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RP-HPLC Analysis of Quercetin in the Extract of Sambong (*Blumea balsamifera* (L) DC) Leaves

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ABSTRACT

Blumea balsamifera (L) DC, known in the Philippines as *sambong*, is an herb valued for its health benefits especially in the management of urolithiasis. Various phytochemicals, including flavonoids such as quercetin, have been determined in sambong leaves. A reversed-phase high-performance liquid chromatographic method (RP-HPLC) was developed for the quantitative determination of quercetin in the methanol extract of sambong leaves obtained from Leyte, Cotabato, and Nueva Ecija, Philippines. The methanol extracts of sambong were prepared by maceration followed by rotary evaporation. The solid phase extraction (SPE) for the sample cleanup involved the use of a C18 SPE packing, a 0.5-mL sample load (50 mg/mL solution), and elution with 4-mL of 80:20 Methanol:0.5% H_3PO_4. The HPLC conditions for the determination of quercetin involved the use of a C18 4.6-mm x 250-mm column maintained at 30°C, 254-nm UV detection, and a mobile phase composition of 25 parts methanol and 75 parts mixture of 0.5% H_3PO_4 and 0.2% triethylamine with a 1 mL/min flow rate in gradient elution.

A good linearity at the concentration range of 3.72–124 µg/mL of quercetin standard (r^2=0.9989) was observed with the limits of detection (LOD) and quantitation (LOQ) at 0.68 ng/mL and 2.28 ng/mL, respectively. The intra-day (n=5-) and inter-day (n=3) precision values were satisfactory (%RSD <2%). The recovery efficiency of the SPE sample cleanup step, which was checked by spiking sambong solution with

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quercetin standard, was 102.41%. The quercetin contents are 0.2337mg, 0.1350mg, and 0.2940mg per gram of the powdered dried leaves of sambong from Nueva Ecija, Cotabato, and Leyte, respectively. This is the first report of quercetin content in the leaves of sambong collected from the Philippines.

Keywords: Sambong, *Blumea balsamifera*, Quercetin, HPLC, Solid Phase Extraction

LAYMAN'S ABSTRACT

Blumea balsamifera (L) DC, known in the Philippines as *sambong*, is an herb valued for its health benefits especially in the management of urolithiasis. Various phytochemicals, including flavonoids such as quercetin, have been determined in sambong leaves collected from other Asian countries using instrumental techniques such as high performance liquid chromatography (HPLC). An HPLC method can be used to generate a chemical fingerprint useful in the accurate authentication and identification of herbal medicines, in the comparison of plant materials grown in different regions, and determination of amount of the chemically characteristic or pharmacologically active components. This study focused on the development of an HPLC method for the determination of quercetin in sambong leaves collected from Nueva Ecija, Cotabato, and Leyte. The methanol extracts of sambong leaves were prepared by maceration and by a solid phase extraction technique developed in this study. A reversed phase HPLC method was optimized and used in the determination of quercetin in the prepared extracts. The quercetin contents determined using the developed methods are 0.2337mg, 0.1350mg, and 0.2940mg per gram of the powdered dried leaves of sambong from Nueva Ecija, Cotabato, and Leyte, respectively. This is the first report of quercetin content in the leaves of sambong collected from the Philippines.

INTRODUCTION

Blumea balsamifera (L) DC, commonly known in the Philippines as *sambong,* is an herb that can grow anywhere in the Philippines and is also abundant in other Asian countries like India, China, and Malaysia (Quisumbing 1978). The leaves of sambong are extensively used in folk medicine to address various conditions including arthritis, rheumatism, headache, chest pains, diarrhea, dysentery, stomach pain, cough, and fever relief. The decoction is used as a diuretic in edema, in expelling kidney

stones, and in the management of urolithiasis (Quisumbing 1978; DOH BFAD 2005). The Philippine National Drug Formulary (PNDF) Essential Drugs List (DOH-NFC 2005) includes sambong in its list of drugs under diuretics, taken orally as a 250 mg or as a 500 mg tablet. Alternative preparations include capsules and bags for infusion. The leaf infusions and dosage forms were used to provide evidence of the pharmacologic activities of *B balsamifera* including intraocular pressure lowering effect (Arroyo and others 1990), antimutagenic activity (Lim-Sylianco and others 1987), and dissolution of calcium/urinary tract stones (Rico 1992).

Phytochemical investigation of sambong leaves reveals a diverse list of chemical constituents present which includes the flavonoids. The high amount of flavonoid content of the crude methanol extracts was attributed for the xanthine oxidase inhibitory and antioxidant activities of *B. balsamifera* (Fazilutan and others 2003; Fazilutan and others 2004). Among the flavonoids found in the leaves of sambong are quercetin, velutin, luteolin, luteolin-7-methyl ether, rhamnetin, tamarixetin, ombuin, 3,3'-dimethyoxyquercetin, 3,7-dimethylquercetin, quercetin-3,7,3.-trimethyl ether, 3,7,4'-trimethylquercetin, persicogenin, and 3',4',5-trihydroxy-3,6,7-trimethoxyflavone (Barua and Sharma 1992; Fazilutan and others 2004; Ali and others 2005).

The use of HPLC has been documented for the determination of flavonoids in extracts of plant materials and food products (Hertog and others 1993; Crozier and others 1997; Zu and others 2006; Andres-Lacueva and others 2008; Lin and others 2008). The research of Fazilutan and others (2005) focused on the HPLC quantitative determination of five major flavonoids in the methanol extract of sambong leaves from five different sources in Malaysia. The flavonoids included in their study are quercetin, dihydroquercetin-7,4'-dimethyl ether, blumeatin, 5,7,3',5'-tetra-hydroxyflavanone, and dihydroquercetin-4'- methyl ether.

An HPLC method can be used to generate a chemical fingerprint useful in the accurate authentication and identification of herbal medicines, in the comparison of plant materials grown in different regions, and the determination of amount of the chemically characteristic or pharmacologically active components. In this study, HPLC was used in the determination of quercetin (Figure 1) in the extracts of sambong leaves collected from Leyte, Cotabato, and Nueva Ecija. At the time of the writing of this manuscript, these are the first reported values of quercetin in sambong samples cultivated in the Philippines.

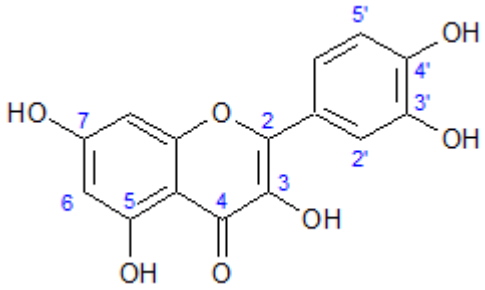

Figure 1. Structure of the flavonoid quercetin determined in *Blumea balsamifera* (L) DC

MATERIALS AND METHODS

Chemicals, Reagents, and Materials

Methanol (RCI Labscan® Limited) and acetonitrile (JT Baker®) solvents used were of HPLC grade. The phosphoric acid 85% (RCI Labscan® Limited) and triethylamine (JT Baker®) used were of analytical reagent (AR) grade. Distilled water was used in the aqueous solutions that were prepared. The quercetin standard was from Sigma-Aldrich®. Solvents used as mobile phase were filtered using a 0.45-µm 47-mm (diameter) nylon membrane filter discs (Whatman®) while solutions injected to the HPLC system were filtered using a 0.45-µm 25-mm (diameter) Puradisc™ 25 NYL nylon membrane with polypropylene housing filter (Whatman®). The solid phase extraction (SPE) was done using a 6-mL capacity SPE tube packed with 500-mg octadecylsilane (C-18) packing (Varian® Bond Elut-C18).

Instruments

The spectrophotometer used in the method developed is a Genesys 10UV Scanning ThermoSpectronic® Spectrophotometer. The HPLC analysis was done using a Perkin-Elmer® S200 HPLC System equipped with a quaternary pump, manual injector, column oven, UV detector, and TotalChrom Workstation® Software. The HPLC column used was a 4.6-mm internal diameter, 250-mm length Brownlee Analytical Perkin-Elmer® column with an octadecylsilane (C1-8) 5-µm particle size packing.

Plant Materials

The healthy and mature leaves of sambong were bought from farms located in three provinces of the Philippines: Nueva Ecija, Leyte, and Cotabato. The drying and comminution of the sambong leaves were done in the processing facility of each area. A Certificate of Analysis for the sambong samples bought from Leyte and Cotabato was provided by Philippine Institute of Traditional and Alternative Heath Care (PITAHC). A voucher specimen for the authentication of the sample from Nueva Ecija has been deposited in the Botany Division of the National Museum.

Sample Preparation

Crude extraction of 50 grams of powdered dried leaves was done by maceration for 18 hours using 500 mL methanol as solvent, with prior heating to 40°C in a water bath for 6 hours. The extract was collected and filtered. The filtrate was dried at 45°C in vacuo using a rotary evaporator.

Sample Extraction and Cleanup

Different solvent volumes and the sonication times for the preparation of the sample solutions were investigated in this study. The optimized conditions of 50 mg/mL sambong solution in methanol, prepared with the aid of sonication for 15 minutes, were used.

Different volumes of methanol extract to be loaded, composition and volume of eluting solvent, and total volume of eluate to be collected were examined. The optimized conditions, described in the succeeding sentences, were used in the sample cleanup. The SPE tube was preconditioned by sequentially passing 6 mL of methanol and 6 mL of 0.5% H_3PO_4. A 0.5 mL portion of the sambong solution was loaded to the same SPE tube and eluted with 4.00 mL of 80:20 methanol:0.5% H_3PO_4. The eluate was collected. Another 0.5 mL portion of the sambong solution was loaded to another separate SPE tube and treated in the same manner. The eluates of each of the two SPE tubes (about 8 mL) were pooled and diluted to 10.0-mL with methanol. The sambong solution was filtered through a 0.45-µm nylon membrane filter and used in the HPLC analysis.

The recovery efficiency of the SPE as a cleanup method was determined by adding 124 µL of 1.26 mg/mL quercetin standard solution to a 1.00 mL aliquot of a 50.12

mg/mL sambong solution. The spiked sambong solution was equally divided and delivered to two SPE tubes as described previously, to achieve a final concentration of 15.62 µg/mL of quercetin standard in the pooled eluate. The quercetin standard solution and unspiked sambong solution were prepared in the same manner. The solutions were filtered and injected to the HPLC system.

Quercetin Standard Solution Preparation

Standard stock solution (1.24 mg/mL) of quercetin was prepared in methanol. Aliquots of the stock solution were diluted with methanol to prepare six concentrations of quercetin standard solution (3.72-124.00 µg/mL). Solutions were filtered through a 0.45-µm nylon membrane filter and used in the HPLC analysis.

Chromatographic Conditions

The conditions that were optimized for the HPLC determination of quercetin were wavelength of detection, and the composition and gradient program of the mobile phase. The optimized procedure, described in the succeeding sentences, were used in the analysis of the sambong solution.

Chromatographic separation was carried out using an octadecylsilane (C18) 5 µm particle size stationary phase packed in a 4.6-mm x 250-mm column maintained at 30°C. The UV detector was operated at 254 nm. The volume of solutions injected to the HPLC was 20 µL. The mobile phase was run with a flow rate of 1 mL/min in a gradient elution (Table 1). The mobile phase was filtered through a 0.45-µm nylon membrane filter and sonicated prior to use.

The chromatographic peak of quercetin in the sambong solution was confirmed by comparing the retention time with those of the quercetin standard solutions. Quantification was made according to the linear calibration curves of the quercetin standard solutions.

Using the data generated by the injection of the six (6) quercetin standard solutions and methanol as blank solution (n=5), an evaluation of the peak areas as a function of the concentration of the solutions was done by calculating the parameters slope, intercept, and coefficient of correlation. The limit of detection (LOD) and limit of quantitation (LOQ) were computed from the same set of data. The intra-day precision

(n=3) for the peak area and retention time of quercetin were reported as % relative standard deviation (%RSD). The intra-day (n=5) and inter-day (n=3) precision of the methods were also tested using the sambong solution and expressed as %RSD.

Statistical Analysis

The quercetin content of the extracts was expressed as mean ± %RSD. A t-test ($p < 0.05$) was performed to determine the significance of the difference between means of the quercetin content of sambong from the different sources.

Table 1. Mobile phase composition and gradient program for the HPLC analysis of solutions

Time (minutes)	Mobile Phase Composition	Elution Type
0.0	25:75 methanol:0.5% H_3PO_4 , 0.2%TEA*	Gradient
0.0 – 10.0	45:55 methanol:0.5% H_3PO_4 , 0.2%TEA	Gradient
10.0 – 30.0	55:45 methanol:0.5% H_3PO_4 , 0.2%TEA	Gradient
30.0 – 40.0	100% methanol	Gradient
40.0 – 50.0	100% methanol	Isocratic

*0.5% H_3PO_4 , 0.2%TEA: This was prepared by adding 1.75 mL of 85% phosphoric acid to water, swirling thoroughly, then adding 1.38 mL of triethylamine (TEA), and diluting to 500.0-mL with water

RESULTS AND DISCUSSIONS

Optimization of the Sample Extraction and Cleanup

Effect of Solvent and Sonication

Different solvent ratios were tested for the preparation of the sample solution from the sambong extracts prepared from maceration and rotary evaporation (Table 2). Although the quercetin standard dissolved in solvents containing 80% or 20% methanol, an incomplete dissolution was observed when these solvents were applied to the sambong extract. Even with the aid of sonication, the amount of insoluble components did not appreciably decrease (Table 3). The sambong solution prepared in pure methanol that was subjected to sonication for 15 minutes was used in this study.

Table 2. Solubility of quercetin standard and sambong extract in different solvents

Solvent	Quercetin Standard	Sambong Extract
100% Methanol	Soluble	Soluble
80:20 Methanol:0.5% H_3PO_4	Soluble; Precipitation was observed after one week	Large portion remained insoluble
50:50 Methanol:0.5% H_3PO_4	Soluble; Precipitation was observed after two days	Large portion of remained insoluble
20:80 Methanol:0.5% H_3PO_4	Insoluble	Large portion of remained insoluble
100% 0.5% H_3PO_4	Insoluble	Insoluble

Table 3. Effect of sonication on the solubility of sambong extracts in various solvents

Solvent	Description of Solution Observed at 5-minute Time Intervals					
	0 min	5 min	10 min	15 min	20 min	25 min
100% Methanol	Small portion undissolved	Small portion undissolved	Very small portion	Complete solubility undissolved	Complete solubility	Complete solubility
80:20 Methanol: 0.5% H_3PO_4	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (dispersed) undissolved	Large portion (dispersed) undissolved
50:50 Methanol: 0.5% H_3PO_4	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (lumps) undissolved	Large portion (dispersed) undissolved

Effect of Volume Loading and Eluting Solvent in SPE

The SPE cleanup procedure in this study was modified from the work of Chen and others (2001) wherein flavonoids and other phenolic compounds in cranberry juice were extracted in a C18 SPE cartridge and analyzed by HPLC. The aim of the SPE in this study was to remove most of the unwanted components including the pigments in the methanol extract while allowing the quercetin to be collected completely.

When 0.3 mL and 0.5 mL volumes of 50 mg/mL sambong solution were loaded to separate SPE tubes, no peak corresponding to quercetin retention time was observed in the HPLC analysis of the eluate. However, at 0.8 mL and 1 mL volumes of the same concentration of sambong solution, the eluates showed a peak corresponding to the retention time of quercetin. The eluates of two SPE tubes (about 4 mL each) loaded with 0.5 mL of the sambong solution were pooled to achieve a quantifiable amount in the HPLC analysis.

Methanol or acetonitrile can remove quercetin completely from the SPE packing. However, the use of pure organic solvents caused the elution of more undesired compounds. The addition of 0.5% H_3PO_4 to methanol increased the polarity of the eluting solvent which enhanced the solubility of the polar quercetin to the eluting solvent. The sambong solution in the SPE tube was eluted with 4.00 mL of 80:20 methanol: 0.5% H_3PO_4. A second 4.00 mL of eluting solvent added to the same SPE tube did not result to additional elution of quercetin.

Recovery Efficiency

The efficiency of extraction of quercetin of the sambong solution treated by SPE was demonstrated by spiking a sambong extract solution with quercetin standard solution and allowing this to pass through the SPE tube (Figure 2). The solutions were analyzed using the optimized HPLC method. A mean recovery of 102.41% (± 1.15) for quercetin was achieved in this study.

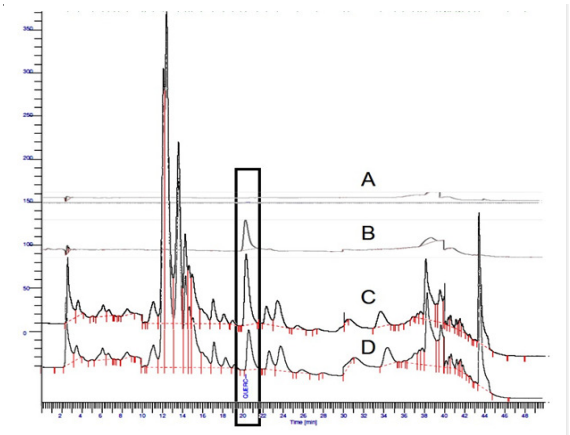

Figure 2. Overlay of chromatograms of solutions that were treated in the SPE tube in the same manner: (A) methanol; (B) quercetin standard solution (15.62 µg/mL); (C) sambong solution spiked with 15.62 µg/mL quercetin standard; and (D) unspiked sambong solution

Optimization of the Chromatographic Conditions

Effect of Wavelength of Detection

Using the UV spectrophotometer, a spectral scan from 200 nm to 350 nm of a quercetin standard solution was done. The three wavelengths that registered

maximum absorbance values were 254 nm, 263 nm, and 274 nm. The wavelength chosen for quercetin determination in this study was 254 nm because quercetin standard exhibited the highest value of area under the curve at this wavelength when tested using the HPLC-UV detector (Table 4).

Table 4. Peak area of quercetin standard tested using an HPLC-UV detector

Wavelength	Mean Peak Area (n=3, ± %RSD)
255 nm	11,342,168.100 ± 2.56
270 nm	7,001,168.213 ± 1.62
360 nm	9,525,722.357 ± 5.27

Effect of Composition and Gradient Program of the Mobile Phase

The HPLC conditions used in this study were modified from the work of Fazilutan and others (2005) where five major flavonoids, including quercetin, were quantitatively determined in the methanol extract of sambong collected from the different areas in Malaysia. Their study used a C18 (250- x 4.6-mm, 5 ìm particle size) column as the stationary phase and a mobile phase consisting of 50:50 methanol: 0.5% H_3PO_4 in an isocratic mode and delivered at 0.9 mL/min. The flavonoids were detected at 285 nm.

Quercetin, a polar molecule, can be eluted from a C18 stationary phase by using a polar mobile phase. This study explored gradient elution of various proportions of methanol and 0.5% H_3PO_4 to increase the resolution of quercetin from adjacent peaks and to improve the peak shape of the compound. The flow rate for all tests was held constant at 1.00 mL/min. Eventually, a gradient that starts with 25:75 methanol: 0.5% H_3PO_4 and the methanol content increasing to 100% in a 50-minute program yielded a satisfactory separation of quercetin (Figure 3A).

The addition of triethylamine was also investigated to improve the peak shape of quercetin in the sambong sample. The concentration of triethylamine in the 25:75 methanol: 0.5% H_3PO_4 mobile phase was varied from 0.1% to 1%. A 0.2% concentration of triethylamine resulted to an acceptable resolution of quercetin from adjacent peaks, as well as an improved peak shape (Figure 3B). Triethylamine, at a suitable concentration, acts as an ion pairing reagent for the stationary phase, which reduced the tailing of quercetin. When the concentration of triethylamine was increased, peak breaking and band broadening resulted (Figure 3C).

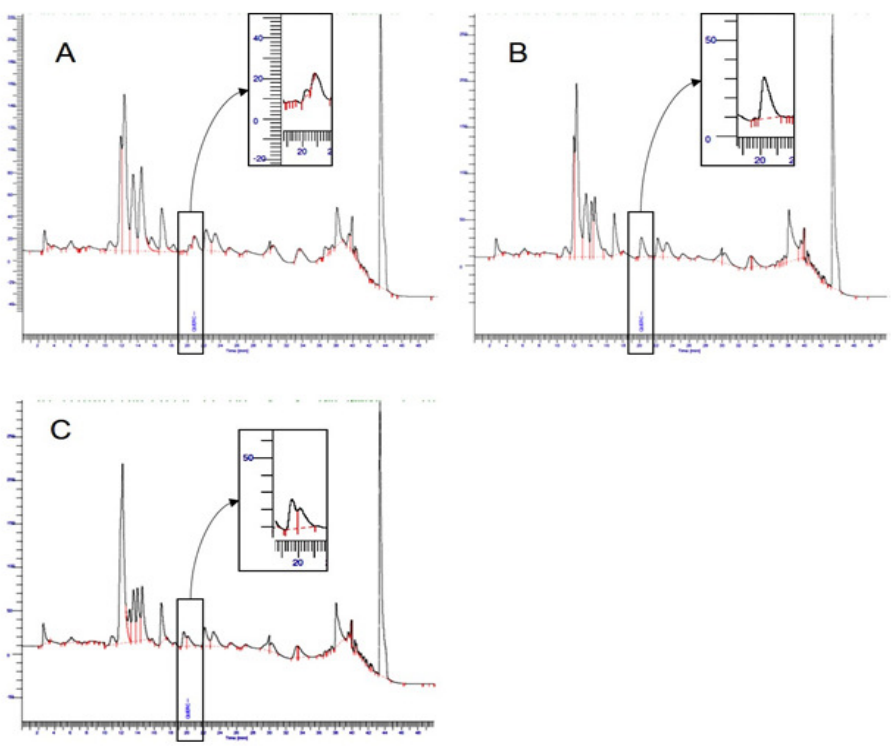

Figure 3. Chromatograms of sambong solutions run in different mobile phase compositions. The mobile phase composition was changing to 100% methanol in a 50-minute gradient program starting with the following solvent combinations: (A) 25:75 methanol:0.5% H_3PO_4; (B) 25:75 methanol:0.5% H_3PO_4 with 0.2% triethylamine; and (C) 25:75 methanol:0.5% H_3PO_4 with 0.5% triethylamine.

The following HPLC conditions were then applied in the analysis of quercetin in the sambong solution: C18,5 µm particle size stationary packed in a 4.6-mm x 250-mm column and maintained at 30°C, 254-nm wavelength of detection, 1.00 mL/min mobile phase flow rate, and a mobile phase and gradient condition as outlined in Table 1.

Linearity, Limit of Detection and Limit of Quantitation, Precision

A series of six quercetin standard solutions (3.72-124.00 µg/mL) was tested to determine the linearity between the concentration and peak areas. A high correlation coefficient of 0.9989 and regression equation of y=102789x-159435 was

generated from the HPLC analysis of the quercetin standard. The chromatograms of the six quercetin standard solutions are provided in Figure 4. The limits of detection and quantitation were evaluated on the basis of a signal-to-noise ratio of 3 and 10, respectively. The detection limit was determined as 0.68 ng/mL, while limit of quantitation was at 2.28 ng/mL. This indicates that the HPLC method is sufficiently sensitive for the determination of quercetin in sambong.

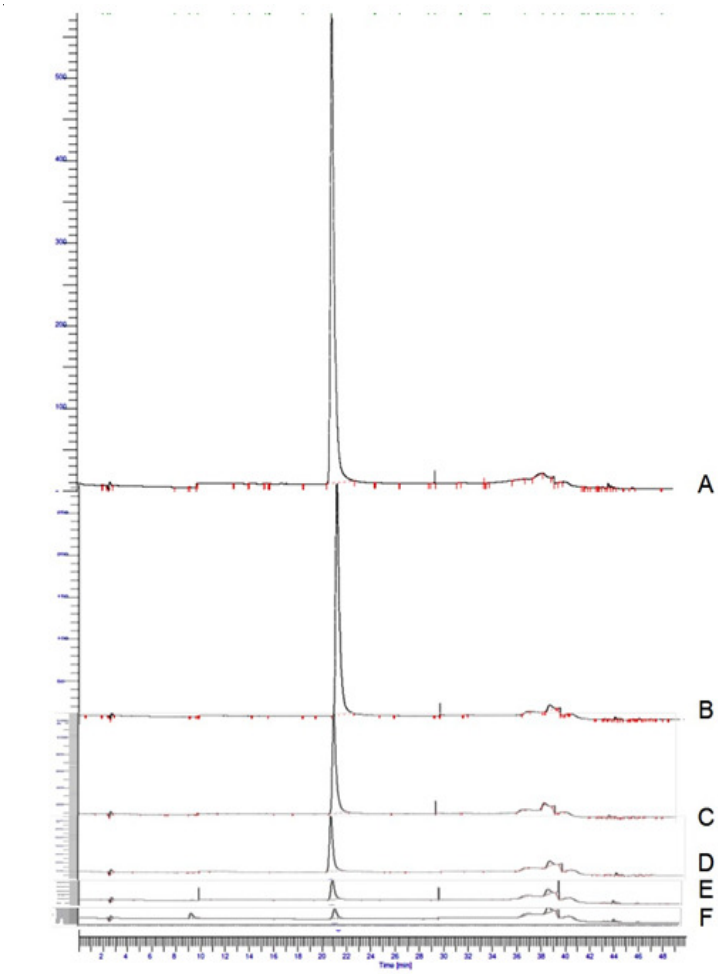

Figure 4. Overlay of chromatograms of the quercetin standard solutions analyzed using the optimized HPLC method: (A) 124.00 µg/mL; (B) 62.00 µg/mL; (C) 31.00 µg/mL; (D) 15.50 µg/mL; (E) 7.44 µg/mL; and (F) 3.72 µg/mL

The %RSD of the peak areas and retention times of the quercetin standards are both quite low which indicated that precision is good (Table 5). The precision was also measured using the peak areas of the quercetin in the sambong solutions. Both the intra-day (%RSD = 1.52%, n=5) and inter-day (%RSD = 1.73%, n=3) precision studies also showed that the method is both repeatable and reproducible.

Table 5. %RSD of the retention time and peak area response of quercetin standard solutions

Quercetin Standard Concentration (µg/mL)	Intra-day %RSD for Retention Time	Intra-day %RSD for Peak Area
124.00	0.07	0.89
62.00	0.13	0.16
31.00	0.65	1.02
15.50	0.88	1.30
7.44	0.64	0.99
3.72	0.21	1.48

Quantitation of quercetin in sambong leaves

The optimized SPE sample preparation and cleanup and HPLC method were applied to the sambong leaves collected from Leyte, Cotabato, and Nueva Ecija. The corresponding chromatograms of the sambong samples are shown in Figure 5. The summary of results on the quantitation is presented in Table 6. The quercetin content ranged from 0.135 to 0.294 mg per gram of powdered dried leaves. At the time of the writing of this article, these are the first reported values of quercetin in sambong samples grown in the Philippines. These results are close to the values reported in the study of Fazilutan and others (2005) where quercetin was reported to be present in the range of 0.021 to 0.958 mg per gram of dried *B. balsamifera* leaves cultivated in the different regions of Malaysia.

The quercetin content of sambong from Cotabato differed significantly (t-test, $p<0.05$) from that found in leaves from Nueva Ecija and Leyte. The quercetin content of samples from Leyte and Nueva Ecija did not differ significantly from each other. The variation in the quantity of quercetin may be due to various factors such as cultivation conditions (soil, temperature, moisture) and agricultural practices (use of fertilizers and pesticides).

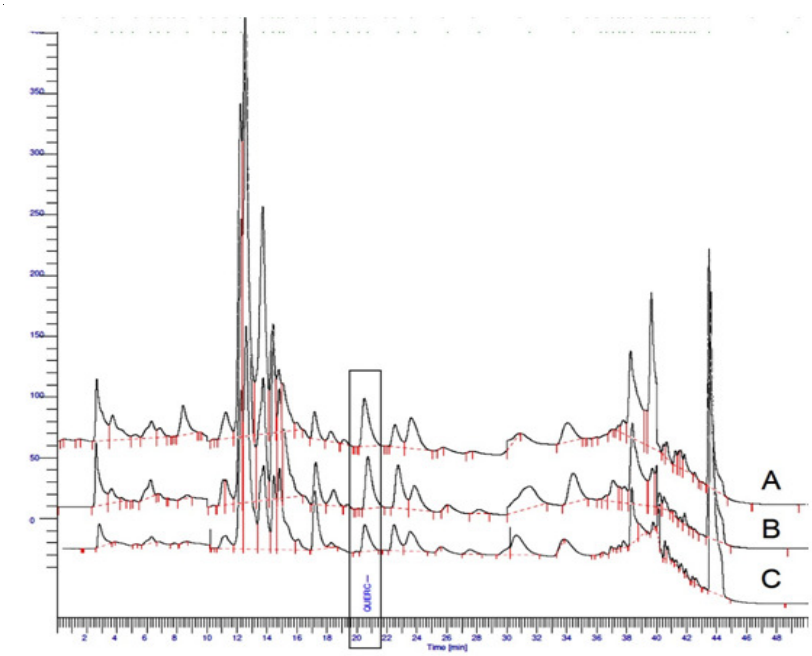

Figure 5. Overlay of chromatograms of the sambong from (A) Leyte, (B) Cotabato, and (C) Nueva Ecija analyzed using the optimized HPLC method

Table 6. Quercetin retention time, peak area, and content as determined by HPLC analysis of the sambong leaves from Leyte, Cotabato, and Nueva Ecija

Source	Retention Time (minutes, n=3, mean ±%RSD)	Peak Area (Au, n=3, mean ±%RSD	Quercetin in mg per gram Powdered Leaves
Leyte	20.67 ± 0.65	1,345,645.36 ± 1.27	0.2940
Cotabato	20.59 ± 0.82	698,866.84 ± 1.05	0.1350
Nueva Ecija	20.57 ± 0.80	1,334,535.34 ± 1.24	0.2337

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