SHORT REPORT

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Determination of gymnemic acid level in *Gymnema inodorum* leaves using multiple reaction monitoring mass spectrometry

Wanwisa Srinuanchai, Rawiwan Nooin, Suwatchai Jarussophon, Kittiwut Kasemwong and Onanong Nuchuchua*

National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Thailand

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Abstract: Gymnemic acid (GA) is known as the antidiabetic phytoconstituent in *Gymnema* species, and is used for quality control and standardization of *Gymnema* products. In Thailand, a number of nutraceutical products of *Gymnema inodorum* (Lour.) Decne. (GI) are commercially available and this number is increasing. However, standardized GA content information and safety usage guidelines for GI have not been published. The aim of this study was to investigate the amount of GA constituent in GI leaves using mass spectrometry. Leaf samples were randomly collected in the Northern part of Thailand. Total GA was extracted using an ethanolic solution. The calculation of total GA was based on the detection of aglycone gymnemagenin using the liquid chromatography/electrospray ionization mass spectrometry with multiple reaction monitoring. The results showed that GI contained total GA content in range of 0.20-1.44 mg per one kilogram dry leaves.

Keywords: *Gymnema inodoru;* gymnemic acid; gymnemagenin; mass spectrometry; electrospray ionization; multiple reaction monitoring.

1. Sample Source

The samples of *G. inodorum* (Lour.) Decne leaves (GI1, GI2, GI3 and GI4) were collected in Thailand. GI1 was a local plant collected from Chiang-Rai province (BK No.070338) authenticated by Plant Varieties Protection Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. GI2 and GI3 were a gift from Chaopraya Abhaiphubejhr Hospital, Prachin Buri Province and Chiangmai Bioveggie Co. Ltd. GI4 was a supplementary diet capsule containing a fine powder of dried *G inodorum* leaves (Gathong Brand, Chiang-Mai, Thailand). *Gymnema sylvestre* leaves were used as a control group in this study. The ground powder of *G. sylvestre* leaves (GS1) were purchased from HERBAL HILLS®, India. GS2 was dietary supplement capsule containing *G. sylvestre* extract (Swanson Health Products, USA). The native *G. sylvestre* extract (GS3) was obtained from Sigma-Aldrich, USA. However, the samples of *G. inodorum* (GI1, GI2, GI3 and GI4) and *G. sylvestre* (GS1, GS2 and GS3) did not specify the gymnemic acid content according to their labels.

2. Previous Studies

Gymnemic acid (GA) is a large group of triterpenoid glycosides found in *Gymnema sylvestre* R. Br. leaves (Ayurvedic medicine). More than fifteen structures of the GA have been elucidated,

Corresponding author: E- Mail:onanong@nanotec.or.th (O. Nuchuchua), Phone +66-2117-6792.

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comprising of two domains: pentacyclic triterpene skeleton of gymnemagenin (GM, Figure 1) and sugar substitutions (e.g., glucose, galactose and glucoronic acid) [1]. GA has shown a great potency in decreasing acute and chronic hyperglycemia as observed in diabetic animals and humans [2 - 5]. GA can delay the absorption of glucose into the blood stream [6]. In addition, Tiwari et al. [7] showed that several structures of GA had different antidiabetic potency using multidisciplinary studies, such as quantitative structure activity relationship (QSAR), molecular docking and pharmacokinetic properties against peroxisome proliferator activated receptor gamma (PPARγ). However, the quantification of each GA derivative is still a limiting factor to antidiabetic activity optimization.

Recently, the content of total GA is typically used for the standardization and quality control of the dried leaves and extract of *G. sylvestre* [8]. Since the derivatives of GA are comprised of the GM skeleton, the quantity of GM is equivalent to that of GA. The detection of GM can be carried out by using thin layer chromatography [9, 10], high performance liquid chromatography with UV or mass detectors [11 - 13]. In addition, Kamble et al. [14] showed that the method of HPLC-MS electrospray ionization with multiple reaction monitoring was a high throughput technique for measuring GM aglycone of total GA derivatives in *G. sylvestre* extract and commercial products. The amount of the GA contained in *G. sylvestre* leaves and its natural products can be in the range of 0.05-4.0%w/w depending on the origin of plant material, processing method and product formulation [11, 15]. In order to develop quality products of *G. inodorum* leaves, information on the GA content of *G. inodorum* is needed.

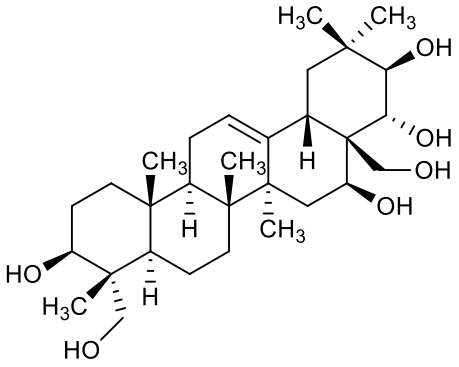

Figure 1. Structure of gymnemagenin

3. Present study

The GA of *G. inodorum* (GI1, GI2, GI3 and GI4) and *G. sylvestre* (GS1, GS2 and GS3) were extracted with 50%v/v ethanol solution in water. The extracts were refluxed with a high concentration of hydrochloric acid and potassium hydroxide to eliminate substitutions on the GM structure. In this study, GM can be monitored by UPLC-ESI-MS/MS with multiple reaction monitoring method. Four MRM transitions of GM ($[M+H]^+$ = 507.72) were selected: $[M+H]^+$ of 489.5, 471.9, 454.0 and 145.4 using collision cell energy of 9 V, 13 V, 17 V and 45 V, respectively. Other mass parameters such as ion source voltage (IS), declustering potential (DP), entrance potential (EP), and collision exit cell (CXP) were 4500 V, 144 V, 9 V, 54 V and 22 V, respectively.

This recent study, the ICH guideline [16] was used as the reference method to develop a reliable LC-MS technique for measuring gymnemic acid. The parameters included linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. Ten different concentrations of GM ranging from 0.001 - 0.480 mg/L were prepared for the calibration plot. The method of LC-MS showed excellent linearity (R^2 = 0.9999) with the regression equation y = 366799x - 815.51 in the above concentration range. The LOD and LOQ were 0.0017 mg/L and 0.0052 mg/L respectively. The GM standard at low (0.048 mg/L) and high concentrations (0.241 mg/L) were used for the determination of precision (intra- and inter-day). The %RSD values of the LC-MS method were found

to be 0.10 and 0.098 at low and high concentrations for intra-day precision while the %RSD of the method was 0.96 and 0.95 at low and high concentrations for inter-day precision respectively. Accuracy of the method was studied using a standard addition of GM in the hydrolyzed samples of GI3 and GS2 and percentage recovery of GM was calculated. Mean percentage recovery for GM was 100.54, 90.87 and 94.64 at low, medium and high levels. The results were shown in Table S1. The validation parameters suggested that the LC-MS method was successfully developed for the determination of GM in the samples of *G. inodorum* and *G. sylvestre*.

The liquid chromatography-mass spectrometry using electrospray ionization and MRM mode was validated and applicable to characterize GM in the leaf samples of *G. inodorum* and *G. sylvestre*. GM in the *G. inodorum* and *G. sylvestre* samples was found at retention time of 9.85 minutes (Figure 2a and 2b) as similar to that of GM standard (Figure 2c). The amount of GM was a representative number of the GA level. The result in Table 1 showed that the leaves of *G. inodorun* and *G. sylvestre* contained the GA in the range of 0.20-1.44 mg and 1.0-2.2 g per kilogram dry matter, respectively. However, the GA in *G. inodorum* leaves was found in a very low level, as compared to the lowest amount of GA found in *G. sylvestre* leaves. In general, the amount of gymnemic acid in *G. sylvestre* can be observed in range of 0.5-2.0 g per one kilogram of dry leaves, depending on cultivation area [13, 17], a part of plant [15], seasonal harvesting and post-harvesting [18]. In case of *G. inodorum*, it is possible that the GA is not a main antidiabetic component. A care should be taken when using gymnemagenin as a chemical standard for the quality control and standardization of *G. inodorum* leaf material and its nutraceutical products.

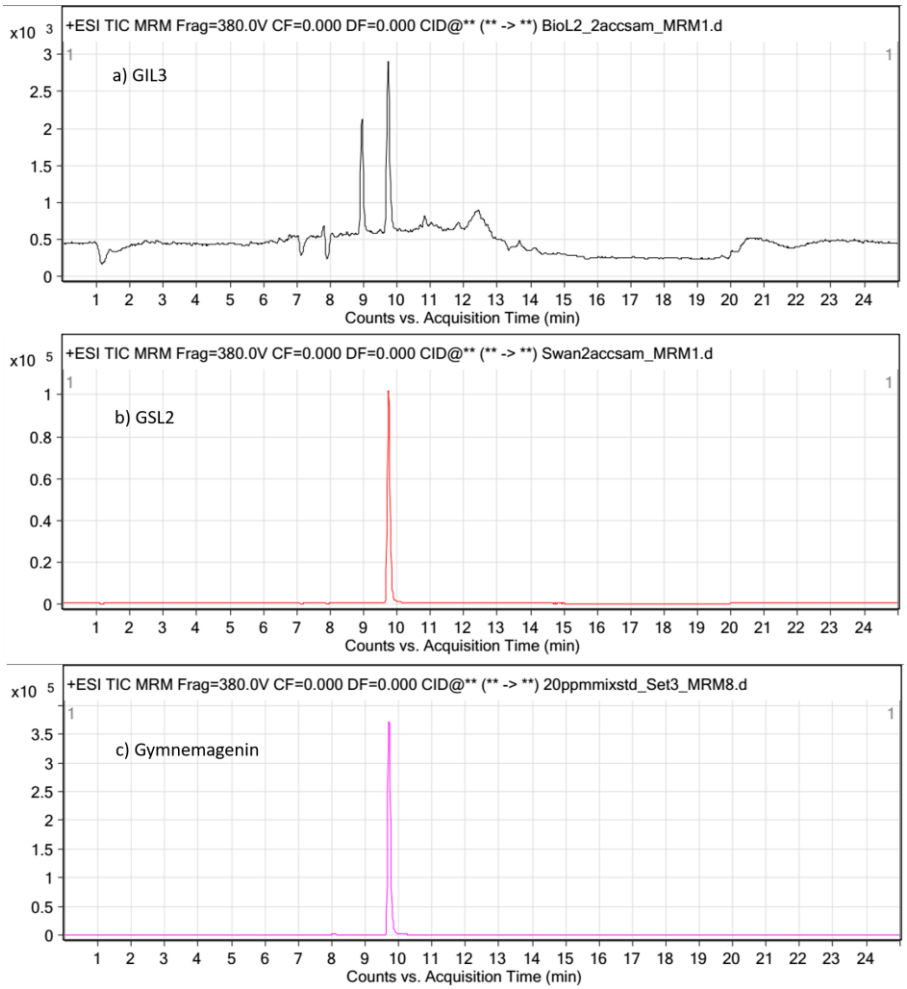

Figure 2. LC-MS Chromatograms of gymnemagenin in *G. inodorum* (GI3) and *G. sylvestre* (GS2)

Table 1. Gymnemic acid content in dried leaf samples of *G. inodorum* and *G. sylvestre* measured by liquid chromatography/electrospray ionization mass spectrometry with multiple reaction monitoring

Plant species	Sample codes	Gymnemic acid* content in extracts (mg/kg)	Gymnemic acid* content in dried leaf samples (mg/kg)
G. inodorum	GI1[a]	0.62 ± 0.08	0.20 ± 0.02
	GI2[a]	2.05 ± 0.10	0.63 ± 0.03
	GI3[a]	2.30 ± 0.04	0.69 ± 0.14
	GI4[b]	4.45 ± 0.08	1.44 ± 0.03
G. sylvestre	GS1[a]	1,084.30 ± 31.16	191.54 ± 13.16
	GS2[c]	2,204.80 ± 45.68	-
	GS3[d]	1,913.44 ± 126.50	-

* Equivalent to the amount of gymnemagenin quantified from mass spectrometry technique.
a) Dried leaf sample.
b) Dietary supplement capsules containing dried leaf powder.
c) Dietary supplement capsules containing an extract.
d) The native gymnema extract (USP standard).

Furthermore, Shimizu et al. [19] found four new triterpene derivatives, which were not constructed from gymnemagenin aglycone and did not suppress sweet taste as compared to gymnemic acid from *G. sylvestre*. Three out of four compounds were able to control blood glucose levels in mice through the inhibition of intestinal glucose uptake as similar to the action of gymnemic acid in *G. sylvestre* [19, 20]. However, the study of Shimizu et al. [19] did not report the contribution of each triterpene component in the *G. inodorum* leaf extract. Currently, the triterpenes in *G. inodorum* leaves have been isolated in our laboratory and being studied to use as a standard chemical for a quality control of *G. inodorum* raw material and its products.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/journal-of-chemical-metrology

ORCID
Wanwisa Srinuanchai: 0000-0002-8688-5780
Rawiwan Nooin: 0000-0002-0512-768X
Suwatchai Jarussophon: 0000-0002-5650-8650
Kittiwut Kasemwong: 0000-0003-2156-8183
Onanong Nuchuchua: 0000-0002-8861-3009

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