Determination of Artepillin-C in Brazilian Propolis by HPLC with Photodiode Array Detector

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Abstract: A method for the determination of artepillin-C using HPLC coupled with photodiode array (PDA) detector was developed. Artepillin-C is abundantly present in Brazilian propolis of *Baccharis* species origin. The chromatographic separation was performed on a Mightysil RP-18 GP II column (150 mm × 4.6 mm, 5.0 μm; Kanto Chemical Company Limited), and column temperature was maintained at 40°C. The mobile phase was a linear gradient elution of 0.5% aqueous acetic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml/min. The chromatogram was monitored at 320 nm. The calibration range of artepillin-C was 0.75-2500 μg/ml (r^2 = 0.9991). The limit of detection (LOD) and limit of quantification (LOQ) were 0.50 μg/ml and 0.75 μg/ml, respectively. The intra-day and inter-day precision of the assay (RSD) were in the range 1.28-5.60% and 1.45-6.75%, respectively. The artepillin-C content in Brazilian propolis is an important factor in ensuring the quality of Brazilian propolis of *Baccharis* species origin. This method was used to determine artepillin-C content in Brazilian propolis.

Keywords: Brazilian propolis, artepillin-C, HPLC, photodiode array detector.

1. INTRODUCTION

Propolis is a resinous product collected by honeybees from various buds and exudates of plants, and is used to protect the beehive from external enemies. Propolis has been widely used in folk medicine for many years due to its varied chemical composition [1]. There is substantial evidence to indicate that propolis has various biological properties such as antibacterial [2], antiviral [3], anti-inflammatory [4], antitumor [5], and antioxidant [6-9]. In Japan, Brazilian propolis extracted from ethanol has been extensively used in food to improve health and prevent diseases, e.g., gingivitis, rheumatism, cold and cancer [10-11].

To date, several researchers have reported that Brazilian propolis possesses characteristic biological properties [3-13]. It is well known that the constituents of propolis depend on its plant origin and the time of collection [14]. Brazilian propolis has been known to contain various phenolic compounds such as flavonoids. Recently, artepillin-C [3-{4-hydroxy-3,5-bis(3-methyl-2-butenyl)phenyl}-2 (E)-propenoic acid; 3,5-diprenyl-4-hydroxycinnamic acid] was reported to have antimicrobial, antioxidant and antitumor activities [15], and can be isolated from Brazilian propolis [16-18]. Kumazawa *et al.* recently reported that Brazilian propolis is derived from *Baccharis dracunculifolia* [19]. Therefore, *Baccharis* species plants are the main source of artepillin-C in Brazilian propolis. Determining the artepillin-C content in Brazilian propolis is an important factor in ensuring the quality of Brazilian propolis of *Baccharis* species origin. Therefore, developing a simple and selective method for its determination in Brazilian propolis of *Baccharis* species origin is required.

Several methods have been described for the determination of the artepillin-C content in Brazilian propolis using high-performance liquid chromatography-ultraviolet (HPLC-UV) detection. The method developed by Han *et al.* is confirmed from chromatograms with good-resolution, but it is time consuming [20]. On the other hand, the method devised by Matsuda and Almeida-Muradian has a shorter retention time, but it cannot be confirmed from chromatograms [21].

We developed a simple and selective method for the determination of artepillin-C in Brazilian propolis of *Baccharis* species origin. Compared with the HPLC methods described above, the proposed method showed high selectivity and rapid speed, and the retention time of artepillin-C was approximately 20 min, and one analysis time was within 45 min, including

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coumn-equilibrating time. Furthermore, the developed method was also used to determine the artepillin-C content in Brazilian propolis.

2. EXPERIMENTAL

2.1. Reagents and Chemicals

Artepillin-C (Figure **1**) and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). All other chemicals used as reagents were of analytical or HPLC grade. Water (18.2 MΩ/cm) was purified on an Autopure WD 500 machine (Nihon Millipore, Tokyo, Japan). An artepillin-C stock solution (5.0 mg/mL) was prepared in methanol. Working solutions were prepared from stock solutions by dilution with methanol. All prepared solutions were stored at 4°C until analyses.

2.2. Brazilian Propolis Samples

Brazilian propolis of *Baccharis* species origin produced in the brazilian districts of Minas Gerais (AF-05 and AF-18; *Baccharis dracunculifolia*) and Paraná (AF-06 and AFG-06; *Baccharis eriocladа* and AF-19; *Baccharis caprarifolia*) was obtained from Amazon Food Ltd. (Tokyo, Japan) along with Brazilian propolis of different species origin (AF-08; *Myrceugenia euosma* and AF-20; *Hyptis divaricata*).

These samples of Brazilian propolis were extracted with ethanol, and the extracts were dried. These dried extracts (5.0 mg/ml) were dissolved in 1.0 ml of methanol, and filtered through a 0.45-µm membrane filter (Millex; Millipore, Billerica, MA, USA) before analyses.

2.3. Chromatography

Measurement was undertaken on a LC-2000 Plus series (JASCO Corporation, Tokyo, Japan) liquid chromatographic system connected to a MD-2015 diode array detector (PDA) (200-500 nm) and controlled by a LC-Net II/ADC machine (JASCO Corporation). Separation was performed on a Mightysil RP-18 GP II column (150 mm × 4.6 mm, 5.0 µm; Kanto Chemical Company Limited, Tokyo, Japan) and the column temperature was maintained at 40°C. The chromatographic separation was performed using a linear gradient elution of 0.5% aqueous acetic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml/min. The linear gradient program was 0-5 min, isocratic 30% B; 5-30 min, linear gradient 100% B; 30-32 min, linear gradient 30%. After each run, the column was re-equilibrated for 10 min at initial conditions before the next injection. The chromatogram was monitored at 320 nm and samples were injected automatically (20 µl). Data were processed by ChromNAV (JASCO Corporation).

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic Conditions

Brazilian propolis of *Baccharis* species origin is known to contain artepillin-C and various phenolic compounds (e.g., flavonoids). Therefore, the chromatographic conditions were optimized to achieve chromatograms with good resolution of adjacent peaks. Two organic solvents commonly used in reversed-phase liquid chromatography were evaluated: acetonitrile and methanol. The former was selected because it provided better resolution than methanol. The effects of adding acetic acid and phosphoric acid to the mobile phase to enhance resolution and eliminate peak tailing were also examined. Good resolution was achieved with the mobile phase comprising solvent A (0.5% aqueous acetic acid) and solvent B (acetonitrile). The analysis time for one sample was set as short as possible. Column type and gradient conditions were examined with the objective

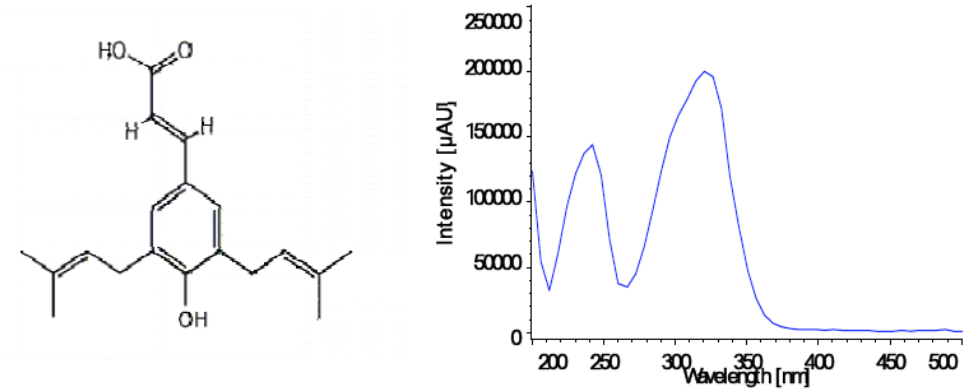

Figure 1: Chemical structure and ultraviolet spectrum of artepillin-C.

that the analysis time should be very short. Two column types were examined: Mightysil RP-18 GP II column (150 mm × 4.6 mm, 5.0 µm) and Mightysil RP-18 GP II column (250 mm × 4.6 mm, 5.0 µm). We selected the Mightysil RP-18 GP II column (150 mm × 4.6 mm, 5.0 µm) because it provided good resolution of adjacent peaks and an appropriate analysis time for artepillin-C. The column temperature was thermostatically controlled at 40°C because increasing the column temperature slightly above room temperature reduced the analysis time and improved resolution.

3.2. Linearity

A calibration curve of artepillin-C was obtained by plotting peak areas versus known concentrations of the standard solution. The calibration curve was constructed by analyzing five concentrations of standard solutions. The curve showed good linearity in the range of 0.75-2500 µg/ml. The coefficient of determination (r^2) was >0.9991.

Limit of detection (LOD) and limit of quantification (LOQ) were the injection concentrations corresponding to the peak heights with a signal-to-noise ratio of 3:1 and 10:1, respectively. Consequently, LOD and LOQ were 0.50 µg/ml and 0.75 µg/ml, respectively.

3.3. Precision Tests

Intra-day precision of the assay was determined using standard solutions at three concentrations (high, medium and low; n = 5) during a single day whereas inter-day precision of the assay was determined over 3 days. The relative standard deviations (RSD) for intra-day and inter-day assays were in the range 1.28%-5.60% and 1.95%-6.75%, respectively (Table **1**).

Table 1: Intra-Day and Inter-Day Precisions of the Assay to Determine the Artepillin-C

Concentration	Intra-day (n = 5)	Inter-day (n = 3)
(µg/ml)	RSD (%)	RSD (%)
5.0	5.60	6.75
100	1.85	2.32
1000	1.28	1.95

3.4. Method Application

Several methods reported for the determination of the artepillin-C content in Brazilian propolis using HPLC-UV [20-21]. Matsuda *et al.* reported the retention time of artepillin-C was 15 min, however, total analysis time did not show clearly. In this proposed method, the condition for chromatographic separation was performed using a linear gradient elution of 0.5% aqueous acetic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml/min. The linear gradient program was 0-5 min, isocratic 30% B; 5-30 min, linear gradient 100% B; 30-32 min, linear gradient 30%. After each run, the column was re-equilibrated for 10 min at initial conditions before the next injection. Therefore, the proposed method of analysis time was within 45 min. The LOD reported in this proposed method is 0.50 µg/ml, which is higher than 0.0036 µg/ml reported in other study [21]. This proposed method is limited to the applications for artepillin-C content in Brazilian propolis.

The proposed method was used to determine Brazilian propolis of *Baccharis* and different species origin. Typical chromatograms obtained from a standard solution of artepillin-C and Brazilian propolis (AF-06) are shown in Figure **2**. Under these chromatographic conditions, baseline resolution was achieved with reasonable retention time and symmetrical peaks. The retention time of artepillin-C was approximately 20 min. The retention time and UV spectra of standard artepillin-C coincided with that in Brazilian propolis (AF-06). Therefore, we can identify artepillin-C in Brazilian propolis by using this proposed method.

Artepillin-C content in Brazilian propolis of *Baccharis* and different species origin were determined by this method using the calibration curve obtained with a standard solution of artepillin-C. The contents of artepillin-C in Brazilian propolis of *Baccharis* and different species origin are listed in Table **2**. Brazilian propolis (5.0 mg/ml) contained artepillin-C in concentrations varying from 0.55 mg/ml to 1.73 mg/ml. The highest content of artepillin-C was found in Brazilian propolis of AF-18 (Minas Gerais). However, artepillin-C was not detected in Brazilian propolis of different species origin (AF-08 and AF-20). Therefore, it is likely that *Baccharis* species are the main source of artepillin-C in Brazilian propolis. These results indicate that the proposed method can be used to determine the artepillin-C content in Brazilian propolis.

4. CONCLUSION

In the present study, a simple and selective method for the determination of artepillin-C was developed using HPLC with Photodiode Array Detector. Artepillin-C in Brazilian propolis of *Baccharis* species origin was completely separated from various chemical

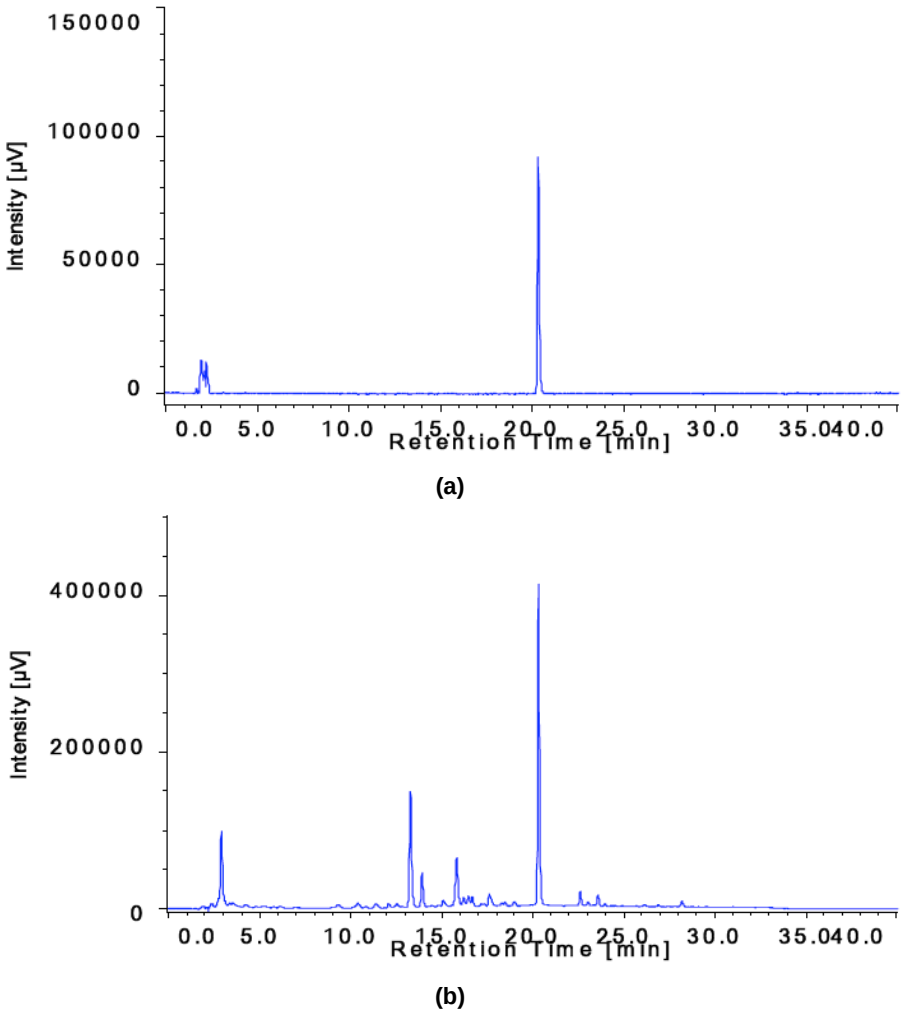

Figure 2: Chromatogram of a standard solution of artepillin-C (100 µg/ml; a) and Brazilian propolis of *Baccharis* species origin (AF-06; b). HPLC conditions; mobile phase, 0.5% aqueous acetic acid (**A**): acetonitrile (**B**), gradient program, 30% B → 100% B (5-30 min) →30% B (30-32 min).

Table 2: Artepillin-C Content in Brazilian Propolis of Different Regions

Sample	Species[a]	Family[a]	State[b]	Concentration (mg/mL)
AF-05	*Baccharis dracunculifolia*	Compositae	Minas Gerais	0.91
AF-06	*Baccharis erioclada*	Compositae	Paraná	0.55
AFG-06	*Baccharis erioclada*	Compositae	Paraná	0.72
AF-08	*Myrceugenia euosma*	Myrtaceae	Rio Grande do Sul	nd
AF-18	*Baccharis dracunculifolia*	Compositae	Minas Gerais	1.73
AF-19	*Baccharis caprarifolia*	Compositae	Paraná	0.69
AF-20	*Hyptis divaricate*	Labiatae	Bahia	nd

nd: not detected.
Note: [a]Major botanical origins in areas where propolis was collected.
[b]Brazilian states where propolis was collected.

compounds. The retention time of artepillin-C was approximately 20 min, and one analysis time was within 45 min. The artepillin-C is an important factor in ensuring the quality of Brazilian propolis of *Baccharis* species origin. We believe that this method will be useful to determine the artepillin-C content in Brazilian propolis.

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