ISOLATION AND STRUCTURE ELUCIDATION OF NATURAL ORGANIC COMPOUNDS ISOLATED FROM THE LEAVES OF *GNETUM GNEMON* L.

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Abstract

The diversity of plants has led to various findings in the medicinal world. The abundance of bioactive existing in the plant is the main factor contributing to its application in pharmaceutical industries. *Gnetum gnemon* L. is an evergreen and perennial gymnosperm belonging to the Gnetaceae family. In Myanmar, it has been consumed as safety foodstuff for centuries. The aim of the present study is to identify the phytochemicals present in the leaves of *G. gnemon*. The crude methanol extract was prepared by maceration method and the obtained extract was concentrated by Rotatory evaporator. The three known constituents were isolated from crude methanol extract through repeated column chromatography separations and elucidated by using UPLC-QTOF/MS, and ^{1}H and ^{13}CNMR spectroscopic technique. The three compounds were elucidated as (1) flavonoidal compound, Vicenin II (2) polyphenol, Piceatannol-3´-*O*-*β*-D-glucopyranoside and (3) Aglycone, Rhapontigenin glucoside by comparison with MS and NMR spectra in the previous literature.

Keywords: *Gnetum gnemon* L., Vicenin II, Piceatannol-3´-O-β-D-glucopyranoside, Rhapontigenin 3´-O-β- D-glycopyranoside

Introduction

The native range of *Gnetum gnemon* L. is Tibet to West Pacific. It is a tree and grows primarily in the wet tropical biome. In Myanmar, it is majorly cultivated and is considered as a vegetable belonging to high status. The leaves (Tanyin-ywet) are mostly used as main constituents in traditionalistic vegetable curry in Tanin-thar- ye Region. Moreover, the female strobilus, the male strobili and the unpeeled seeds (Hin-byin- thee) are used as constituents in a dish in upper regions of Myanmar. The phytochemical screening of *Gnetum gnemon* L. revealed the presence of phenol, flavonoids, alkaloids, tannin, phytosterols and carbohydrates for different extract prepared from leaves. (Preetham *et al*., 2015). Vicenin 2 is a potent inhibitor of alpha glucoside. It has been reported to have a range of pharmacological values which includes antioxidant, hepatoprotective, anti-inflammatory and anticancer (Yang *et al*.,2018). Stilbenoids, Piceatannol is classified as phenolic acid derivatives. It is well known for pharmacological effects such as antioxidant, antimicrobial, anti-inflammatory and antiproliferative effects (Akinwumi *et al*.,2018). Rhapontigenin is aglycone of rhaponticin and it has to be a potent anti-allergic, anticoagulant, and anti-inflammatory compound (Roupe *et al*., 2005). In this study, there has been no reported literature related to phytochemical components in the leaves of *G. gnemon* L. in Myanmar. Therefore, the goal of this study is to determine the chemical composition of *G. gnemon* leaves using spectroscopic techniques.

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Materials and Methods

I. Plant material Collection and Identification

The fresh aerial parts of the plant *Gnetum gnemon* L. were collected from Ye Phyu Township; Thanintharye Division, Myanmar during the flowering period of March 2019–2020. The voucher specimen was deposited at the Plant Extract Bank of Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea.

II. Preparation of plant extracts from dry leaves of *Gnetum gnemon* L.

Firstly, the leaves were washed with distilled water and were dried at 40 °C for 72 hours. The dried leaves were pulverized to a homogeneous powder using an electric blender. The powdered samples were stored in airtight containers to preserve the biomolecules present in the plant and stored at room temperature. Then, the crude plant extract was prepared by the maceration method. The dried samples (5 kg) were extracted with methanol at room temperature, filtered, and evaporated using a rotary evaporator (50°C, 250 rpm) to obtain the total extract (714.5g).

III. Isolation of organic compounds from the methanol extract from dry leaves of *Gnetum gnemon* L.

General experimental procedures

The *Gnetum gnemon L.* methanol extract (250.0g) was fractionated into five fractions using open column chromatography (C.C) using HP-20 resin. The fractionated sample (G2) that contained the targeted peak were isolated by a preparative liquid chromatography system. Furthermore, the peaks were isolated by preparative HPLC to get a pure peak. The preparative HPLC was performed using C_{18} columns, YMC-PACK ODS-AQ-HG, (250 × 20 mm, 10 μm, YMC, Kyoto, Japan), Acclaim Polar Advantage II, (21.2 × 250 mm, 5.0 μm, Dionex, Sunnyvale, CA, USA), and Kinetex Biphenyl (20 × 250 mm, 5.0 μm, Phenomenex, Torrance, CA, USA) and MPLC system: Spot Prep II System using C_{18} column (220g, 250 × 60 mm, 10 μm, YMC). The chemical structure of isolated compounds was elucidated using a JEOL ECZ500R [^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz, (JEOL, Tokyo, Japan)], and ultra-performance liquid chromatography (ACQUITY UPLC, Waters, Milford, MA, USA) equipped with a photodiode array detector (PDA, Waters) coupled to a quadrupole time of flight mass spectrometer (Micromass QTof Premier mass spectrometry, Waters) to collect spectroscopic data such as UV, MS, MS/MS, HRESIMS. AQUITY BEH C_{18} column (1.7 μm, 2.1 × 100 mm, Waters) was used on UPLC-PDA-QTof-MS operated at 35°C with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was as follows: 0.0 min (A: B = 95:5 υ/υ) → 1.0 min (95:5, υ/υ) → 8.0 min (60:40, υ/υ) → 8.3 – 10.0 min (0:100, υ/υ), → 10.30 – 12.0 min (95:5, υ/υ). The flow rate was 0.4 mL/min and the injection volume was 2 μL. MS data were obtained in negative ion mode under the following conditions; source temperature 110 °C, desolations temperature 350 °C, capillary voltage 2.3 kV, and cone voltage 40 V. Qualitative analysis and tentative identification of major constituents from methanol extracts of *G. gnemon* were carried out by ACQUITY UPLC (Waters) system connected to Micromass Q-TOF Premier mass spectrometry (Waters) with an electrospray ionization device, operating in the negative ion mode that were acquired using Mass Lynx software (ver. 4.1, Waters).

Results and Discussion

I. Morphological Characters of Gnetum *gnemon* L.

Scientific Name - *Gnetum gnemon* L.
Local Name - Hyinbyin, Tanyin-ywe
English Name - Melinjo
Family - Gnetaceae
Flowering and Fruiting period - October to February

Evergreen dioecious trees. Leaves simple, opposite, lanceolate in young leaves and elliptic in mature leaves, the exstipulate. Male spikes axillary or terminal, one male spike contain 5-9 involucre collars, each collar contains 12-50 male flower and 3-9 globose sterile female flowers. Male flowers with a claw-shaped perianth, stamen one, stamen adnate to the base of the perianth, synangia (anther) bifid. Female spikes axillary or terminal, one female spike contains 5-9 collars, each collars with 3-9 female flowers. Female flower globose, without perianth, othotropous ovule, ovoid, three integuments. Seed large, drupe-like, pink or red in mature, the fleshy coats enclosing the hard seed, with longitudinally ribbed.

Figure.1 Habit of *Gnetum gnemon* L.

Figure.2 Leaves as seen

Figure.3 Male Strobilus of *Gnetum gnemon* L.

Figure 4 Female Strobilus of *Gnetum gnemon* L.

Figure 5 Fruits as seen

(1) Identification of compound 1 from the methanol extract of leaves of *Gnetum gnemon* L.

The molecular formula of compound **1** was deduced as $C_{27}H_{30}O_{15}$ from the deprotonated ion peak at *m/z* 593.1505 $[M - H]^-$ in HR-ESI-MS data. For the precursor ion at *m/z* 593, the presence of di-*C*-glucosidic linkages were assumed based on the two product ion peaks at *m/z* 473 [M – H – 120] and *m/z* 353 [M – H – 120 – 120] in the fragmented MS spectrum. Vitexin is a well-known, naturally occurring flavonoid that has the apigenin skeleton (Kim *et al*., 2005). The ^1H and ^{13}C NMR spectra of compound 3 were similar to those of Vitexin, except for the presence of an additional *β*-anomeric signal [δ_H 4.74 (H-1''') and δ_C 74.2 ppm (C-1''')]. Compound 1 was identified as Vicenin 2 based on comparisons with the NMR and MS spectroscopic data in the reported literature (Islam *et al*., 2014 and Wang *et al*., 2021).

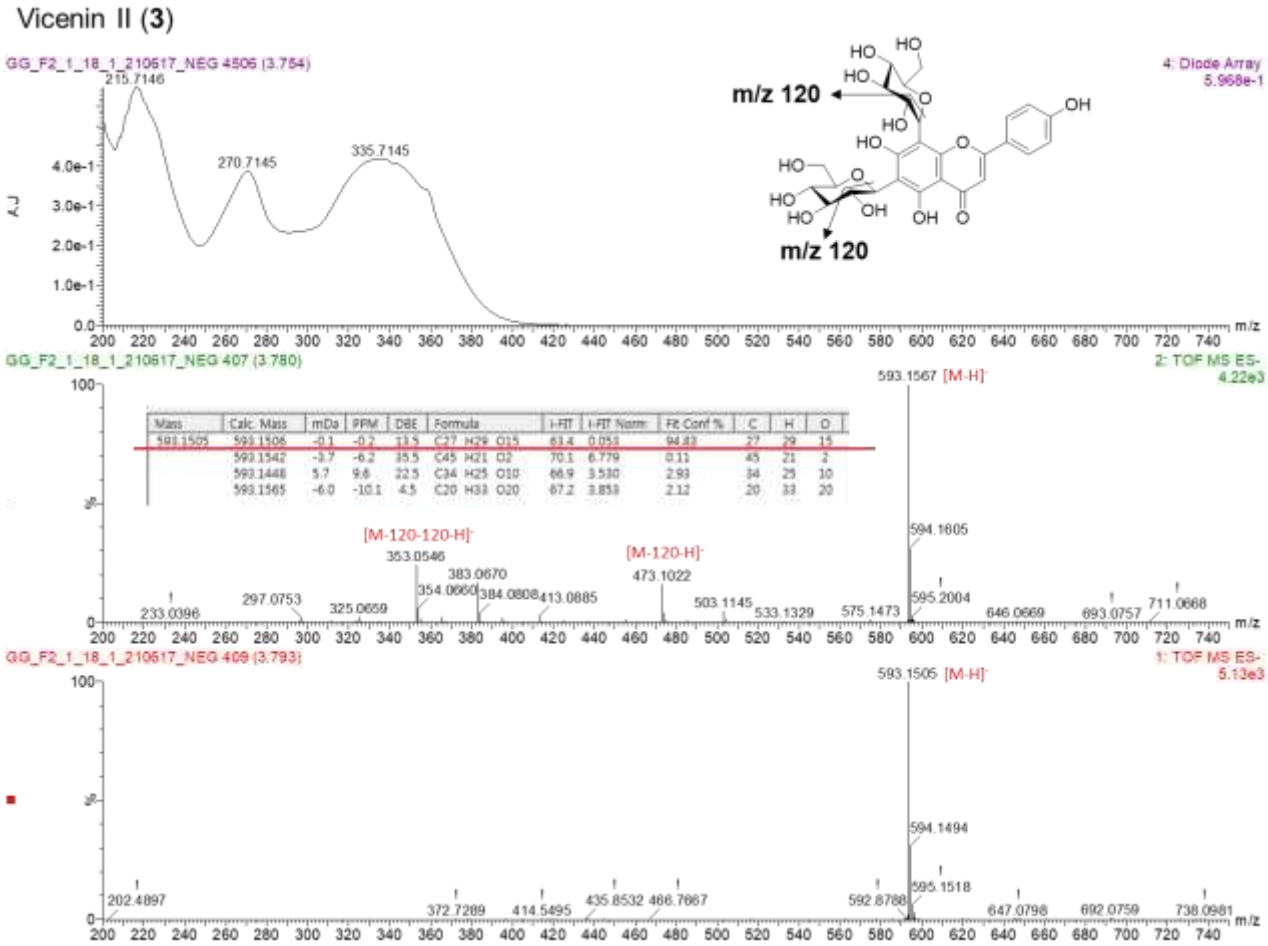


Figure. 6 UV and UPLC-QTOF-MS spectra of Vicenin II

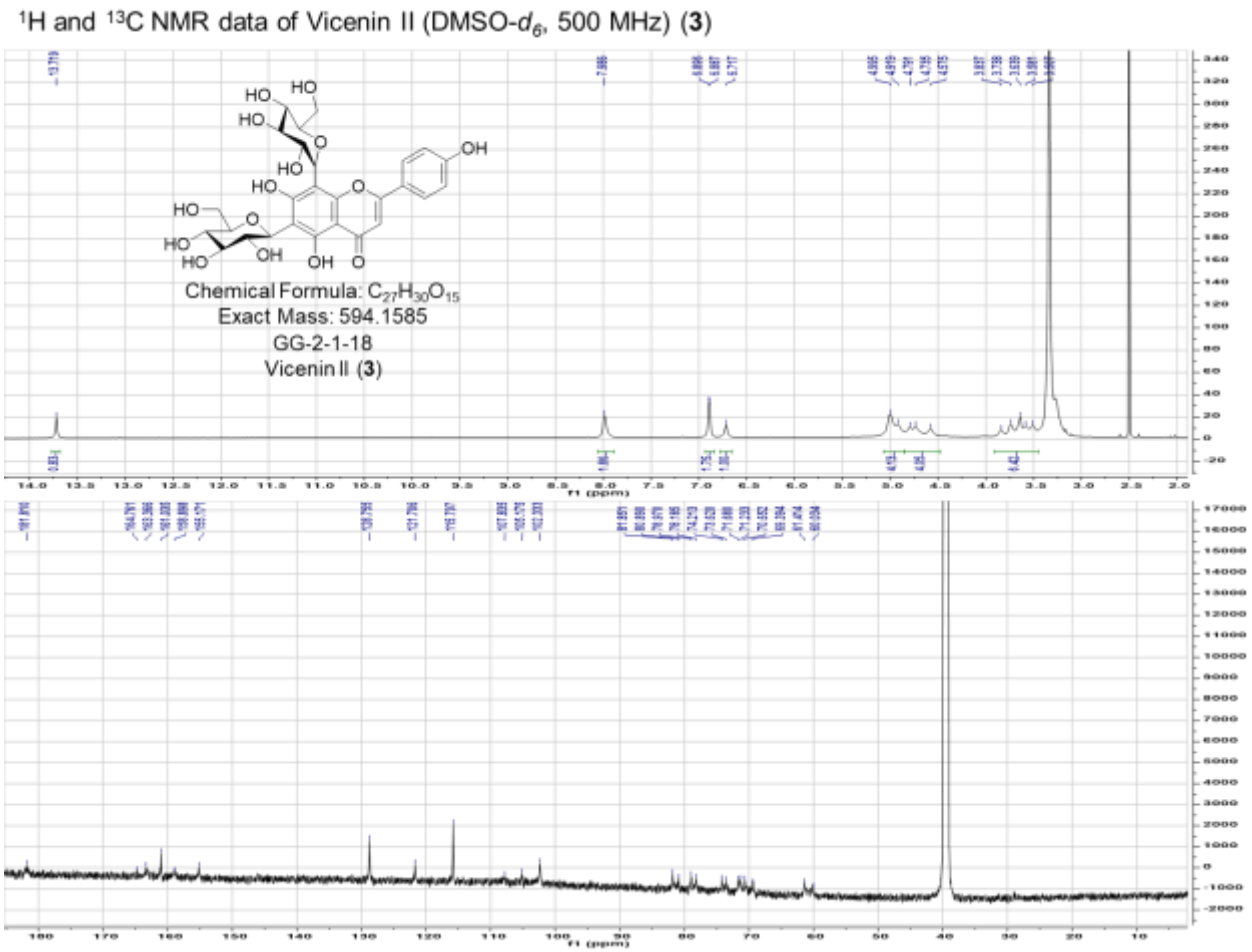


Figure.7 ^{1}H and ^{13}C NMR (500 MHz, methanol-d_4) spectrum of Vicenin II

(2) Identification of compound 2 from the methanol extract of leaves of *Gnetum gnemon* L.

The molecular formula of compound 2 was determined to be $C_{20}H_{22}O_9$ by analyzing a pseudomolecular ion peak at *m/z* 405.1230 in the HR-ESI-MS spectrum and the fragmented MS spectrum of the parent ion at 405 produced ions at *m/z* 243 [M – hexose]$^-$. The ^{1}H NMR data of compound 2 displays *trans*-olefinic protons [δ_H 6.90, 6.85 (d, J = 16.0 Hz)], 1,3,4-trisubstituted aromatic ring signals [δ_H 7.08 (1H, d, J = 8.0 Hz); 7.05 (1H, d, J = 2.0 Hz); 6.93 (1H, dd, J = 8.0, 2.0 Hz)], 1,3,5-trisubstituted aromatic ring signals [δ_H 6.39 (2H, d, J = 2.0); 6.12 (1H, t, J = 2.0 Hz)], and glucose resonances [δ_H 4.69 (1H, d, J = 7.0, H-1"); 3.17–3.72 (6H, m)]. The HMBC correlation of anomeric proton (H-1") to C-3' (δ_H 145.2) placed the glucose group at the 1,3,5-trisubstituted aromatic group. Comparison of these MS and NMR spectral data with those previously reported in the literature (Kashiwada *et al*., 1990) suggested that compound 2 was Piceatannol-3'-*O*-*β*-D-glucopyranoside.

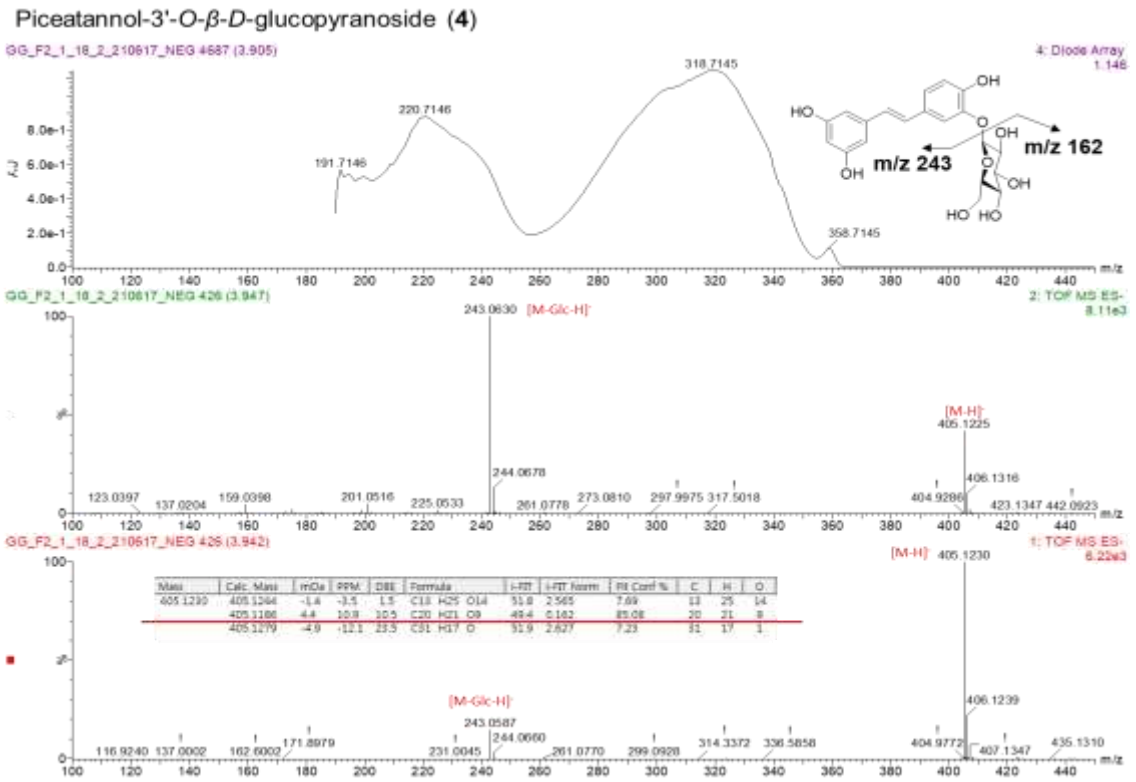


Figure 8. UV and UPLC-QTOF-MS spectra of Piceatannol-3'-*O*-*β*-*D*-glucopyranoside

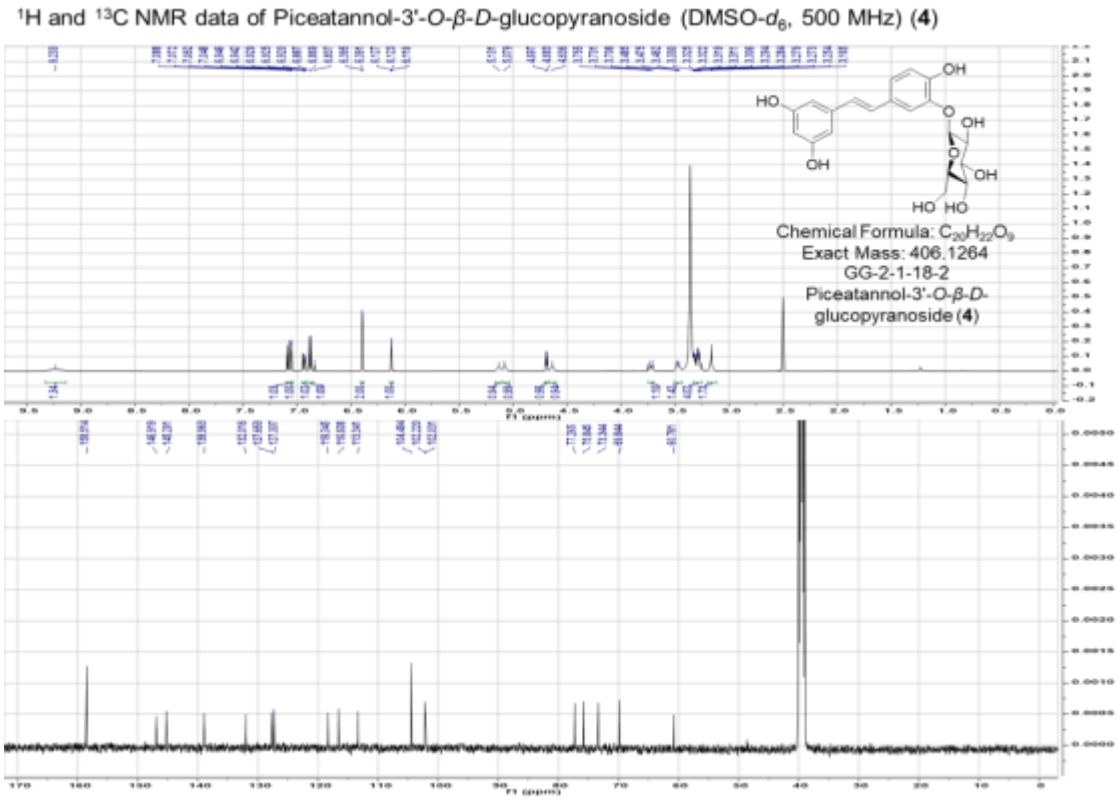
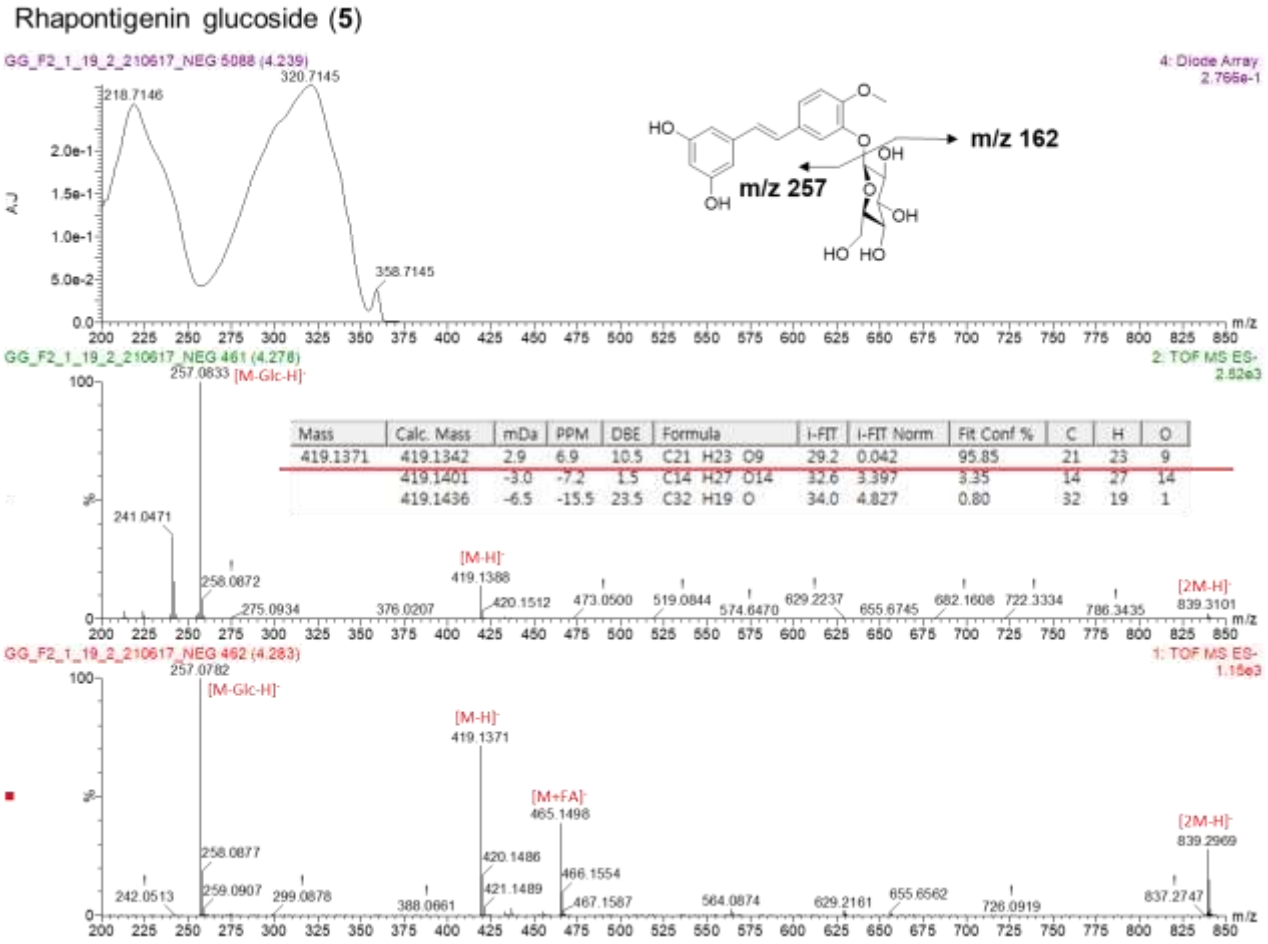


Figure 9. ^{1}H and ^{13}C NMR (500 MHz, DMSO-d_6) spectrum of Piceatannol-3'-*O*-*β*-*D*-Glucopyranoside

(3) Identification of compound 3 from the methanol extract of leaves of *Gnetum gnemon* L.

The molecular formula of compound 3 was deduced as $C_{21}H_{24}O_9$ from deprotonated ion peak at *m/z* 419.1371 in the HR-ESI-MS data. The UV, HRMS, ^{1}H and ^{13}C NMR spectroscopic data were similar to those of Piceatannol-3'-*O*-*β*-D-glucopyranoside, except for additional methoxy group [δ_H 3.82, (3H, s, OCH_3-4'); δ_C 55.7]. The position of the methoxy group was determined to be at C-4' (δ_C 149.1) based on the HMBC cross-peak between OCH_3-4' and C-4'. Therefore, compound 3 was assigned to be Rhapontigenin-3'-*O*-*β*-D-glucopyranoside (Kashiwada *et al*., 1990).



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
419.1371	419.1342	2.9	6.9	10.5	C21 H23 O9	29.2	0.042	95.85	21	23	9
	419.1401	-3.0	-7.2	1.5	C14 H27 O14	32.6	3.397	3.35	14	27	14
	419.1436	-6.5	-15.5	23.5	C32 H19 O	34.0	4.827	0.80	32	19	1

Figure 10. UV and UPLC-QTOF-MS spectra of Rhapontigenin-3'-*O*-*β*-D-glucopyranoside

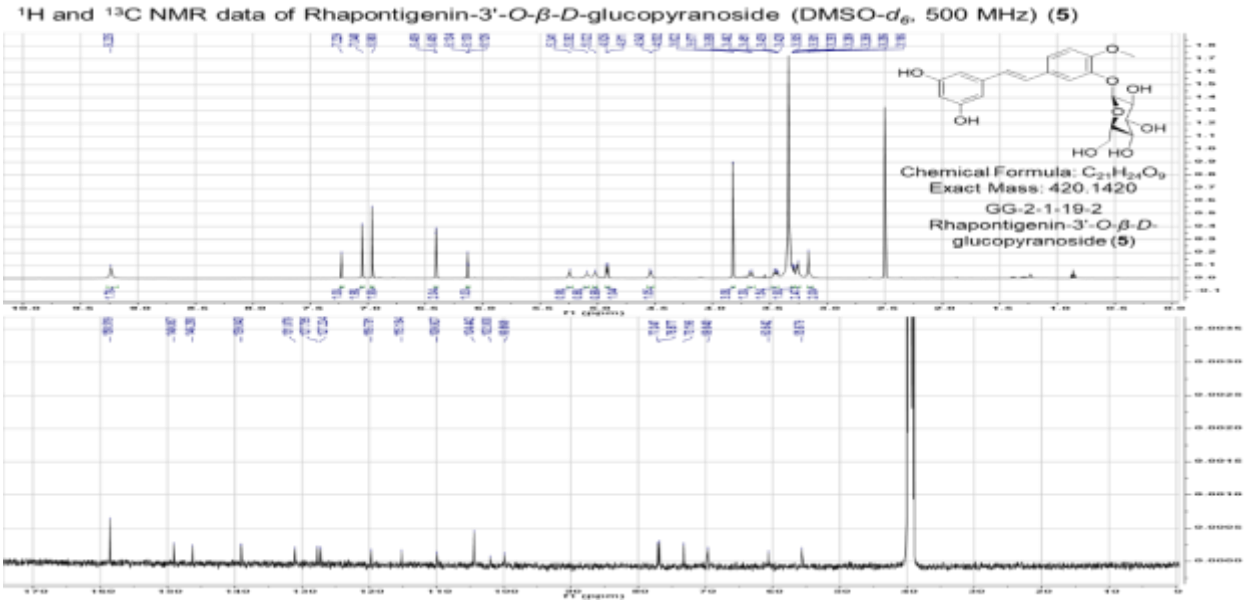


Figure. 11 ^{1}H and ^{13}C NMR (500 MHz, DMSO-d_6) spectrum of Rhapontigenin-3'-*O*-*β*-*D*-glucopyranoside

Conclusion

The leaves of *Gnetum gnemon* L. was selected to isolate pure organic compounds. The isolated three compounds are Vicenin II, Piceatannol-3´-O-β-D-glycopyranoside, Rhapontigenin-3´-O-β-D-glycopyranoside. The molecular formula of compound 1**,** Vicenin II was deduced as $C_{27}H_{30}O_{15}$ from the deprotonated ion peak at *m/z* 593.1505 $[M - H]^-$ in HR-ESI-MS data, whereas the molecular formula of compound 2, Piceatannol-3´-O-β-D-glycopyranoside was determined to be $C_{20}H_{22}O_9$ by analyzing a pseudomolecular ion peak at *m/z* 405.1230 in the HR-ESI-MS spectrum. The molecular formula of compound 3 was deduced as $C_{21}H_{24}O_9$ from deprotonated ion peak at *m/z* 419.1371 in the HR-ESI-MS data.

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