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Comparative Study on Different Extraction Methods for Stilbenoids: Maceration and Supercritical Fluid Extraction (SFE) of *Anisoptera laevis* Ridl.

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Maceration is a conventional extraction method that is considered simple and inexpensive but takes a lengthy extraction period. Supercritical fluid extraction (SFE) is among the modern extraction techniques to extract natural chemical components such as flavonoids, essential oils, carotenoids, and fatty acids, and it is a more sustainable alternative to conventional extraction procedures. In this study, the stem bark of *Anisoptera laevis* was extracted using SFE and maceration techniques. Stilbenoids are a class of polyphenolic compounds that are rich in bioactivities. It is a secondary metabolite that plays an important role as a defensive mechanism in plants. This research aimed to extract stilbenoids from the stem bark of *A. laevis* using maceration and SFE techniques and compare the extraction of stilbenoids using both approaches. The LOTUS and MassBank of North America (MoNA) databases identified seventy peaks from each maceration and SFE crude extract with MS/MS values. In maceration and SFE crude extracts, nine stilbenoids were found, with two more peaks detected in maceration crude extract. The results for both extraction procedures are almost comparable, implying that SFE could be considered an option for the greener extraction method with a shorter period.

Keywords: *Anisoptera laevis*; Dipterocarpaceae; maceration; supercritical fluid extraction; stilbenoids

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Maceration is a traditional extraction method that is considered simple and inexpensive but takes a lengthy extraction period [1]. This method is reported as the oldest technique, which was enlightened in the 11th century and paved the way for advanced extraction techniques [2]. This simple technique is carried out in a closed vessel, involving the soaking of plant materials in a specific solvent for a period of time [3]. The soaking process softens the cellular structure, enabling solvent penetration to extract the intracellular components [4]. The solubility and effective diffusion of bioactive compounds into the solvent determines the efficacy of maceration. Therefore, utilizing abundant solvent is inevitable, even though the process is aided by elevated temperature and stirring [5].

As a sustainable substitute for conventional extraction procedures, supercritical fluid extraction (SFE) is one of the modern extraction techniques used to extract natural chemical components such as flavonoids, essential oils, carotenoids, and fatty acids [6]. This technique works by pressurizing and heating carbon dioxide (CO_2) above its critical pressure and temperature to change its state from gas to super-critical fluid. It stands out from the other modern extraction techniques since the penetration power (towards the sample), and the elution power (towards the analyte) of supercritical fluid can be done by simply adjusting the pressure and temperature of CO_2 during the extraction process. Moreover, CO_2 is non-flammable and "green"; thus, it has no significant environmental impact. Besides, it can reduce energy and solvent consumption, is a fast process, has minimal analyte degradation, and has simple sample

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preparation and clean-up steps [7]. SFE is often used to extract non-polar compounds, but polar compounds can be extracted by adding a polar modifier such as ethyl acetate, ethanol, and water [8]. The low density and viscosity of supercritical CO_2 and high diffusivity can increase the extraction efficiency of desired compounds [9].

The Dipterocarpaceae family is well-known for its high economic importance in timber production due to its wood quality [10]. It consists of 16 genera, namely *Anisoptera*, *Cotylelobium*, *Dipterocarpus*, *Dryobalanops*, *Hopea*, *Marquesia*, *Monotes*, *Neobalanocarpus*, *Pakaraimaea*, *Parashorea*, *Shorea*, *Stemonoporus*, *Upuna*, *Vateria*, *Vateriopsis* and *Vatica*. *Anisoptera* is the minor genus in this family, with only 10 species identified, namely *A. aurea*, *A. costata*, *A. curtisii*, *A. grossivenia*, *A. laevis*, *A. marginata*, *A. megistocarpa*, *A. reticulata*, *A. scaphula* and *A. thurifera* [11]. This genus is distributed in Malaysia and Indonesia, only in Kalimantan and Sumatra [12]. Although the forests of Malaysia are mainly dominated by Dipterocarps, only a few studies have been conducted on *Anisoptera* species [12-16]. The family of this genus is well-known to contain abundant sources of stilbenoids, starting from monomer to a high degree of polymerization, such as resveratrol **(3)**, ampelopsin A, and vaticanol B **(7)** [17]. These compounds have been reported to possess a broad spectrum of biological activities such as anticancer [18], antimicrobial [19], antioxidant [20], anti-osteoporosis [21], antiviral [22], and hepatoprotective effects [23].

This study involves sample collection of *A. laevis,* followed by two different extraction methods (maceration and SFE). Maceration is a simple and economical method but requires a large amount of organic solvents. Meanwhile, SFE uses supercritical CO_2, providing a green alternative that does not require organic solvents [24]. However, a slight modification was introduced by adding a polar solvent, ethanol, to extract polar compounds. The crude extracts' chemical profile was determined by Liquid Chromatography-Mass Spectrometry (LC-MS). To the best of our knowledge, no researcher has reported on the chemical constituents of *A. laevis* or the use of a green extraction technique. This study focused on the LOTUS and MassBank of North America (MoNA) databases.

EXPERIMENTAL

Sample Collection

The stem bark of *A. laevis* was collected from Kuala Keniam UiTM-PERHILITAN Research Station, Taman Negara, Pahang (4.517975384691467, 102.4743349109 0645) in September 2020. This species was identified by Mr. Imin Kamin, a botanist from Forest Research Institute Malaysia (FRIM), and a voucher specimen (Fri 94375) was deposited at FRIM herbarium. The stem barks were cut into small pieces and air-dried for two weeks.

Extraction of Sample – Maceration

Before drying, the mass of fresh stem bark of *A. laevis* was 3 kg. The sample was air-dried for two weeks and ground into granulated form. Solvent extraction was performed by soaking 900 g of granules in 5 L of acetone for 24 hours. After the duration, the sample was filtered and re-soaked in acetone for another 24 hours. The soaking process was repeated for a total of 3 times of extraction. Then, the extract was concentrated using a rotary evaporator to obtain crude acetone extract, which was dark brownish semisolid with a mass of 65.42 g.

Extraction of Sample – SFE

The SFE extraction was performed following the method by Zachová et al. (2018) with a slight modification [25]. The plant material (30.4 g) was placed in an extraction column, heated to the desired temperature (50°C), and pressurized to 30 MPa by a high-pressure pump for liquid CO_2. Ethanol (20%) was continuously added to the stream of CO_2 as a polar modifier with a flow rate of 8 mL/min. The extraction was conducted in a dynamic mode for 4 hours. The extract was collected in a pre-weighed conical flask. Ethanol was evaporated using a rotary vacuum evaporator to afford brown extract (0.51 g).

Analytical Procedures

LC-MS/MS Data Acquisition and Processing

The separation process was performed using a Thermo Scientific C_{18} column (AcclaimTM Polar Advantage II, 3 × 150 mm, 3um particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was performed at a flow rate of 0.4 mL/min and 40°C column temperature using H_2O + 0.1% formic acid (A) and 100% acetonitrile (B) with 22 minutes total run time. The injection volume of the sample was 5 µL. The gradient started at 5% B (0-3 min), 80% B (3-10 min), 80% B (10-15 min), and 5% B (15-22 min).

High-resolution mass spectrometry was carried out following the method by Bisson et al. (2011) and Mazlan et al. (2018) with slight modification using a MicroTOF QIII Bruker Daltonic using an ESI positive ionization with the following settings: capillary voltage: 4500 V; capillary temperature: 300°C; nebulizer pressure: 2.0 bar; drying gas: 8 L/min. The mass range was 50-1500 m/z [26,27].

The accurate mass data of the molecular ions, provided by the TOF analyzer, were processed by Compass Data Analysis software (Bruker Daltonik GmbH).

The identification of the peaks was achieved based on the interpretation of MS/MS values using the LOTUS Natural Products database. The web link https://lotus.naturalproducts.net/ was accessed in July and August 2023. The database consists of more than 270,000 patterns of known compounds. It is an open-source project for Natural Products (NPs) storage, search, and analysis. LOTUS is one of the biggest and best annotated resources for NPs occurrences, free of charge and without restriction [28]. The spectra of the unknown components of *A. laevis* crude for both maceration and SFE were compared with the standard mass spectra of known components stored in the library MassBank of North America (MoNA). The web link https://mona.fiehnlab.ucdavis.edu/ was accessed in July and August 2023. It is a metadata-centric, auto-curating repository designed to store and query mass spectral records efficiently. It intends to serve as the framework for a centralized, collaborative database of metabolite mass spectra, metadata, and associated compounds. MoNA currently contains 2,052,966 mass spectral records from experimental and *in-silico* libraries as well as from user contributions.

Peaks with MS/MS data are selected to be identified using LOTUS and MoNA database. For both extraction methods, 70 peaks have been thoroughly analyzed with the value of 5 for search ppm. Nine stilbenoids were identified for maceration and SFE crude, respectively, while others are unknown compounds.

RESULTS AND DISCUSSION

Liquid Chromatography – Mass Spectroscopy (LC-MS) Profiling

The extraction efficiency of the maceration technique (7.27%) is higher than that of SFE (1.69%). The overlay of maceration and SFE with expansion for retention time between 7.5 to 12.5 minutes is shown in **Figure 1**.

Expansion of the chromatogram of maceration overlay with SFE in **Figure 1** revealed 22 peaks discovered in maceration and 20 peaks detected in SFE crude extracts. Peak intensity in maceration crude extract is somewhat more significant than in SFE. In both crude extracts, 20 peaks were comparable. Meanwhile, there were two further peaks in maceration at 9.3 and 10 minutes that were minor compounds (peaks 36 and 53). This demonstrates that the crude extract from SFE is nearly equal to that from maceration.

Based on the LOTUS and MoNA databases, nine stilbenoids were identified in both maceration and SFE crude extracts, namely *trans*-piceid **(1)**, *cis*-piceid **(2)**, resveratrol **(3)**, upunoside C **(4)**, diptoindonesin A **(5)**, pauciflorol B **(6)**, vaticanol B **(7)**, shoreaketone **(8)** and upunaphenol L **(9)**.

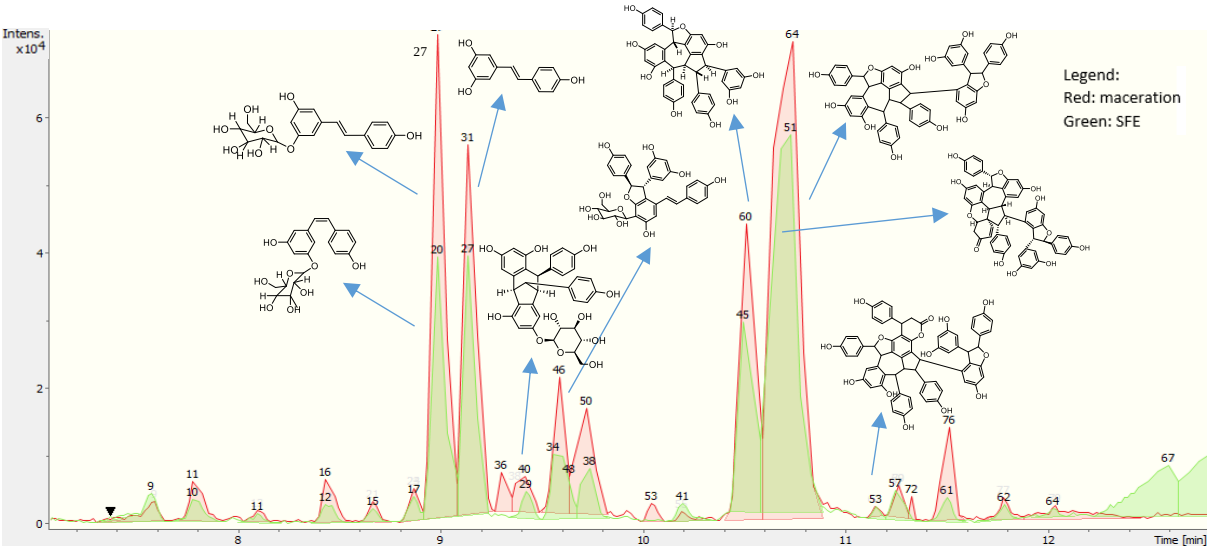

Figure 1. LC-MS Chromatogram of maceration (red) overlay with SFE (green) at a retention time of 7.5-12.5 minutes.

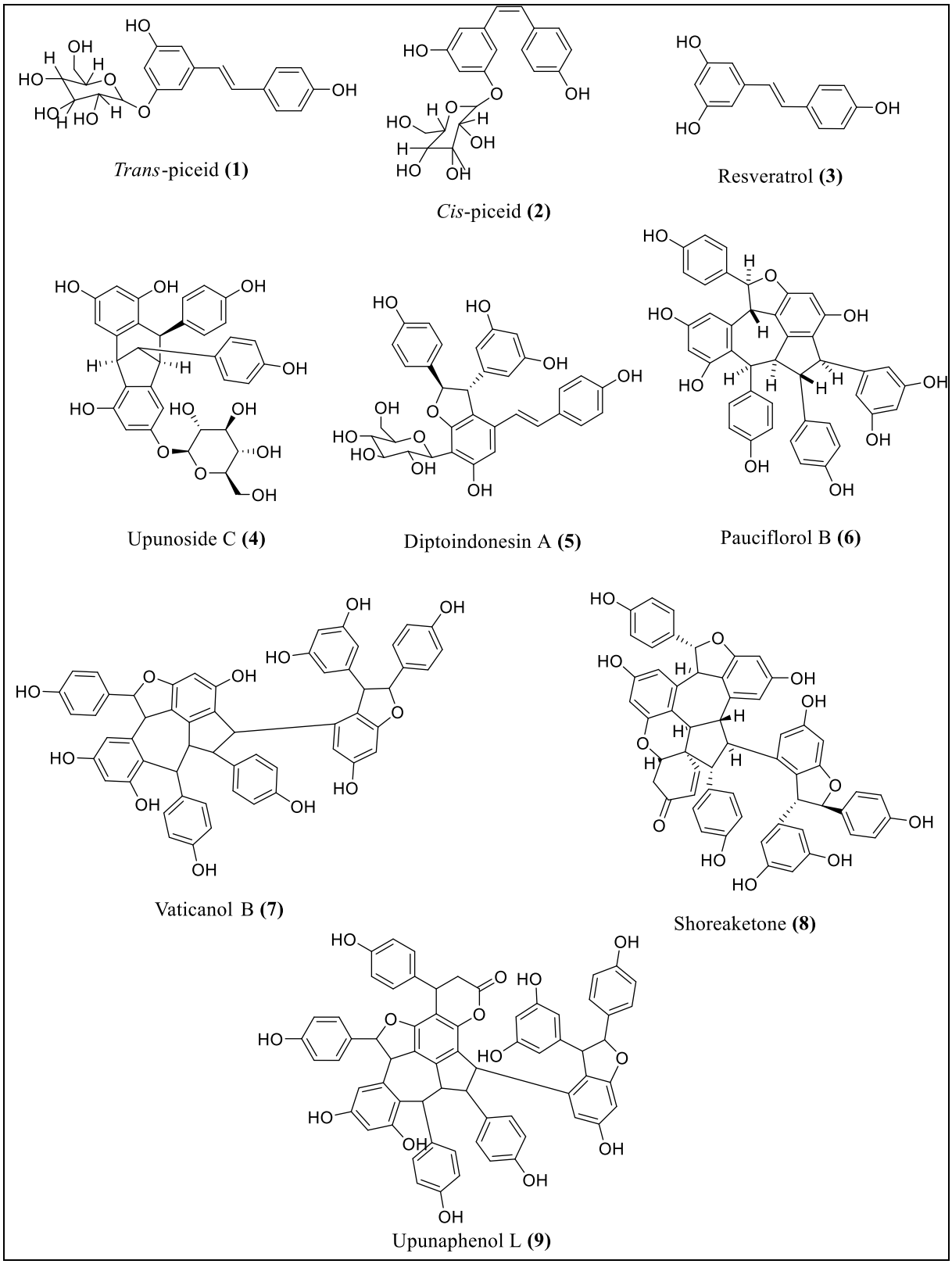

Figure 2. Stilbenoids identified from MS/MS data of maceration and SFE.

These nine stilbenoids displayed in **Figure 2** are first detected from the stem bark of *A. laevis*. Furthermore, based on previous studies, vaticanol B **(7)** was also found in other *Anisoptera* species, such as *A. marginata* and *A. thurifera* [12, 14].

Stilbenoids identified from MS/MS data of maceration and SFE techniques are listed in **Table 1.** Based on the putative stilbenoids identified from the MS/MS values, compared to SFE crude extract, only two unknown compounds did not match the crude

extract from the maceration, indicating the SFE method is almost comparable with the maceration method. Due to that, the SFE method is suitable to be used as an alternative for the extraction technique, which is greener and high technology. Adding ethanol as a polar modifier to the CO_2 fluid that is non-polar has increased the polarity of the solvent, which leads to the capability to extract the polar stilbenoid compounds. The extraction solvents used in this work were chosen due to their practical use with respective techniques and to reduce the extraction of tannin that could affect the purification of stilbenoids.

Table 1. Stilbenoids identified from MS/MS data of maceration and SFE methods.

RT (min)	Name of compound	Molecular formula	*m/z*	Fragment ions
9.0	*Trans*- piceid **(1)** *Cis*-piceid **(2)**	$C_{20}H_{22}O_8$	390.13154	229.08597 241.08597 253.08597 271.09654
9.2	Resveratrol **(3)**	$C_{14}H_{12}O_3$	228.07869	107.04917 119.04917 135.04408 183.08049 211.0754
9.4	Upunoside C **(4)**	$C_{34}H_{32}O_{11}$	616.19457	107.04917 199.0754 215.07031 239.07031 361.10711 373.10711 385.10711 467.149 497.15957
9.6	Diptoindonesin A **(5)**	$C_{34}H_{32}O_{11}$	616.19457	199.0754 215.07031 227.07031 255.06522 349.10711 455.149
10.5	Pauciflorol B **(6)**	$C_{42}H_{32}O_9$	680.20475	199.0754 215.07031 239.07031 331.09654 359.09145 481.12825 493.12825
10.7	Vaticanol B **(7)**	$C_{56}H_{42}O_{12}$	906.26778	199.0754 215.07031 229.08597 331.09654 347.09145 441.13334 477.13334
10.7	Shoreaketone **(8)**	$C_{56}H_{42}O_{12}$	906.26778	199.0754 215.07031 229.08597 331.09654 347.09145 359.09145 477.13334
11.3	Upunaphenol L **(9)**	$C_{57}H_{42}O_{13}$	934.26269	199.0754 229.08597 331.09654 735.18619

CONCLUSIONS

The findings of a comparative study comparing maceration and Supercritical Fluid Extraction (SFE) for extracting stilbenoids from *Anisoptera laevis* Ridl. indicated that both techniques are equally effective. A total of nine stilbenoids were identified using both methodologies, with the maceration crude extract exhibiting two additional peaks. These results indicated that SFE might be a viable alternative for a shorter-lasting and more environmentally friendly extraction method.

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