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Simultaneous HPLC Analysis of Three Flavonoids in the Extracts of *Artocarpus heterophyllus* Heartwoods

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Abstract – A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of three antibacterial flavonoids, artocarpanone, artocarpin, and cycloartocarpin in ethyl acetate extracts from *Artocarpus heterophyllus* heartwoods. Separation was achieved using a TSK-gel ODS-80Tm column (5 μm, 4.6 × 150 mm) at 25 °C with a gradient elution system of methanol and water as follows: 0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v, at a flow rate of 1 mL/min, and a quantitative UV detection at 285 nm. The method was validated by measuring the key parameters, including specificity, linearity, sensitivity, accuracy, repeatability and reproducibility. A high degree of specificity and sensitivity was achieved. The calibration curves for all three flavonoids showed good linearity with a coefficient of determinations (R^2) of ≥ 0.9995. The recoveries of the method were from 98-104%, with good reproducibility and repeatability (RSD values of less than 2%) were also achieved. Ethyl acetate was the best solvent for extraction of these three flavonoids using the heat reflux conditions for 1 h. This optimized sample preparation and HPLC method can be practically used for a routine standardization process of the extracts from the *A. heterophyllus* heartwoods.
Keywords – *Artocarpus heterophyllus*, Artocarpanone, Artocarpin, Cycloartocarpin; HPLC

Introduction

The heartwoods of *Artocarpus heterophyllus* Lam. (Moraceae) have been used as a popular folk medicine in several Asian countries for the treatment of bacterial and fungal infection as well as some other diseases associated with inflammation, malarial fever and diabetes.[1,2] The flavonoid compounds isolated from the heartwoods of this plant possess many biological activities including anticarcinogenic, antioxidant and tyrosine inhibitory activities.[3-5] Recently, we have isolated some flavonoid compounds with antibacterial activity, i.e. artocarpanone, artocarpin and cycloartocarpin (Fig. 1) from the heartwoods of *A. heterophyllus*.[6] Artocarpanone also possessed anti-inflammatory activity and had an inhibitory effect on melanin biosynthesis[7,8], while artocarpin possessed anticancer activity and inhibited neuraminidase[9,10], while cycloartocarpin possessed antiplatelet activity.[11] These three flavonoids were therefore considered to be the best bioactive markers used for standardization of the heartwood extracts of *A. heterophyllus* before being used for medicinal purposes.

A standardized method for assessing the quality and effectiveness of herbal medicines is becoming a hot issue for many people including those in the herbal medicine industries. In order to obtain a high and consistent quality of the heartwood extracts of *A. heterophyllus*, a simple quantitative analytical method for the active compounds in the extracts should be developed. Recently, a reversed-phase HPLC method has been popularly used for standardization of herbal extracts due to its high sensitivity, selectivity and accuracy.[12] In this study there has been a focus on establishing a validated quantitative HPLC method for the simultaneous determination of artocarpanone, artocarpin and cycloartocarpin as well as determining the most suitable solvent for sample preparation.

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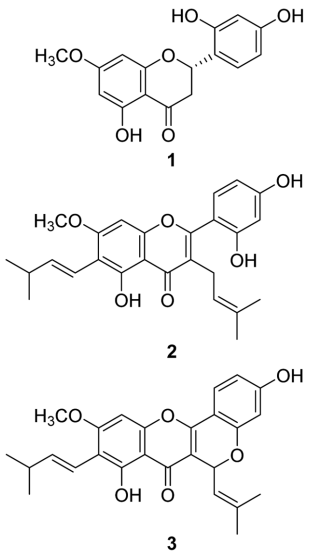

Fig. 1. Chemical structures of artocarpanone (**1**), artocarpin (**2**), and cycloartocarpin (**3**).

Experimental

Chemicals and reagents – Standard artocarpanone, artocarpin and cycloartocarpin had been previously purified.[6] Methanol (HPLC-grade) was from Merck (Bangkok, Thailand). Analytical-grade solvents (hexane, chloroform, ethyl acetate and methanol) were from Labscan limited (Songkhla, Thailand). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Plant material – *A. heterophyllus* heartwoods were collected from Songkla Province, Thailand, in June 2012. The voucher specimen (SKP 117 01 08 01) was deposited at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant material was washed and dried at 60 ºC in a hot air oven for 24 h, then ground and passed through a No. 45 sieve.

Standard solution – Stock solutions of artocarpanone, artocarpin, and cycloartocarpin were prepared in methanol. The working solution of the combined standard compounds was subsequently prepared in methanol and diluted to give a series of standard solutions of 6.25, 12.5, 25, 50 and 100 μg/mL. Calibration curves were constructed for each of the target analytes.

Sample preparation – The *A. heterophyllus* heartwood powder (1 g) was extracted with ethyl acetate (250 mL) under reflux conditions for 1 h. The extract was concentrated and dried under reduced pressure. The dried extract (5 mg) was dissolved and adjusted to 10 mL with methanol, and then filtered through a 0.45 μm membrane filter, and analyzed immediately after extraction in order to avoid possible chemical degradation.

HPLC analyses – HPLC analysis was carried out using the Waters 1500 series equipped with a Waters 2998 photodiode-array detector (PDA) and Waters 2707 auto sampler. For analysis of the data, Waters Chemstation for Empower software was used. Separation was achieved at 25 ºC on a TSK-gel ODS-80Tm column (150 × 4.6 mm i.d.) (Tosho Bioscience, Japan). The mobile phase consisted of methanol and water, with a gradient elution system (0 - 8 min, 60:40; 8 - 27 min, 80:20; 27 - 35 min, 60:40, v/v) at a flow rate of 1 mL/min. The sample injection volume was 20 μL, and detection was by UV at a wavelength of 285 nm.

Validation of method – Validation of the analytical method was achieved by following the guidelines of the International Conference on the Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use.[13] The HPLC method was validated for linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) of the analytes.

Linearity – Calibration curves of the three standard flavonoids were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations (6.25 - 100 μg/mL), and plotting peak areas against the concentrations of each reference standard. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area. The coefficient of determination (R^2) of the regression line should be not less than 0.999.

Accuracy – Sample portions were fortified with known quantities of standards (6.25, 25, and 100 μg/mL) in order to assay the accuracy of the data. Prior to fortification of the analyte, the background levels of artocarpanone, artocarpin and cysloartocarpin in extracts of *A. heterophyllus* heartwood were determined so as to calculate actual recoveries. The amount of each analyte was determined in triplicate, and the percentage recoveries were then calculated.

Precision – Precision experiments were conducted for intraday and interday. A solution of sample was used to test for repeatability. The repeatability (intraday precision) data was obtained from six injections of one sample solution performed on the same day. The data were used to calculate the % RSD for intraday precision (less than 2%). The inter-day precision of the extraction procedure was validated by repeating the extraction procedure on the same sample of *A. heterophyllus* heartwood. This parameter was evaluated by repeating the extraction in triplicate on 3 different days with a freshly prepared mobile phase and samples. An aliquot of each extract was then injected and

quantified. The data were used to calculate the % RSD (less than 5%) for the interday precision.

Specificity – Peak identification was carried out using the authentic standards and by scanning the UV spectrum of each peak using a photodiode-array detector. The UV spectra were taken at various points of the peaks and at least three different points to check for the peak homogeneity.

LOD and LOQ – Serial dilutions of a sample solution were made with methanol and analyzed by the HPLC method. The LOD and LOQ were obtained as the ratio of the signal to noise ratio equal to 3 and 10, respectively.

Determination of solvent for extraction – To optimize the solvent for extraction, *A. heterophyllus* heartwood powder (1 g) was separately extracted with methanol, ethyl acetate, chloroform and hexane (250 mL) under reflux conditions for 1 h. The extracts were then filtered and the solvents were evaporated under reduced pressure. The dried extracts (5 mg) were dissolved in methanol, and volume adjusted to 10 mL, and subjected to HPLC analysis. This experiment was performed in triplicate.

Statistical analysis – Data are presented as mean values ± SD. The data were analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's HSD post hoc test that denoted the presence of a statistically significant difference and was considered to be significant at the $P < 0.05$ level.

Result and Discussion

A reversed-phase gradient HPLC system was established for the simultaneous quantitative determination of three bioactive flavonoids: artocarpanone, artocarpin and cycloartocarpin in extracts from *A. heterpohyllus* heartwoods. These three flavonoids showed a high UV absorption at 285 nm therefore this wavelength was used for their quantitative determination. Mixtures of methanol and water were examined as the mobile phases, with different ratios as well as a gradient elution system were optimized. The most suitable gradient elution system was 0 - 8 min, 60:40; 8 - 27 min, 80:20; 27 - 35 min, 60:40, v/v. All three flavonoids were eluted within 25 min with a satisfactory resolution (Fig. 2). Artocarpin was detected as the major

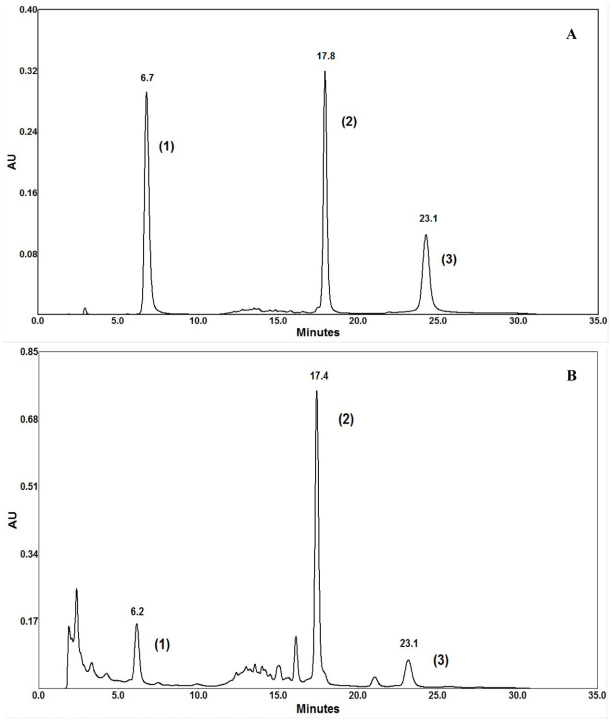

Fig. 2. HPLC chromatograms of (A) authentic compounds and (B) heartwood extract of *A. heterophyllus.* (**1**: Artocarpanone, **2**: Artocarpin, **3**: Cycloartocarpin).

flavonoid in the ethyl acetate extract, while artocarpanone and cycloartocarpin were minor components. This HPLC method is relatively simple and also slightly faster than a previous HPLC method for determination of artocarpanone in a *A. heterophyllus* heartwood extract that has been described by Zheng et al. (2009).[14]

The HPLC method was validated by means of determination of the important parameters, including linearity, accuracy, precision, specificity, LOD and LOQ. Linearity was evaluated using the standard samples over five calibration points (6.25 - 100 μg/mL) with six measurements for each calibration points. Three calibration curves were obtained by plotting the peak areas against concentrations. Artocarpanone, artocarpin and cycloartocarpin all exhibited an excellent linearity with the coefficient of determinations (R^2) of 0.9997, 0.9998 and 0.9995, respectively (Table 1). The results of the LOD and LOQ indicated that

Table 1. HPLC calibration data for artocarpanone, artocarpin, and cycloartocarpin

Compounds	Linear range (μg/mL)	t_R (min)	Equation[a]	Linearity (R^2)	LOD (μg/mL)	LOQ (μg/mL)
Artocarpanone	6.25 - 100	6.7	$Y = 52290X - 35882$	0.9997	0.06	0.2
Artocarpin	6.25 - 100	17.8	$Y = 47287X - 44018$	0.9998	0.04	0.2
Cycloartocarpin	6.25 - 100	23.1	$Y = 25630X - 37434$	0.9995	0.2	0.4

[a] $Y = aX + b$, where Y is a peak area and X is the concentration of the analyzed material

Table 2. Recovery data for the three flavonoids spiked into *A. heterophyllus* heartwood extracts

Compounds	Spiked level (μg/mL)	% Recovery (Mean ± SD)
Artocarpanone	100	101.9 ± 0.83
	25	98.2 ± 1.10
	6.25	103.4 ± 0.30
Artocarpin	100	103.5 ± 0.75
	25	101.7 ± 0.20
	6.25	101.6 ± 0.74
Cycloartocarpin	100	102.1 ± 0.63
	25	103.8 ± 0.73
	6.25	102.4 ± 0.55

Table 3. Intraday and interday precision data for the quantitative determination of the three flavonoids

Compounds	RSD (%)	
	intra-day (n = 6)	inter-day (n = 3)
Artocarpanone	0.76	1.23
Artocarpin	0.78	1.49
Cycloartocarpin	0.64	1.27

RSD = Relative standard deviation

this HPLC method was sensitive for determination of these flavonoids in the heartwood extract, even though at a very low concentration (Table 1). The specificity of the analytical method was determined using the UV absorption spectra at three points of each peak. When compared with authentic samples, the results showed that all peaks were homogenous without any impurity. The accuracy of the analytical method was studied by the spiking technique. The percentage recoveries in the ranges of 98-104% were obtained for all analytes (Table 2). This result indicated that the established HPLC method possessed a good accuracy. The precision of the HPLC method was evaluated by assessing the RSD values of intraday and interday analysis. Intra-day precision was performed using

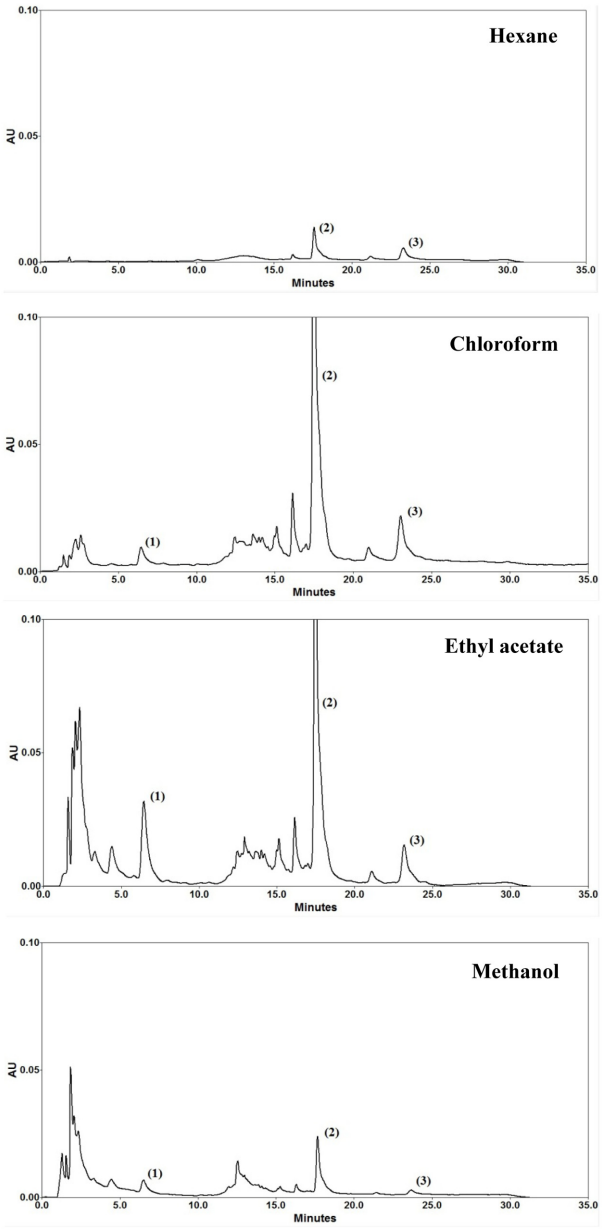

Fig. 3. HPLC chromatograms of hexane, chloroform, ethyl acetate, and methanol extracts from of *A. heterophyllus* heartwoods.

Table 4. Content of the three flavonoids in different solvents for extracting *A. heterophyllus* heartwood

Solvents	Yield of dried extracts (% w/w; mean ± SD)	Content (%w/w of dried extract; mean ± SD)		
		Artocarpanone	Artocarpin	Cycloartocarpin
Hexane	2.2 ± 0.07*	n.a.	2.84 ± 0.16*	2.62 ± 0.02*
Chloroform	5.4 ± 0.15*	1.03 ± 0.03*	21.72 ± 0.57	5.51 ± 0.12
Ethyl acetate	7.2 ± 0.20	4.95 ± 0.01	21.84 ± 0.60	5.75 ± 0.20
Methanol	25.6 ± 1.47*	1.09 ± 0.05*	3.01 ± 0.10*	1.09 ± 0.03*

*Significant difference ($p < 0.05$) when compared with ethyl acetate in the same column. n.a. = not analyzed due to it being lower than the limit of quantification.

six injections in the same day, and the RSD values for all three flavonoids were less than 1% (Table 3). Analysis of three independently prepared samples on three different days estimated the inter-day precision. The RSD values for all three flavonoids were less than 2% (Table 3). These results indicated that the HPLC method enabled the quantitative determinations of artocarpanone, artocarpin and cycloartocarpin in *A. heterophyllus* heartwood extracts with a high degree of precision.

Four solvents with different polarities were used to decide the best for extraction and sample preparation included hexane, chloroform, ethyl acetate and methanol. Among the three flavonoids, artocarpanone was the most polar compound, and was therefore sparingly extracted with hexane. An increased polarity of the solvent used for extraction resulted in an increase of the flavonoid content of the extracts. Although, methanol gave the highest yield of the crude extract, but the lowest contents of these three flavonoids due to its nonselective extractability (Fig. 3). The most suitable solvent for extraction of all three flavonoids should have partial polarity. Although both ethyl acetate and chloroform produced extracts with higher levels of artocarpin and cysloartocarpin than the other two solvents, only ethyl acetate gave a high amount of artocapanone (Table 4). Therefore, ethyl acetate was considered to be the most suitable solvent for sample preparation of the flavonoid extract from *A. heterophyllus* heartwoods and was used for quantitative HPLC analysis.

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