372021, 98.586666
Samut Songkhram	13.360500, 99.935431	Thai Mueang	8.532966, 98.234293
Nakhon Pathom	13.818346, 100.041351	Khura Buri	9.147416, 98.366300
Chachoengsao	13.603952, 101.080709	Surat Thani	9.143549, 99.323124
Chon Buri	13.443104, 100.986322	Krabi	9.067481, 98.930995
Trat	12.360549, 102.550273	Muang Nakhon Si	8.552798, 100.010743
Chumphon	10.225869, 99.120080	Pak Phanang	8.442782, 100.154938
Muang Nakhon Si	8.479845, 100.014828	Trang	7.155590, 99.721490
Pak Phanang	8.329921, 100.129269		
Khanom	9.205330, 99.813308		

Chemicals and solvents

Verbascoside USP reference standard (99% purity) was purchased from Sigma-Aldrich (USA). HPLC-grade acetonitrile and water, as well as analytical-grade butanol, ethanol, and ethyl acetate, were sourced from B and J (South Korea). Analytical-grade formic acid was acquired from Merck (Germany), and distilled water from Puris, Expe-CB Ele10 Water System (South Korea).

Extraction of plant materials

The ethanol-to-water ratio was optimized for efficient extraction of verbascoside from the plant materials. The resulting extract was then used for HPLC-UV analysis and isolation.

For HPLC-UV analysis, approximately 50 mg of finely powdered *A. ebracteatus* or *A. ilicifolius* (accurately weighed) was sonicated with 2 mL of a mixture containing equal volumes of ethanol and water in a water bath for 30 min. The mixture was centrifuged at 4,000 rpm at 25°C for 5 min. The supernatant was transferred to a new 2 mL tube and evaporated to dryness at 50°C under reduced pressure. The residue was dissolved in 2 mL of a mixture of equal volumes of ethanol and water. The solution was filtered through a nylon membrane with a 0.45 µm porosity. The filtrate (200 µL) was transferred to an HPLC vial, diluted with 800 µL of water, and mixed thoroughly.

For verbascoside isolation, approximately 25 g of finely powdered *A. ebracteatus* (accurately weighed) was sonicated with 1 L of a mixture containing equal volumes of ethanol and water in a water bath for 30 min. The sample was evaporated to dryness at 50°C under reduced pressure before CPC isolation.

Determination of verbascoside content

The verbascoside content of each extract was determined using HPLC-UV (Thermo Scientific Dionex Ultimate^{TM} 3000 Basic Systems, USA) with an Acclaim® 120-C18 column (3 µm x 2.1 mm x 150 mm). The mobile phase consisted of aqueous 0.1% formic acid and acetonitrile, flowing at approximately 0.18 mL/min in a gradient mode: starting at 95:5 (water) from 0 to 25 minutes, changing to 60:40 from 25 to 26 minutes, holding at 5:95 from 26 to 31 minutes, returning to 95:5 from 31 to 36 minutes, and re-equilibrating at 95:5 from 36 to 46 minutes. The column temperature was maintained at 40°C, and detection was performed at 322 nm using an ultraviolet spectrophotometer. The robustness was performed at pH 2.5, 2.7, and 2.9 or at column temperature of 35, 40, and 45°C.

The chromatographic system's suitability was evaluated based on three parameters: resolution between the verbascoside peak and the nearest peak (≥ 1.5); tailing factor for the verbascoside peak (≤ 1.5), and theoretical plate number (≥ 2,000).[11]

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For standard preparation, 10 mg of verbascoside reference standard was dissolved in a mixture of equal volumes of ethanol and water to prepare a stock solution of approximately 1 mg/mL. This stock solution was diluted in a stepwise manner with the ethanol:water mixture to obtain six dilutions with concentrations of 1.25, 5, 10, 20, 30, and 50 µg/mL. Approximately 5 µL of each standard preparation was injected separately into the chromatograph, the chromatograms were recorded, and the responses for the major peaks measured. The readings were plotted, and the best-fit standard curve was prepared. Thereafter, 5 µL of the sample preparation was injected into the chromatograph, the chromatogram recorded, and the response for the major peak measured. Using the standard curve as a reference, the content of verbascoside in the sample was calculated.

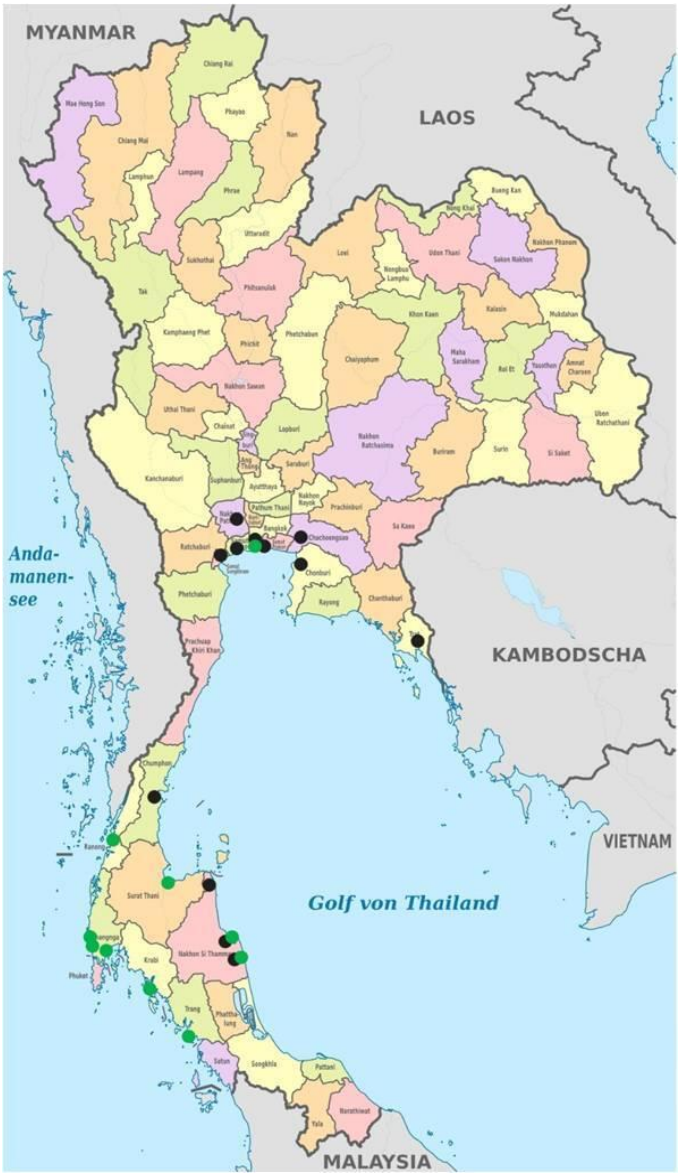

Figure 3: Map of Thailand showing the locations of plant samples collection. Black spots indicate collection points for *A. ebracteatus*, while green spots denote collection points for *A. ilicifolius*.

Isolation of verbascoside

The extract (1 g) was dissolved in 10 mL of mobile phase and injected into a CPC column using a PLC 2250 Purification System controlling a Gilson CPC 250 PRO column. The solvent system used was water, ethyl acetate, and butanol in a ratio of 10:10:2 v/v/v. Elution and extrusion rates were set at 12 mL/min and 30mL/min, respectively. The column rotation speed was 2,000 rpm, and detection was performed at a wavelength of 332 nm.

The compound was identified via mass spectrometry (MS) using a Bruker AmaZon SL ESI-ITMS instrument (USA) in negative mode, recording a mass range from m/z 100 to 1,000. The capillary voltage was set at 4,500 V, and the drying gas temperature was 200°C with a flow rate of 7.0 L/min. Nebulizer pressure was maintained at 2 bars. Data analysis was done using the Compass 1.3 SR2 program.

Statistical analysis

The data were presented as mean ± standard deviation (SD). Differences between means were evaluated by t-test using IBM SPSS Statistics 22 (2013).

Results and Discussion

Optimal extraction solvent for extracting verbascoside

Sonication is an extraction method that can be easily performed in the laboratory. It requires less time than conventional methods and is suitable for heat-labile samples since the extraction temperature is usually low. The optimal ethanol-to-water ratio for extracting verbascoside from *A. ebracteatus* was determined to be 1:1 (Table 2). Sonication duration was 30 minutes, indicating an efficient extraction process. Additionally, verbascoside is known to degrade via hydrolysis, which may explain the lower verbascoside content reported in previous study using a 1:1 ethanol-to-water ratio. Water alone proved to be an inadequate extraction solvent due to its polarity, consistent with previous findings.[12-13] While ethanol alone yielded high verbascoside content, its higher cost compared to water makes the ethanol-water mixture the most cost-effective choice for verbascoside extraction.

Validated HPLC-UV method for verbascoside quantification

Previous studies have reported using the quantitative HPLC method to separate and analyze verbascoside in various plant species, such as *Verbena officinalis*, *Euphrasia officinalis*, and *Radix scrophulariae*.[14-16] However, different matrices contain different secondary metabolites, making it necessary to optimize suitable conditions for separating verbascoside in *A. ebracteatus* and *A. ilicifolius*. Since the samples in this study were from two species and collected from various locations, a full method validation was required to ensure consistent results.

Several parameters were considered for optimizing the HPLC-UV analysis. The first challenge was determining the appropriate mobile phase. Different compositions of aqueous 0.1% formic acid and acetonitrile were tested. The most suitable peak was observed when the gradient was performed as described in the method. The retention time of verbascoside was 17.1 minutes.

The standard curve of verbascoside was constructed and demonstrated linearity over the concentration range of 1.25-50 µg/mL, with a correlation coefficient of 1. The method proved to be highly sensitive, with LOD and LOQ values of 0.0078 and 0.0313 µg/mL, respectively. The accuracy of the method was evaluated by analyzing samples spiked with known concentrations of the standards. Prior to spiking, the background level of verbascoside in the samples was analyzed to calculate actual recoveries. Mean recoveries ranged from 99.01% to 105.81%. The precision of the method, expressed as the %RSD value, was obtained from three replicate analyses at three concentrations of verbascoside, and was found to be between 0.21% and 1.58% (Table 3). The method was considered to be both accurate and precise, ensuring reliable results.

Robustness was assessed by introducing slight variations from the optimum conditions, including changes in pH and column temperature. When the pH was adjusted from 2.5 to 2.9 or the temperature from 35°C to 45°C, a minimal variation in the retention time of verbascoside was observed (17.0 -17.1 minutes), with a resolution greater than 1.5 and a tailing factor less than 1.5 (Table 4). The results demonstrate the reliability of the analysis under deliberate variations.

System suitability was assessed across 22 samples. The resolution of the verbascoside peak in all samples was ≥1.5, with a tailing factor consistently <1.5, and a high theoretical plate number was observed in each sample (Table 5).

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Table 2: Extraction solvent optimization

Solvent ratio (ethanol:water)	Verbascoside content in plant material (%w/w)
2:0	0.056 ± 0.0004
1:1	0.060 ± 0.0004*
0:2	0.022 ± 0.0002*

Values are mean ± standard deviation (SD), *Significantly different from ethanol:water 2:0 at $p < 0.05$ using Student's t-test.

Table 3: Validated HPLC-UV method for verbascoside quantification

Marker	Linear equation	LOD (µg/mL)	LOQ (µg/mL)	%Recovery (Mean ± SD)	%RSD
Verbascoside	Y = 68.749X + 7.9385	0.0078	0.0313	102.97(2.76)	0.21-0.46 (intra)
(*A. ebracteatus*)					0.33-1.58 (inter)
Verbascoside	Y = 68.749X + 7.9385	0.0078	0.0313	99.59(0.50)	0.21-0.46 (intra)
(*A. ilicifolius*)					0.33-1.58 (inter)

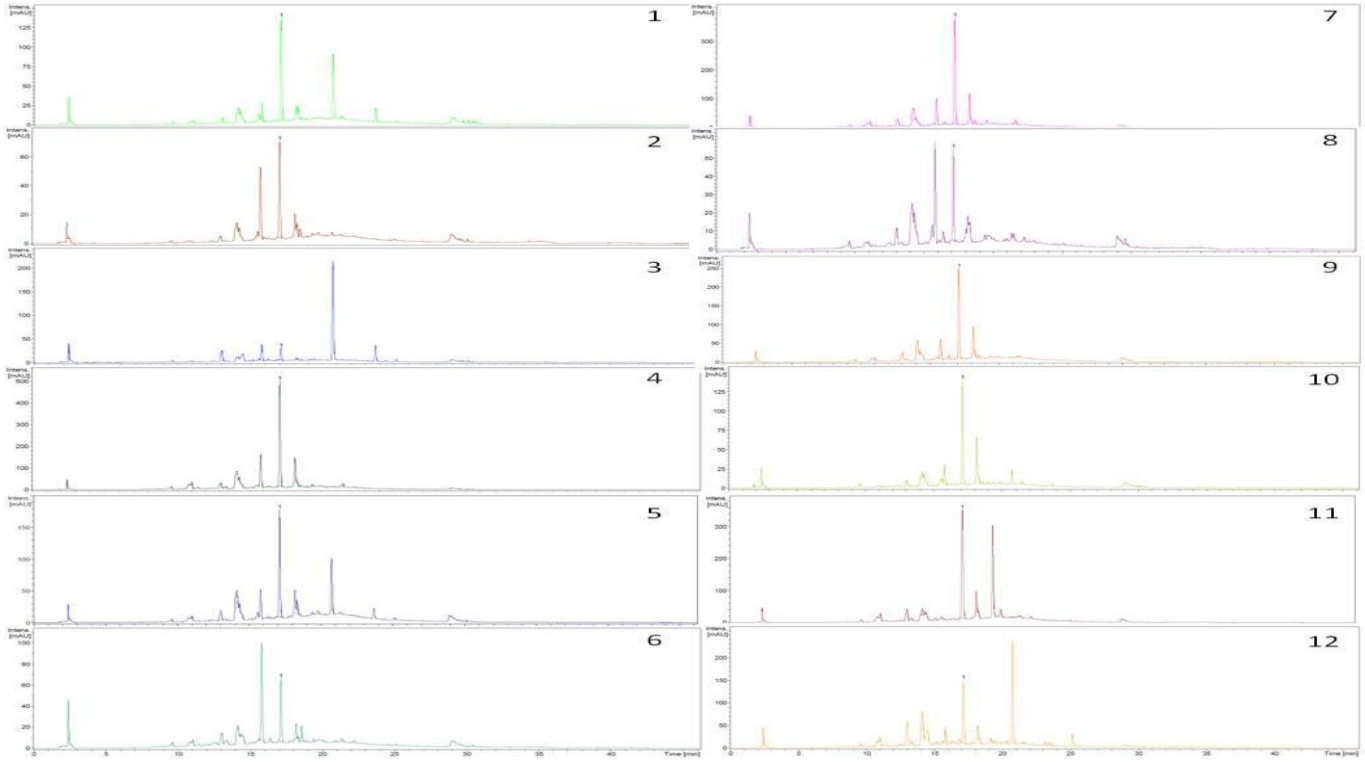

Figure 4: Chromatographic fingerprints of *A. ebracteatus* from 12 locations: 1-Bang Kachao, 2-Phra Pathom Chedi, 3-Samut Sakhon, 4-Samut Songkhram, 5-Nakhon Pathom, 6-Chachoengsao, 7-Chon Buri, 8-Trat, 9-Chumphon, 10-Muang Nakhon Si, 11-Pak Phanang,12-Khanom.

Verbascoside content of A. ebracteatus and A. ilicifolius from different locations

Verbascoside was detected in all samples, with its content in *A. ebracteatus* and *A. ilicifolius* from 22 locations detailed in Table 5. In *A. ebracteatus*, verbascoside content ranged from 0.01% to 0.18% w/w, while in *A. ilicifolius*, the range was 0.02% to 0.08% w/w.

From the present study, it was found that the average content of verbascoside in *A. ilicifolius* was lower compared to *A. ebracteatus*. From March to August of plant collections, the highest verbascoside content was observed in *A. ebracteatus* from Samut Songkhram, followed by Nakhon Si Thammarat (Pak Phanang) and Chon Buri (0.18%, 0.16%, and 0.14% w/w, respectively), while the lowest was recorded in Samut Sakhon (0.01% w/w). In terms of resolution, the sample from Nakhon Si Thammarat (Pak Phanang) showed the highest (5.79), despite having lower verbascoside content (0.05% w/w). The sample from Samut Songkhram followed with a resolution of 5.50, where fewer major peaks were detected. *A. ebracteatus* from Samut Songkhram was selected as the material for isolating verbascoside in high quantities using CPC.

Table 4: Robustness data for the developed HPLC-UV method

Parameters	Conditions (pH or ºC)	Retention time	Tailing factor	Resolution
pH	2.5	17.0	1.0	3.84
	2.7	17.1	0.9	5.71

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	2.9	17.1	1.1	4.00
Column temperature (ºC)	35	17.0	1.0	5.50
	40	17.1	0.9	5.71
	45	17.1	0.9	6.25

Table 5: System suitability of the developed HPLC-UV method and verbascoside content in plant materials from different locations

Samples	**Verbascoside content (%w/w) (Mean ± SD)**	**System suitability parameters**		
		Theoretical plate number	**Tailing factor**	**Resolution**
A. ebracteatus				
Bang Kachao	0.03 ± 0.001	96664	1.0	5.00
Phra Pathom Chedi	0.06 ± 0.001	96664	1.0	5.24
Samut Sakhon	0.01 ± 0.001	144400	1.0	5.49
Samut Songkhram	0.18 ± 0.004	96664	1.0	5.50
Nakhon Pathom	0.05 ± 0.001	116964	1.0	5.25
Chachoengsao	0.02 ± 0.001	116964	1.0	3.33
Chon Buri	0.14 ± 0.002	116964	1.2	3.45
Trat	0.02 ± 0.001	116964	1.1	3.33
Chumphon	0.09 ± 0.001	144400	1.1	3.45
Muang Nakhon Si	0.05 ± 0.001	116964	1.0	5.79
Pak Phanang	0.16 ± 0.001	52593	1.1	4.00
Khanom	0.05 ± 0.002	116964	1.0	1.50
A. ilicifolius				
Samut Sakhon	0.08 ± 0.001	116008	1.2	3.69
Ranong	0.04 ± 0.002	74419	1.2	3.74
Muang Phang Nga	0.02 ± 0.001	116281	1.4	3.78
Thai Mueang	0.02 ± 0.001	116281	1.5	3.36
Khura Buri	0.03 ± 0.001	116281	1.5	2.40
Surat Thani	0.03 ± 0.001	128538	1.5	5.22
Krabi	0.03 ± 0.001	116417	1.4	4.80
Muang Nakhon Si	0.06 ± 0.004	105723	1.2	5.02
Pak Phanang	0.02 ± 0.001	96324	1.5	3.33
Trang	0.07 ± 0.001	81225	1.4	3.83

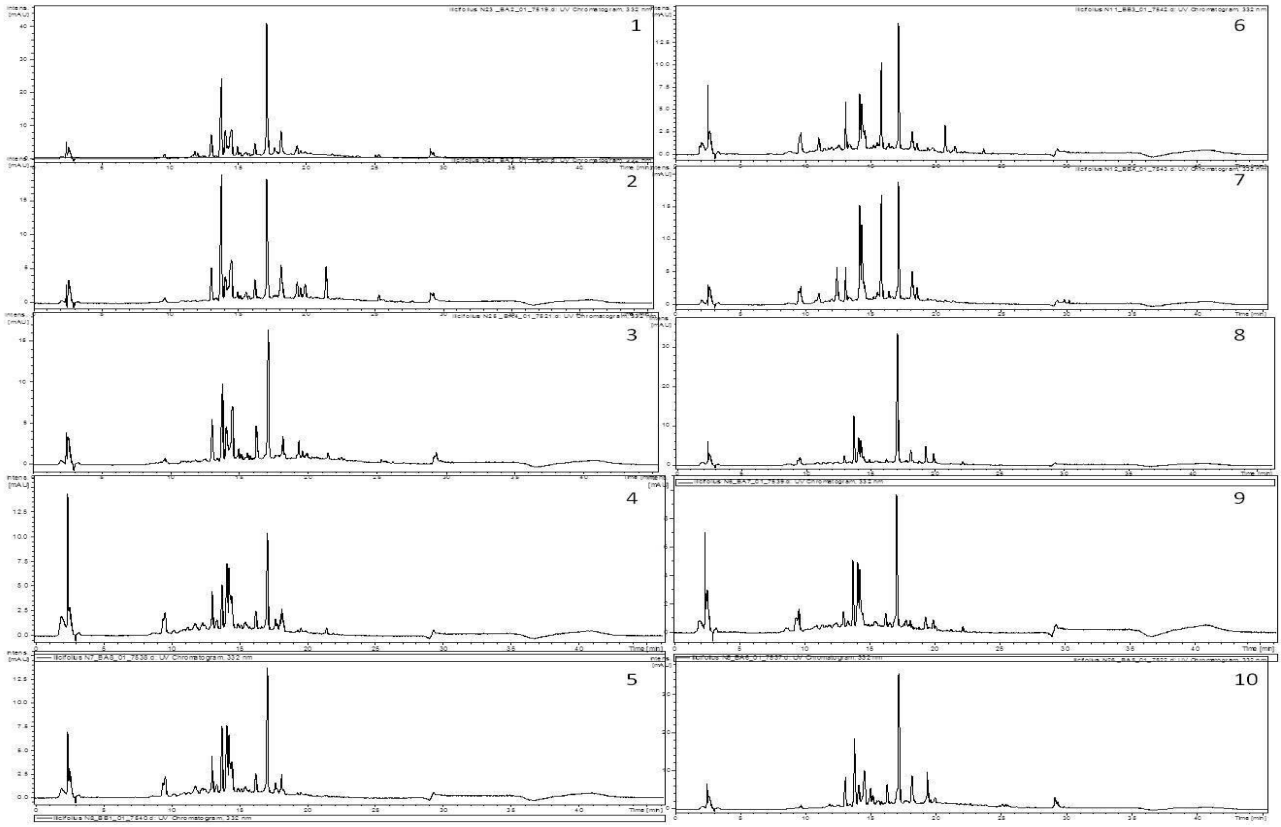

Figure 5: Chromatographic fingerprints of *A. ilicifolius* from 10 locations: 1-Samut Sakhon, 2-Ranong, Muang Phang Nga, 4-Thai Mueang, 5-Khura Buri, 6-Surat Thani, 7-Krabi, 8-Muang Nakhon Si, 9-Pak Phanang, 10-Trang.

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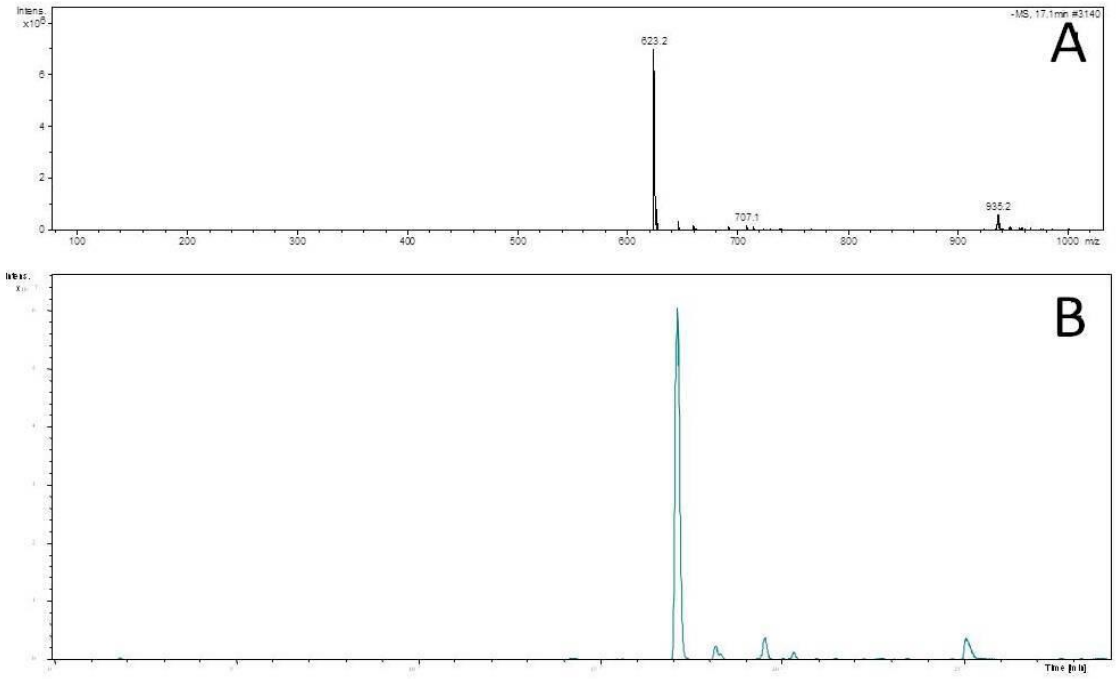

Figure 6: Mass spectrum of isolated verbascoside (A) and its purity based on LC-MS chromatogram (B)

Chromatographic fingerprints of A. ebracteatus and A. ilicifolius

Chromatographic fingerprints are shown in Figures 4 and 5, revealing a nearly identical pattern between both species. In *A. ebracteatus*, major peaks were consistently observed at retention times of 13.0, 14.1, 15.8, 17.1, and 18.1 minutes, with an additional peak at 20.8 minutes exclusively in samples from Nakhon Pathom, Nakhon Si Thammarat (Muang and Khanom), Samut Prakan (Bang Kachao), and Samut Sakhon. In *A. ilicifolius*, samples from Surat Thani and Krabi exhibited a peak at 15.8 minutes, while the 20.8-minute peak was present only in Surat Thani samples. Furthermore, a higher abundance of peaks around 13-15 minutes was noted in *A. ilicifolius*.

The validation of methods and system suitability is standard practice for HPLC-UV analysis. Furthermore, the data can be beneficial in the isolation of verbascoside, as demonstrated by the differing chromatographic fingerprints observed in *A. ebracteatus* and *A. ilicifolius* from various locations. Samples with lower resolution suggest the presence of other compounds with polarity or partition coefficient values similar to verbascoside. Therefore, if a sample contains high verbascoside content but low resolution, isolating high-purity verbascoside may prove challenging. Conversely, selecting samples with high verbascoside content and good resolution may offer a better choice. Despite differing methods between analysis and isolation, adjustments can enhance the isolation process, while polarity and partition coefficient values remain constant and significantly influence both analytical and isolation procedures. Moreover, starting material selection plays a crucial role in effective compound isolation.

Isolation of verbascoside

In this study, the isolation process was completed in 2 hours 15 minutes, with verbascoside found in the fraction 25-26. Twenty-five grams (25 g) of plant material yielded 2 g of extract, from which 11 mg of verbascoside was isolated from *A. ebracteatus*. Identification was confirmed by comparing mass spectra to the USP reference standard with m/z 623.2. The purity, determined based on MS data, of verbascoside isolated from *A. ebracteatus* was 79% (Figure 6). By combining selection of verbascoside-rich plant material and utilizing the higher speed of the CPC rotor, a significant amount of verbascoside was obtained through one-step purification. However, some verbascoside was lost during the process. While the amount and purity of verbascoside cannot be directly compared to previous studies due to differences in plant materials and instruments, this study offers verbascoside with higher purity with shorter isolation time than the previous studies.[4] The verbascoside isolation from selective *A. ebracteatus* using CPC was an alternative strategy for isolating compounds from natural products, emphasizing selective plant material usage over random sourcing.

Conclusion

Two *Acanthus* species were collected from 22 different locations across Thailand. Production of verbascoside and other secondary metabolites varied naturally depending on geographical location. Careful selection of *A. ebracteatus* and *A. ilicifolius* for compound isolation is crucial to maximize yield, particularly of verbascoside, Future studies should explore variations in extraction methods and parameters to further optimize the process.

Conflict of interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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