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RESEARCH ARTICLE

Quantitative Analysis of Quercetin Content of *Blumea balsamifera* L. DC Dichloromethane Leaf Fraction Using HPLC-RP-PDA and Direct TLC-Bioautography

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ABSTRACT

Quercetin, a flavonoid compound which is widely distributed in plants are considered as beneficial physiologically due to attributed bioactivity such as anti-cancer, immunomodulatory, antidiabetic, and anti-inflammatory. In this study, the quercetin content from the dried *Blumea balsamifera* L. DC dried leaf was macerated with 95% ethanol and the concentrated extract was purified using Modified Kupchan method and flash chromatography. All fractions were tested for the presence of flavonoids using phytochemical screening and the selected dichloromethane fraction were further purified using another round of flash chromatography. All resulting fractions and pooled samples were tested for the antioxidant property using the developed Thin Layer Chromatography (TLC)-Bioautography and separated compounds were derivatized with DPPH. Using the optimized TLC-Bioautography method, the quercetin content in the dichloromethane fraction was analyzed and compared with a reversed phase high performance liquid chromatography hyphenated with photodiode array detector (RP-HPLC-PDA). The calculated quercetin content from the pooled sample using TLC-bioautography method is 2.25 mg/ml and from RP-HPLC-PDA is 2.02 mg/ml which was not comparable statistically using unpaired t-test ($p<0.05$, $\alpha=0.05$).

Keywords: *direct TLC-Bioautography, Quercetin, Blumea balsamifera L. DC , Raw Material Quality Control, HPLC-RP-PDA*

Introduction

Herbal drug preparation is an integral part of the Philippines' Primary Health Care Program due to the readily accessibility and affordable cost [1]. While the use of synthetic medication for therapeutic purposes has proven to be safe and effective, the cost of the active pharmaceutical ingredient (API) significantly contributes to the increase of the pharmaceutical cost of production together with the quality testing which leads to lack of access medicines which continues to represent as a national health burden [2,3]. Furthermore, the steady demand of the propagation and utilization of herbs and herb-based preparations in the drug industry indicates that there is a need for an enhance the current quality control method of herbal raw materials and finished products [4,5].

Herbal remedies derived from the simple crude extract or isolate of active constituents are employed as important component of healthcare system as a source of over-the-counter preparation, home remedies, and key ingredients in different pharmaceutical products in the developing and industrialized countries [6]. Herbal drug industry represents a competitive portion of the global market and majority of the rural areas from the developing countries such as the Philippines rely on herbs as its chief source of primary healthcare system [7]. Thus, the quality control testing of raw materials is a crucial component to ensure the safety and efficacy, and protect the public from potential harm through misuse and improper consumption. Evaluation of the herbal drug is challenging to the pharmaceutical industry due to the complex matrix of the crude and partial purified extracts. Also, identification of the active components is ideal for quality monitoring [8,9]. Several studies have shown the different approach in quantifying polyphenols present in B.

balsamifera such as UV-Vis, HPLC, and FTIR [10,11]. However, the methods require sophisticated analytical equipment. Thin layer chromatography (TLC) is one of the mostly used quality control test which identify and analyze the purity of official plant parts in the Philippine Pharmacopeia 1 (Pp1) [12]. While bioautography analysis utilized planar chromatography to detect the potential bioactivity of the test compound [13]. Therefore, there is a need to develop a fast and highly sensitive analytical method that can quantify chemical markers from partially purified extract [14].

Blumea balsamifera L. DC or locally known as sambong (Family: Asteracea) is one of the "Sampung Halamang Gamot" recommended by the Department of Health (DOH) for home remedy is reported to exhibit various therapeutic efficacy in different ailments such as in management of hypertension, urinary tract infection, and treatment of uritholithiasis [15]. The plant material has been used as a folkloric medicine in South-East Asia like China, Philippines, and Malaysia for the treatment of various ailments like eczema, rheumatism, and relieve pain due to the presence of diverse phytocompounds [16-19]. In the Philippines, sambong is widely cultivated and propagated in the rural and urban areas as immediate source of herbal material in the community [20,21]. Some of the compounds successfully isolated from the plant material were polyphenols such as terpene analogues and flavonoids [19]. Different polyphenolic compounds including quercetin have been reported present in *B. balsamifera DC* leaf extract and have been found to be potent antioxidants activity, which is comparable to vitamin E and synthetic antioxidants [19,22]. Quercetin (3,3',4',5,7-pentahydroxyflavone), a ubiquitous plant pigment which is found mostly in berries, broccoli, and citrus fruits, is now utilized as a chemical marker for the identification of plants under its family [23]. Aside from being a potent antioxidant, it is also considered as one of the vital bioflavonoids present in some plant and is known for its anti-hypertensive, anti-infective, antiviral, anti-diabetic, immunomodulatory, and anti-inflammatory effects [24]. Thus, this study aims to extract the quercetin from *Blumea balsamifera L. DC* dichloromethane fraction and determined using direct TLC-bioautography and the results were compared with RP-HPLC-PDA.

Methodology

Collection and authentication of plant material:

Fresh *Blumea balsamifera* leaves were obtained from University of the Philippines Los Baños in Laguna (UPLB) and authenticated by the National Museum. Plant materials were air dried for 3 days and oven dried at 55ºC to remove the water content until crisp to touch [12]. The resulting dried plant was reduced to a coarse powder using Retsch Rotor-Beater Mill.

B. balsamifera extraction:

The course *B. balsamifera* leaf was macerated with 95% ethanol for 8 hours, allowed to stand to complete the 24 hours and the mixture was filtered [12] Then, the concentrated filtrate was pass through different organic solvents (Modified Kupchann method) to remove the pigments [26].

Quality control of plant material:

Prior to extraction, the processed raw material must pass Raw Material Quality Control tests indicated in the official pharmacopeia [12,26].

Modified Kupchann method:

The ethanolic extract (85.5 ml) was separated with an equal volume of n-hexane twenty-seven times (27X) in a separatory funnel and the ethanol fraction was concentrated using a rotary evaporation under reduced pressure. The components of the resulting aqueous fraction were separated with dichloromethane, chloroform, and ethyl acetate, respectively. After which, the resulting fractions were concentrated using rotary evaporator [26]. Percentage yield recovered from the extracts were noted by evaporating the solvent to dryness.

Phytochemical screening of polyphenols:

The concentrated extract of n-hexane, dichloromethane, chloroform, ethyl acetate, and water were assessed using Wilstater test and Bate-Smith and Metcalf method and all samples were re-dissolved in their previous solvents [27].

Column chromatography

Flash column chromatography was performed using 10.5 inches column with an internal diameter of 10 cm. The pressure applied was -10 kPa and normal phase silica gel 60 was introduced with 130.70 mg of sample diluted in ethyl acetate and dried to run in a 7 inches layer of silica. The solvents used were (20 ml) hexane, EA, methanol, and deionized water, respectively. The column was equilibrated using 100 % hexane and each solvent had an interval dilution of 20 from 100% to 80% and so on every 20 ml from high to

low polarity with the last solvent having dilution of (50:50) methanol: water28. The fractions (2 ml) were collected and assayed using TLC (6.5 cm x 20 cm), with a solvent run of 5.5 cm. Fractions which exhibited antioxidant activity with DPPH reagent were pooled and used in the succeeding tests.

Thin layer chromatography-bioautography

Mobile phase:

The 95:5 (v/v) ethyl acetate and methanol mixture was considered as the optimized ratio of mobile phase for thin layer chromatography (TLC) to carry out quantitative bioautography based on the separation of the bands in the normal phase TLC (2 X 10 cm). The TLC plates were read at 254 nm and 366m nm using UV chamber [25].

Direct TLC-bioautography using DPPH solution:

The pooled sample was diluted to 1:5 using ethanol as solvent to be used for quantitative analysis in comparison with the standard. The standard concentrations (5-point) of quercetin used were 0.1 mg/ml to 0.5 mg/ml. Five microliters of the diluted pooled samples and the standard concentrations were applied to pre-coated silica gel 60 TLC (10 x 60 cm). The plate was developed in a TLC chamber pre-calibrated with optimized mobile phase, air-dried, subjected to UV detection (366 nm), and sprayed with 2% DPPH to detect the phenolic compounds present [25]. The 1:5 dilution pooled sample was analyzed for quercetin content by extrapolating the quercetin standard curve and Bio-Rad Quantity One version 4.6.5 software was employed to convert the intensity of the developed spots into quantitative analysis [30].

Chromatographic system:

After the optimization of the adopted method of Jiang G. and Zhang T, (2010) for the quercetin quantitation from sambong, the analysis was performed using Thermoscience with quaternary pump equipped with autosampler and photodiode array (PDA). Purospher star RP-18 (150 cm X 4.6 cm, 5µm) was used for the chromatographic separation using a gradient mixture of acetonitrile (Solvent A) and water (Solvent B) as mobile phase. The gradient program was set to (Time/Solvent A%/Solvent B%) 0min/2%/98%, 10min/5%/95%, 16min/2%/98% and 20min/2%/98% with 10 µL as injection volume at a flow rate of 1.0 ml/min. The analytes' elution was monitored at 220 nm, 254 nm, and 280 nm. Data acquisition was processed by Xcalibur software [31].

Results and Discussion

The powdered *B. balsamifera* leaf in the study successfully passed the acceptable limit by the official pharmacopeias as shown in table 1 which suggest the absence of contaminants such as carbonates and silica in the processed plant material. The results also ensure that the plant sample was fit for use in the method development [32]. The aqueous portion of the ethanolic extract was further purified using liquid-liquid extraction to remove the plant pigments. The qualitative presence of antioxidant compound(s) in each resulting fraction with a concentration of 1 mg/ml was determined using DPPH as spray reagent. A percentage recovery of 1.77% (n-hexane), 0.32% (dichloromethane), 0.04% (chloroform), and 0.17% (ethyl acetate) was recorded from 200 grams of plant material. Dichloromethane fraction was selected for column chromatography due to high color intensity of color change in Wilstater "cyanidin" test in comparison with all the fractions.

Fractions (2 ml) that were collected from flash chromatography were applied in the normal phase TLC plate in TLC chamber containing the pre-equilibrated optimized mobile phase. After the plates were dried, DPPH reagent was sprayed to visualize the presence of antioxidants compounds. The yellow color in violet background corresponds to positive result. Fractions 11 to 20 (Figure 1) were pooled together and subjected to quantitative analysis for quercetin content.

Table 1. *Quality control test conducted in the B. balsamifera leaf powder*

Particulars	Acceptable Limit	Actual Result	Remarks
Foreign Matter	not more than 2%	0.00 %	Passed
Loss on Drying	not more than 15%	4.32%	Passed
Alcohol Soluble Extractives	not less than 5%	5.14%	Passed
Water Soluble Extractives	not less than 15%	16.41%	Passed
Total Ash	not less than 13%	9.74%	Conforms

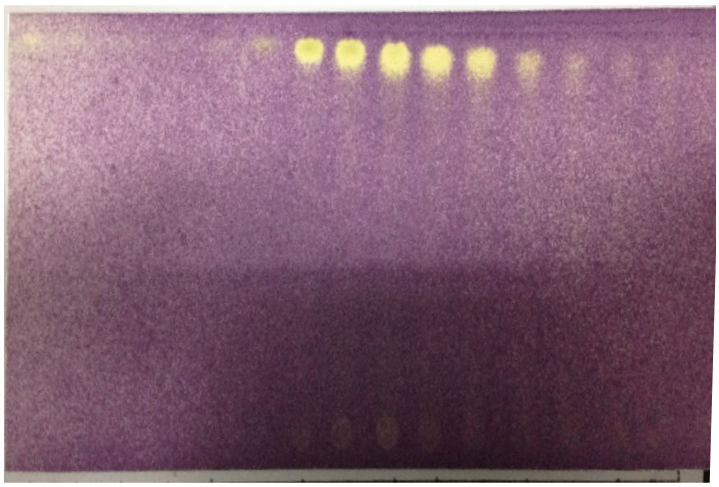
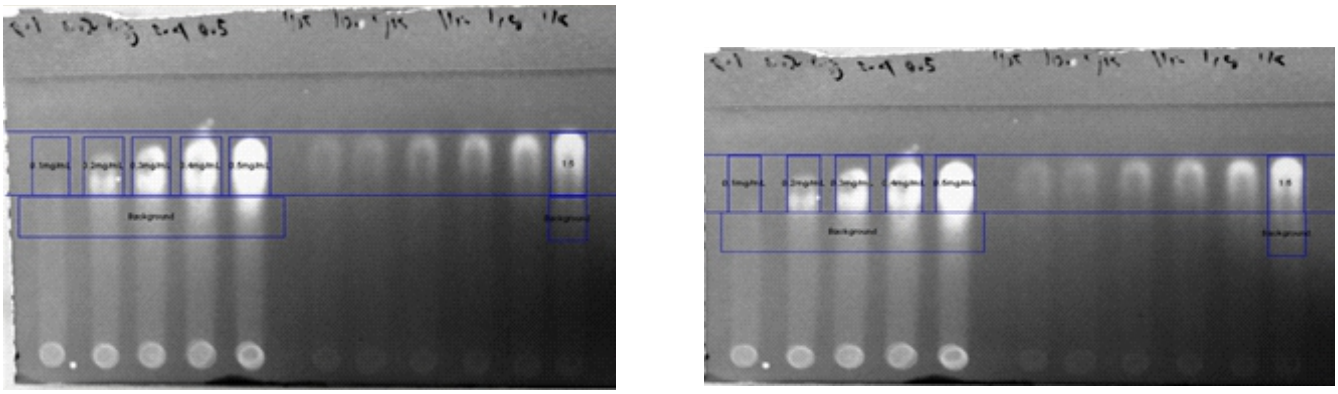


Figure 1. *Developed TLC plates of fractions 11-20 sprayed with DPPH reagent*



2A **2B**

Figure 2. *TLC bioautography-guided A) trial 1 and B) trial 2*

A direct thin-layer chromatography (TLC) bioautography method was created to quantitatively analyze the quercetin content of the sample by using the antioxidant property of quercetin towards the DPPH radical. It was found that the ideal solvent system to be used was the mixture of ethyl acetate: methanol (95:5). Bio-Rad Quantity One software was used to analyze the intensity of the spots detected in the TLC plates as shown in figure 2. Duplicate analyses were performed yielding two standard curves with r^2 of 0.98 and 0.99, respectively. The calculated concentration of the 1:5-diluted pooled sample was computed to be 0.34 mg/ml and 0.41 mg/ml.

To further confirm the presence of quercetin from the pooled sample and compare the result of the TLC method, an RP-HPLC-PDA analysis was conducted. The compound having an antioxidant property present in the pooled was confirmed to be quercetin due to the same retention time, 7.30 minutes as noted in the chromatogram (Figure 3). The pooled sample was spiked with quercetin and the peak of intertest exhibited an increase in peak height at retention time 7.29 minutes (Figure 3-C). This confirmed the presence of quercetin in the sample. Fraction 36 (Figure 3-E) was also analyzed using the optimized HPLC conditions and it was confirmed that the sample also contained a small amount of quercetin as shown in spiked fraction 36 (Figure 3-F). Quan browser was used to analyze the chromatograms obtained for the sample and standards. The area under the curve was manually integrated to calculate the concentration of quercetin.

Based on the standard curve generated, the concentration of the 1:5-diluted pooled sample was 0.34 mg/ml. While the average concentration of quercetin using the two trials is 0.38 mg/ml. The dilution factor for the samples used is 120µl/20µl. Thus, the final concentration of quercetin in the dichloromethane phase of the ethanol extract for HPLC is 2.02 mg/ml while for the TLC it is 2.25 mg/ml. The unpaired t-test of the quercetin quantitation using TLC-Bioautography, and HPLC-RP-PDA analysis revealed that the two methods were

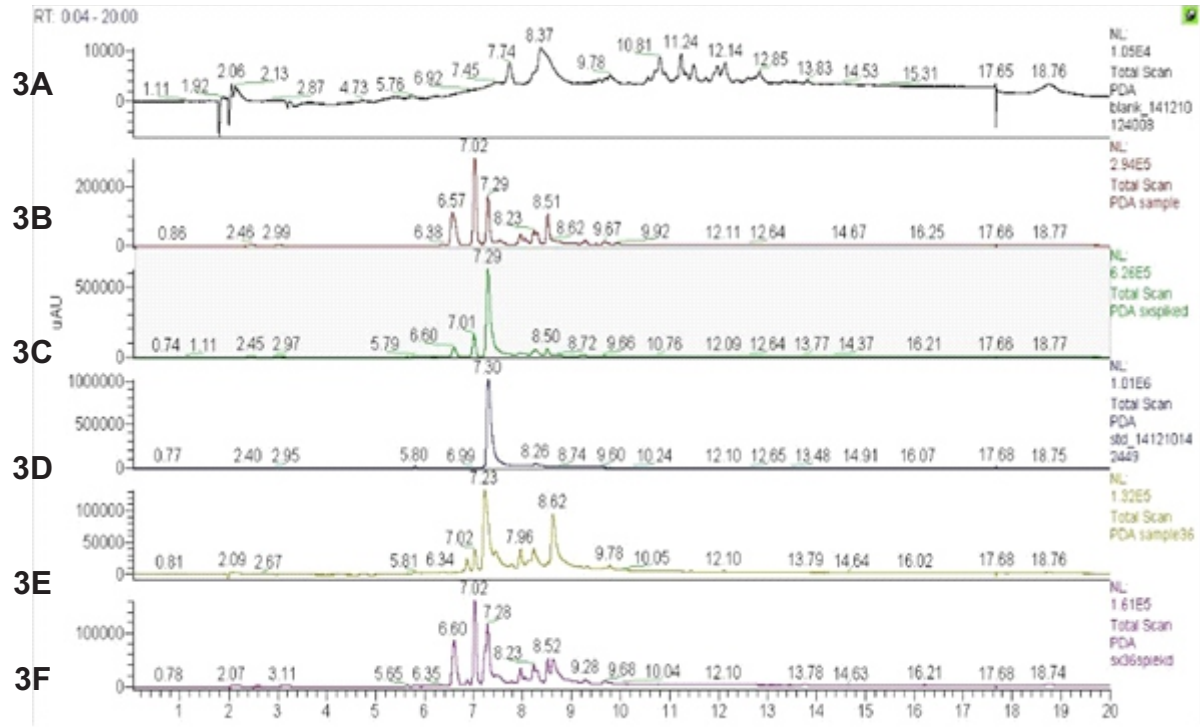


Figure 3. *A) Blank, B) Pooled sample C) Pooled sample spiked with quercetin D) Quercetin standard E) Fraction 36 and F) Fraction 36 spiked with quercetin (Legend: X-axis- intensity of the signal expressed in uAU and Y axis is the time in minutes)*

statistically significant ($p<0.05$). This outcome can be attributed to the factors such as the difference in the efficiency of separating quercetin from other impurities of reversed phase column in HPLC and normal phase silica of thin layer chromatography.

Several analytical methods are commonly used for routine testing and research purpose such as HPLC and automated version of thin layer chromatography, the high-performance thin layer chromatography (HPTLC). An HPLC-RP method performed by Toralba, J., Quiming, N., and Palacapac, J., (2015) using the methanolic extract cleaned up by solid phase extraction confirmed the presence of quercetin from sambong obtained from Nueva Ecija, Cotabato, and Leyte with reported recovery values of 0.23 mg, 0.14 mg, and 0.29 mg per gram of the powdered dried leaves, respectively. The separation was achieved with the use of C18 column maintained at 25C and mobile phase of 25:75 of 0.3% phosphoric acid and 0.2% triethylamine and a flow rate of 1 ml/min (gradient mode)32. A gradient mode separation is highly recommended for the separation of polyphenol in a reverse phase column. The results in both studies demonstrated that the careful selection of binary solvent system is an important parameter to be considered for achieving the desired resolution of other peaks. One of the approaches proposed in this study was to reduce the time of analysis from 60 minutes to 20 minutes by using a simple solvent system 33. Another published reports of quercetin detection and quantitation using high performance thin layer chromatography (HPTLC) was conducted by Jain et al (2021) using hydroalcoholic and methanolic extract of Phoenix sylvestris L Roxb, a member of the family Asteraceae. The developed HPTLC method employed the validation parameters like accuracy, precision, detection limit, and linearity which was the next goal of this study. With r2 = 0.996, the calibration curve for Quercetin was determined to be linear. The computed LOD and LOQ for Quercetin in leaf methanolic extract were within the detectable range. The TLC densitometry method was precise and repeatable, as seen by the low RSD values. The average recoveries were found to be 97.87–99.16 % at three distinct doses of Quercetin, demonstrating that the procedure was accurate. Quercetin concentration in leaf methanolic extract was determined to be 6.14 mg/g 34 . In quality control testing of plant material, the identification of genuine species, the harvesting of the highest quality raw materials, the evaluation of post-harvesting management, the assessment of intermediates and finished products, and the detection of dangerous or poisonous substances all need the use of chemical markers. The medicinal components of herbal medicines should be novel chemical indicators. However, the therapeutic components of most

herbal medications have yet to be thoroughly explained or monitored 35. Further study is suggested to elucidate the role of quercetin in the mechanism of action of sambong.

Conclusion

In this preliminary study, the antioxidant activity of the B. balsamifera DCM fractions using direct TLC bioautography derivatized with DPPH solution was determined to quantitate the quercetin content and the results were compared by using RP-HPLC-PDA. It has been shown that the quercetin content using RP-HPLC-PDA and direct TLC Bioautography was not comparable based on unpaired t-test ($p<0.05$) at $\alpha=0.05$. Although both method is not comparable based on the statistical approach, it is important to perform the complete validation process to accurately compare the two techniques in terms of specificity, precision, linearity, accuracy, limit of detection, and stability.

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