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HPLC-DAD-MS method for Simultaneous quantitation of flavonoids in *Hypericum formosanum* and antiglycation activity

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Abstract
Hypericum formosanum (HF) is widely used as traditional and folklore medicine in Taiwan. In the present study, ultrahigh performance liquid chromatography equipped with photodiode array detection-mass (UPLC–DAD-MS) method was developed for the separation, identification, and quantification of bioactive flavonoids. The developed method was also validated for accuracy, precision, limit of detection and quantification (LOD and LOQ). In this method, four flavonoids, viz., hyperoside, astiblin, quercitrin, and quercetin, were quantified in linearity range of 10–200 (µg/mL) with correlation coefficient of greater than 0.997. A high recovery (87.55–92.11%) and good reproducibility was obtained for four flavonoids with the relative standard deviation (RSD) ranging from 1.75-2.26 % (intra-day) and 1.99-2.27% (inter-day). Hence, the proposed method for simultaneous quantification of four bioactive flavonoids in the extract and fractions of *H. formosanum* using UPLC–DAD-MS detection under the specified conditions is accurate and validated. Besides that, ethyl acetate (EtOAc) fraction showed the best anti-glycation and its IC_{50} was observed at 69.5µg/mL.

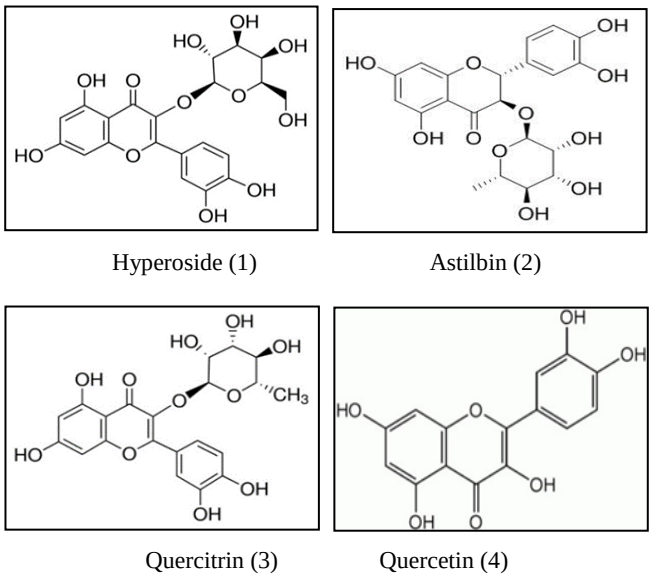
Keywords: UPLC, *Hypericum formosanum*, anti-glycation, flavonoids

Introduction
The Hypericum genus of plants is widely distributed throughout Europe, Asia, and North America, and some of them are well known as medicinal herbs. As reported previously Hypericum genus mainly contains xanthones, chromenes, flavonoids, dipeptide derivatives and phloroglucinol derivatives [1-4]. Flavonoids are a group of polyphenols abundant in Hypericum species. Many studies have reported on the wide range of pharmacological activities that flavonoids exhibit, including anti-oxidant, anti-bacterial, anti-inflammatory, anti-diabetic, and anti-glycation activities [5-9]. *Hypericum formosanum* (HF) is native to Taiwan where it is regarded as a valuable folk herb. However, there have been few reports published regarding its bioactivity and phytochemistry. Proteins can be modified by reducing sugars through the glycation reaction, resulting in the formation of advanced glycation end-products (AGEs). The relationship between glycation and Alzheimer's disease and diabetes has been demonstrated [10]. It has been reported that flavonoids have inhibitory effects on the glycation processes [11-12].
LC–MS has been becoming a powerful analytical technique for qualitative and quantitative analysis of natural products [13-14]. Although few studies have demonstrated the chemical analysis of Hypericum spp.by HPLC, but until now, to the best of our knowledge, no data have been reported on the simultaneous determination of these flavonoids in HF. Thus, for the purpose of developing an efficient and validated method for analysis flavonoids in HF is necessary. In this study, a simple and efficient UPLC-DAD-MS method is proposed for the quantification of the major flavonoids in extract and fractions of HF.

Materials and Methods
Reagents
HPLC-grade methanol, acetonitrile water, and chemicals were purchased from Merck, Mumbai, India. Reference standards of hyperoside (≥97%), astilbin (≥99%), quercitrin (≥97%), and quercetin (≥97%) were purchased from Sigma-Aldrich (St Louis, MO., USA). The chemical structures of the four constituents were listed in Fig. 1.

Plant Material and Preparation of Extract and Fractions
The tissues of *H. formosanum* including the leaves, stem, and root were harvested (Chiayi, Taiwan). The plant material was identified by Dr. Chang, San-xian, the botanist of the Hualien

District Agricultural Research and Extension Station Council of Agriculture. The plant materials were washed with water and air-dried in the room temperature for 5 days. The collected sample was then oven-dried at 55 °C for 2 days and ground to a fine powder by an electronic blender. The dried *H. formosanum* (15.8 kg) was mechanically ground to a fine powder and then sieved through a 10 mesh sieve. The obtained powder was extracted with methanol (3 × 120 L) at room temperature. The combined methanol (MeOH) extracts (644 g) were successively partitioned between ethyl acetate (EtOAc, 38.2 g) and H_2O. The latter fraction was repartitioned between n-butanol (n-BuOH, 68.1g) and H_2O (506.8 g).



Hyperoside (1) Astilbin (2)

Quercitrin (3) Quercetin (4)

Fig 1: Structures of the four flavonoids in *Hypericum formosanum*.

UPLC-DAD-MS analysis

The fine powders of four samples were accurately weighed (0.1 g) and placed in100 mL volumetric flasks. They were extracted with 100mL of methanol in an ultrasonic bath for 30 min at room temperature. Additional methanol was added to make up to the volume of 10 ml. The supernatant was filtered through a 0.22 μm micropore membrane prior to analysis.

The samples, dissolved in methanol, was separated on RP-C18 column (XBridge2.5μm, C18, 100×2.1mm)by UPLC equipped with Waters Acquity system consisting of a column oven, sample manager, solvent manager and DAD detector(Waters Corp., Milford, MA, USA).The flow rate was 0.4 mL/min. The solvent gradient for UPLC was a mixture of 0.1% formic acid/H_2O (solvent A) and 0.1% formic acid/*acetonitrile* (solvent B): 85% A from 0 to3 min, 65-45% A during 3 to 5min, 45-0%A during 5 to 8 min. The column temperature was fixed at 40°C. The 270 nm wavelengths were employed to quantify the flavonoids. Identification of the constituents was carried out with a Waters Acquity QDa mass with an Electro Spray Ionization (ESI) source and Empower software was used for data analysis. Negative ion mass spectra were recorded in the range *m/z*200–650. Probe temperature was kept at 600°C and Cone Voltage 30V.

Method validation

The method was validated for linearity, accurate, precision, limits of detection(LOD) and quantification (LOQ).Standards at the concentration range of 10-200 (μg / mL) were prepared. Solutions containing four standards at five different concentrations were injected in triplicate. Linear regression equations were constructed by establishing calibration graph with the peak area (y), concentration (x, μg / mL). The mixed standards solution was further diluted to a certain concentration to explore the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ were determined at a signal-to-noise ratio of 3 and 10, respectively. The intra- and inter-day precisions were determined by continuously injecting the sample solution for three replicates on the same day and by measuring it once a day for three consecutive days, respectively. The recovery test for reflecting accuracy was conducted by the standard addition approach. The recovery yield was carried out according to the following formula: recovery yield (%) = [(amount detected − original amount)/amount spiked] × 100%, and RSD (%) = (SD/mean) × 100%. The repeatability was estimated on the ground of relative standard deviation (RSD).

Analysis of flavonoids ingredient in HF

The extract and different fractions of HF were analyzed by UPLC-DAD-MS. The identification of flavonoids in HF was completed by comparing the retention time and MS spectra with standards. The peak area of each component in the extract and fractions of HF was acquired from its chromatogram and the abundance of each compound was calculated from its corresponding calibration curve. Experiments were conducted in triplicate and the resulting data were expressed as the mean ± SE and the unit was represented as mg/g.

***In vitro* anti-glycation assay**

AGEs were produced *in vitro* using a method described previously [15]. In brief, BSA (20 mg/ml) in phosphate buffered-saline solution(PBS, pH 7.4) containing 0.02% sodium azide was incubated with glucose (500 mM) at 37°C for 0, 7, 14, 21, and 28 days in the absence (control) and presence of each of the extracts (100, 200, and 400 μg/ml) or AG (200 μg/ml). Each solution was kept in the dark in a capped vial, and incubation was allowed to proceed in triplicated vials. For time course experiments involving fluorescent AGE formation, we measured the fluorescence (370-nm excitation wavelength and 440-nm emission wavelength) using an F-2500 Spectrofluorometer (Hitachi, Tokyo, Japan). The percentage inhibition was calculated using the following formula:

% inhibition = 1-[(fluorescence of test sample)/ (fluorescence of control)]× 100.

The effective concentration for 50% inhibition (IC_{50}) was obtained by interpolation of the linear regression analysis. All determinations were performed in triplicate, and the results were averaged and compared using the Duncan's multiple range test ($P < 0.05$).

Results and Discussion

Optimization of UPLC-DAD-MS conditions

To achieve good resolution with short analysis time, the mobile phase was optimized through comparison s of different optimize UPLC conditions, preliminary experiments were conducted to test the chromatographic column. The Acquity UPLC XBridge column (2.5μm, C18, 100×2.1mm) was selected for UPLC-DAD-MS analysis based on its good separation ability and short analytical time. Under these optimized MS conditions, the flavonoids exhibit stronger signal responses in negative ion mode than positive ion mode. To obtain higher sensitivity, the cone voltage was optimized to generate protonated molecules. The Fig 2 showed the total ion chromatograms in negative mode of four flavonoids and

possessing their own characteristic fragmentation patterns. Table I lists the retention time and MS data for the standard flavonoids. The results showed under specified conditions were applicable for separation and identification of the mixture of hyperoside, astilbin, quercitrin, and quercetin.

Table 1: Peak assignments of four standard flavonoids.

Peak number	Retention time (tR)	[M-H] (m/z)	Identification
1	3.3	463	Hyperoside
2	3.7	449	Astibin
3	4.1	447	Quercitrin
4	5.3	301	Quercetin

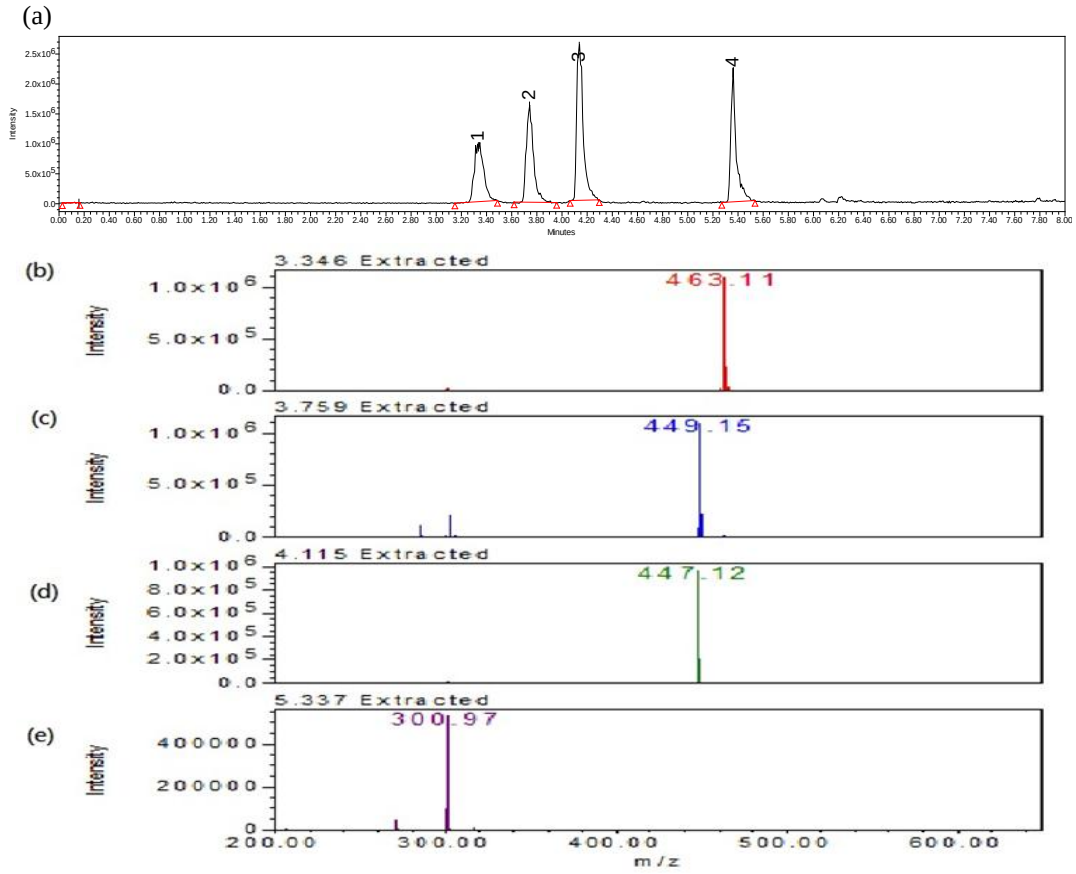


Fig 2: The MS spectra of four standard flavonoids (ESI/MS). (a) Total ion chromatograms.1 = Hyperoside; 2 = Astilbin; 3=Quercitrin; 4= Quercetin. (b) MS spectrum of Hyperoside, (c) MS spectrum of Astibin, (d) MS spectrum of Quercitrin, (e) MS spectrum of Quercetin.

Method validation of quantitation four flavonoids by UPLC-DAD

Four compounds showed excellent linearity with r^2 >0.997and results are shown in Table II. The limits of detection (LOD) of four compounds were 1.1, 0.9, 0.7, and 0.5µg/ml for hyperoside, astilbin, quercitrin, and quercetin based on a signal-to-noise ratio of 3:1, respectively. Precision was acceptable that RSD values ranged between 1.75 % and 2.26 % and between 1.99 % and 2.27 % for intra and inter-day variation, respectively. The results are displayed in Table III. Recovery experiments were performed at three concentration levels by adding an appropriate amount of standards solution to the blank samples. Recovery values of the four constituents added were in the acceptable range, hence the validated method was accurate (Table III).

Table 2: Linear regression equations, coefficients, linear range, limit of detection and limit of limit of quantification of four standard flavonoids.

Compound	Regression equation	R^2	Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Hyperoside	y = 5431.1x -10341	0.998	10-200	1.1	3.3
Astibin	y = 4384.6x – 4307.3	0.999	10-200	0.9	2.7
Quercitrin	y = 10181x - 32961	0.997	10-200	0.7	2.1
Quercetin	y = 8237x –8773.2	0.999	10-200	0.5	1.5

Table 3: Precisions and recovery yields of intra-day and inter-day experiments of the four flavonoids.

Compound	Precision (%) RSD		Recovery (%)	
	Intra-day (n=3)	Inter-day (n=3)	Average	RSD, %
Hyperoside	1.95	2.27	91.77	2.71
Astibin	1.99	2.12	87.55	2.55
Quercitrin	2.26	1.99	90.33	2.11
Quercetin	1.75	2.01	92.11	2.03

Sample analysis

This validated method was successfully applied to the quality control of HF extract and fractions, which provided particularly important information for production and application. The UPLC-DAD chromatograms of the standard solution, HF extract and fractions are shown in Fig. 3. The described UPLC-DAD-MS method was applied to analysis and quality evaluation of extract and fractions of *H. formosanum* through simultaneous determination of four

marker compounds. The quantitative analytical results (Table IV) indicated their contents distributed in these samples. The contents of hyperoside, astilbin, quercitrin, and quercetin in the extract and fractions were in the range of 0.82-66.09 mg/g. In this results, quercitrin with the highest abundance (66.09 mg/g) among four flavonoid compounds were simultaneously obtained. The content of bioactive markers was affected by different solvent and extraction condition. To ensure the stability, safety and efficacy for clinical use, guidelines and quality control for *H. formosanum* preparations should be standardized in the future.

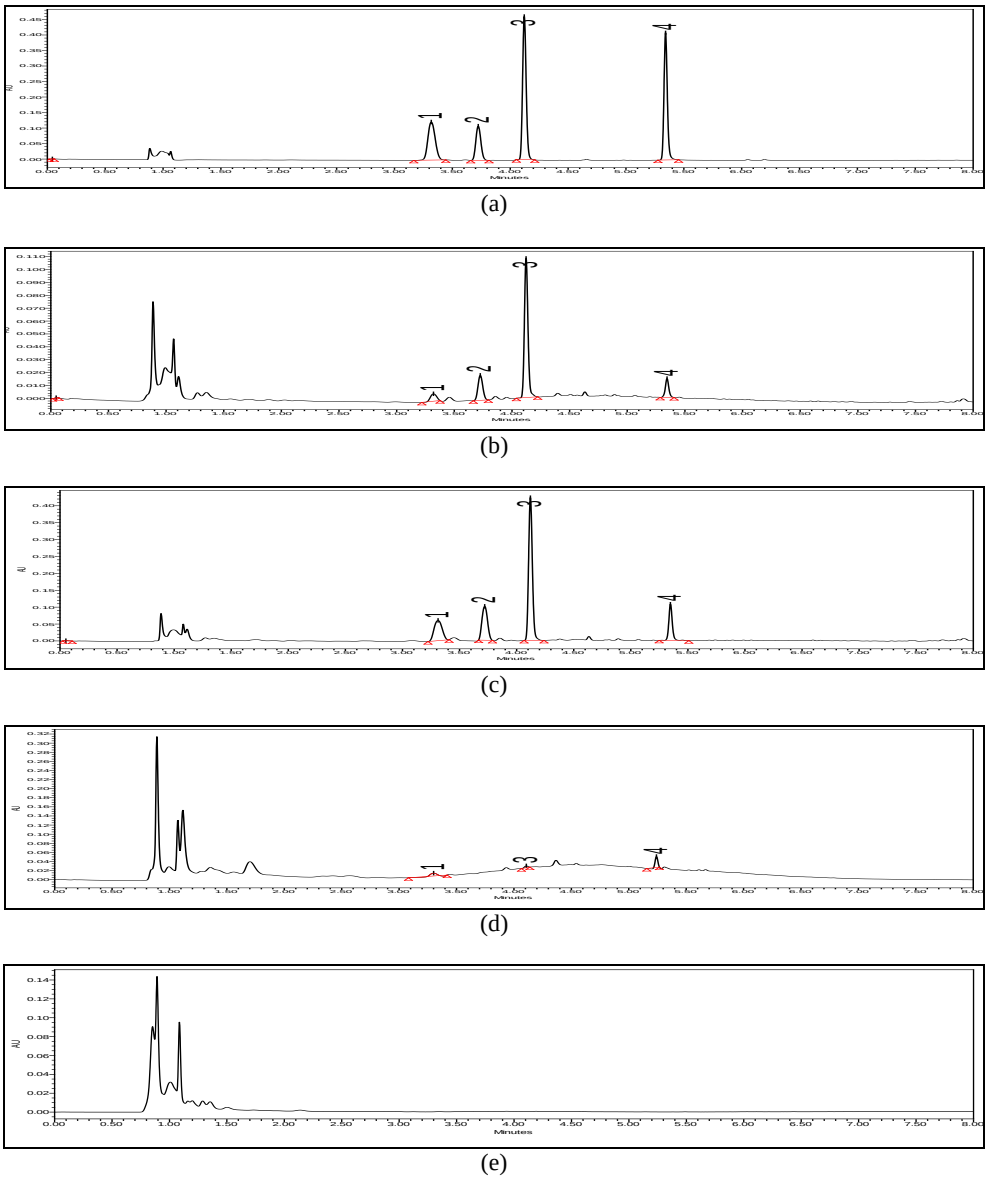


Fig 3: UPLC-DAD chromatograms obtained at 270 nm from (a) standard mixture, (b) methanol extract of HF, (c) EtOAc fraction , (d) BuOH fraction and (e) H_2O fraction. 1 = Hyperoside; 2 = Astilbin; 3=Quercitrin; 4= Quercetin.

Table 4: Contents of the four flavonoids in extract and fractions from *Hypericum formosanum*

Compound	[a]MeOH(mg/g)	[a]EtOAc(mg/g)	[a]BuOH(mg/g)	[a] H_2O (mg/g)
Hyperoside	2.27± 0.05	36.42± 0.05	1.13± 0.05	[b]ND
Astibin	4.33± 0.05	53.83± 0.05	ND	ND
Quercitrin	8.56± 0.05	66.09± 0.05	0.82± 0.19	ND
Quercetin	1.53± 0.05	18.54± 0.05	1.26± 0.75	ND

[a] Data represented as mean (mg/g) ± SD, n = 3.
[b] ND = not detected

Anti-glycation activity

Antiglycation activity of the MeOH extract and different solvent soluble fractions of *H. formosanum* was presented in the Table V. The MeOH extract and EtOAc fraction exhibited strong AGEs formation inhibitory activity with the IC_{50} value of 93.1 and 69.5 µg/ml compared to the positive control aminoguanidine with the IC_{50} value of 190.2µg/ml. On the other hand, n-BuOH and H_2Ofractions did not exhibit anti-glycation activity within the test concentrations. A previous study revealed that phenolic and flavonoid compounds could affect the glycation process [16]. In addition, a previous study illustrated that flavonoids might serve as one of the potent AGE inhibitors for the prevention of ageing and diabetes. In the present study, the MeOH extract and EtOAc fraction of *H. formosanum* demonstrated stronger anti-glycation activity which might be attributed to the abundant flavonoids

(hyperoside, astilbin, quercitrin, and quercetin). In the future, *H. formosanum* could be used as nutraceutical in the treatment of diabetes.

Table 5: Anti-glycation activities of Aminoguanidine and extract and fractions from *Hypericum formosanum.*

Samples	IC_{50} (µg/mL)
MeOH	93.1 ± 0.2
EtOAc	69.5 ± 0.2
BuOH	>400
H_2O	>400
Aminoguanidine	190.2 ± 0.2

IC_{50} values represent the mean ± SD (n = 3).

Conclusion

We have successfully developed a rapid and accurate UPLC-DAD-MS method to determine the four major flavonoids in *H. formosanum*. Based on validation results, the developed method can be useful for detection of flavonoids (hyperoside, astilbin, quercitrin, and quercetin) under the specified conditions. The EtOAc fraction of *H. formosanum* showed stronger anti-glycation activity might be attributed to these abundant flavonoids. Hence, this method can be applied for quantification of these marker compounds in the *Hypericum spp.* and marketed formulations.

Acknowledgments

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