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QUANTIFICATION OF CATECHIN IN LEAVES AND STEMS OF MALAYSIAN *Uncaria gambir* (HUNTER) ROXB. BY HPLC-DAD

(Kuantifikasi Catechin dalam Daun dan Batang *Uncaria gambir* (Hunter) Roxb. dari Malaysia Menggunakan KCPT-PSD)

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Abstract

Recently, we reported the isolation of a novel flavonoid named uncariechin along with epicatechin and epiafzelechin from the leaf extract of *Uncaria longiflora* variety *pteropoda* (Miq.) Ridsd. of the family *Rubiaceae*. Continuing our investigation on the *Uncaria* genus, the identification and quantification of its phytoconstituents was carried out. The species of particular interest is the Malaysian *Uncaria gambir*. This species is distributed mainly in Malaysia and Indonesia and has been cultivated for the flavonoid catechin in Indonesia. Hence, the objective of this study is to determine the quantity of catechin in hexane (Hx), dichloromethane (DCM) and methanol (MeOH) extract in both stem and leaf parts of the plant via HPLC-DAD. Finding result indicates that catechin is present in higher amounts in the MeOH extract [8.64 % (leaves); 5.12 % (stems)] compared to the DCM extract [0.77 % (leaves); 0.92 % (stems)] with no catechin found in the hexane extract. This is the first report of the quantification of catechin from Malaysian *U. gambir* using HPLC-DAD. The method can be used for the quantification of flavonoids from other *Uncaria* and related genus and is useful for targeted isolation of interest flavonoids.

Keywords: *Uncaria gambir*, catechin, flavonoid, high performance liquid chromatography-diode array detecto

Abstrak

Kami telah melaporkan pengasingan satu flavonoid novel iaitu uncariechin bersama-sama dengan epicatechin dan epiafzelechin daripada ekstrak daun *Uncaria longiflora* var. *pteropoda* (Miq.) Ridsd. yang tergolong dalam famili *Rubiaceae.* Lanjutan terhadap penyelidikan dalam genus ini, pengenalan identiti terhadap sebatian serta kuantitinya dijalankan, Ia memberi pengkhususan terhadap spesies *Uncaria gambir* dari Malaysia. Spesies ini banyak dijumpai di Malaysia dan Indonesia dan telah ditanam di Indonesia untuk flavonoid catechin. Maka, objektif penyelidikan ini adalah penentuan kuantiti catechin dalam ekstrak heksana (Hx), diklorometana (DCM) dan metanol (MeOH) dari batang dan juga daun *Uncaria gambir* dengan menggunakan KCPT-PSD. Hasil penyelidikan mendapati bahawa catechin banyak dijumpai di dalam ekstrak metanol [8.64 % (daun); 5.12 % (batang)] berbanding di dalam ekstrak diklorometana [0.77 % (daun); 0.92 % (batang)] tetapi tidak dikesan dalam ekstrak heksana. Ini merupakan laporan pertama bagi kuantifikasi catechin daripada *U. gambir* tempatan melalui KCPT-PSD. Kaedah ini boleh digunakan untuk kuantifikasi flavonoid dari spesies *Uncaria* lain dan genus berkaitan, malah berguna untuk pengasingan flavonoid sasaran.

Kata kunci: *Uncaria gambir*, catechin, flavonoid, kromatografi cecair prestasi tinggi – pengesan susunan diod

Introduction

Uncaria genus comes from family of *Rubiaceae* and has been known for their medicinal and therapeutic properties. This genus represents 34 species which is widely distributed in tropical regions and about 14 species are available in Malaysia [1]. The most popular among local *Uncaria* species is *Uncaria gambir*. Malaysian *Uncaria* species have been reported to have antioxidant properties due to their flavonoids [2]. Catechin is a flavonoid frequently used as a chemical marker in the analysis of flavonoids in *Uncaria*. The choice of catechin as a marker flavonoid is due to its abundancy in *Uncaria gambir* [3]. Previous study on the quantification of flavonoids from crude the extract sample showed five flavonoids are present in *Uncaria gambir* which are (-)-epigallocatechin, (-)-gallocatechin, (+)-catechin hydrate, (-)-epicatechin and (-)-epicatechin gallate [4]. Quantification of catechin on *Uncaria gambir* especially Malaysian's *Uncaria gambir* has not been reported. There has been some report on quantification of catechin in the leaves of *Uncaria gambir* collected from West Sumatera but no report has been found on the stems and leaves of Malaysian *Uncaria gambir*.

Thus, the objectives of this study are to obtain HPLC profiles and to quantify the amount of catechin in hexane, dichloromethane and methanol extracts of leaves and stems of *Uncaria gambir*. This study provides beneficial information regarding catechin content in the leaves and stems of Malaysian *Uncaria gambir* and knowledge on quantification of flavonoids by High Performance Liquid Chromatography (HPLC).

Materials and Methods

Chemicals and raw materials

The reference compound, catechin was purchased from Sigma-Aldrich (India). The purity of the reference compound was over 95 %. HPLC-grade acetonitrile was purchased from RCL Labscan Limited (Bangkok, Thailand). HPLC-grade methanol and formic acid 98 % pure were purchased from Merck (Darmstadt, Germany). HPLC-grade hexane and HPLC-grade dichloromethane were purchased from Merck (USA) and Fisher Scientific (UK). Ultra-pure water, 18 MΩ-cm resistances, filtered through a 0.2 µm filter by Thermo Scientific (referred to here as deionized water). Trifluoroacetic acid (TFA), 99 % pure was purchased from Merck (Hohenbrunn, Germany).

Uncaria gambir was collected from Bukit Diman, Ajil, Terengganu, Malaysia. Voucher specimens (HTBP 4320) were deposited at the Herbarium Taman Botani, Putrajaya, Malaysia.

Preparation of reference and sample solutions for quantification and identification

Standard catechin was accurately weighed and dissolved in 0.05 % formic acid in 70 % methanol to make a stock solution with concentration of 1.0 mg/ml. Appropriate volumes of the stock standard was added into the appropriate volume of 0.05 % formic acid in 70 % MeOH to prepare a mix working standard. The mixed standard was stored at 4 ^{o}C in the dark.

For sample preparation, dried leaves and stems of *U. gambir* were ground into fine powder. The finely ground plants materials were accurately weighed and macerated individually with MeOH for 72 hours at room temperature. The solvent was evaporated off under reduced pressure. A 10 g of crude methanolic *Uncaria gambir* extract (stems and leaves) was dissolved in H_2O: MeOH (1:1) and was put in a separatory funnel. The mixture was then subjected to liquid – liquid partitioning using Hx and DCM to yield Hx, DCM and MeOH extracts after solvent evaporation. The weight of the extracts was recorded. The extracts were stored at 4 ^{o}C until they were subjected to HPLC analysis.

For quantification, an amount 1.0 mg of each extracts was accurately weighed and dissolved in 1.0 ml of 0.05 % formic acid in 70 % MeOH. The solutions were then filtered through a 0.2 µm membrane filter before being injected into HPLC for analysis.

HPLC-DAD quantitative analysis

HPLC quantitative analysis was performed on an Agilent series 1200 LC system (Agilent Technologies, USA) equipped with a quaternary solvent delivery system, an on-line degasser, a diode-array detector (DAD) system performing the wavelength scanning from 190 to 400 nm, a injector with 20 µL sample loop and a column compartment. Separation was carried out using a Zorbax Eclipse XDB-C18 column (250 mm x 4.6 mm, i.d. 5 µm), from Agilent Technologies (USA). Column temperature was maintained at 25 ^{o}C. Elution was conducted using mobile phase A (0.1 % TFA in 5 % acetonitrile) and mobile phase B (0.1 % TFA in acetonitrile) with a gradient elution program at a flow rate of 0.8 mL/min. The following gradient was applied: 0-10 min 88 – 82 % A, 10-15 min isocratic gradient 82 % A, 15 – 30 min 50 % A. The UV absorbance was monitored at wavelength of 280 nm. The solvents for the mobile phases were filtered through a 0.45 µm membrane filter. 10 µL of each sample and standard was injected manually to the HPLC system. The data were processed with Agilent ChemStation software (Agilent Technologies, USA).

Method validation

Linearity was measured at six concentration levels for each standard. Each solution was tested in triplicates. Calibration curves were constructed by plotting the peak area versus concentration of each standard. Acceptability of linearity data was determined by the correlation coefficient, r^2. Limits of detection (LOD) and quantification (LOQ) for each standard were calculated based on linear regression method. Accuracy was determined by analyzing the percentage recovery for the catechin flavonoid in the *U. gambir* leaf extract by spiking the raw material with reference standards at three levels. Three replicates were performed for each analysis. Variations of all the tests were expressed in terms of relative standard deviation (% RSD).

Results and Discussion

Validation of HPLC method

The HPLC method for quantification was validated and the results are summarized in Tables 1 – 2, respectively. As seen in Table 1, the calibration curve demonstrated good linearity with $r^2 \geq 0.981$. The LODs and the LOQs were found to be 2 mg/L and 6 mg/L, respectively, showing good sensitivity of the method. The recovery test represented in Table 2 ranged from 80.1 – 88.1 %, showed good accuracy of the method.

Table 1. Figure of merits of catechin

Analyte	Concentration range (mg/L)	Regression equation	Correlation coefficient (r^2)	Limit of detection (mg/L)	Limit of quantification (mg/L)
Catechin	2 – 100	$y = 8.7935x + 22.615$	0.981	2	6

Table 2. Recovery of catechin

Analyte	Concentration level (mg/L)	Percent recovery (%)
Catechin	25	88.10 ± 1.13
	50	80.10 ± 0.71
	75	82.30 ± 0.71

Quantification of flavonoids

Catechin was successfully determined and quantified from the hexane, dichloromethane and methanol leaf and stem extracts of Malaysian *U. gambir* using the developed HPLC-DAD method. The chromatogram was shown in Figure 1 and Figure 2, respectively. The peak from the extracts was determined by comparison of the UV-vis spectra and the retention times with the authentic standard. The purity of the peak was ascertained by the singularity of the peak component.

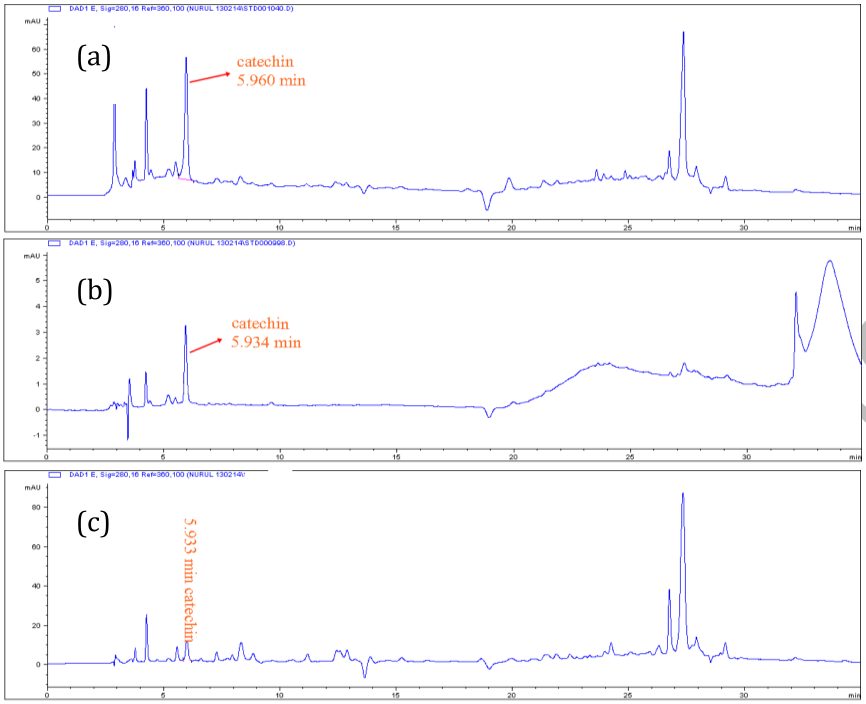

Figure 1. HPLC chromatograms *of U. gambir* (a) MeOH extract (b) Hx extract and (c) DCM extract of the leaves

Quantification of catechin was performed using the external standard method. The relationship between the amount of standard solution (concentration) and detector responses (peak area) were plotted to construct the calibration curve. The data was fitted by linear least squares to determine response equation.

Recently, a study done by Amir et al. [5] on *U. gambir* collected in Indonesia found that the estimated total flavonoid content of this plant using the aluminium chloride colorimetric method to be 5.82 ± 2.23 mg rutin equivalents (RE)/g dry weight. In addition, the catechin content of four sub-species of Indonesian *U. gambir* known as gambir cubadak, gambir udang, gambir riau mancik and gambir riau gadang had been earlier determined by HPLC [6]. They had found that the catechin content of the respective sub-species was 104.5, 101.2, 99.4 and 108.5, 104.5 µg/ml, respectively. However, the initial concentration of the extract was not specified. Hence, the content of catechin in µg/mg could not be determined for comparison.

As far as our knowledge, the quantification of the catechin in *Uncaria gambir* from the Malaysian population has not yet been determined by HPLC methods. The contents of catechin obtained in each leaf and stem extracts are shown in Table 3 and Table 4, respectively. Catechin was present in higher amounts in the MeOH extract compared to Hx and DCM extracts. It was suggested that polar compounds in plant matrix would be easier to extract with more polar solvent such as MeOH. The amount of catechin present in MeOH leaf extract was 86.35 mg/g while in MeOH stem extract was 51.20 mg/g. The DCM extract showed that reasonable amounts of catechin can be extracted from the leaves and stems with 7.71 mg/g and 9.19 mg/g respectively. This is because DCM is a moderately polar compound, thus it has the ability of 'like dissolve like' between the polar compounds. The Hx extract indicated that the amount of catechin cannot be quantified since the peak area was below limit of quantification. Furthermore, by comparing stems and leaves of *Uncaria gambir*, the amount of catechin present in leaf extracts are higher than in the stem extracts with an average amount of 94.06 g/kg and 60.39 g/kg plant material correlating to 9.406 % and 6.039 %, respectively.

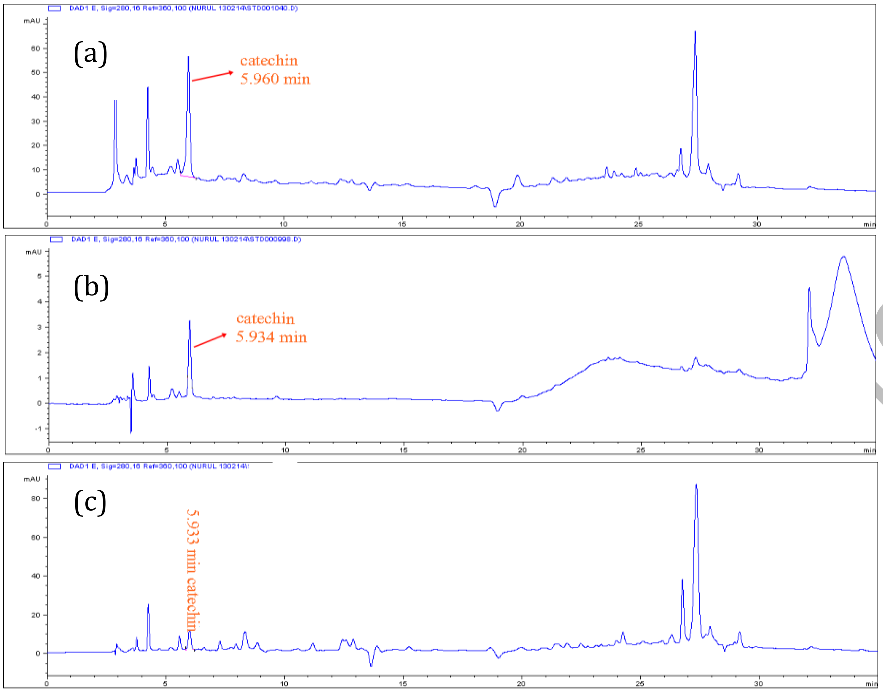

Figure 2. HPLC chromatograms *of U. gambir* of (a) MeOH extract, (b) Hx extract and (c) DCM extract (C) of the stems

Table 3. Contents of catechin in leaf extract of *U. gambir* (mg/g)

Sample	**Retention times (min)**	**Area (mAU* s)**	**Amount of catechin (mg/g)**	**Percentage of catechin (%)**
MeOH extract	6.027 ± 0.020	782.3 ± 3.5	86.35 ± 0.35	8.635
DCM extract	5.942 ± 0.070	90.4 ± 1.4	7.71 ± 0.16	0.771
Hx extract	5.913 ± 0.050	11.0 ± 0.8	N/A	N/A

± is standard deviation value, N/A is non-applicable (below the limit of quantification)

Table 4. Contents of catechin in stem extract of *U. gambir* (mg/g)

Sample	**Retention times (min)**	**Area (mAU* s)**	**Amount of catechin (mg/g)**	**Percentage of catechin (%)**
MeOH extract	5.901 ± 0.0800	473.05 ± 1.06	51.20 ± 0.14	5.120
DCM extract	5.918 ± 0.110	103.40 ± 1.06	9.19 ± 0.12	0.919
Hx extract	5.920 ± 0.020	22.60 ± 0.49	N/A	N/A

± is standard deviation value, N/A is non-applicable (below the limit of quantification)

Conclusion

A HPLC-DAD method was successfully developed and validated for the analysis of catechin in Malaysian *U. gambir*. The developed LC method is proven to be an efficient, selective and rapid analytical method for the quantification of catechin in the hexane, dicloromethane and methanol leaf and stem extracts of the plant. Catechin can be found in each of the three extract in different amounts. This is the first report on the quantification of flavonoids from Malaysian *U. gambir*. The method can be used for the quantification of flavonoids from other *Uncaria* and related genus and useful for targeted isolation of interest flavonoids.

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