Phytochemistry 150 (2018) 12–22

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Discrimination of different geographic varieties of *Gymnema sylvestre*, an anti-sweet plant used for the treatment of type 2 diabetes

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ARTICLE INFO

Article history:
Received 2 December 2017
Received in revised form
2 February 2018
Accepted 20 February 2018

Keywords:
Gymnema sylvestre
Asclepiadaceae
Geo-diversity
Morphology
Anatomy
ITS sequences
Gymnemoside
3β-glucuronide oleane-triterpene
Glucose uptake

ABSTRACT

Gymnema sylvestre (Retz.) R.Br. ex Sm. (Asclepiadaceae) is a well-known Ayurvedic anti-sweet plant for the treatment of type 2 diabetes mellitus. Although it was previously proposed that G. *sylvestre* exhibits chemical variation based on geography, most research on *G. sylvestre* has used material originating from India. Morphological and anatomical descriptions, ITS1-5.8S-ITS2 DNA sequencing, and acid hydrolysis analyses showed that *G. sylvestre* samples from Vietnam are distinguishable from those of Indian origin and thus suggest a dissimilarity among *G. sylvestre* samples with different geographic distributions. An LC-MS-guided strategy targeting 3β-glucuronide oleane-triterpenes in the Vietnamese *G. sylvestre* variety led to the isolation of four known compounds and nine previously undescribed compounds, named gymnemosides ND1-ND9. None of the isolated compounds were reported in the Indian sample, further supporting the geo-diversity of *G. sylvestre*. Three compounds, gymnemosides ND7-9, exerted significant stimulatory effects on the uptake of 2-NBDG in 3T3-L1 adipocyte cells and thus have potential as lead molecules for anti-diabetes agents.

1. Introduction

In recent years, a growing awareness of the relationship between functional foods and health has led to increased interest in the development of physiological functional plants due to their potential health benefits (Zhao et al., 2017). *Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Asclepiadaceae) is a well-known medicinal plant with a long history of use in Ayurvedic traditional medicine and has been studied extensively for its effectiveness in the treatment of type 2 diabetes mellitus (T2DM) (Pothuraju et al., 2014). This plant has been used in formulations such as a simple tea brew, tea bags, beverages and confectioneries (Tiwari et al., 2014) and has also been applied in various food preparations for the regulation of sugar homeostasis and the control of obesity and blood cholesterol levels. *G. sylvestre* has been blended with wheat (*Triticum aestivum*), legumes, non-fat dry milk, vegetable oils and spices to formulate suitable dietary supplements or meal alternatives for non-insulin-dependent diabetes patients (Shobana et al., 2007).

Most studies of *G. sylvestre* have been performed using material from India, and its main active components are a group of gymnemic acids with a β-glucuronic acid at C-3 and a hydroxyl substitution at C-23 on an oleane triterpene-type aglycone (Pothuraju et al., 2014). These gymnemic acids have long been recognized for their role in selectively suppressing sweet taste sensations in humans (Warren and Pfaffmann, 1959) (Frank et al., 1992) (Gent et al., 1999). Kashima et al. recently suggested that the subjective sweet taste intensity was decreased among volunteers administered *G. sylvestre* compared with a control group and revealed the role of an extract of *G. sylvestre* in delaying postprandial gastrointestinal blood flow and gastric emptying, which might affect the

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https://doi.org/10.1016/j.phytochem.2018.02.013
0031-9422/

subsequent glycemic metabolism (Kashima et al., 2017).

An LC-MS analysis of extracts of *G. sylvestre* from different geographic distributions (India, Vietnam and China) subjected to acid hydrolysis revealed similarities in the LC patterns between samples from Vietnam and China but significant discrepancies with the samples of Indian origin. Specifically, gymnemagenin, a 23-hydroxyl triterpene aglycone, was found in the Indian sample but not in the Vietnamese and Chinese samples (Fig. S7, Supplementary data). This result is consistent with the proposed chemical variation in *G. sylvestre* varieties from China, which are characterized by the absence of a 23-hydroxyl functional group in their oleanane-type triterpene glycosides (Ye et al., 2001b). Variations in the chemical composition of a medicinal plant can influence its pharmacological activity, safety and standardization. Thus, in this study, we investigated the discrimination of two varieties of *G. sylvestre* using different approaches, including morphological and anatomical analyses and ITS1-5.8S-ITS2 DNA sequence comparisons. Furthermore, an isolation process targeting 3β-glucuronide oleanane-triterpenes from *G. sylvestre* collected from Vietnam was performed and resulted in the purification and elucidation of nine previously undescribed compounds, named gymnemosides ND1-ND9 (**1**–**9**), and four known compounds (**10**–**13**). All the isolates were evaluated to assess their effect on glucose uptake in differentiated 3T3-L1 adipocyte cells using 2-NBDG as a fluorescent-tagged glucose probe with the aim of identifying the potential of the Vietnamese *G. sylvestre* variety for the treatment of T2DM.

2. Results and discussion

2.1. Morphological and anatomical analysis of two Gymnema sylvestre varieties

Detailed descriptions of the macroscopic and microscopic characteristics of two samples, Vietnamese *G. sylvestre* variety (GS-V) and Indian *G. sylvestre* variety (GS-I), revealed many similar morphological traits matching those of *G. sylvestre* (Retz.) R.Br. ex Sm., described in Flora of China (Wu and Raven, 1995) (Figs. S1 and S2, Supplementary data). Despite these similarities, some distinctive characteristics could be used to differentiate the two samples (Fig. 1A): (1) young branchlets that were glabrescent or pubescent in GS-V but densely pubescent in GS-I; (2) leaf blades were diverse and varied from obovate to ovate in both samples but were more likely to be obovate and thickly papery in GS-V but obovate and thinner in GS-I; (3) adaxial and abaxial leaves that were nearly glabrous and slightly pubescent at the mid-vein in GS-V but pubescent at the midvein in GS-I; (4) four to five pairs of lateral veins in GS-V, in contrast to three to four pairs in the venation system of GS-I, with three more prominent veins converging at the base; and (5) follicle fruits that were broadly lanceolate with an acuminated beak on top in GS-V but smaller fruits with a narrowly lanceolate shape and no beak in GS-I (see Fig. 2).

To confirm the scientific names of these two samples, we further compared their morphological characteristics with TYPE specimens of *G. sylvestre* deposited at the Museum National d'Histoire Naturelle, Paris, France. The GS-V sample was similar to the HOLOTYPE specimen MNHN-P-P04256786 collected in Tonkin, Vietnam (Fig. S3, Supplementary data), whereas the GS-I sample was comparable to the "TYPE" specimen MNHN-P-P00645841 from India (Fig. S4, Supplementary data). Another SYNTYPE specimen, MNHN-P-P00442712 (Fig. S5, Supplementary data) from Madagascar (Africa), also matched GS-I. All type specimens mentioned above were identified as *G. sylvestre*. Although the two studied samples were determined to be the same species, their differences were sufficiently obvious, indicating that the samples represent two different varieties of *G. sylvestre* (Retz.) R.Br. ex Sm.

2.2. ITS1-5.8S-ITS2 sequence analysis of different Gymnema accessions

The ITS region encompasses two noncoding regions, ITS1 and ITS2, separated by the highly conserved 5.8S rRNA gene (White et al., 1990). A multiple alignment analysis of 21 samples also illustrated the conservation of the 5.8S region, with only two single nucleotide polymorphisms (SNPs). Variations between samples mainly occurred in the ITS1 and ITS2 regions (Fig. S6-A, Supplementary data), promising significant separation among closely related species. Accordingly, the neighbor-joining phylogenetic tree showed clear divisions among all the samples at the inter-species level, with pairwise genetic distances based on identity that varied from 90.9% (between the *G. sylvestre* Indian variety and *G. latifolium*) to 96.4% (between the *G. sylvestre* Vietnamese variety and *G. yunnanense*) (Fig. S6-B, Supplementary data). At the intra-species level, the *G. sylvestre* samples were divided into two groups (Fig. 1B) that strongly referred to the native origins of Vietnam and India. The molecular differences between the two groups of *G. sylvestre* samples were consistent with the morphological analysis and further supported the discrimination of the two varieties.

2.3. Isolation and structural elucidation of compounds from the Vietnamese Gymnema sylvestre variety

Through LC-MS in the positive mass fragmentation mode, 3β-glucuronide oleane-triterpenes can be effectively discriminated from other triterpenes in *G. sylvestre* based on a neutral loss of 176 Da (corresponding to glucuronic acid). Thus, an LC-MS-guided strategy was used to isolate the target glucuronide triterpenes from *G. sylvestre* with the following procedure: (1) extraction of *G. sylvestre* leaves with 60% EtOH under ultrasonic conditions; (2) column chromatography (CC)-based separation using macroporous resin; (3) open silica gel CC to obtain the enriched triterpenoid fraction; (4) purification using RP-18 (CC), Sephadex LH-20 (CC) and semi-preparative HPLC in a successive manner; and (5) structural elucidation by MS, NMR and acid hydrolysis/HPLC analysis. As a result, nine previously undescribed compounds, named gymnemoside ND1-ND9 (**1**–**9**), and four known compounds (**10**–**13**) were identified.

Gymnemoside ND1 **(1),** obtained as an amorphous powder with α_D^{25} -24.5^0 (*c* 0.2, MeOH), has the molecular formula $C_{42}H_{66}O_{16}$, as determined by the deprotonated molecular ion peak at *m/z* 825.4315[M−H]$^-$ (calcd for $C_{42}H_{65}O_{16}$, 825.4278), and 10 indices of hydrogen deficiency. The IR spectrum showed strong absorptions at 3399, 2943 and 1706 cm^{-1}, indicating the presence of hydroxyl and carbonyl functionalities. In the ^{1}H NMR spectrum, six methyl singlets at δ_H 0.81, 0.97, 0.99, 1.26, 1.38 and 1.58 (each 3H, s) were observed. Furthermore, one olefinic proton signal at δ_H 5.31 (1H, br s) and two anomeric protons at δ_H 4.98 (d, J = 7.5 Hz) and δ_H 5.37 (d, J = 8.0 Hz) were apparent. The ^{13}C NMR spectrum showed signals for 42 carbons, including two carboxyl groups at δ_C 181.6 and 172.5, two olefinic carbon signals at δ_C 143.6 and 123.5, two anomeric carbons at δ_C 107.0 and 106.1, and 11 oxygenated carbons in the range from δ_C 62.7 to 89.3. The above spectroscopic data suggested that **1** is an oleane-type triterpene with two sugar moieties (Yoshikawa et al., 1998). The carboxylic acid at δ_C 181.6 was assigned to C-29 through its HMBC correlations with H-30 (δ_H 1.58), H-19 (δ_H 2.70), and H-21 (δ_H 2.52). Through a comparison to the literature and an HMBC analysis, the oxygenated methylene protons at δ_H 4.41 (d, J = 10.3 Hz) and δ_H 3.75 (d, J = 10.3 Hz) were attached to C-28 (δ_C 68.2), and the oxygenated methine proton at δ_H 4.81 (dd, J = 12.0, 4.9 Hz) was posited at C-16 (δ_C 67.0) (Ye et al., 2000). The relative configuration of the aglycone was analyzed