Two-Dimensional NMR Analysis of *Angiopteris evecta* Rhizome and Improved Extraction Method for Angiopteroside

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ABSTRACT:

Introduction - The rhizome of *Angiopteris evecta* is of academic interest in Kalimantan, Indonesia, from an ethnobotanical perspective. Angiopteroside is a substance of pharmaceutical importance that is found in the rhizome of *A. evecta*.

Objective - The aims of this research are to improve the extraction method for angiopteroside from the rhizome, compared to that in a previous report, and to determine the yield of angiopteroside from the rhizome of *A. evecta*, as well as to obtain precise data for extractives from the rhizome of *A. evecta* by using two-dimensional NMR spectroscopy and liquid chromatography–mass spectrometry (LC-MS).

Methodology - We investigated the chemical constituents of the whole rhizome by means of two-dimensional NMR (heteronuclear single quantum coherence or HSQC) spectroscopy, neutral sugar analysis using the alditol acetate method, and lignin analysis using alkaline nitrobenzene oxidation and Klason lignin methods. LC-MS revealed the purity of the angiopteroside. Antimicrobial assays were also performed for the purified angiopteroside by using a broth microdilution method.

Results - Angiopteroside was isolated by Soxhlet extraction with aqueous acetone followed by preparative thin-layer chromatography (eluent: 20% methanol/dichloromethane). LC-MS revealed that angiopteroside can be found in the rhizome of *A. evecta* in 9.9% yield, which is an extremely high yield for a plant extractive.

Conclusion - HSQC analysis is a powerful tool for surveying compounds in plant materials, such as the whole rhizome of *A. evecta*. Soxhlet extraction with aqueous acetone is an effective method for extracting glycosides from plant materials.

Keywords: *Angiopteris evecta*; Rhizome; Angiopteroside; Soxhlet extraction; Two-dimensional NMR spectroscopy

INTRODUCTION

Angiopteris species are known as medicinal plants (Chen, Tao, Lian, Wang, Zhao, Jiang and Zhang 2010, Winter and Amoroso 2003). We surveyed medicinal plants in Central Kalimantan, Indonesia, from the viewpoint of ethnobotany, and ascertained that rhizomes of *Angiopteris evecta* (G. Forst.) Hoffm. (Marattiaceae) are used as a local medicine by the Dayak people for liver function disorders and to treat fever (Garuda 2011). In fact, the Roengsumran group have reported that a glycoside extract of *A. evecta,* angiopteroside shows significant activity for inhibition of HIV-1 reverse transcriptase (Taveepanich, Kamthong, Sawasdipuksa and Roengsumran 2005). A leaf extract of the plant was also reported to possess anti-tuberculosis activity against *Mycobacterium tuberculosis* H37Rv (Mohamad, Zin, Wahab, Ibrahim, Sulaiman, Zahariluddin and Noor 2011).

By contrast, the Japanese food *Akaboshi zenmai*, consisting of dried leaves of *Osmunda japonica* Thunberg and the Vermont royal fern *Osmunda regalis* var. *spectabilis* (Willd.) A. Gray, was shown to contain the hydroxypentenolide glycoside osmundalin (Hollenbeak and Kuehne 1974). The study of the chemical constituents of the fern *Osmunda japonica* was reportedly prompted by a possible correlation between the unusually high incidence of gastric tumours in Japan and unusual factors in the Japanese diet (Hollenbeak and Kuehne 1974). In addition, osmundalin has been shown to have strong feeding-inhibition activity (80% inhibition at a concentration of 0.2%) for the larvae of the yellow butterfly, *Eurema hecabe mandarina*

(Lepidoptera: Pieridae) (Numata, Takahashi, Fujiki, Kitano, Kitajima and Takemura 1990). Although the carcinogenicity of both angiopteroside and osmundalin has not yet been established (Hseu 1981), some unsaturated lactones and their glycosides have been noted to be cytotoxic and antibiotic agents, for example, ranunculin (Benn and Yelland 1968, Hill and Vanheyningen 1951), parasorbic acid (Reynolds 1975), and tuliposide and parasorboside (Tschesche, Hoppe, Snatzke, Wulff and Fehlhaber 1971).

The aglycone of angiopteroside, (5*S*,6*S*)-5,6-dihydro-5-hydroxy-6-methyl-2H-pyran-2-one, is an epimer of that of osmundalin, (5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-methyl-2H-pyran-2-one (osmundalactone). Although the chemical structures of angiopteroside and osmundalin are epimers (Fig. 1), angiopteroside has not been well studied; only a few reports are available (Hseu 1981, Taveepanich, Kamthong, Sawasdipuksa and Roengsumran 2005).

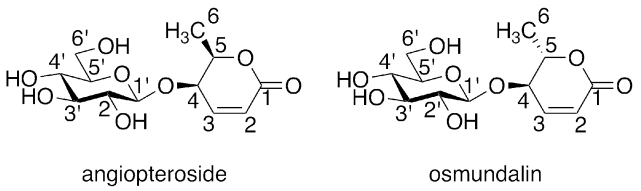

Figure 1. Chemical structures of angiopteroside and osmundalin

Moreover, the extraction method used by the Roengsumran group (Taveepanich, Kamthong, Sawasdipuksa and Roengsumran 2005) is time-consuming: a methanol extract was successively re-extracted with *n*-hexane and ethyl acetate. The ethyl acetate extract was fractionated by silica gel column chromatography and elution with a stepwise gradient of *n*-hexane/ethyl acetate. Angiopteroside was obtained from elution of the silica gel column with 100% ethyl acetate and purified by recrystallization from methanol to obtain crystals. In addition, the yield of angiopteroside has not been described in any previous reports (Hseu 1981, Taveepanich, Kamthong, Sawasdipuksa and Roengsumran 2005).

On the other hand, the Soxhlet method has been reported for the extraction of phenolic components from plant material (Julkunen-Tiitto 1985). This prompted us to research the extraction of angiopteroside from *A. evecta* rhizomes by this simple and efficient method. We examined Soxhlet extraction with aqueous acetone (acetone/water, 7/3–10/0 (v/v)) to obtain angiopteroside in good yield.

Herein, the purification process for angiopteroside from rhizomes of *A. evecta* is discussed, along with analytical methods such as liquid chromatography–mass spectrometry (LC-MS) and heteronuclear single quantum coherence (HSQC) spectroscopy. The neutral sugar and lignin contents of rhizomes of *A. evecta* have also been investigated. Moreover, antimicrobial assays have been performed for the purified angiopteroside against gram-negative (*Escherichia coli*), and gram-positive (*Klebsiella pneumoniae* and *Bacillus subtilis*) bacteria by using a broth microdilution method to confirm whether this compound is responsible for the antimicrobial activity of the *A. evecta* extract.

EXPERIMENTAL

Plant material

A. evecta (G. Forst.) Hoffm. (Marattiaceae) was collected in Central Kalimantan, Indonesia, on March 9th, 2011. The plant was identified by Dr. Ruliyana Susanti and a voucher specimen was deposited at the Herbarium Bogoriense, LIPI (Indonesian Institute of Science), Cibinong, Indonesia, under the collection number RS 209. Fresh rhizomes of *A. evecta* were cut into small pieces, freeze-dried, and then pulverized with a blender. The pulverized rhizomes were used for chemical and spectroscopic analyses.

General measurements

NMR spectra were acquired on Varian 500 and Varian INOVA 300 NMR spectrometers at room temperature. Chemical shifts are referenced to the signals of the solvents used [δ_H/δ_C: methanol, 2.04/29.8 ppm; dimethylsulfoxide (DMSO), 2.49/39.5 ppm]. The standard Varian implementations of one- and two-dimensional (gradient-selected correlation spectroscopy (COSY), HSQC and heteronuclear multiple bond correlaion (HMBC)) NMR experiments were used for structural assignments of purified angiopteroside and its acetylated samples. Adiabatic 2D-HSQC ('GHSQCAD') experiments for the crude rhizome preparations were carried out by using the parameters optimized by Mansfield, Kim, Lu and Ralph (2012). Spectral processing was performed with Bruker's Topspin 3.2 software (Bruker Biospin, Billerica, MA, USA) and used typical matched Gaussian apodization in F2 (LB = -0.8, GB = 0.001) and squared cosine-bell and one level of linear prediction (16 coefficients) in F1. LC-MS was performed on a Shimadzu Prominence LCMS-2020 system (Shimadzu, Kyoto, Japan) by using following conditions: column: Cosmosil-$^5C_{18}$-ARII (Nacalai Tesque, Japan, 4.6 mm internal diameter × 150 mm); column oven: 40 °C; mobile phase: acetonitrile/H_2O containing 0.1% formic acid with a linear gradient from 5% to 30% acetonitrile for 15 min at a 0.3 mL/min flow rate; detection: electrospray ionization-mass spectrometry (ESI-MS) (negative ion mode; total ion monitoring: *m/z* 100–1000; single ion monitoring: *m/z* 335.1). Quantification of angiopteroside in the crude rhizome extracts was based on the single ion peak area monitored at *m/z* 335.1. UV/Vis spectrometry was carried out on a JASCO V-560 spectrophotometer (JASCO, Tokyo, Japan) by using a 1 cm quartz cuvette.

Extraction and purification of angiopteroside

Freeze-dried *A. evecta* rhizomes were pulverized with a blender. The pulverized *A. evecta* rhizomes were dried in a desiccator in vacuo. Crude angiopteroside was isolated from the

pulverized *A. evecta* rhizomes (1.018 g) by Soxhlet extraction with 70–100% aqueous acetone (7/3–10/0 (v/v), 100 mL) for 24 h. The crude extract (273.8 mg) from the *A. evecta* rhizomes obtained with 80% aqueous acetone (8/2 (v/v)) was further purified by precipitation (from ethanol at 4 °C, twice) to give an ethanol-soluble fraction (83.2 mg). The ethanol-soluble fraction was purified by preparative thin-layer chromatography (TLC) (eluent: 20% methanol/dichloromethane) to yield essentially pure angiopteroside (16.4 mg). The yield and purity of the angiopteroside from the rhizomes of *A. evecta* was determined by means of LC-MS analysis. The retention time of angiopteroside was 5.55 min.

Whole rhizome preparation for NMR analysis

Pulverized *A. evecta* rhizomes (~200 mg) were further ball-milled (5 × 10 min milling with a 5 min cooling cycle) with a Fritsch P6 plenary mill (Fritsch GmbH, Idar-Oberstein, Germany) vibrating at 600 rpm with a ZrO_2 vessel (80 mL) containing ZrO_2 ball bearings (10 mm in diameter × 15). The recovered ball-milled cell walls (~60 mg) were then transferred into an NMR tube, dissolved in DMSO-d_6/pyridine-d_5 (4/1 (v/v), 600 µL) under sonication, and then subjected to 2D-HSQC experiments (Mansfield, Kim, Lu and Ralph 2012).

Antibacterial activity assay (CLSI 2006)

Determination of the minimum inhibitory concentration (MIC) was performed by the broth microdilution method that has been validated by the National Committee for Clinical Laboratory Standards (CLSI 2006). The assays were done in triplicate in 96-well microplates. The tested bacterial isolates used in this assay were *Escherichia coli* InaCC B5 (Indonesian Culture Collection B5), *Klebsiella pneumoniae* BCC 1758 (Balitvet Culture Collection 1758), and *Bacillus subtilis* InaCC B1, and the cultures were grown at 35 °C in liquid Muller Hinton broth

(Difco). The population density used for antimicrobial testing was 1–5 × 10^5 cfu/mL. The angiopteroside was dissolved in DMSO and a stock solution was prepared with a double concentration in yeast-malt agar (YMA) medium. The concentrations of isolated compounds and reference antibiotics used for the determination of MIC values were in the range of 128.0–0.25 µg/mL with 5 % DMSO (highest concentration and decrease after serial dilution). Determination of MIC values was achieved by addition of 15 µL (0.5 mg/mL) of iodonitrotetrazolium chloride (Sigma). Two commercial antibiotics, chloramphenicol (Sigma) and erythromycin (Sigma), were used as references.

Chemical Analysis

The neutral sugar composition was determined by the alditol acetate method (Borchardt and Piper 1970). Alkaline nitrobenzene oxidation was conducted according to a modified method (Katahira and Nakatsubo 2001).

Angiopteroside

^{1}H NMR (500 MHz, CD_3OD): δ = 1.47 (d, 3H, *J* = 6.5 Hz, -CH_3), 3.19 (dd, 1H, *J* = 8.0 and 9.0 Hz, H-2'), 3.27 (dd, 1H, *J* = 8.5 and 10.5 Hz, H-4') 3.29–3.32 (m, 1H, H-5'), 3.35 (t, 1H, *J*=9.0 Hz, H-3'), 3.66 (dd, 1H, *J* = 6.0 and 12.0 Hz, H-6'), 3.91 (dd, 1H, *J* = 6.0 and 12.0 Hz, H-6'), 4.43 (d, 1H, *J* = 8.0 Hz, H-1'), 4.49 (dd, 1H, *J* = 3.5 and 5.5 Hz, H-4), 4.72 (ddd, *J* = 3.5, 6.5, and 13.0 Hz, H-5), 6.13 (d, *J* = 10.0 Hz, H-2), 7.17 ppm (dd, *J* = 5.0 and 10.0 Hz, H-3). (See Fig. S1 in the Supporting Information)

^{13}C NMR (125 MHz, CD_3OD): δ = 16.2 (C-6), 62.9 (C-6'), 68.6 (C-4), 71.7 (C-4'), 74.9 (C-2'), 78.0 (C-3'), 78.2 (C-5'), 78.3 (C-5), 102.4 (C-1'), 123.6 (C-2), 145.3 (C-3), 165.9 ppm (C-1). (See Fig. S2 in the Supporting Information)

Acylated angiopteroside

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (d, 3H, *J* = 6.8 Hz, -CH_3), 3.65–3.75 (m, 1H, H-5'), 4.15–4.30 (2H, H-6'), 4.35 (ddd, 1H, *J* = 0.6, 3.6, and 5.1 Hz, H-4), 4.64 (ddd, *J* = 3.6, 6.6, and 13.2 Hz, H-5), 4.67 (d, 1H, *J* = 7.8 Hz, H-1'), 5.02 (dd, 1H, *J* = 7.8 and 9.8 Hz, H-2'), 5.10 (t, 1H, *J* = 9.6 Hz, H-4'), 5.22 (t, 1H, *J* = 9.9 Hz, H-3'), 6.20 (d, *J* = 9.9 Hz, H-2), 6.90 ppm (dd, *J* = 4.5 and 9.9 Hz, H-3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.5 (C-6), 20.5, 20.6, 20.7 (CO<u>C</u>H_3), 61.8(C-6'), 67.8 (C-4), 68.2 (C-2'), 71.0 (C-4'), 72.1 (C-5'), 72.4 (C-3'), 76.4 (C-5), 98.2 (C-1'), 124.3 (C-2), 141.5 (C-3), 162.6 (C-1), 169.2, 169.4, 170.2, 170.5 ppm (<u>C</u>OCH_3).

RESULTS AND DISCUSSION

Soxhlet extraction

Soxhlet extraction with an acetone/water mixed solvent system is common for the extraction of phenolic compounds (Julkunen-Tiitto 1985). We tried the mixed solvent system with various compositions of the two solvents from 70 to 100% acetone to afford extracts A_{70}, A_{80}, A_{90}, and A_{100}, as shown in Figure 2. The yield of extractives decreased from approximately 30% to 20% as the percentage of acetone in the mixed solvent system was increased from 70% to 100% (Fig. 3). We evaluated the yield of pure angiopteroside, extract B, in the extracts by means of LC-MS (Fig. 4). Extracts A were purified by precipitation and preparative TLC to yield extract B for NMR spectroscopic and LC-MS analyses (Figs. S1, S2, 4, and 5C).

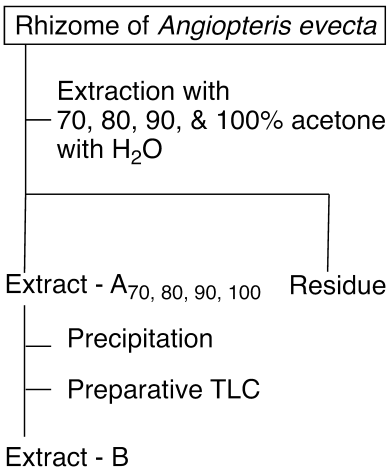

Figure 2. Fractionation of rhizomes of *A. evecta*

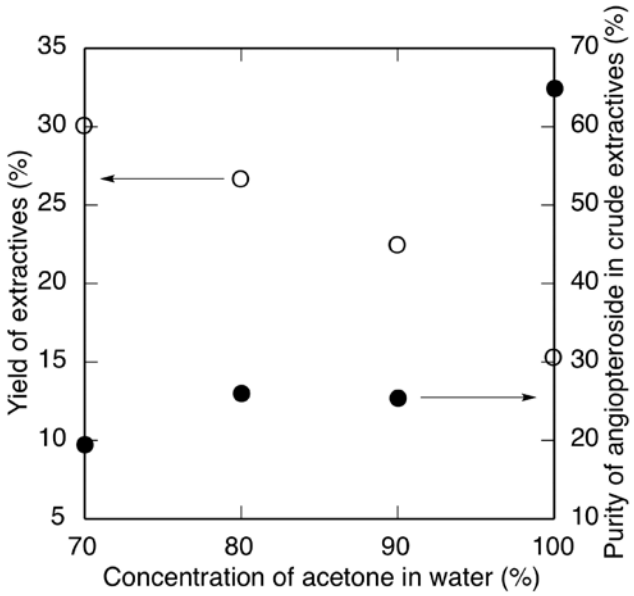

Figure 3. Yield of extractives (extracts A) by Soxhlet extraction with an aqueous acetone solvent system. ○: yield of extractives; ●: purity of angiopteroside in crude extractives

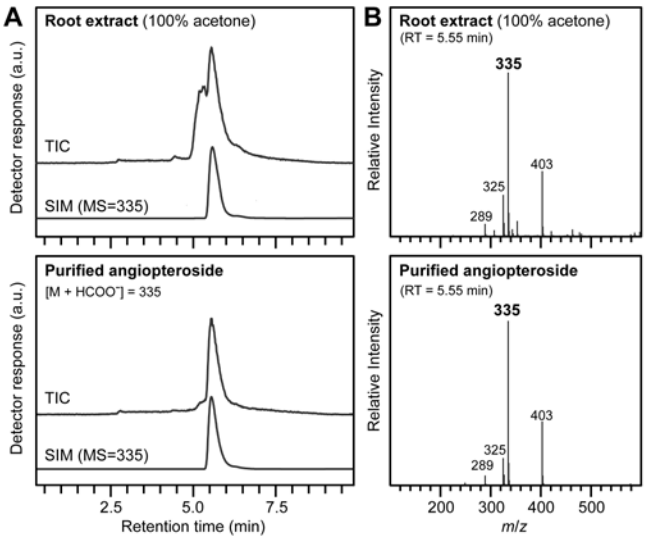

Figure 4. (A) Total ion chromatographs (TICs) and (B) selective-ion monitoring (SIM) for rhizome extract (extracted with 100% acetone) and purified angiopteroside (extract B)

LC-MS analysis of extractives from rhizomes of *A. evecta*

The TICs and SIM for rhizomes extracted with 100% acetone (extract A_{100}) and purified angiopteroside (extract B) are shown in Figure 4. The compound with a retention time of 5.55 min shows *m/z* 335, which indicates a formic acid adduct of angiopteroside, $[M + HCOO]^-$. The compound with a retention time of 5.33 min exhibits *m/z* 343 and is probably an unidentified glycoside. The compound with a retention time of 5.20 min exhibits *m/z* 225 and 179 for the formic acid adduct and $[M - H]^-$ of a hexose, respectively.

The total ion chromatograph of the rhizome extract shows that extract A_{100} contains angiopteroside and some impurities; glucose was the main impurity (confirmed by the alditol acetate method; data not shown). The LC-MS spectrum of purified angiopteroside indicates that the purified sample contains no impurities.

By using the purified angiopteroside, we calculated the weight percentage of angiopteroside (extract B) in the crude rhizome extract (extract A) with the acetone/water solvent system on the

basis of a standard curve of the SIM peak height at *m/z* 335. From the results of LC-MS analysis of extract A_{100} (Soxhlet extraction with 100% acetone), angiopteroside (extract B) in the rhizome of *A. evecta* was found in 9.9% yield (Fig. S3 in the Supporting Information).

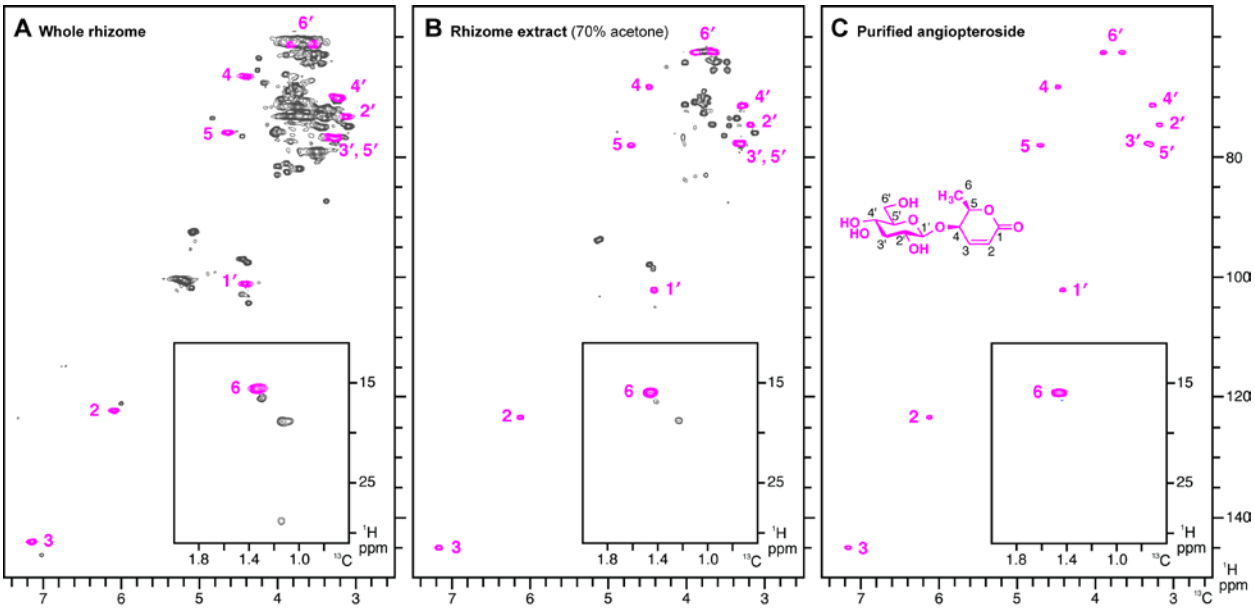

Figure 5. HSQC spectra of (A) a whole rhizome, (B) a rhizome extract (extract A_{70}), and (C) the purified angiopteroside (extract B)

Two-dimensional NMR analysis

The HSQC spectra of a whole rhizome, a rhizome extract (extract A_{70}), and the purified angiopteroside (extract B) are shown in Figure 5. The whole rhizome includes angiopteroside and some carbohydrates (Fig. 5A). Soxhlet extraction of the rhizome of *A. evecta* with acetone/water (70/30 (v/v)) gave a mixture comprising angiopteroside and probably a few carbohydrates (Fig. 5B). After purification of the extract with TLC, the HSQC spectrum (Fig. 5C) indicates that the purified fraction contains no impurities. Thus, HSQC spectroscopy was useful not only to evaluate the purification process of the natural compound but also to elucidate the extractives included in the crude plant material.

Structural analysis of a purified angiopteroside

The aglycone of angiopteroside, (5*S*,6*S*)-5-hydroxy-6-methyl-5,6-dihydro-2*H*-pyran-2-one (Murayama, Sugiyama and Yamashita 1986), is an epimer at the C-5 position of osmundalactone, (5*R*,6*S*)-5-hydroxy-6-methyl-5,6-dihydro-2*H*-pyran-2-one (Hollenbeak and Kuehne 1974, Murayama, Sugiyama and Yamashita 1986). Osmundalactone is the aglycone of osmundalin (Hollenbeak and Kuehne 1974), a hydroxypentenolide glucoside, and the NMR spectra of these two compounds were compared to determine the chemical structure. Hollenbeak and Kuehne (1974) reported that 4,5-substituted Δ^2-pentenolides can be assigned by comparison with analogous pentenolides of *trans* (J = 9.0 Hz) (Rosenbrook and Carney 1970) and *cis* (J = 2–3 Hz) (Achenbach and Wittmann 1970, Argoudelis and Zieserl 1966, Evans, Ellestad and P. 1969, Yamamoto, Suide, Hemmi and Yamano 1970) 4,5-substituted structures. The coupling constant, $J_{4,5}$ = 3.5 Hz, of our compound indicates the *cis* configuration, which means that our compound is angiopteroside, not osmundalin.

Akita and co-workers reported the synthesis of (-)-tetra-*O*-acetylosmundalin (Ono, Zhao, Shida and Akita 2007). Thus, we acetylated our purified compound, angiopteroside, for comparison of the NMR spectra. The protons at C-4 and C-5 of (-)-tetra-*O*-acetylosmundalin appeared at δ = 4.27 (ddd, J = 8.0, 2.4, and 2.0 Hz) and 4.42 ppm (qd, J = 8.0 and 6.0 Hz), respectively. By contrast, those of the acetylated angiopteroside appeared at δ = 4.35 (ddd, J = 5.1, 3.6, and 0.6 Hz) and 4.64 ppm (ddd, J = 13.2, 6.6, and 3.6 Hz), respectively. These data clearly indicate that (-)-tetra-*O*-acetylosmundalin and our acetylated pure compound are not identical, which confirms that our purified extract is angiopteroside.

Preliminary results for the antibacterial activity of angiopteroside

The purified angiopteroside was evaluated for its antibacterial activity against the pathogenic bacteria *E. coli* InaCC B5, *K. pneumoniae* BCC1758, and *B. subtillis* InaCC B1. The results showed that the angiopteroside possessed weak antibacterial activity against the tested bacteria, as shown in Table S1 in the Supporting Information.

Sugar and lignin analyses of *A. evecta* rhizome

We also investigated the neutral sugar and lignin contents in the rhizome of *A. evecta* (Table S2 in the Supporting Information). Glucose was the major sugar component. The Klason lignin content was 8.77%. Although no syringaldehyde was found, *p*-hydroxybenzaldehyde and vanillin were obtained in 0.56 and 2.77% based on Klason lignin, respectively.

CONCLUSION

HSQC analysis (Fig. 5) is a powerful tool for surveying compounds in crude plant materials, such as the whole rhizome of *A. evecta*. Soxhlet extraction method with aqueous acetone is a simple and effective method to extract glycosides from plant materials. The analysis provides evidence that angiopteroside is a major component, 9.9%, in the rhizome of *A. evecta*. The pure angiopteroside could be obtained by Soxhlet extraction with acetone, re-precipitation with ethanol, and preparative thin-layer chromatography. Investigation into the medicinal and biological effects of the rhizome of *A. evecta* is now in progress.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online under the Supporting Information tab for this article.

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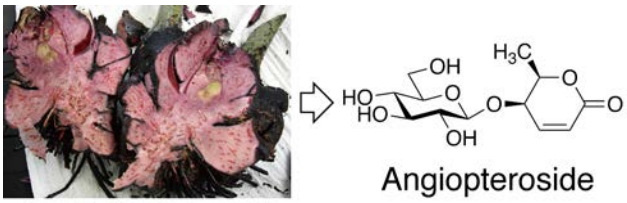

Angiopteris evecta

Short abstract (up to 80 words)

Two-dimensional NMR analysis was determined to be a powerful tool for surveying compounds in crude plant materials, such as the whole rhizome of *Angiopteris evecta*. Soxhlet extraction with acetone was a simple and effective method for extracting glycosides of medicinal interest, such as angiopteroside, from the rhizome of *A. evecta*. Liquid chromatography–mass spectrometry revealed that angiopteroside can be found in the rhizome of *A. evecta* in 9.9% yield, which is an extremely high yield for a plant extractive.