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Development of a HPLC Method for Quantification of Amentoflavone in Leaf Extracts of Three *Calophyllum* Species

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This study describes the development and validation of a method that can quantify amentoflavone (AF) in methanol extracts of leaves of three Malaysian *Calophyllum* species *via* a high performance liquid chromatography (HPLC) technique. Chromatographic analysis was conducted using a reverse phase C_{18} column with water/acetonitrile (55:45) as the mobile phase with a flow rate of 1.0 mL/min and detection by UV at 270 nm. The method was validated in terms of linearity, precision and accuracy in accordance with International Conference on Harmonization (ICH) guidelines. The calibration curve showed good linearity ($r^2 > 0.9998$) in a concentration range of 2.5 – 100 μg/mL with low detection and quantification limits of 1.33 and 4.02 μg/mL, respectively. The coefficient of variation for intra-day and inter-day precision was less than 2%, while recovery was more than 80%, indicating this method has good precision and accuracy. The developed HPLC method was found to be suitable and reliable for its intended application. Using this method, the amount of AF quantified from *C. incrassatum*, *C. canum* and *C. rubiginosum* leaf extracts was 9.42, 30.39 and 24.23 μg/mL, respectively.

Key words: Amentoflavone; HPLC; quantification; *Calophyllum*; extracts

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Calophyllum, also known in Malaysia as *'bintangor'*, is a pantropical genus that belongs to the family of Guttiferae and is widely distributed in the Indo Pacific region, China and America. This genus comprises approximately 180 –200 species. It is the largest genus in the sub-family Calophylloideae and is identified by opposite leaves with close and regular veins that alternate with latex canals [1]. *Calophyllum* has been commonly employed in traditional medicine to treat ailments such as bronchitis, gastric inflammation, diabetes, hypertension, diarrhea, rheumatism, varicose veins, hemorrhoids, chronic ulcer and to prevent wound infections [2]. *Calophyllum incrassatum* M. R. Henderson & Wyatt-Smith is widely scattered in East Malaysia, Borneo and Sulawesi [3]. *C. incrassatum* can grow up to 36 metres tall, and is commonly found in mixed dipterocarp lowland forests or swampy areas. Its other features include a latex that appears white or pale yellow in colour, the lamina elliptic to suboblong, persistent bracts, smooth surface of barks and a prominent midrib[4].

Calophyllum canum, locally known as '*bintangor merah*', is a large evergreen tree which can grow up to 36 metres tall and is a source of timber [3]. The species is widely distributed in Malaysia, Sumatera and Northwestern Borneo. The distinctive features of this species include the non-buttress trunk, no spurs and a yellowish outer bark with lines of lenticles closed to each other [1]. *C. canum* can be characterized by a plump terminal bud, leaf blades which appear to have undulate margins and brown-vinaceous on the surface, and a midrib that is less prominent and gradually narrowed from the bottom [1]. *Calophyllum rubiginosum* M. R. Henderson & Wyatt-Smith, locally known as '*bintangor daun karat*', is usually widely distributed in the Southern Malay Peninsula, Sumatera and Borneo [3]. This species can grow up to 39 metres tall and is usually found in lowland-colline forest with a minimum altitude of 30 to 500 metres. The species has a non-buttress trunk and small spurs, a brownish outer bark that later changes to grayish and yellowish, white latex, flattened twigs and the lamina appears to be ovate or subovate [1]. The flowers usually have four to eight petals, numerous stamens and ovate anthers. The apex of the fruit is rather sharply pointed and prominently wrinkled on the surface [1].

One of the few interesting bioactive compounds isolated from *Calophyllum* species is amentoflavone (AF) (**Figure 1**). AF is a naturally-occurring biflavonoid, with a molecular formula of $C_{30}H_{18}O_{10}$ and the chemical name 8-[5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2-hydroxyphenyl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one [5]. Previous studies on the isolation of AF from several *Calophyllum* species e.g. *C. inophylloide* [6],

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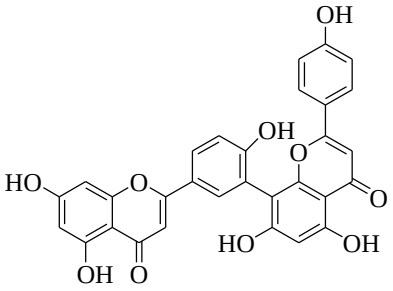

Figure 1. Chemical structure of amentoflavone (AF)

C. calaba [7], *C. brasiliense* [8], *C. venulosum* [9], *C. inophyllum* [10], *C. pinetorum* [11] and *C. flavoramulum* [12] have proposed AF as one of the bioactive chemical markers for the *Calophyllum* species. AF also shows interesting bioactivities such as anti-inflammation [13], antioxidant, antifungal [14], and anti-senescence [15] effects on many important reactions in the cardiovascular and central nervous system [5]. Thus, the *Calophyllum* species has the potential to be developed as a herbal product for alternative medicine.

However, there are impediments to the wide acceptance and utilization of herbal products, especially in terms of the safety and efficacy of these products due to the lack of quality control standards. One of the parameters of quality control standards for herbal products is the standardization of crude extracts. For many herbs, the active constituents are not known, thus the crude extract may be standardized to certain chemical marker compounds to guarantee the herbal product's content and its authenticity [16]. This standardization will lead to consistency in the chemical composition of the herbal product. Therefore, a validated quantification method is crucial and this can be aided by chromatographic techniques such as High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) or Capillary Electrophoresis (CE) [17]. HPLC is widely used in phytochemical and analytical chemistry to identify, quantify and purify the individual components in a mixture [18]. The resolving power of HPLC is ideally suited to the rapid processing of complex samples on both analytical and preparative scales. The presence of AF as the chemical marker in the extract must be quantified for further application as a herbal product. However, to our knowledge there has been no reported validated quantification method for AF from any *Calophyllum* species that can serve as a reference. Thus, this study aims to develop a validated method for the quantification of AF from the leaf extracts of three Malaysian *Calophyllum* species, *C. incrassatum*, *C. canum* and *C. rubiginosum,* by HPLC.

MATERIALS AND METHODS

1. Materials

The leaves of *C. incrassatum*, *C. canum* and *C. rubiginosum* were collected from Hutan Gunung Belumut, Kluang, Johor, Malaysia on October 1, 2014, identified by Dr. Shamsul Khamis and deposited at Herbarium Universiti Putra Malaysia (UPM). The AF standard used in this study was previously isolated from extracts of *C. symingtonianum* [19], *C. ferrugenium* [20] and *C. incrassatum* [4] leaves in pure form. The HPLC-grade solvents used for analysis i.e. acetonitrile, water and methanol were purchased from Qrec.

2. Extraction of *Calophyllum* species

The leaves were air-dried before being ground with a laboratory mill. The dried leaves (100 g) were extracted with analytical grade methanol (Qrec) for 16 hours using the Soxhlet extraction technique. The extracts were filtered to remove plant material with Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator to yield the crude extracts. All crude extracts were kept in vials at 4°C for further use in HPLC analysis. The percentage yield of each extract was reported previously by Ramli *et al.* [21].

3. Preparation of Standard and Sample Solutions

AF standard stock solution was prepared by accurately weighing 10 mg of AF standard which was then dissolved in 10 mL HPLC grade methanol to afford a concentration of 1000 µg/mL. From this stock solution, five working standard solutions with concentration of 100, 50, 25, 5 and 2.5 µg/mL were prepared. Sample solutions of *C. incrassatum*, *C. canum* and *C. rubiginosum* extracts were prepared by dissolving 1 mg of extract in 1 mL of HPLC grade methanol to afford a concentration of 1000 µg/mL.

4. Instrumentation and Chromatographic Separation Parameters

Chromatographic analysis for the detection of AF was performed using an HPLC equipped with Chromera software, LC pump, UV-PDA detector and Brownlee Analytical C_{18} column with particle size 5 µm and dimension of 150 mm × 4.6 mm (Perkin Elmer). Chromatographic conditions were optimized in order to reach baseline separated peaks of the target analytes. For this purpose, a mobile phase of isocratic elution with various ratios of H_2O:ACN (48:52, 55:45, 60;40, 65:35), mobile phase flow rates (0.5, 0.8, 1.0 mL/min), injection volumes (5, 10, 20 µL) and detection wavelengths (254, 260, 270, 280, 330 nm) were optimized. The column conditioning and equilibration were performed in 5 minutes before attaining the initial conditions. The standard and sample solutions were filtered using 0.45 µm polytetrafluoroethylene (PTFE) membrane filters prior to injection.

5. Method Validation

5.1. Linearity test

Five standard concentrations of AF (2.5, 5, 25, 50 and 100 µg/mL) were used in the preparation of a calibration curve. Three replicates (n = 3) at each concentration were analysed using HPLC with the chromatographic conditions mentioned previously. Linear regression analysis was done on the calibration curve to determine its linearity. The significance of linear regression was confirmed using a one-way ANOVA test.

5.2. Sensitivity test

The sensitivity for detection of AF was determined by evaluating the limit of detection (LOD) and limit of quantification (LOQ) of the analyte. According to ICH guidelines, the LOD of the analyte is the lowest amount of analyte in the sample that can be detected, while the LOQ should be at least 10 times the response compared to a blank response (noise) with suitable accuracy and precision. The LOD and LOQ of the analyte were calculated using the following equations:

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where, σ is the standard deviation of the intercept of the calibration plot and S is the average of the slope of the corresponding calibration plot [22].

5.3. Precision

Intra-day (n = 3) and inter-day (n = 3) variation studies were determined by evaluating repeatability and reproducibility over one and three days, respectively. Three standard concentrations of AF (5, 25 and 100 µg/mL) were prepared and analysed in triplicate using the HPLC method as described above. The coefficient of variation (%CV) was calculated for each sample analysed in order to evaluate the closeness of the analyte response obtained from the same measurement procedures and conditions. The %CV was calculated using the following formula:

$$\%CV = (\sigma/\mu) \times 100$$

where, σ is the standard deviation and µ is the sample mean.

5.4. Accuracy

The accuracy of the method was determined by standard addition. 100 µg/mL solutions of spiked sample, unspiked sample and standards were analysed in triplicate (n = 3). The spiked sample was prepared by adding 200 µg/mL of AF standard (500 µL) into 1000 µg/mL extract (500 µL). The mean recovery was calculated using the following formula:

$$\%Recovery = (Spiked\ sample - Unspiked\ sample) / Standard \times 100$$

6. Statistical analysis

All data obtained from triplicate analysis were reported as mean ± standard deviation. Calculation of mean, standard deviation, %CV, linear regression analysis and one-way ANOVA analysis were accomplished by using Microsoft Excel software.

RESULTS AND DISCUSSION

The HPLC method was developed to select the best chromatographic parameters including the ratio and flow rate of the mobile phase, the detection wavelength and the sample injection volume. For this purpose, preliminary trials were performed by varying the conditions of each parameter. Various mobile phase ratios for H_2O:ACN (48:52, 55:45, 60:40 and 65:35) and mobile phase flow rates (0.5, 0.8 and 1.0 mL/min) were tested using the isocratic elution mode. In this study, isocratic elution mode was chosen instead of gradient elution mode in order to develop a simple HPLC method. Different sample injection volumes (5, 10 and 20 µL) and wavelengths for detection (254, 260, 270, 280 and 330 nm) were also tested for optimization. The optimized chromatographic conditions (**Table 1**) were applied for the method validation according to ICH guidelines.

Table 1. Optimized HPLC Parameters.

Parameters	***Value***
Wavelengths	270 nm
Flow rate	1.0 mL/min
Injection volume	10 µL
Mobile phase composition	H_2O:ACN (55:45)

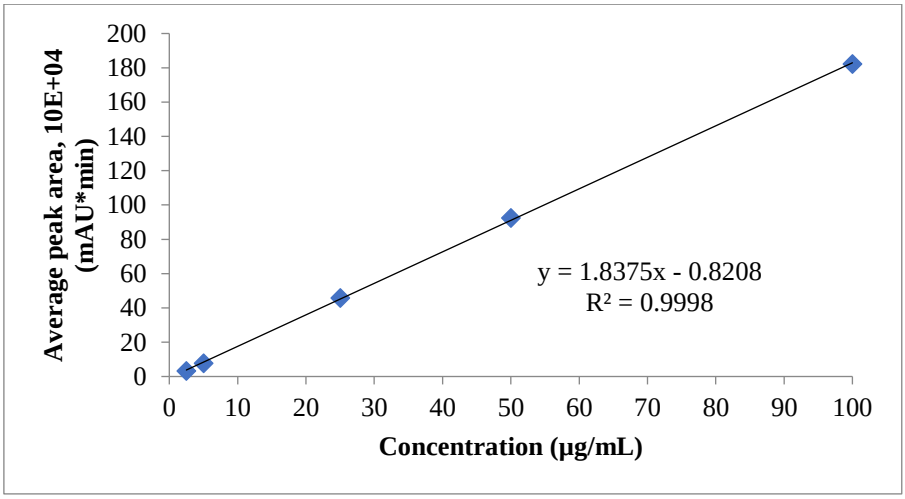

Figure 2. Calibration Curve of AF standard

The calibration curve was constructed using the five standards of AF as described in the previous section against the mean peak areas. A good linear relationship with equation of y = 1.8375x – 0.8208 was established between the peak areas and AF concentrations over the concentration range tested (**Figure 2**). It was confirmed by a regression coefficient value close to 1 (R^2 = 0.9998) indicating a strong positive linear correlation between AF concentration and peak area. One-way ANOVA of triplicate analysis of linear regression also showed that the positive regression model is statistically significant ($p < 0.05$) to predict the response. The LOD and LOQ of AF determined from the standard deviation of the regression line were found to be 1.33 µg/mL and 4.02 µg/mL respectively.

The %CV of intra-day and inter-day precision for AF were both found to be between 0.86 – 1.91 %. In both cases, the values of %CV were found to be within acceptable limits according to ICH specifications, i.e. less than 2 % [23], as shown in **Table 2**. Therefore, the method is demonstrated to be precise and reproducible with low variability in the results. The accuracy of the method also was determined using triplicate analysis of the standard addition method. According to ICH guidelines, accuracy is the closeness of agreement between the known concentration of the analyte and the values obtained. The average percentage recovery obtained for all *Calophyllum* extracts are tabulated in **Table 3**. The average percentage recovery obtained for *C. incrassatum*, *C. canum* and *C. rubiginosum* were 84.33 %, 91.62 % and 86.60 %, respectively. According to the ICH guidelines [23], recoveries should be higher than 80.00 %, therefore, these results were acceptable.

Table 2. Precision results for determination of AF.

Concentration (µg/mL)	**Intraday (n = 3)**			**Interday (n = 3)**		
	R_t ± SD (min)	**MC ± SD (µg/mL)**	**%CV**	**R_t ± SD (min)**	**MC ± SD (µg/mL)**	**%CV**
5	2.78 ± 0.02	4.66 ± 0.05	1.08	2.77 ± 0.02	4.69 ± 0.09	1.91
25	2.80 ± 0.01	25.56 ± 0.22	0.86	2.80 ± 0.01	24.82 ± 0.37	1.51
100	2.84 ± 0.02	100.53 ± 1.29	1.28	2.84 ± 0.02	100.23 ± 0.93	0.93

R_t: Retention time; MC: Mean concentration; SD: Standard deviation; %CV: coefficient of variation percentage

Table 3. Recovery for determination of AF in *Calophyllum* leaves extracts.

Calophyllum species	Nominal Concentration of AF in Extract (µg/mL)	Recovery ± SD (%)
C. incrassatum	100	84.33 ± 2.57
C. canum	100	91.62 ± 3.75
C. rubiginosum	100	86.60 ± 2.59

SD: Standard deviation

Quantification of a single compound from a plant extract is challenging due to the complex composition of phytochemical compounds in the extract. A chromatographic investigation of AF was done by comparing the UV spectra of the corresponding peaks in the *Calophyllum* extracts with those of the AF standard (**Figure 3**). The retention times and amount of AF in each extract quantified through triplicate analysis are shown in **Table 4**. The peaks of AF were detected at a retention time of 2.67 – 2.64 min, thus indicating this method is fast. Among the three *Calophyllum* species, AF was highest in *C. canum* followed by *C. rubiginosum* and *C. incrassatum*. It suggests *C. canum* and *C. rubiginosum* as reliable plant sources to isolate AF for further development studies.

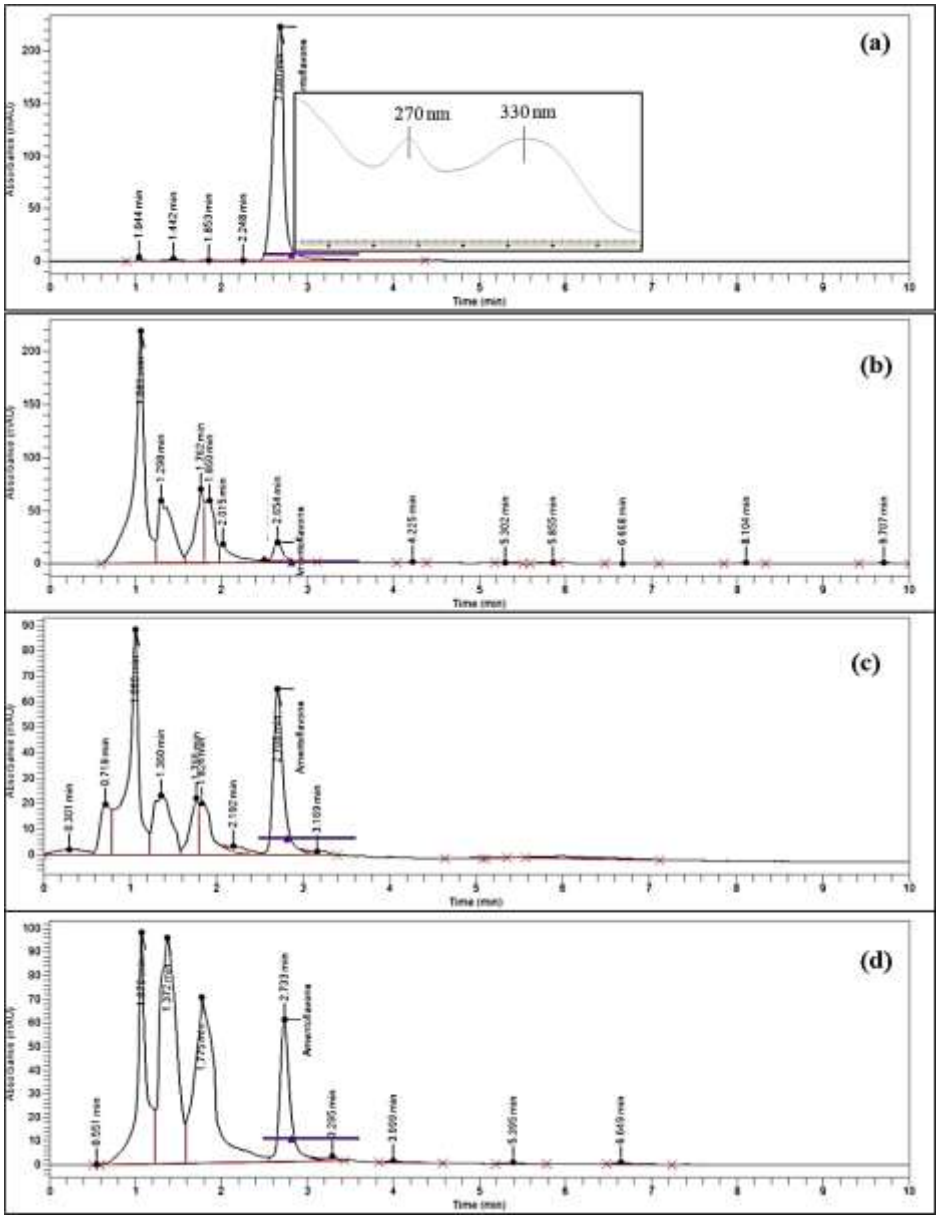

Figure 3. Chromatograms of (a) AF standard; (b) *C. incrassatum* extract; (c) *C. canum* extract; (d) *C. rubiginosum* extract

Table 4. Quantification of AF in the *Calophyllum* extracts.

***Calophyllum* species**	**MC of AF ± SD (µg/mL)**	**R_t ± SD (min)**
C. incrassatum	9.42 ± 0.08	2.67 ± 0.01
C. canum	30.39 ± 3.69	2.73 ± 0.01
C. rubiginosum	24.23 ± 0.23	2.74 ± 0.01

R_t: Retention time; MC: Mean concentration; SD: Standard deviation

CONCLUSION

The proposed HPLC method was successfully developed and validated according to ICH guidelines and was found to fulfill the requirements and exhibit acceptable recovery values. The method is simple, fast, precise and accurate for the quantification of AF in leaf extracts of *Calophyllum* species. This method can be used in the future to determine the AF content in a herbal product or standardized *Calophyllum* extract for quality control purposes.

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