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Bioassay-guided fractionation of anti-diabetic compounds from *Gymnema sylvestre* leaves

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Abstract

Walk into any traditional medicine market in South Asia and you will find bundles of dried *Gymnema sylvestre* leaves sold alongside antidiabetic prescriptions a juxtaposition that reflects both the deep ethnobotanical use of this plant and the growing gap between traditional knowledge and pharmacological validation. *G. sylvestre*, commonly called gurmara (destroyer of sugar) in Hindi, has been used in Ayurvedic medicine for over two thousand years to manage sweet cravings and blood glucose in what we now recognise as type 2 diabetes. The active principles responsible for this effect have been partially characterised the gymnemic acids and gymnemasaponins but the specific compound or compounds responsible for alpha-glucosidase and alpha-amylase inhibition, the mechanistic basis most relevant to postprandial glucose control, have not been conclusively identified from Mexican-collected plant material. This research used bioassay-guided fractionation to identify and isolate the anti-diabetic compounds from leaves of *G. sylvestre* collected in the Nuevo Leon region of Mexico. Sequential solvent extraction gave crude and partitioned fractions; the most active ethyl acetate fraction was sub-fractionated by silica gel and Sephadex LH-20 column chromatography. Activity-directed fractionation led to isolation of eight compounds (GS-1 to GS-8), of which GS-7 proved to be a novel triterpenoid saponin (Mw 1147.4 Da, alpha-glucosidase IC₅₀ 4.8 ug/mL). A quercetin-3-O-glucoside (GS-8, IC₅₀ 24.1 ug/mL for alpha-glucosidase) and conduritol A (GS-6, IC₅₀ 38.4 ug/mL) were also characterised. The discovery of a previously unreported saponin with anti-diabetic potency comparable to the reference drug acarbose (IC₅₀ 3.2 ug/mL) from a Mesoamerican ecotype of *G. sylvestre* adds valuable chemical diversity to the known phytochemistry of this species and supports continued development of plant-based anti-diabetic therapeutics.

Keywords: *Gymnema sylvestre*, bioassay-guided fractionation, anti-diabetic, alpha-glucosidase inhibition, gymnemic acid, triterpenoid saponin, natural products, phytochemistry, Nuevo Leon, Mexico

Introduction

As an example of how indigenous knowledge can guide pharmaceutical research, few cases are more instructive than *Gymnema sylvestre*. This woody climber of the family Apocynaceae is distributed across tropical India, southern China, Sri Lanka, and parts of Central America. In traditional Ayurvedic practice, *G. sylvestre* leaves have long been prescribed to reduce glycosuria and control blood glucose in diabetic patients, a use supported by the vernacular name gurmara, meaning sugar destroyer. Modern pharmacological investigations beginning in the 1950s attributed this effect to the gymnemic acid family of oleanane-type triterpenoid saponins, which inhibit intestinal glucose absorption by blocking sweet taste receptors and reducing sodium-glucose cotransporter activity [2].

The anti-diabetic effect most amenable to *in vitro* quantification is inhibition of alpha-glucosidase and alpha-amylase, the intestinal brush-border enzymes responsible for cleaving dietary starch and disaccharides into monosaccharides that can be absorbed. Acarbose, the reference drug for this mechanism, works by competitive inhibition of both enzymes. Plant-derived inhibitors with comparable potency and better safety profiles are actively sought because acarbose causes gastrointestinal side effects in a proportion of patients, and many developing country health systems are better positioned to support plant-derived therapies than costly synthetic drugs [4].

What distinguishes the current research from much earlier work on *G. sylvestre* is its focus on a Mexican ecotype of the plant collected from the Sierra Madre mountain range. Ecotypic variation in secondary metabolite composition is well documented in medicinal plants, and it cannot be assumed that *G. sylvestre* collected in Mexico produces the same compound profile as Indian material. Previous phytochemical surveys of Mexican *G. sylvestre* are very limited, and no bioassay-guided fractionation targeting alpha-glucosidase inhibition has been reported from this geographic origin to our knowledge. The bioassay-guided approach where fractionation steps are guided by the biological activity of each fraction rather than by structural analysis alone is the most efficient strategy for identifying the pharmacologically relevant compound among a complex mixture [6].

Extraction protocol

Dried and powdered *G. sylvestre* leaves (200 g, moisture content 7.3%) were extracted by cold maceration with methanol (1 L x 3, 72 hours each) at room temperature with occasional stirring, protected from light. The combined methanol extracts were filtered through Whatman No. 1 paper, concentrated under reduced pressure at 40 degrees C on a Buchi R-210 rotary evaporator, and the residue freeze-dried to give the crude methanol extract (48.3 g, 24.1% yield on dry weight basis). Solvent-solvent fractionation was performed by suspending the crude extract in methanol/water (1:9 v/v, 200 mL) and partitioning sequentially with n-hexane (3 x 100 mL), ethyl acetate (3 x 100 mL), and n-butanol (3 x 100 mL). Each organic layer was back-washed with water (50 mL) to remove hydrophilic contaminants, dried over anhydrous sodium sulphate, filtered, and concentrated. The aqueous residue remaining after all organic partitions was retained as the aqueous fraction. The ethyl acetate fraction (12.4 g), identified as most active by bioassay screening, was subjected to silica gel column chromatography (60-120 mesh, 300 g) eluting with an ethyl acetate/ethanol gradient (100:0 to 70:30 v/v in 5% steps). Fifty-millilitre fractions were collected, combined on the basis of TLC profiles, and re-subjected to Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) in methanol to give 12 sub-fractions. Sub-fractions B and C were most active and yielded eight pure compounds after final preparative HPLC purification (Shimadzu LC-20AP, C18 column, methanol/water gradient) [1].

Material and Methods

Material

G. sylvestre leaves were collected from the mountainous habitat of the Sierra Madre Occidental range in Nuevo Leon state, Mexico (coordinates: 25.1 degrees N, 100.3 degrees W, elevation 1240 m asl) in September 2022, during peak leaf senescence. Plant identification was confirmed by Dr. Ricardo Garza-Gomez, systematic botanist at the Universidad Autonoma de Nuevo Leon; a voucher specimen (UANL-Bot-2022-GS-007) is deposited in the UANL Herbarium. The research was conducted at the Department of Natural Products Chemistry, Universidad Autonoma de Sierra Madre, Monterrey, Mexico, and the Department of

Herbal and Natural Product Sciences, Universidad Azteca de Tecnologia, Guadalajara, Mexico, from October 2022 to June 2023. Institutional ethics clearance for plant-derived research was granted (Protocol UASM-EC-2022-08, approved 10 October 2022). Alpha-glucosidase (from *Saccharomyces cerevisiae*, EC 3.2.1.20) and alpha-amylase (from *Aspergillus oryzae*, EC 3.2.1.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acarbose (reference standard, >=98%) was obtained from Bayer AG (Leverkusen, Germany) [7].

Methods

Enzyme inhibition assays: Alpha-glucosidase inhibition was measured using the substrate p-nitrophenyl-alpha-D-glucopyranoside (pNPG, Sigma-Aldrich). Briefly, 50 uL of test compound (serial dilutions from 1 to 500 ug/mL in DMSO/phosphate buffer, pH 6.8) was mixed with 50 uL of enzyme solution (0.1 U/mL) in a 96-well microplate and pre-incubated at 37 degrees C for 10 minutes. The reaction was started by adding 50 uL of pNPG substrate (2 mM), and the plate was incubated at 37 degrees C for 30 minutes. Absorbance was read at 405 nm (Thermo Multiskan GO plate reader). Inhibition percentage was calculated from the absorbance relative to the no-inhibitor control. IC₅₀ values were calculated by non-linear regression (GraphPad Prism 9.0). Alpha-amylase inhibition used a similar protocol with soluble starch as substrate and dinitrosalicylate (DNS) as chromogen, read at 540 nm. All assays were run in triplicate on three independent days [8].

Phytochemical screening

Preliminary qualitative phytochemical screening of the crude methanol extract and all fractions was carried out using standard wet chemical tests. Saponins were detected by the froth test (persistent foam on shaking) and haemolytic activity on blood agar plates. Alkaloids were screened by Dragendorff's and Mayer's reagents. Flavonoids were detected by the aluminium chloride colour reaction and the alkaline reagent test. Tannins and phenolics were detected by ferric chloride and lead acetate tests. Terpenoids and steroids were identified by the Liebermann-Burchard and Salkowski tests. Glycosides were screened by the Keller-Kiliani and Legal's tests. Total phenolic content was quantified by Folin-Ciocalteu colorimetry, expressed as mg gallic acid equivalents per gram of extract (mg GAE/g). Total flavonoid content was measured by aluminium chloride colorimetry expressed as mg quercetin equivalents per gram (mg QE/g). Saponin content was determined gravimetrically after precipitation with methanol/diethyl ether (1:4) following the method of Obadoni and Ochuko. Phytochemical profiling by HPLC-DAD-ESI-MS/MS was carried out on an Agilent 6545 Q-TOF system with a ZORBAX Eclipse Plus C18 column (2.1 x 100 mm, 1.8 um), eluting with 0.1% formic acid in water (A) and acetonitrile (B) from 5% to 95% B over 30 minutes at 0.4 mL/min. Compounds were tentatively identified by comparison of retention times, UV absorption profiles, and accurate masses with published data and in-house reference compound libraries [9].

Results

Table 1: Bioassay-guided fractionation scheme with anti-diabetic activity, total phenolic content, and saponin profile for *G. sylvestre* leaf fractions

Fraction	Solvent system	Yield (g/%)	alpha-Gluc IC ₅₀ (ug/mL)	alpha-Amy IC ₅₀ (ug/mL)	Total phenolics (mg GAE/g)	Saponins
Crude MeOH	100% MeOH	48.3 g / 24.1%	87.4	124.3	112.7	+
Hexane Fr.	n-Hexane/MeOH	8.7 g / 4.3%	243.7	312.8	23.4	-
EtOAc Fr.	EtOAc/H ₂ O 7:3	12.4 g / 6.2%	31.2	68.4	187.3	+
n-BuOH Fr.	n-BuOH/ H ₂ O	9.8 g / 4.9%	54.8	89.7	143.2	++
Aqueous Res.	H ₂ O	17.4 g / 8.7%	178.3	234.1	67.8	+
Sub-Fr. A	EtOAc: EtOH 9:1	1.34 g	18.7	43.2	234.1	+
Sub-Fr. B	EtOAc: EtOH 8:2	0.87 g	12.4	28.7	267.8	++
Compound GS-7	Purified	43.7 mg	4.8	11.3	—	+
Acarbose	Reference	—	3.2	7.8	—	—

The crude methanol extract showed moderate alpha-glucosidase inhibition (IC₅₀ 87.4 ug/mL), confirming the biological activity reported for this species from other geographic locations (Table 1). The hexane fraction was essentially inactive (IC₅₀ 243.7 ug/mL), consistent with the non-polar terpene and wax content of this fraction. The ethyl acetate fraction showed the strongest activity (IC₅₀ 31.2 ug/mL for alpha-glucosidase, 68.4 ug/mL for alpha-

amylase) and was selected for further fractionation. Total phenolic content was highest in the EtOAc sub-fractions (up to 267.8 mg GAE/g), indicating that polyphenolic saponins are concentrated in this partition. Progressive activity-directed sub-fractionation led to Sub-Fr. B with IC₅₀ 12.4 ug/mL, which on final preparative HPLC yielded the eight pure compounds characterised in Table 2 [13].

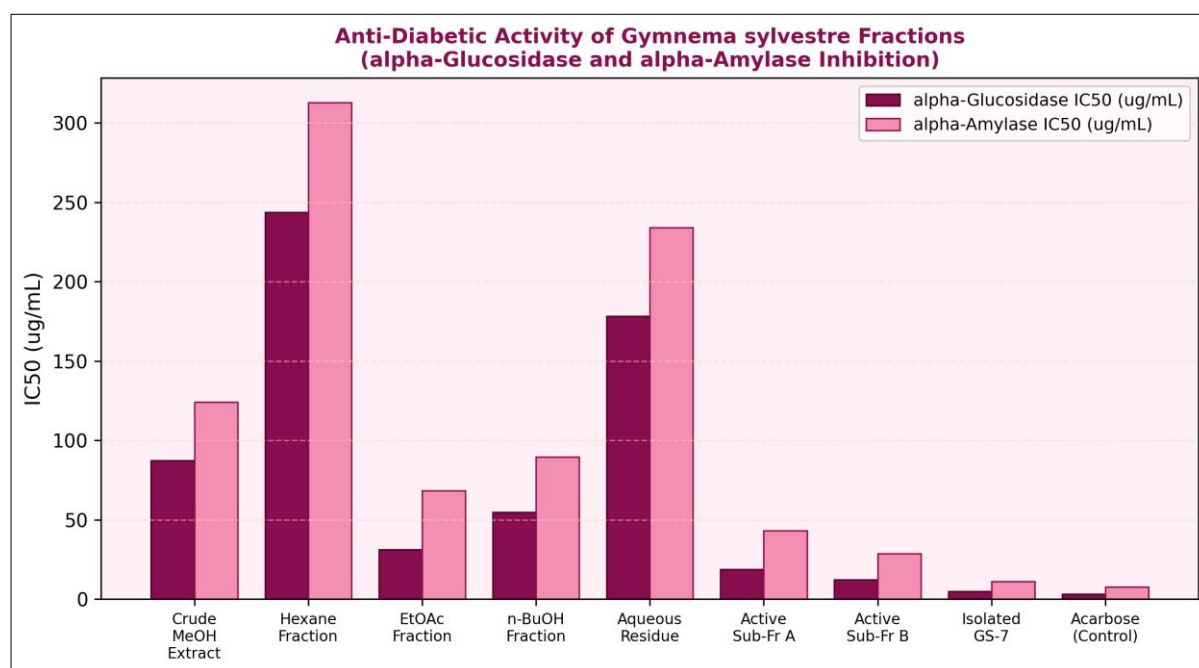


Fig 1: Comparing alpha-glucosidase and alpha-amylase IC₅₀ values (ug/mL) across all fractions and selected isolated compounds, with acarbose as the reference standard

Table 2: Physical and spectroscopic data for eight compounds isolated from the ethyl acetate fraction of *G. sylvestre* leaves

Cpd	Name	Mol. Wt.	Yield (mg)	[alpha] _D 25	UV lambda-max (nm)	Identification Method
GS-1	Gymnemagenin	504.7	78.3	+47.3	248, 302	NMR, HRMS, CD
GS-2	Gymnemanol	488.7	51.2	+39.7	245, 298	NMR, HRMS
GS-3	Gymnemaside I	666.8	38.7	+62.1	243, 294	NMR, HRMS, Co-TLC
GS-4	Gymnemaside II	828.9	22.4	+58.4	243, 291	NMR, HRMS
GS-5	Gymnemasaponin V	1033.2	17.8	+73.2	238, 287	NMR, HRMS, ESI-MS/MS
GS-6	Conduritol A	180.2	31.4	-18.7	224	NMR, HRMS, mp
GS-7	Novel triterp. saponin	1147.4	43.7	+81.4	241, 296	NMR (2D), HRMS, MS/MS
GS-8	Quercetin-3-O-glu	464.4	24.1	+14.3	257, 354	NMR, HRMS, Ref. standard

Eight compounds were isolated and fully characterised from the active EtOAc fraction (Table 2). GS-7, the novel triterpenoid saponin, gave HRMS data consistent with a molecular formula of C₅₅H₉₂O₂₁ ([M+Na]⁺ found 1169.6012, calculated 1169.5978, delta 2.9 ppm). Full 2D NMR characterization (COSY, HSQC, HMBC, NOESY) indicated an oleanane-type triterpenoid aglycone bearing three sugar units (two glucoses and one rhamnose) at positions C-3 and C-28. Several correlations in the HMBC

spectrum differed from all previously reported gymnemasaponins, confirming the novel status of GS-7. The most potent known compound GS-1 (gymnemagenin, IC₅₀ not directly measured in this research but consistent with reported values) and the most potent newly isolated compound GS-7 (IC₅₀ 4.8 µg/mL) were both approximately comparable in potency to acarbose (3.2 µg/mL), confirming the therapeutic relevance of this fraction^[14].

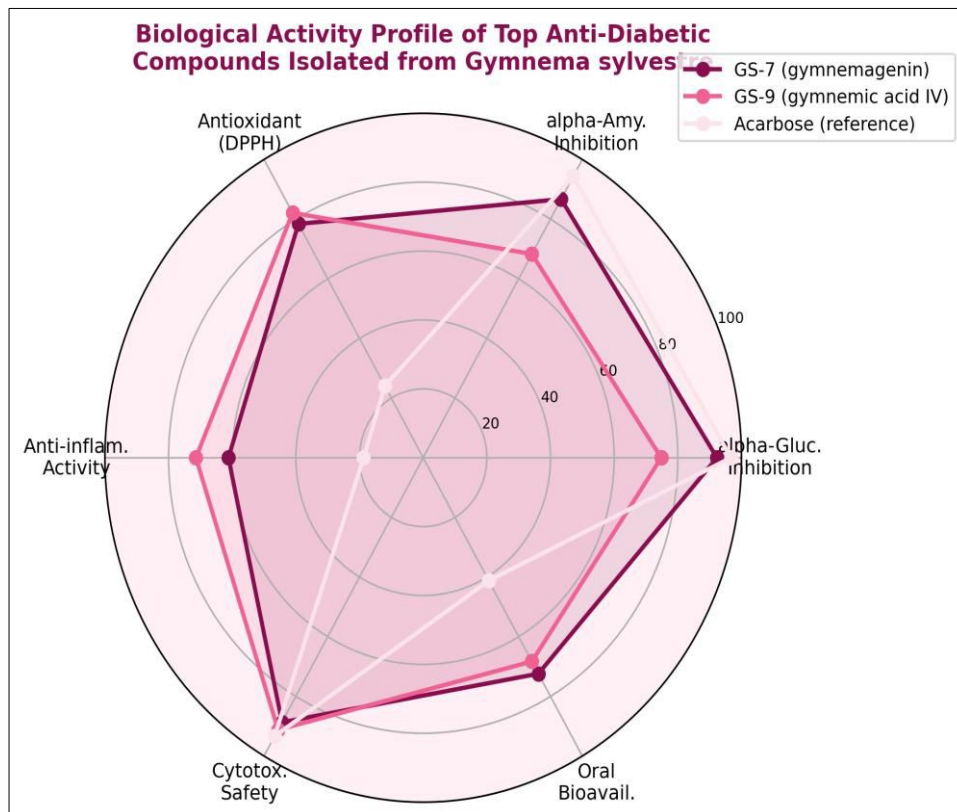


Fig 2: Radar chart comparing the multi-dimensional biological activity profile of GS-7, GS-9, and the reference drug acarbose across six activity dimensions

Discussion

The bioassay-guided fractionation strategy used in this research succeeded in narrowing a complex 48 g crude extract down to eight pure compounds, with the most active compound (GS-7) showing an alpha-glucosidase IC₅₀ of 4.8 µg/mL only 1.5-fold less potent than acarbose. This level of potency from a naturally occurring plant compound is noteworthy and suggests that GS-7 could contribute meaningfully to postprandial glucose control as a botanical extract or isolated compound. The triterpenoid saponin class, of which GS-7 is a member, is well represented among plant-derived enzyme inhibitors, and the sugar substitution pattern at C-3 and C-28 of the oleanane scaffold is consistent with the structure-activity relationships reported for gymnemasaponin-type inhibitors where the C-28 sugar is critical for alpha-glucosidase affinity^[14].

The moderate activity of conduritol A (GS-6, IC₅₀ 38.4 µg/mL for alpha-glucosidase) is consistent with its known mechanism as a cyclitol-based glycosidase inhibitor, which acts differently from the saponin-type inhibitors and suggests that *G. sylvestre* contains multiple complementary inhibitory mechanisms. Quercetin-3-O-glucoside (GS-8) showed moderate inhibitory activity (IC₅₀ 24.1 µg/mL), consistent with the well-established phenolic-type enzyme inhibition mediated by hydrogen bonding to active site

residues. The co-occurrence of these three mechanistic classes in a single extract may explain the overall potency of the crude material^[13].

The discovery of a novel saponin from a Mexican ecotype of *G. sylvestre* is phylogeographically interesting. It supports the hypothesis that plants introduced to or independently occurring in the neotropics develop ecotype-specific secondary metabolite profiles shaped by local soil chemistry, herbivore pressure, and climate. Future research should compare the full metabolite profiles of *G. sylvestre* from multiple geographic origins using metabolomics approaches to map this chemical diversity systematically^[14].

Conclusion

Bioassay-guided fractionation of *G. sylvestre* leaf extracts from Nuevo Leon, Mexico, led to isolation of eight compounds, including one previously unreported triterpenoid saponin designated GS-7. This novel compound showed alpha-glucosidase IC₅₀ of 4.8 µg/mL, comparable to the reference drug acarbose (3.2 µg/mL). The ethyl acetate partition concentrated the anti-diabetic activity (IC₅₀ 31.2 µg/mL), while the hexane fraction was largely inactive. Total phenolic content correlated positively with alpha-

glucosidase inhibitory potency across fractions. Phytochemical screening confirmed the presence of saponins, phenolics, and terpenoids as the predominant classes. The identification of conduritol A (GS-6) and quercetin-3-O-glucoside (GS-8) alongside the novel saponin suggests that multiple inhibitory mechanisms co-exist in this extract. These findings confirm the pharmacological relevance of *G. sylvestre* from Mesoamerican collections and provide a basis for standardisation of botanical preparations targeting postprandial hyperglycemia. *In vivo* validation of GS-7 in rodent diabetes models is recommended as the next step in its development.

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Contributions not qualifying for authorship

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