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Relationships between Phenotypes and Chemotypic Characteristics of Local *Gymnema inodorum* Plants in Northern Thailand

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Abstract: *Gymnema inodorum* (Lour.) Decne or Chiang Da, the local northern Thai vegetable, is renowned for its anti-diabetic and hypoglycemic properties and is highly sought after by the functional food industry. This research investigated the relationship between the physiological characteristics, genetic variation, and chemical compositions of different Chiang Da accessions. Two commercial lines (COM1 and COM2) and eight local accessions (BAC1-8) of Chiang Da were gathered and maintained in the same study plot for the investigation of their morphological characteristics, genetic variation, and nutritional and phytochemical constituents. From the morphological data, the two commercial lines were clearly distinctive with their closely related leaf structures. Random amplification of the polymorphic illustrated the genetic diversity between the local accessions and commercial strains in a similar pattern as described by their morphology characteristics and proximate contents. The phytochemical characteristics, nonetheless, projected the two commercial lines in separate groups. The outcome of this study could be beneficial toward the selection of the Chiang Da strains with specific traits for plant breeding programs of functional food interest.

Keywords: antioxidant; genetic diversity; gymnemic acid; principal component analysis; RAPD

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1. Introduction

Gymnema inodorum (Lour.) Decne., known as "Chiang Da" in Thai, is a climbing vegetable that is cultivated throughout Southeast Asia [1]. In Thailand, young shoots and flowers are employed in the local recipe and incorporated as folk medicine for diabetic management [2]. It has broad lanceolate leaves with silky margins and green stems that

turn brown. Some taxa have no or minute stipules and are fleshy or cactus-like [3] with pentamerous bisexual and actinomorphic blossoms. The pistil consists of two styles and a five-lobed stigma. Rarely alternate, simple leaves are arranged in opposite or whorled clusters [4,5]. The leaves of Chiang Da contain several phytochemical components, mainly triterpenoids, including gymnemic acids and saponins [6–10]. Chiang Da has been promoted as the economic vegetable in Southeast Asia, and the demand in the functional food market has ramped up recently [11]. In the global scale, Chiang Da and the relating *Gymnema* species together has contributed largely to the diabetic food segment; thus, it requires a cost-effective and simple technique of cultivation to meet the growing demand [12]. The extracts from the edible parts illustrate the powerful antioxidant action due to the polyphenols, which consist of phenolic and flavonoid constituents [9,10,13,14]. Along these same lines, it is likely that Chiang Da may have a favorable effect on reducing the amount of reactive oxygen species (ROS), as well as inflammation [9]. Additionally, their anti-diabetic and hypoglycemic properties were also reported [15], which relates to mainly triterpenoid saponin, such as gymnemic acid and oleanane. It was recommended that drinking Chiang Da tea (dried leaves steeped in hot water) 15 min prior to meals could dramatically lower peak plasma glucose levels. Moreover, the long-term ingestion of Chiang Da tea for 28 days had no influence on fasting hypoglycemia or hepatotoxicity [16]. Chiang Da leaves have been processed into a variety of products, including processed food, herbal tea, powders, and capsules, in response to recent demands in the functional food markets, particularly where diabetics are concerned. Thus, the cultivation of *G. inodorum* is intriguing, as it is expected to become a revenue source in the future to satisfy economic need [17].

There are several species in the genus *Gymnema*, some of which are unclassified due to the presence of identically physical characteristics among their genetic relatives [18]. Chiang Da is locally found in the north of Thailand with a variable range of morphological traits. The commercial varieties with high gymnemic acid and antioxidant contents have been bred by Rajamangala University of Technology, Lanna Lampang, Thailand (RMUTL) [17]. The data on their chemotype and morphology characteristics, nonetheless, are lacking. Chemometrics is a valuable tool in plant genotyping and breeding programs, as it allows for the analysis of complex data sets and descriptions of desirable traits. Principal component analysis (PCA) was successfully used to analyze the relationship between the morphological traits and phytochemical compositions of different *Andrographis paniculate* landraces [19]. The technique was also applied to illustrate the correlation of the volatile constituents of *Zanthoxylum* species collected from various locations. The obtained data can be used to select the best plant source for commercialization and implementation in a variety of value-added sectors [20]. To serve the need, in the future, the development of commercial Chiang Da variety, which not only produces high yield but also potentially displays substantial amounts of the active ingredients, relationships between the phenotypic and chemical characteristics ought to be evaluated. The purpose of this study was to examine the relationship between the physical characteristics and chemical compositions of various Chiang Da lines in order to fill the mentioned gap. This study also utilized the phenotypes and chemotypic characteristic tools for the analysis of the complex biological data, which enhanced a deeper understanding of the interrelationship between these two features. The outcome of this study would ideally support the use of local food or medicinal plants, which will lead to substantial applications in the functional food industries.

2. Materials and Methods

2.1. Raw Materials

Two commercial *G. inodorum* (GI) lines (COM1-2) and eight local GI lines (BAC1-8) were evaluated in this study. They were previously selected from different regions of Northern Thailand, which were Chiang Mai, Phrae, Phayao, Nan, and Lamphun provinces, and maintained in the plant breeding program collection of the Agricultural Technology Research Institute, RMUTL, with their assigned collection numbers. The plants were cloned by cutting, and 3-month-old plants, after cutting, were transplanted into 12-inch pots using

a mixture of commercial soil, cocopeat, and husk at a ratio of 50:25:25. New accession numbers were also given to mitigate the variations of their habitats. They were raised in an open greenhouse with 50% shade and watered daily for 10 min with a springer system (~70% relative humidity) for 4 months (December to March) and supplemented with fertilizer in a 15:15:15 ratio before being pruned at a height of 5 inches above the ground.

2.2. Reagent and Chemicals

Genomic DNA Extraction Kit for Plants material was purchased from RBC Bioscience (New Taipei City, Taiwan). Polymorphic DNA primer sequences were supplied by Integrated DNA Technologies (Coralville, IA, USA). Taq DNA polymerase 2× Master Mix RED and High Range DNA Ladder were purchased from Ampliqon PCR Enzymes and Reagents (Stenhuggervej, Odense M, Odense, Denmark). Novel Juice (DNA Staining Reagent) was supplied by Bio-Helix (New Taipei City, Taiwan). TBE buffer was supplied by Invitrogen (Waltham, MA, USA). Standard gymnemic acid (Gymnemagenin) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). Gallic acid monohydrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt, (+)—catechin hydrate (sum of enantiomers, HPLC) and 2,2-Diphenyl-1-picrylhydrazyl were from Sigma-Aldrich (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent was supplied by Merckmillipore (Billerica, MA, USA). Sodium hydrogen carbonate ($NaHCO_3$), sodium nitrite ($NaNO_2$), sodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl), aluminum chloride hexahydrate ($AlCl_3$ $6H_2O$), sodium hydroxide (NaOH), potassium dihydrogen orthophosphate anhydrous (KH_2PO_4), potassium persulfate, acetonitrile (C_2H_3N), formic acid, and methanol (LCMS-grade solvents) were purchased from RCI Labscan Limited, Bangkok, Thailand.

2.3. Plant Morphological Characteristics

After 30 days of cultivation, the morphological characteristics were recorded, including tip length (cm), stem diameter [21], number of leaf pairs (pair), leaf width and leaf length (cm), and leaf area index one month after planting [22].

2.4. Genetic Variation

2.4.1. DNA Extraction

A shoot tip (3 cm in length) of the GIs was sampled after 30 days of cultivation and extracted using the Genomic DNA Extraction Kit for Plants [8] with some modifications [23]. The 50 mg of sample was pulverized using liquid nitrogen in a microcentrifuge tube before adding 400 µL of GP1 buffer and 5 µL of RNase A. The mixture was then heated at 65 °C for 10 min, and 100 µL of GP2 was added and vortexed before being placed on ice for 3 min. The mixture was added to a filter column and then added to a collection tube before centrifuging at 15,000 rpm for 3 min to get the supernatant. The 750 µL of GP3 buffer was also added to the supernatant, and the mixture was vortexed for 5 sec in a fresh microcentrifuge tube for DNA binding. The mixture was added to the genomic DNA [11] column and then added to the collection tube before being centrifuged for 2 min at 15,000 rpm. The GD column was filled with 400 µL of W1 buffer for washing and then centrifuged for 30 s at 15,000 rpm. The GD column was then washed again with 600 µL of wash buffer. The GD column matrix was rehydrated by centrifuging at 15,000 rpm for 3 min and transferred to a 1.5 mL clean microcentrifuge tube with 50 µL of preheated elution buffer, and the solution was left to stand for 5 min. The purified DNA was obtained by centrifuging at 15,000 rpm for 30 s. The DNA samples were stored at −20 °C prior to random amplified polymorphic deoxyribonucleic acid (RAPD) analysis.

2.4.2. DNA Quantification

The quantity of DNA was evaluated using a Nano Drop Spectrophotometers, Thermo Scientific/NanoDrop 2000C (AG-BIO PERDO, Bangkok, Thailand), following a method of

Sunanta et al. [24]. The DNA solution was then diluted with deionized water and kept at −20 °C until used to determine the molecular marker.

2.4.3. RAPD-PCR

Random amplified polymorphic deoxyribonucleic acid analysis by polymerase chain reaction (RAPD-PCR) was applied as the molecular marker technique for the sequencing analysis of genomic DNA. The 15 primers were selected for random amplification. Similar research was conducted by Smita and Keshavachandran [25], who investigated genetic fingerprinting using RAPD in *G. sylvestre*, as presented in Table 1. The PCR was conducted by mixing 12.5 µL of Taq 2× Master Mix RED (Ampliqon), 1 µL of 25 µM RAPD primer, 1 µL of genomic DNA, and 10.5 µL of deionized water. All reactions were operated on a K960 Thermal cycler (Heal Force) using the following program: 1 cycle, 95 °C, 5 s; 40 cycles, 95 °C, 30 s; 37 °C, 1 min; 72 °C, 30 s; 1 cycle, 72 °C, 5 min [25]. Then, the PCR products were stained with Novel Juice and placed on a 1% agarose gel that was submerged in 10× TBE buffer. The system was run at 135 V for 25 min [26] using submarine electrophoresis (MupidTM-ONE, Advance). The gel was accordingly visualized using the LED transilluminator (TT-BLT-470; Hercuvan).

Table 1. Random amplified polymorphic DNA primer sequence used in genetic variation experiment of *Gymnema inodorum*.

Primer Name	Sequence	Reference
OPA 11	CAATCGCCGT	[25]
OPA 13	CAGAACCCAC	[25]
OPA 14	CTCGTGCTGG	[25]
OPA 15	TTCCGAACCC	[25]
OPA 17	GACCGCTTGT	[25]
OPA 18	AGGTGACCGT	[25]
OPAH 12	TCCAACGGCT	[25]
OPAH 17	CAGTGGGGAG	[25]
OPE 14	TGCGGCTGAG	[25]
OPE 15	ACGCACAACC	[25]
OPE 17	CTACTGCCGT	[25]
OPE 18	GGACTGCAGA	[25]
OPF 13	GGCTGCAGAA	[25]
OPF 14	TGCTGCAGGT	[25]
OPF 19	CCTCTACACC	[25]

2.5. Phytochemical Analyses

2.5.1. Sample Preparation and Extraction

Leaves of GI (above the fifth pair) were collected to be washed with tap water about 2–3 times, cut into small pieces, dehydrated using a hot-air oven at 60 °C for 15 h, and finely ground for further analysis [27]. The serial extraction method reported by Wisetkomolmat et al. [28] was followed with some modifications. One gram of the GI powder was preliminarily extracted with 10 mL of 95% dichloromethane (DCM) at room temperature for 24 h before being centrifuged at 10,000 rpm for 3 min to remove fat-soluble content that may interfere with the analyses. Afterward, the residue was extracted three times with 80% ethanol (EtOH) in an ultrasonic bath (VEVOR, Rancho Cucamonga, CA, USA) at room temperature for 30 min, centrifuged, and concentrated. This was used as the EtOH crude fraction. Both fractions were lyophilized and stored at 4 °C for further phytochemical analysis.

2.5.2. Proximate Analyses

The proximate composition analyses of GI powder were carried out according to the methods of the Association of Official Analytical Chemists (AOAC) [29]. The sample's moisture content was determined using the oven drying method at 105 °C. The crude

fat content of GI powder was estimated by extracting the sample in a Soxhlet apparatus with petroleum ether as the solvent. To determine the amount of crude fiber, the defatted sample was digested with 1.25% (w/v) sulfuric acid and the same concentration of sodium hydroxide. The AOAC method 923.03 for dry ashing was used to determine the amount of ash in the sample [30]. Total carbohydrate contents were thereafter calculated using the following equation:

$$\% \text{ Carbohydrate} = 100 - (\% \text{ moisture content} + \% \text{ crude protein} + \% \text{ ash} + \% \text{ crude fat} + \% \text{ crude fiber} + \% \text{ nitrogen}) \quad (1)$$

2.5.3. Phenolic Content

The Folin–Ciocalteu technique was used to analyze the total phenolics in each extract [31]. The 30 µL EtOH crude extract (0.1 g crude) was mixed with 60 µL of Folin–Ciocalteu reagent and 210 µL of a 6% w/v $NaCO_3$ solution before being left in the dark for 90 min. The solution was then measured at 725 nm using a UV-Vis spectrophotometer (SPECTROstar, BMG LABTECH, Offenburg, Germany). At different gallic acid concentrations (0–1000 mg/mL), a gallic acid calibration curve was constructed. The results were represented in milligrams of gallic acid equivalents per gram of dry weight.

2.5.4. Flavonoid Content

The total flavonoid content was determined following a method of Sunanta et al. [24]. with some modifications. A total of 25 µL of ethanolic extract was reacted with 125 µL of deionized water and 7.5 µL of a 5% $NaNO_2$ solution. At room temperature, the mixture was allowed to react for 5 min prior to the addition of 15 µL of a 10% $AlCl_3 \cdot 6H_2O$ solution. After an incubation period of 6 min, 50 µL of a 1 M NaOH solution and 27.5 µL of distilled water were added. The solution was then measured at 510 nm using a UV-Vis spectrophotometer. The catechin calibration curve was constructed using concentrations ranging from 0 to 1000 mg/mL. Total flavonoid content was reported in milligrams of catechin equivalents per gram of dry weight.

2.5.5. Determination of DPPH Radical Scavenging Activity

Antioxidant scavenging activity was determined using the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) with the following modifications: Blois [32] and Sunanta et al. [24]. A total of 20 µL of the sample was combined with 250 µL of a 0.2 mM methanolic DPPH solution and incubated at 25 °C for 30 min. The absorbance of the mixture was measured at 517 nm using a UV-Vis spectrophotometer, and the DPPH radical scavenging activity was calculated using the following equation;

$$\%\text{DPPH Inhibition} = 100 \times (A_{(\text{control})} - A_{(\text{sample})})/A_{(\text{control})} \quad (2)$$

where $A_{(\text{control})}$ is a DPPH absorbance and $A_{(\text{sample})}$ is the sample absorbance.

2.5.6. Determination of ABTS Radical Scavenging Activity

The radical scavenging ability of the samples for the radical cation 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) was evaluated according to Re et al. [33]. ABTS was prepared by combining a 7 mM stock solution at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, and 154 mM NaCl) with 2.5 mM potassium persulfate and storing it at room temperature in the dark for 16 h before use. The ABTS solution was prepared by diluting 1.0 mL of ABTS with 60 mL of 80% methanol to achieve an absorbance of 0.70 ± 0.02 units at 734 nm. A total of 10 µL of the EtOH sample extract and 200 µL of the ABTS working solution was added to each well of the microplate, shaken, and incubated for 30 min at room temperature. The absorbance was accordingly measured at 734 nm. The ABTS scavenging activity was estimated using the following equation:

$$\%\text{ABTS Inhibition} = 100 \times (A_{(\text{control})} - A_{(\text{sample})})/A_{(\text{control})} \quad (3)$$

where $A_{(control)}$ is the absorbance of ABTS radical mixed with 80% methanol; $A_{(sample)}$ is the absorbance of ABTS radical reacted with sample extract/standard.

2.5.7. Gymnemic Acid and Saponin Contents

The crude GI extract prepared by DCM and EtOH was dissolved in 95% methanol to a final concentration of 0.1 mg/mL and analyzed for gymnemic acid and saponin using high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) [34] with an automatic injection (SIL-20ACHT), diode array detection (CTO-20AC), pump (LC-20AD), and automatic control at a wavelength detector of 210 nm (SPD-M20A, LC-20AD). Separations were performed using a Luna C-18 column (250 mm × 4.6 mm × 5 µM) at a column temperature of 26 °C. The mobile phase was composed of acetonitrile (A) and water [20] in a ratio of 80:20 at a flow rate of 1 mL/min. The following equation was used to convert standard gymnemagenin (ChromaDex) and saponin to gymnemic acid and total saponin: C = X (809.0/506.7) and C = X (1223.3/1223.3) [35], where C signifies the quantity of gymnemic acid and total saponin in the sample, X denotes the amount of gymnemagenin and saponin in the sample, 506.7 denotes gymnemagenin's molecular weight, and 1223.3 denotes saponin standard molecular weight, and 809.0 denotes gymnemic acid's molecular weight, and 1223.3 denotes saponin molecular weight.

2.6. Statistical Analysis

The analyses of physical and chemical data were carried out at least in biological and technical triplicates. Data were analyzed using a one-way analysis of variance and Duncan's test. Differences in values were considered significantly different when the *p*-value was <0.05. The statistical analysis was performed using IBM SPSS program v. 24. The XLSTAT (Addinsoft, New York, NY, USA) was used to perform principal component analysis (PCA) and a dendrogram on all sample types with morphology and phytochemical attributes. The NTSYSpc2.2 program (Applied Biostatistics Inc., New York, NY, USA) was used to analyze DNA fingerprint data, and cluster analysis used a dendrogram to generate the level of similarity.

3. Results

3.1. Morphology

The morphological data of all GI lines at day 30 after recutting were illustrated in Table 2. Overall, the leaf appearance of all GI samples was the opposite arrangement, pinnate venation, entire edge, and glabrous surface. The general characteristics of the leaves were their ovate and elliptic shape, cuspidate apex, as well as an obtuse and rounded base. The highest and shortest lengths of the tip were statistically found in the COM2 line (55.50 ± 42.59 cm) and the BAC6 (15.26 ± 2.76 cm), respectively. As for the stem diameter, the widest and narrowest sizes were the BAC2 line (5.06 ± 0.53 mm) and the BAC6 line (3.65 ± 0.49 mm), respectively. Meanwhile, the largest average sizes of leaf width were the lines of BAC4 (6.94 ± 1.32 cm), while the BAC3 line had the smallest average leaf width (4.8 ± 1.82 cm). For leaf length, the BAC2 line had the highest average leaf length (12.48 ± 1.44 cm), while the lowest average leaf length (7.24 ± 2.36 cm) was the BAC3 line. The average number of leaf pairs in the BAC2 line was 6.24 ± 0.72 pairs, which was the highest number of leaf pairs found. On the other hand, the BAC3 line had the least number of leaf pairs at 3.62 ± 1.18 pairs. To clearly understand, the appearance of all GI lines was analyzed, and their relationship was displayed on the dendrogram and multivariate plots using principal component analysis (PCA), as shown in Figure 1. The entry data were unevenly distributed into two clusters (Figure 1A). The C2 cluster was mostly led by BAC3, BAC7, and COM1, while BAC1, BAC8, and COM2 were somewhat separated into a distinctive cluster (C1). To comprehend the influence of the factors, a biplot analysis was conducted (Figure 1B). The analysis depicted 87.55% of all data, of which 68.58% in PC1 and 18.97% in PC2 were considered. In accordance with the dendrogram pattern, clear separation among the samples was observed, with the sample group leading with BAC1,

BAC2, and BAC8, and most of the COM2 lines were shaded in purple, and the others were in green. The samples in the shade of purple appeared to have a long tip and a large stem diameter, whereas COM1 and the other samples appeared to have a wider leaf width, longer leaf length, and a greater number of leaf pairs.

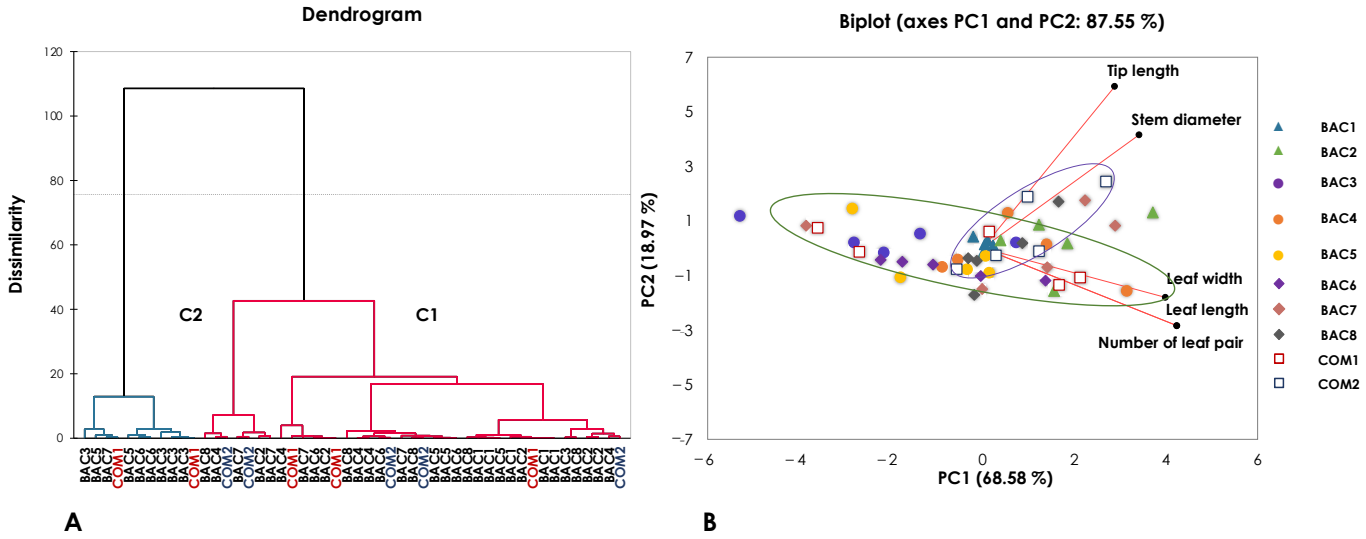


Figure 1. (**A**) The dendrogram of *Gymnema inodorum* samples generated from their physical characteristics. (**B**) The principal component (PC) analysis of the *G. inodorum* sample based on their physiological characteristics. C1 and C2 represent different clusters displayed on the dendrogram, and the same clusters (color) are shown in the biplot.

3.2. Genetic Variation of G. inodorum Samples

From the results, RAPD-PCR fingerprints were generated from the DNA extract of all GI samples using 15 randomly chosen 10-oligonucleotide primers, as shown in Table 1. The 146 fragments based on the RAPD (Supplementary Figure S1) were used to create a dendrogram, as shown in Figure 2. The dendrogram divided GI samples into two groups: the first cluster included BAC1, BAC2, BAC3, BAC4, BAC5, BAC6, and COM1. The second cluster contained BAC7, BAC8, and COM2. The two commercial lines were genetically distinct by a 47% coefficient. In line with the morphological result, we would then figure out the genetic patterns that contribute vastly to the physiological appearance of GI.

Table 2. Morphological appearances of different Gymnema inodorum lines.

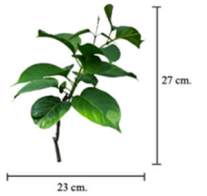
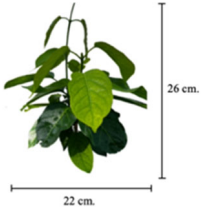
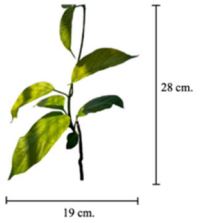
Collection Number	Accession Number	Appearances	Description	General Characteristics	Picture	Tip Length (cm)	Stem Diameter (mm)	Leaf Width (cm)	Leaf Length (cm)	Number of Leaf Pair (Pair)
CM014	BAC1		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, obtuse leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Obtuse		33.50 ± 6.07 ^{ab}	4.52 ± 0.22 ^{abc}	5.60 ± 0.15 ^{abc}	10.70 ± 0.47 ^{ab}	5.35 ± 0.24 ^{ab}
CM016	BAC2		Woody climbing shrub, light green leaves, elliptic shape, cuspidate leaf apex, rounded leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Elliptic Leaf apex: Cuspidate Leaf base: Rounded		49.78 ± 33.35 ^{ab}	5.06 ± 0.53 ^{a}	6.26 ± 0.61 ^{abc}	12.48 ± 1.44 ^{a}	6.24 ± 0.72 ^{a}
CM025	BAC3		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, obtuse leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Obtuse		17.30 ± 12.74 ^{b}	3.79 ± 0.72 ^{bc}	4.80 ± 1.82 ^{c}	7.24 ± 2.36 ^{b}	3.62 ± 1.18 ^{b}
CM058	BAC4		Woody climbing shrub, light green leaves, oval shape, cuspidate leaf apex, obtuse leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Obtuse		30.60 ± 20.36 ^{ab}	4.49 ± 0.76 ^{abc}	6.94 ± 1.32 ^{a}	10.78 ± 1.95 ^{a}	5.39 ± 0.98 ^{a}

Table 2. *Cont.*

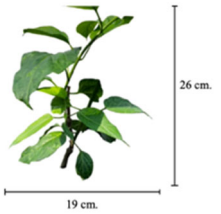
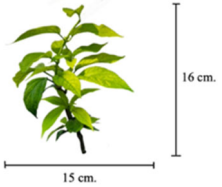
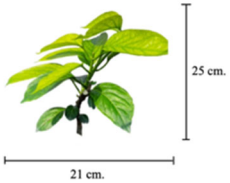
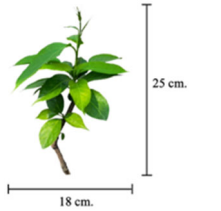
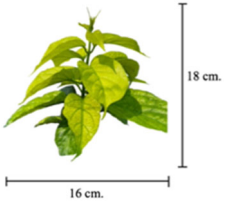
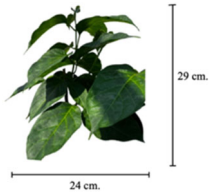
Collection Number	Accession Number	Appearances	Description	General Characteristics	Picture	Tip Length (cm)	Stem Diameter (mm)	Leaf Width (cm)	Leaf Length (cm)	Number of Leaf Pair (Pair)
CM064	BAC5		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, rounded leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Rounded		18.20 ± 3.83 ^{ab}	3.82 ± 0.77 ^{bc}	5.42 ± 0.94 ^{abc}	9.42 ± 2.17 ^{ab}	4.71 ± 1.09 ^{ab}
PR049	BAC6		Woody climbing shrub, dark green leaves, elliptic shape, cuspidate leaf apex, obtuse leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Elliptic Leaf apex: Cuspidate Leaf base: Obtuse		15.26 ± 2.76 ^{b}	3.65 ± 0.49 ^{c}	5.06 ± 1.17 ^{bc}	10.48 ± 1.71 ^{a}	5.24 ± 0.85 ^{a}
PY005	BAC7		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, rounded leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Rounded		50.40 ± 40.84 ^{ab}	4.28 ± 0.91 ^{abc}	5.64 ± 1.52 ^{abc}	11.16 ± 3.21 ^{a}	5.58 ± 1.60 ^{a}
N018	BAC8		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, rounded leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Rounded		42.48 ± 34.82 ^{ab}	3.97 ± 0.75 ^{bc}	6.68 ± 1.03 ^{ab}	10.46 ± 0.23 ^{a}	5.23 ± 0.12 ^{a}
LP004	COM1		Woody climbing shrub, light green leaves, oval shape, cuspidate leaf apex, obtuse leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Obtuse		18.80 ± 7.20 ^{ab}	4.20 ± 0.93 ^{abc}	5.58 ± 1.28 ^{abc}	9.92 ± 3.75 ^{ab}	4.96 ± 1.87 ^{ab}

Table 2. *Cont.*

Collection Number	Accession Number	Appearances	Description	General Characteristics	Picture	Tip Length (cm)	Stem Diameter (mm)	Leaf Width (cm)	Leaf Length (cm)	Number of Leaf Pair (Pair)
LP006	COM2		Woody climbing shrub, dark green leaves, oval shape, cuspidate leaf apex, rounded leaf base, umbel inflorescence, green calyx, yellow petals, single, round, long fruit.	Leaf shape: Ovate Leaf apex: Cuspidate Leaf base: Rounded		55.50 ± 42.59 ^{a}	4.75 ± 0.91 ^{ab}	5.98 ± 0.52 ^{abc}	11.06 ± 0.68 ^{a}	5.53 ± 0.34 ^{a}

Data expressed as mean ± standard deviation. Different superscription letters in each row denote a statistically significant difference at the 95% confidence level ($p < 0.05$). CM; Chiang Mai province, PR; Phrae province, PY; Phayao province, N; Nan province, LP; Lamphun province.

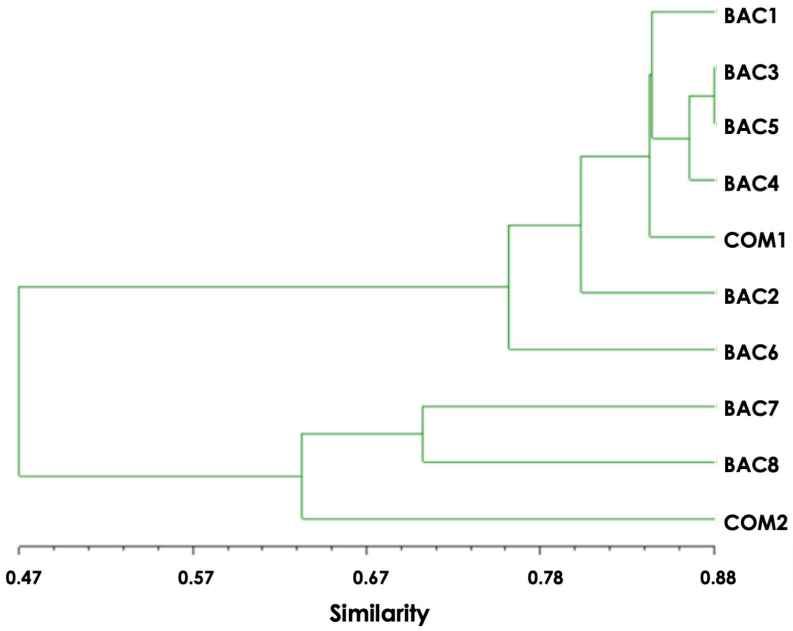


Figure 2. The dendrogram of *Gymnema inodorum* samples generated from the similarity matrix of RAPD data.

3.3. Proximate Compositions

The proximate analysis of GI from different lines is illustrated in Table 3. For the proximate compositions, the highest contents of nitrogen (4.27% ± 0.28), moisture (8.94% ± 0.24), protein (26.70% ± 1.73), and carbohydrate (37.57% ± 2.28) were the lines of BAC6. The BAC4 line presented the highest values in fiber (31.94% ± 2.52) and ash (3.68% ± 1.08), while the BAC1 line had the highest value in fat (5.69% ± 0.37).

The relationship of proximate compositions of all GI lines was analyzed and displayed on the dendrogram (Figure 3A) and multivariate plots using PCA (Figure 3B). The biplot PCA was used to determine the factors' influence. The analysis depicted 70.73% of all data, of which 48.89% in PC1 and 21.84% in PC2 were considered. The entry data were unevenly separated into three clusters. The C1 cluster in the shade of yellow was dominantly led by BAC1, BAC3, BAC4, BAC8, and COM2. The green shade of the C2 cluster was led by BAC2, BAC5, BAC7, and COM1, while BAC6 was in a separate cluster in red (C3). It appeared that samples in the yellow group had a high percentage of fat, ash, and fiber. Meanwhile, the other samples, except for BAC6, had a high percentage of carbohydrates. Apparently, BAC6 had a high percentage of protein, nitrogen, and moisture.

Table 3. Proximate compositions of Gymnema inodorum.

Specimen Number	BAC1	BAC2	BAC3	BAC4	BAC5	BAC6	BAC7	BAC8	COM1	COM2
Nitrogen (%)	3.27 ± 0.11c	3.84 ± 0.21ab	3.39 ± 0.12bc	3.48 ± 0.08bc	3.86 ± 0.52ab	4.27 ± 0.28a	3.76 ± 0.39b	3.43 ± 0.17bc	3.72 ± 0.02bc	3.27 ± 0.01c
Moisture (%)	7.37 ± 0.22c	8.41 ± 0.53ab	7.95 ± 0.34bc	8.25 ± 0.25ab	8.89 ± 0.64a	8.94 ± 0.24a	8.55 ± 0.73ab	7.39 ± 0.42c	8.69 ± 0.03ab	8.43 ± 0.06ab
Protein (%)	20.42 ± 0.71c	23.99 ± 1.29ab	21.21 ± 0.76bc	21.76 ± 0.50bc	24.15 ± 3.24ab	26.70 ± 1.73a	23.48 ± 2.42b	21.46 ± 1.04bc	23.23 ± 0.11bc	20.46 ± 0.09c
Fat (%)	5.69 ± 0.37a	3.43 ± 1.11ab	2.87 ± 1.71b	3.12 ± 1.17ab	2.77 ± 1.11b	1.49 ± 0.47b	3.24 ± 2.05ab	3.52 ± 2.63ab	4.17 ± 1.13ab	3.75 ± 0.22ab
Fiber (%)	30.14 ± 0.11b	28.05 ± 2.93b	30.70 ± 2.99b	31.94 ± 2.52b	29.09 ± 1.85b	19.75 ± 0.12a	30.23 ± 1.87b	30.66 ± 5.02b	30.82 ± 0.13b	30.53 ± 0.59b
Ash (%)	2.82 ± 0.95ab	2.97 ± 0.60ab	2.49 ± 0.61b	3.68 ± 1.08a	1.93 ± 0.34bc	1.278 ± 0.21c	2.49 ± 0.19b	2.86 ± 0.54ab	2.23 ± 0.32bc	2.33 ± 0.11bc
Carbohydrate (%)	30.02 ± 1.68a	29.31 ± 4.64a	31.38 ± 1.34a	27.76 ± 0.86a	29.32 ± 4.16a	37.57 ± 2.28b	28.24 ± 3.43a	30.68 ± 3.49a	27.15 ± 0.91a	31.23 ± 0.78a

Data expressed as mean ± standard deviation. Different letters in each column denote a statistically significant difference at the 95% confidence level ($p < 0.05$).

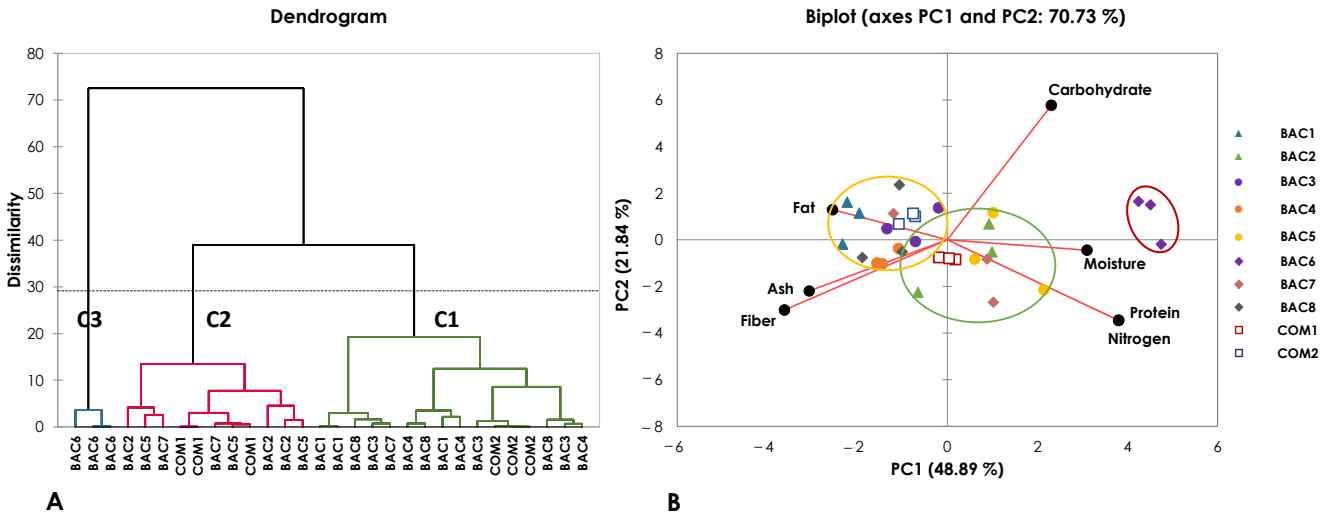


Figure 3. (**A**) The dendrogram of different *Gymnema inodorum* samples assessed from their proximate compositions. (**B**) The principal component (PC) analysis of the *G. inodorum* based on their proximate compositions. C1–C3 represent different clusters displayed on the dendrogram (**A**), and the same clusters (color) are shown in the biplot (**B**).

3.4. Phytochemicals

The phytochemicals of GI extract are shown in Table 4. The highest quantities of saponin (81.60 ± 1.30 mg/g DE) and gymnemic acid (0.57 ± 0.02 mg/g DE) were the EtOH extract of BAC4 and BAC3. Additionally, the greatest levels of total phenolic compound (21.16 ± 1.98 mg/g DE) and total flavonoid content (33.55 ± 1.12 mg/g DE) were found in the BAC3 and BAC7 lines. The lines of COM2 and BAC3 likewise contained high antioxidant potencies of 92.02% ± 0.42 ABTS and 88.65% ± 0.16 DPPH, respectively. The antioxidant capacities of ABTS and DPPH were 59.80% and 73.59%, respectively.

The relationship of phytochemicals of all GI lines was analyzed and displayed (Figure 4C,D). In the analysis, the data depicted 63.36% of all data, of which 42.63% in PC1 and 20.73% in PC2 were considered. The entry data was unevenly separated into two clusters. The C1 cluster in the shade of green was dominantly led by BAC1, BAC3, BAC5, BAC6, BAC7, COM1, and COM2. Meanwhile, the other samples in the shade of blue were the C2 cluster. It appeared that samples in the green group (C1) had a high saponin, ABTS, DPPH, phenolic acid, and flavonoid content. The samples in the C2 cluster were of greater gymnemic acid content. It is interesting to note that gymnemic acid did not correspond with saponin content, antioxidant activity (ABTS and DPPH), and phenolic and flavonoid contents.

Table 4. Phytochemicals and antioxidant activities of *Gymnema inodorum*.

Specimen Number	BAC1	BAC2	BAC3	BAC4	BAC5	BAC6	BAC7	BAC8	COM1	COM2
Gymnemic Acid (mg/g DE)	0.55 ± 0.03a	0.55 ± 0.05a	0.33 ± 0.02c	0.57 ± 0.02a	0.33 ± 0.02c	0.49 ± 0.02b	0.34 ± 0.01c	0.55 ± 0.00a	0.51 ± 0.01b	0.33 ± 0.01c
Saponin Content (mg/g DE)	47.58 ± 1.59bc	55.15 ± 4.85b	81.60 ± 1.30a	34.50 ± 8.80d	75.33 ± 5.04a	51.07 ± 1.41bc	76.91 ± 4.24a	32.15 ± 3.44d	43.08 ± 6.44c	52.17 ± 4.28b
Total Phenolic Content (mg/g DE)	20.19 ± 1.12ab	18.80 ± 1.65ab	21.16 ± 1.98a	17.72 ± 1.37b	18.72 ± 2.10ab	19.34 ± 1.16ab	18.75 ± 2.70ab	18.44 ± 0.88ab	20.12 ± 1.52ab	18.86 ± 0.08ab
Total Flavonoid Content (mg/g DE)	30.24 ± 2.52ab	24.30 ± 3.20d	27.50 ± 2.96ab	16.54 ± 2.03e	24.92 ± 1.56cd	28.13 ± 2.43bc	33.55 ± 1.12a	16.48 ± 1.59e	30.88 ± 0.85ab	19.52 ± 0.71e
ABTS (%)	90.82 ± 0.56ab	90.03 ± 1.50ab	90.25 ± 1.23ab	88.67 ± 1.81b	91.03 ± 0.70ab	90.46 ± 1.28ab	88.91 ± 1.59b	89.74 ± 4.02ab	92.02 ± 0.66a	92.02 ± 0.42a
DPPH (%)	88.02 ± 0.75a	88.36 ± 0.91a	88.65 ± 0.16a	83.21 ± 3.74b	85.23 ± 4.28ab	88.56 ± 0.72a	86.75 ± 0.83ab	84.46 ± 2.97ab	86.97 ± 0.75ab	84.89 ± 2.46ab

Data expressed as mean ± standard deviation. Different letters in each column denote a statistically significant difference at the 95% confidence level ($p < 0.05$).

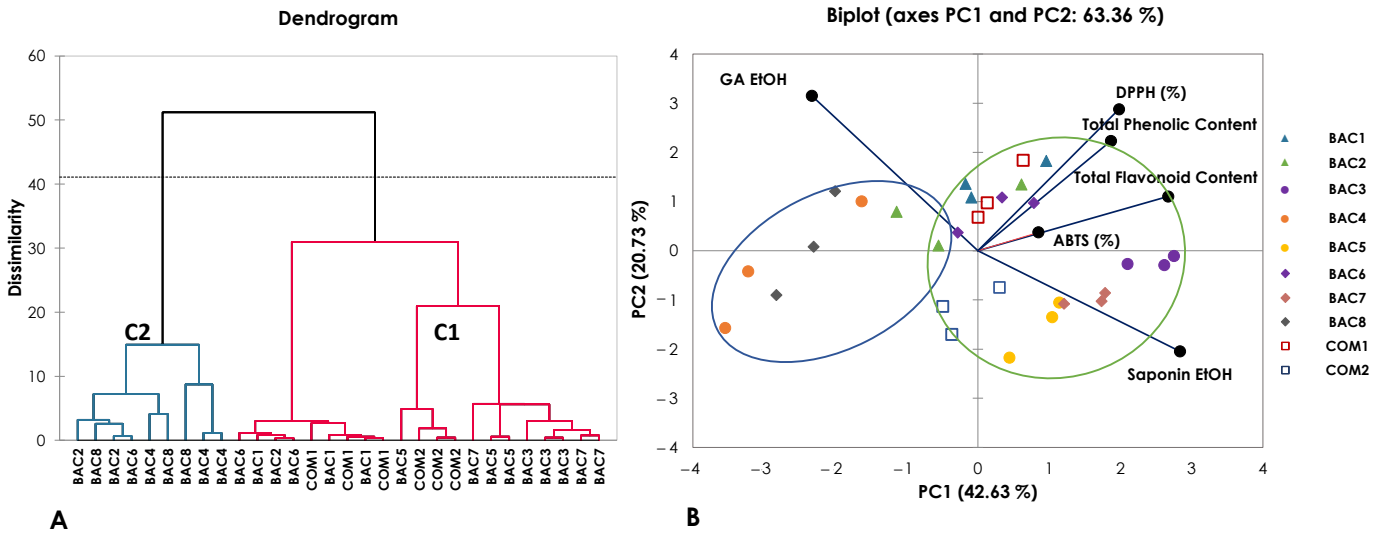


Figure 4. (**A**) The dendrogram of different *Gymnema inodorum* samples assessed from their phytochemicals. (**B**) The principal component (PC) analysis of the *G. inodorum* based on their phytochemicals. C1 and C2 represent different clusters displayed on the dendrogram (**A**), and the same clusters (color) are shown in the biplot (**B**). GA; gymnemic acid.

4. Discussion

In spite of being reclassified as the same species, the morphology of the GI plants obtained from diverse sources revealed that their morphology was distinct. External and internal growth factors contribute to plant appearance differences. Ramar et al. [36] reported that the discrepancies in leaf morphology of 35 lines of *G. sylvestre* obtained from different assessions may have resulted from environmental and genetic interactions. Similar morphological variations were revealed by Nair and Keshavachandrann [37]. Even though physical attributes frequently vary across the populations of a species, phenotypic traits typically exhibit covariation patterns that can result from gene and environment interactions [38]. Puttawarachai et al. [39] found that within the pool of 101 non-selective GI lines, there were 48 plants with above-average leaf length, as well as 47 plants with above-average shoot length, and 57 plants with the highest average number of leaf pairs per tip length. In a plant breeding program for a single species, there is always one line that is better than the others [40]. This goes along with the research on genetic variability and character association in GI [41], which suggests that the phenotypic traits could be considered selection indices for the commercial breeding program. Regarding the production of GI as a vegetable, the edible portion quantity should be taken into consideration when selecting GI lines for the breeding program. Our data confirmed that the two commercial lines were bred to provide a high volume of edible parts, i.e., shoots and leaves.

Such variation in phenotypes within and between populations is primarily attributed to evolutionary processes. The evolutionary interpretation of complex-trait variation is greatly facilitated by the identification of the genes that underlie phenotypic variation, along with a mechanistic understanding of the molecular, biochemical, and developmental mechanisms responsible for this variation [42]. Therefore, it is of great relevance to elucidate the genetic variation among the samples. A molecular study is basically useful for confirming the genetic diversity based on morphological characteristics and for an in-depth comprehension of the physiological characterization of these landraces. It could also highlight the availability of these genetic resources for future breeding plants [43]. The data from genetic variation is in correspondence to the report of Tung et al. [44], who investigated the taxonomical variation of plants within the genus *Gymnema* and determined that the classification within the species was consistent with their morphological traits. Genetics and environment are the main determinants of plant morphological characteristics [45–47]. They play important roles during plant evolution and the dynamics

of populations, communities, and ecosystems [48,49]. In addition, it is generally known that the genetic diversity within the same plant species could mainly be attributed to the diverse agroclimatic conditions [50]. Sometimes, accessions from different regions were closely related, or those cultivated in the same region had a distinct genetic background. In a similar way, Shahnawaz et al. [51] conducted an experiment on *G. sylvestre* of different accession and found that geographical remoteness may be one of the causes of great genetic differences. In contrast, Nair and Keshavachandran [37], who investigated the genetic variability of *G. sylvestre* in Kerala, India, revealed that the morphological and biochemical characteristics of *Gymnema* accessions are highly variable. We are convinced that plant habitats and agronomical conditions may contribute to these great variations.

In addition to evaluating the morphology and genetic characteristics of GI plants, chemical composition analyses were conducted to ascertain the relationship between appearance and variation in chemical composition, which included proximate compositions and bioactive compounds. Variations in the substance's concentration of *Gymnema* spp. have been identified in previous studies. Sharma et al. [52] reported that the leaves of *G. sylvestre* have a suitable amount of carbohydrates (54.8%), protein (10.94%), and crude fiber (11.50%). In contrast, *G. lactiferum* showed lower fiber content (3.25%) than crude fat (1.05%) but higher moisture content (77.52%) [30]. Regarding its high crude protein, crude fiber, and carbohydrate content, GI is also an excellent choice for vegetables or ingredients in the development of functional foods [53,54]. In *G. sylvestre* leaf extracts, the total phenolic content was 19.87 mg of GAE/g [55]. Praveen et al. [56] reported that the content of gymnemic acid in *G. sylvestre* was consistent with their antioxidant activity. Jeytawan et al. [14] also reported that the amount of gymnemic acid in baked leaves of *G. inodorum* was approximately 0.1020 ± 0.0122 µg/mg. From our study, saponin and gymnemic acid content were high in the ethanolic extracts. EtOH was found to be the suitable choice of solvent to extract the phytochemical substances because of its polarity, which can dissolve the remaining polar substances [57]. Our study advised that the active ingredients with anti-diabetic and hypoglycemic properties dissolved much better in this semi-polar substance. However, many parameters contribute to the wide range of phytochemicals among GI samples, such as heredity, biology, maturation stage, and environment. Though there was a firm pattern between the morphological appearance and genetic characteristics, GI lines were not clearly predicted. This is because the actual synthesis and accumulation of secondary metabolites are often induced or modulated by multiple environmental factors simultaneously [58–60]. Secondary metabolites are natural tools that plants use to deal with stressors and the environment [61,62]. These secondary chemicals protect plants from abiotic and biotic stresses and may help them adapt to their local environment. This is consistent with the findings of a study by Verma et al. [63], which concluded that genetics could not establish a connection between the bioactive ingredient in *G. sylvestre*.

5. Conclusions

In this study, we analyzed the genotypes of *Gymnema inodorum* and their relations to the phenotypes of several landraces. Principal component analysis (PCA) was employed to reduce the dimensionality of large datasets and identify the most important variables or markers that are associated with the traits. The finding indicated that the differentiation of genetics was related to morphological and nutritional properties, while the chemical constituents of *G. inodorum* were uncorrelated with the genetics. From the research, all accessions were separated into two groups, including those of extending edible parts and those of higher phytochemical composition. More study is needed to ensure that future correlation studies are reliable. This result could be used in the selection of the *G. inodorum* strain with specific traits for yield and potential for both consumption and functional property improvement. For future correlation studies to be more precise, additional research should be conducted. In addition, genes responsible for the target trait should be identified through various methods, such as gene mapping, sequencing, or bioinformatics analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9040484/s1, Figure S1: The random amplified polymorphic DNA (RAPD)-PCR fingerprints of all *Gymnema inodorum* samples using 15 primers.

Author Contributions: Conceptualization, S.R.S.; methodology, P.N.a. and M.W.; validation, T.T., S.W., and M.W.; formal analysis, P.N.a.; investigation, P.N.a.; resources, P.N.a., P.S., T.J. and C.L.; data curation, P.N.a.; writing—original draft preparation, P.N.a., writing—review and editing, T.T., M.W. and S.R.S.; visualization, M.W. and S.R.S.; supervision, S.R.S.; project administration, T.J. and C.L.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

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