*Research article***ANTIOXIDANT POTENTIAL AND PHENOLIC COMPOSITION OF COLUMN-CHROMATOGRAPHIC FRACTIONS FROM *GYMNEMA INODORUM* LEAF EXTRACT**

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ABSTRACT

Gymnema inodorum (Lour.) Decne, a plant traditionally used in Northern Thailand for its antidiabetic properties has shown potential as a source of bioactive compounds with antioxidant activity. Despite its traditional use, the phenolic profile and specific antioxidant contributions of *Gymnema inodorum* fractions remain inadequately characterized. Given the growing interest in identifying plant-based molecules to combat oxidative stress, further exploration is necessary. This study aims to explore the antioxidant properties and phenolic profile of column chromatographic fractionated *G. inodorum* leaf extract. The ethanolic reflux extracts of *G. inodorum* leaves were subjected to column chromatography for the isolation of bioactive molecules. Total phenolic and flavonoid contents were assessed using Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively. Total antioxidant activity was evaluated by employing different antioxidant assays, including DPPH, ABTS scavenging assay and FRAP assay. High-Performance Liquid Chromatography (HPLC) with a C18 column was employed to determine the level of gymnemic acid, while Liquid Chromatography Quadrupole Time-of-Flight/Mass Spectrometry (LC-QTOF/MS) was used to identify potential phenolic compounds. The three separated fractions obtained after column chromatographic separation were designated as Fraction A (fraction 1-4, eluted with low polarity mobile phase), Fraction B (fractions 5-10, intermediate polarity), and Fraction C (fractions 11-12, higher polarity) based on UV absorbance profiles. These fractions exhibited remarkable antioxidant activity, with total phenolic content of 0.294 ± 0.056 , 0.085 ± 0.039 , and 0.227 ± 0.041 mg QE/mL, and total flavonoid content of 0.274 ± 0.050 , 0.309 ± 0.110 , and 0.384 ± 0.007 mg QE/mL, respectively. Fraction A exhibited the highest DPPH and ABTS scavenging activity, while fraction C demonstrated significantly higher ferric-reducing power. HPLC analysis revealed a highest concentration of gymnemic acid in fraction C (0.393 ± 0.003 mg/g) compared to fraction B (0.159 ± 0.003 mg/g) and fraction A (0.142 ± 0.000 mg/g). The LC-QTOF/MS phenolic screening

showed seven potential phenolic compounds in fraction A, seven compounds in fraction B, and four compounds in Fraction C. Pearson correlation analysis using the individual replicate measurements underlying Table 1 and Table 2 ($n = 9$) showed a significant positive correlation between total phenolic content and DPPH scavenging ($r = 0.68$, $p = 0.044$), and significant correlations between total flavonoid content and DPPH ($r = -0.79$, $p = 0.012$), ABTS ($r = -0.95$, $p < 0.001$), and FRAP ($r = 0.81$, $p = 0.009$), suggesting that phenolic and flavonoid compounds are important contributors to the antioxidant activity of *G. inodorum*.

1. Introduction

Apocynaceae is a family of plants that includes the herb *Gymnema* spp. It is extensively dispersed in tropical and subtropical areas of Asia and Africa, and currently, more than 50 species are listed (*Gymnema* - The Plant List, 2023). *Gymnema inodorum*, a medicinal plant locally known as Chiang-da in Northern Thailand (Dunkhunthod et al., 2021), has a wide range of therapeutic uses. In northern Thailand, Chiang-da is mainly used as a vegetable for cooking and as a key botanical ingredient in various dishes, undergoing essential preparation methods like boiling and stir-frying. It is also air-dried for tea production, especially in the countryside. Traditionally, it has been used for treating diabetes and obesity. Because of the plant's numerous therapeutic benefits, including its possible antioxidant action, Ayurvedic medicine has long been used (Kasote et al., 2015).

Gymnema sylvestre, a common *Gymnema* species found in Asia has been extensively researched for its antioxidant and medicinal properties (Dai & Mumper, 2010). Many studies have reported the medical usage of this species. This species is also studied for potential phytoconstituents, including triterpene saponins known as gymnemic acids, *Gymnemasaponins*, and polyphenols (Ounjaijean et al., 2021).

G. inodorum belongs to the same genus as *G. sylvestre* and occupies a same ecological niche. However, despite being cultivated in regions same to *G. sylvestre*, *G. inodorum* has received comparatively limited scientific attention. Previous research has demonstrated that *G. inodorum* extract possesses glucose absorption inhibiting activity, primarily due to gymnemic acids (Suwan et al., 2022). Further, *G. inodorum* leaf extracts contain gymnemic acid and ginsenosides responsible for anti-

fungal and antioxidant activity (Jeytawan et al., 2022). Additionally, *G. inodorum* has been investigated for phytoconstituents and gymnemic acid and is believed as one of the potential phytochemicals that act as a blood sugar regulator (Chiabchalard et al., 2010) along with antimalarial properties (Boonyapranai et al., 2022). Nuchuchua et al. (2023) studied the relationship between phytochemical and antioxidant activities in *G. inodorum* leaf extracts, reporting significant antioxidant capacity in the crude ethanol extract, with polyphenolic compounds identified as key contributors. Similarly, (Haideri et al., 2024) compared different extraction techniques for *G. inodorum*, noting that while the crude extracts showed promising phenolic profiles, specific bioactive compounds responsible for antioxidant activity remained insufficiently characterized (Cianciosi et al., 2018). These studies underscore a critical gap: the reliance on crude extracts limits the identification and attribution of antioxidant activity to specific phenolic fractions.

Plants' phenolic chemicals have been reported to be the source of their antioxidant action (Baba & Malik, 2015). Phenolic compounds have been extensively researched due to their non-toxic nature, efficacy at low concentrations across various biological activities, eco-friendly extraction methods at low cost, showcase diverse pharmacological benefits (Gil et al., 2002). They exhibit potent antioxidants, anti-inflammatory properties, and robust inhibition against cardiovascular diseases, cancer, and diabetes (Phanjaroen et al., 2024). Phytochemicals have been widely utilized in healthcare and studied for their antioxidant potential, reactive ability of compounds to stop the oxidation of free radicals

(Tiwari et al., 2014). Exploration into extracting antioxidants from plant tissues has garnered significant interest due to their natural availability and cost efficiency. Enhancing foods, beverages, or medicinal items with additional antioxidants presents a promising approach to mitigating the adverse impacts of oxidative stress (Abu et al., 2017). The limited prior research on the active polyphenols of *G. inodorum* responsible for antioxidant activity underscores the importance of our current study.

As far as we know, our study is the first to explore fractions of *G. inodorum* extract because most literature received is of crude extract. The crude extract provides limited information about phenols activity and fractionation is done to enhance the activity of extract as well as to identify the essential phenols responsible for higher activity. Hence, the screening and investigation of the *G. inodorum* extract, which bears a resemblance to the well-known *G. sylvestre*, along with limited information on its phenolic profile, becomes imperative. The detection and measurement of phenolic compounds in the leaf extract of *G. inodorum* can shed light on the compound's potential health benefits, as phenolic compounds have considerable antioxidant qualities (Miliauskas et al., 2004).

Various extraction methods have been studied for purification of phytoconstituents, but *Gymnema* leaf contains various other compounds, creating difficulty in screening specific phytochemicals. An additional column separation technique was applied to overcome this shortcoming and find targeted polyphenols, which assisted in screening polyphenols in *Gymnema* leaf extract.

The primary objective of this study was to investigate the phenolic profile and antioxidant activity of *G. inodorum* leaf extract. The study focused on specific column separation technique for fractionation of crude extract to find out potential phenolic compounds, for the analysis of antioxidant capacity, as well as quantification of the total phenolic compounds, total flavonoid contents, and gymnemic acid levels. The results support the plant's potential polyphenols as specific phytochemical constituents in the

extract and antioxidant potential, highlighting the prospective use in pharmaceutical and nutraceutical applications.

2. Materials and Methods

2.1 Chemicals and reagents

The solvent methanol, chloroform, acetonitrile, chemicals and gymnemic acid standard of analytical grade (AR) were obtained from Sigma Chemical Company (Sigma-Aldrich, St. Louis MO, USA).

2.2. Plant material and preparation of extract

The shoot and first three pairs of *G. inodorum* leaves were harvested from Sanmahaphon Organic Herbs Community Enterprise, Chiang-da Garden, Chiang Mai, Thailand. Subsequently, the leaves were washed thoroughly under running tap water with to remove dust and soil particles and rinsed with distilled water, followed by air-drying at room temperature under shade conditions. The dried leaves were ground and sieved through a sieve shaker using 60-mesh sieve size to yield the particle size 150 - 250 μm . Dried leaves were powdered, and 10 g was packed into a cellulose thimble. Next, a 95 percent ethanol solution (100 mL) was added to the thimble at a ratio of 1:10 (w/v). The extraction process was performed thrice at 80 °C using an automatic Soxhlet extractor, each running for 2 h. After this extraction, the resulting solution was subjected to evaporation using a rotary evaporator to yield the crude extract. Subsequently, the extract was desiccated in a chemical hood, reweighed, and stored at 4°C for further analysis (Gini & Jeya Jothi, 2018). Based on the same extraction protocol applied to the same source material and reported separately (Dewanto et al., 2002), the corresponding crude ethanol reflux extract yield was 20.12% (w/w) relative to dry leaf powder.

2.3. Column chromatographic fractionation of extract

The crude extract was subject to column chromatography for fraction separation using slurry method for column packing. 20 g of 'silica gel 60' was packed into transparent (80 cm long,

5 cm diameter) glass column, as a stationary adsorbent without introducing air bubbles. Petroleum ether and methanol (2:1) was used as mobile phase in the column. The column was passed with mobile phase before adding silica slurry, and then the mobile phase was gradually increased to develop a packed solid column. Subsequently, 2 g of crude extract was added from top without disturbing the solid phase. Sea sand with a particle size 0.3 – 0.9 mm was applied to settle the layers properly, separating the crude extract and mobile phase. After proper layer formation between the sample and mobile phase, stopcock was opened to run the mobile phase down through column. The mobile solvent was gradually added to column to develop gradient elution. Fractions of the extract were received 4 mL, each with specific time interval. 12 fractions were collected in glass tubes with a flow rate of 0.99 mL/min (Islam et al., 2023). Each fraction was scanned and grouped by similarity of its whole UV-Vis absorbance profile (λ_{max} ranging ~250–670 nm across fractions), then later re-checked at 450 nm and 660 nm and pooled into the final 3 fractions (A=1–4, B=5–10, C=11–12) were passed through rotary evaporator to remove the organic solvent, followed by lyophilization to dry for further analysis.

2.4. Phytochemical Analysis

The fresh fraction extract was analyzed for detecting phytochemicals, total phenolic content (TPC) and total flavonoid content (TFC) through spectroscopy.

2.4.1. Total Phenolic Content

Quantitative analysis of phenolic content was analyzed using the Folin-Ciocalteu colorimetric method (Jeenoo et al., 2022), using Quercetin as the standard reference. To perform the analysis, a mixture of 20 μL of *G. inodorum* fraction extract and 20 μL of the diluted (10 times in distilled water) Folin-Ciocalteu solution was prepared. The mixture was combined with 80 μL of 7% Na_2CO_3 and 200 μL of DW. The plate was observed for any turbidity, and immediately at 760 nm, the absorbance was recorded by spectrophotometer to calculate total phenolic content. The findings were expressed

in mg of quercetin equivalents (mg QE/g sample) or milligrams per gram of sample.

2.4.2. Total Flavonoid Content

Total flavonoid content was determined by using quercetin as standard through colorimetric assays (Arnao et al., 2001). To perform this, 25 μL of the sample, blank or various concentrations of standard solution were added in a 96-well microtiter plate with 12.5 μL of DW and 7.5 μL of 7% NaNO_3 solution. The mixture was allowed to settle at ambient temperature for 5 min. Afterwards, 10% AlCl_3 15 μL solution was added and thoroughly mixed. The plate was stored for another 5 min at room temperature. Finally, 27.5 μL of distilled water and 50 μL of 1 M NaOH solution were added and incubated at room temperature for an additional 5 min. At 510 nm, the absorbance was measured using water as a reference, and the results were reported in terms of the amount of quercetin equivalents per g of sample (mg QE/g sample). The calibration curve (quercetin, 0.00625–0.4 mg/mL) followed $y = 8.346x + 0.217$ ($R^2 = 0.952$); estimated LOD and LOQ were 0.003 and 0.009 mg/mL. Quercetin was also used as the TPC calibration standard (Section 4.3.1) to keep a single reference compound across the phenolic and flavonoid assays; TPC is therefore expressed as quercetin equivalents rather than the more conventional gallic acid equivalents.

2.5. Antioxidant Analysis

The fractions of *G. inodorum* leaf extract were evaluated for their potential antioxidant capacity and free radical scavenging activities.

2.5.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to assess the free radical scavenging capabilities of *G. inodorum* extracts. The procedure involved creating a DPPH stock solution in methanol and adding 195 μL of the DPPH solution with 10 μL of *G. inodorum* extract in a 96-well micro-titer plate. After a 30-minute dark incubation period, the absorbance at 517 nm was obtained using a SPECTROstar nano microplate reader (BMG LABTECH) (Chaves et al., 2020).

2.5.2. ABTS Decolorization Assay

Arnao's described method (Arnao et al., 2001) was used to evaluate the antioxidant potential of *G. inodorum* fraction extract using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay. The method involved preparing a 7mM ABTS reagent using 2,2'-azino-bis-6-sulfonic acid, diammonium salt in H₂O and 2.45 mM of K₂S₂O₈ (potassium persulfate), which was kept in dark incubation for 12 h at room temperature. 80% ethanol was added to dilute the solution in a 96-well microtiter plate along with 10 µL of *G. inodorum* fraction extract. After a 30-minute dark incubation at 734 nm wavelength, the absorbance was measured and reported as the 50% inhibition concentration value, which reflects the correlation between the % inhibition of the test sample concentration and ABTS. The inhibition was measured at 734 nm absorbance, determined as a percentage and calculated by comparison with Trolox as a standard reference.

2.5.3. FRAP Assay

The method described by Aljadai (Devi & Jain, 2014) was used to determine the ferric-reducing antioxidant power (FRAP) value of *G. inodorum* extract. The process involved preparing the FRAP reagent by mixing 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) with 2.5 mL of 20 mM ferric chloride (FeCl₃), 40 mM HCl, and 25 mL of 300 mM acetate buffer at pH 3.6. The *G. inodorum* fraction extract was then diluted two times with DW (distilled water), and ten microliters of the *G. inodorum* fraction extract was added with 190 microliters of the FRAP standard reagent in a 96-well microtiter plate for a 30-minute dark incubation period. Finally, at 593 nm, the sample's absorbance was observed using a SPECTROstar nano microplate reader and calculated by comparison with Trolox as a standard reference.

2.6. Detection and Quantification of Gymnemic Acid

The quantification of gymnemic acid was conducted according to the validated HPLC method described by Devi and Jain (Devi & Jain, 2014) and modifications by Kamble et al.

For the detection of gymnemic acid, 10 mg of the crude gymenema extract was dissolved in 50 mL of a 50% (V/V) methanol solution for chromatographic analysis. To this solution, after adding 10 mL of 11% KOH, heated under reflux for one hour in a boiling water bath before being cooled. After that, 9 mL of 12 N hydrochloric acid (HCl) was mixed and refluxed for another hour in the water bath. After lowering the temperature, the solution was mixed with 50% (V/V) methanol marked 100 mL final volume and further subjected to analyzation with HPLC (Shimadzu LC, Kyoto, Japan). The percentage of gymnemic acid was calculated using the sample's area under the curve, and it was compared to the standard value of deacyl gymnemic acid. Because gymnemic acid lacks a readily available analytical reference standard, deacyl gymnemic acid was used for calibration, consistent with the method of Devi and Jain (Bajpai et al., 2016); gymnemic acid content reported in this study is therefore expressed as deacyl gymnemic acid equivalents. Chromatography was conducted using LC systems, HPLC with analytical C18 column (250 x 4.60 mm i.d. x 5.0 µm), mobile phase consisting of (A) Acetonitrile solution and (B) Type 1 water (50:50) at 30°C column temperature, a flow rate of 2.0 mL/min, and detected at the wavelength of 218 nm. Deacyl gymnemic acid was used as a standardization concentration (3.91-500 µg/mL), and a duplicate sample was injected along with 20 µL of the sample solution to perform the HPLC estimation (Manthey, 2000). Because gymnemic acid was quantified against a deacyl gymnemic acid standard, in accordance with Devi and Jain (Pietta, 2000), all gymnemic acid concentrations reported in this study are expressed as deacyl gymnemic acid equivalents. The standard injection series (40–80 µL) gave a linear peak-area response ($R^2 = 0.993$) across the validated range.

2.7 Characterization of Active Phytochemicals

The characterization of active phytoconstituents from *G. inodorum* leaf extract fractions was performed by a quadrupole time-

of-flight mass spectrometer, following the method described by Chiwat (Zhang et al., 2015). Initially, the collected fractions were dissolved in methanol, followed by cleaning with dispersive C18 SPE. Before analysis, the solution was filtered through HPLC micro-filters (0.22 μm). Further analysis was conducted by utilizing the series of Agilent 1290 Infinity II in conjugation with a 6546 LC-QTOF/MS apparatus with resolution 1.3 Da and 3 ppm accuracy of m/z determination. The LC conditions involve the use of a ZORBAX Eclipse Plus C18 column Shimadzu (HPLC-UV) with an injection quantity of 10 μL , a flow rate of 0.2 mL/min, and a mobile gradient system starting with a mixture of 95% water (1% formic acid) and 5% acetonitrile, gradually increasing to a final composition of 95% acetonitrile. Chromatographic separation was achieved through mass spectrometry conditions that include a positive mode electrospray ionization (ESI) probe, a nebulizer pressure of 20 psi, a flow rate of 7 L/min of N_2 , a capillary temperature of 300°C, a flow rate of 8 $\mu\text{L}/\text{min}$, a mass-to-charge ratio range of 50-1000 m/z , a capillary voltage of 4500 V, and a temperature of 280°C dry heater. The phenolic compounds were identified using the database EMBL – EBI (Bajpai et al., 2016).

3. Results and Discussion

3.1. Results

The preliminary screening of ethanol reflux extract based on Thin Layer Chromatography (TLC) indicated active compounds in the *G. inodorum* leaf extract. The active compounds in the extract were separated on the TLC plate and compared with the R_f values of employed standards (Quercetin, Gallic Acid, Caffeine, and Trolox). The column chromatographic fractionation of crude extract yielded 12 fractions in the best-suited solvent system of TLC as a mobile phase and silica gel as a stationary phase. Solvent-system screening identified ethyl acetate:methanol:water (10:1:1) as the system giving the clearest separation on

silica gel, with R_f values of 0.93–0.975 (two spots) for the extract, 0.93–0.97 for quercetin, and 0.812 for gallic acid; caffeine remained at the origin ($R_f = 0$) under this system, visualized with vanillin sulfuric acid spray. The 12 fractions were assessed by using a 218 nm UV spectrophotometer and produced 3 pool fractions based on UV absorbance values. Newly named as Fraction A (fraction 1 - 4 of column chromatography), Fraction B (fraction 5 – 10), and Fraction C (fraction 11 – 12). TLC screening prior to column chromatography tested seven mobile-phase systems on the ethanol reflux extract against Quercetin, Gallic acid, and Caffeine standards, visualized with vanillin–concentrated H_2SO_4 spray; ethyl acetate:methanol:water (10:1:1 v/v/v) gave the cleanest multi-spot resolution (sample $R_f \approx 0.93$ –0.98; Quercetin $R_f \approx 0.93$ –0.97; Gallic acid $R_f \approx 0.81$) and was used to select the mobile-phase polarity range for the column gradient.

The phenolic and flavonoid content of the three fractions derived from the leaf extract of *G. inodorum* were examined. Total phenolic content revealed that fraction A has the highest at 0.294 ± 0.056 mg QE/mL, followed by fraction C (0.227 ± 0.041 mg QE/mL) and fraction B (0.085 ± 0.039 mg QE/mL), as shown in (Table 1).

The analysis of total flavonoid content in the fractions of *G. inodorum* found that fraction C has the highest flavonoid content at 0.384 ± 0.007 mg QE/mL, followed by fraction B and A (0.309 ± 0.110 mg QE/mL, 0.274 ± 0.050 mg QE/mL), respectively (Table 1). The statistical analysis showed significant differences ($p < 0.05$) among fractions for phenolic, flavonoid, and antioxidant activities. Summing the individual fraction yields in Table 1 gives a total recovery of 63.94% (w/w) of the crude extract mass loaded onto the column, with the remainder attributable to material retained on the column and losses during evaporation and lyophilization.

Table 1. The total phenolic and flavonoid content and percent yield of the *G. inodorum* leaf extract fraction.

Samples	Total Phenolic Content (mg QE/mL)	Total Flavonoid Content (mg QE/mL)	% Yield (w/w)
Fraction A	0.294 ± 0.056 ^a	0.274 ± 0.050 ^b	12.60
Fraction B	0.085 ± 0.039 ^c	0.309 ± 0.110 ^{ab}	39.93
Fraction C	0.227 ± 0.041 ^b	0.384 ± 0.007 ^a	11.41

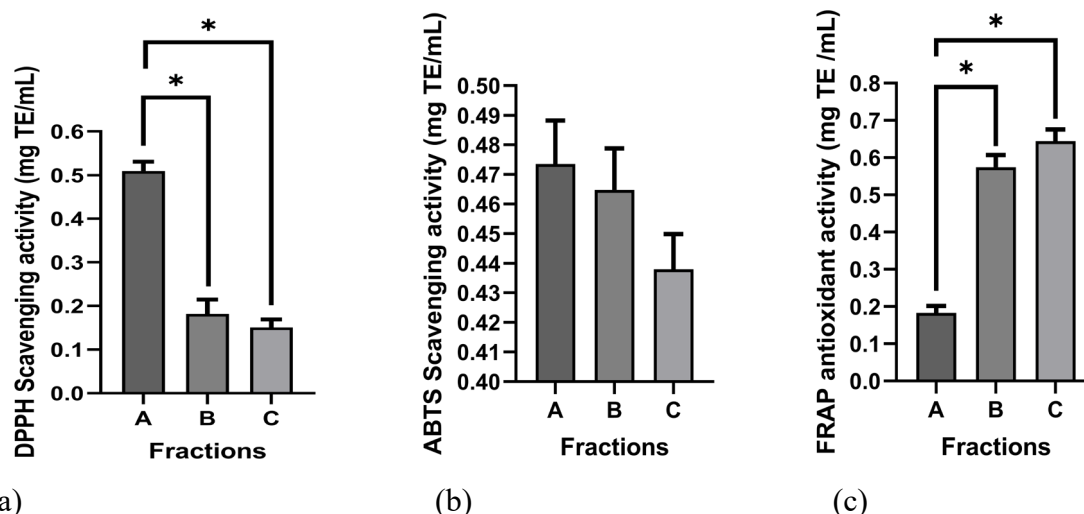


Figure 1. The bar graph shows the antioxidant activities of 3 different pool fractions (A, B, and C): (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, and (c) Ferric reducing antioxidant activity. The statistical significance were determined using one-way ANOVA, followed by Duncan's multiple range test at $p < 0.05$ and represented by star marks. The Star marks represent the significant mean difference between the fractions (A; fraction 1 - 4 of column chromatography, B; fraction 5 - 10, C; fraction 11 - 12). Data are expressed as mean $\pm$ SD ($n = 3$).

Total phenolic content was quantified using the Folin-Ciocalteu method and expressed as mg quercetin equivalents (QE) per milliliter of extract (mg QE/mL). Total flavonoid content was measured using a colorimetric method and also expressed as mg quercetin equivalents (QE) per milliliter of extract (mg QE/mL). Statistical significance ($p < 0.05$) was assessed using one-way ANOVA followed by Duncan's multiple range test. Results are presented as mean $\pm$ SD ($n = 3$) for total phenolic content, total flavonoid content, and % yield (w/w) with distinct superscript letters (a,b,c) to highlight significant differences.

The antioxidant activity of the three fractions received from the leaf extract of *G. inodorum* was assessed by employing DPPH, ABTS, and FRAP assays. In the DPPH assay, it was observed that fraction A exhibited the most pronounced antioxidant activity (71.25 ± 3.15

mg TE/mL), followed by fraction B (31.57 ± 6.42 mg TE/mL), with the lowest activity observed in fraction C (27.92 ± 3.64 mg TE/mL). Summing the percent yields of Fractions A, B, and C (12.60%, 39.93%, and 11.41% w/w, respectively) gives a total recovery of 63.94% (w/w) of the crude extract loaded onto the column.

Likewise, the ABTS assay yielded results indicating that fraction A displayed the highest radical scavenging activity (95.45 ± 0.89 mg TE/mL), closely followed by fraction B (93.46 ± 1.43 mg TE/mL), whereas fraction C demonstrated activity of a comparable magnitude (87.29 ± 0.45 mg TE/mL).

Conversely, the assessment of reducing properties using the FRAP assay unveiled that fraction C exhibited the most robust antioxidant activity (2.97 ± 0.25 mg TE/mL). In comparison, fractions B and A also demonstrated

considerably lower ferric ion reducing power (2.65 ± 0.26 mg TE/mL and 0.85 ± 0.14 mg TE/mL, respectively) (Figure 1). The three fractions were analyzed through HPLC with C18 column to quantify the Gymnemic acid level. After analyzing the sample, fraction C showed the highest gymnemic acid

concentration at 0.3933 ± 0.003 mg/g, followed by fractions B and A at 0.1591 ± 0.003 mg/g and 0.1417 ± 0.000 mg/g, respectively (Table 2). The HPLC-C18 chromatogram in Figure 2 shows the extract's standard and gymnemic acid peaks.

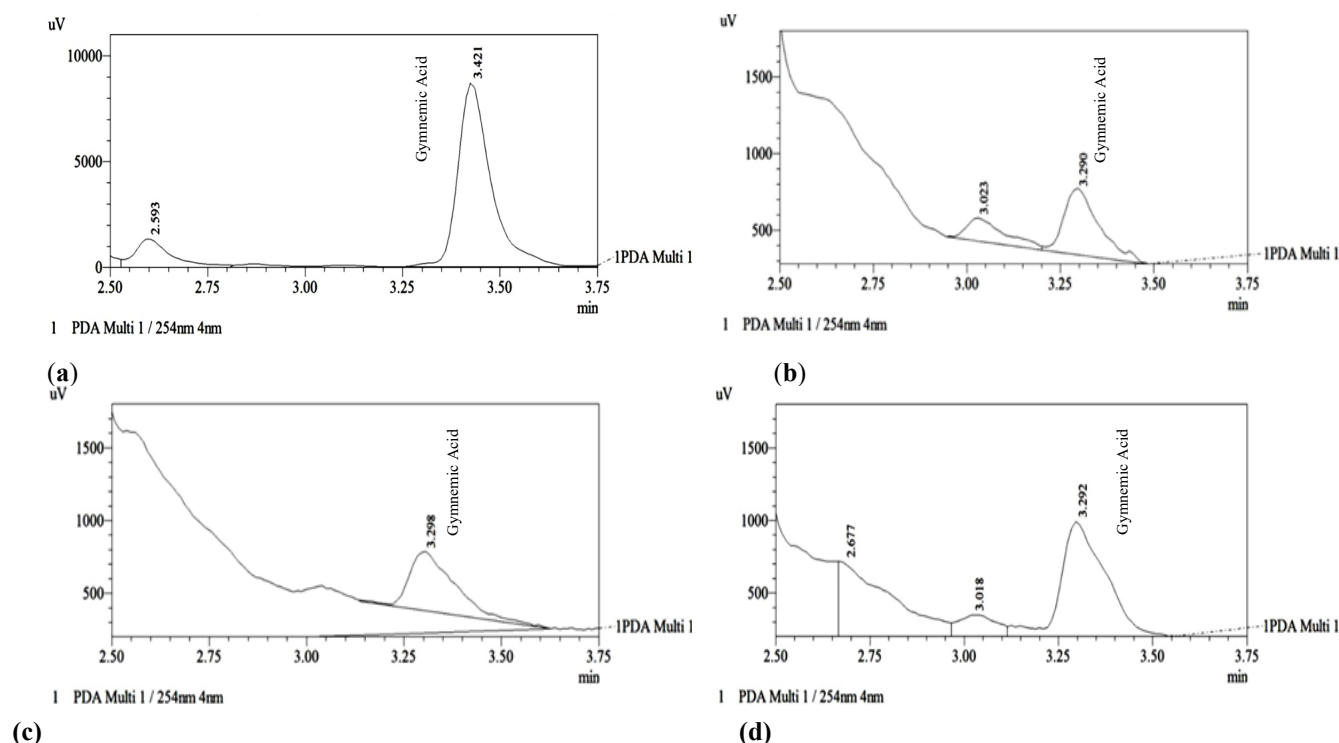


Figure 2. HPLC-UV chromatograms of gymnemic acid. Figure (a) shows the chromatogram of a gymnemic acid standard at 100 µg/mL. Figure (b), (c), and (d) display gymnemic acid content in fractions A, B, and C, respectively. All analyses were performed at a detection wavelength of 218 nm, matching the method used for gymnemic acid detection in Section 3.6. The presence of distinct peaks in each fraction shows isolation and quantification of gymnemic acid, indicating variability in concentration across the fractions.

Table 2. The gymnemic acid concentration in fraction of *G. inodorum* leaf extract.

Fractions	Gymnemic Acid (mg/g)
Fraction A	0.1417 ± 0.000^c
Fraction B	0.1591 ± 0.003^b
Fraction C	0.3933 ± 0.003^a

The concentration of gymnemic acid was determined using HPLC technique, with quantified using authentic standard and expressed as milligrams per gram of extract. The statistical significance was at $p < 0.05$ expressed as mean $\pm$ SD ($n = 3$) denoted by different superscript letters (a, b, c) within the column.

Phenolic compounds were screened in fractions of *G. inodorum* leaf reflux extract by LC-QTOF/MS. The LC-QTOF/MS analyses revealed phytochemical compounds in fractions. From the LC-QTOF/MS result for each fraction, the compounds were analyzed for active phenolic compound screening and the database EMBL – EBI and ChemSpider by Royal Society of Chemistry was used to authenticate the compounds. Fraction A contained the phenolic compounds Bufotalinin, Erysothiopine, Schisanhenol B, Zhebeiresinol, Kanzonol P, 8-[(2Z)-3,6-Dimethyl-2-hepten-1-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one, and Toyocamycin based on tentative database matching; other potential antioxidants listed include fatty acids and drugs.

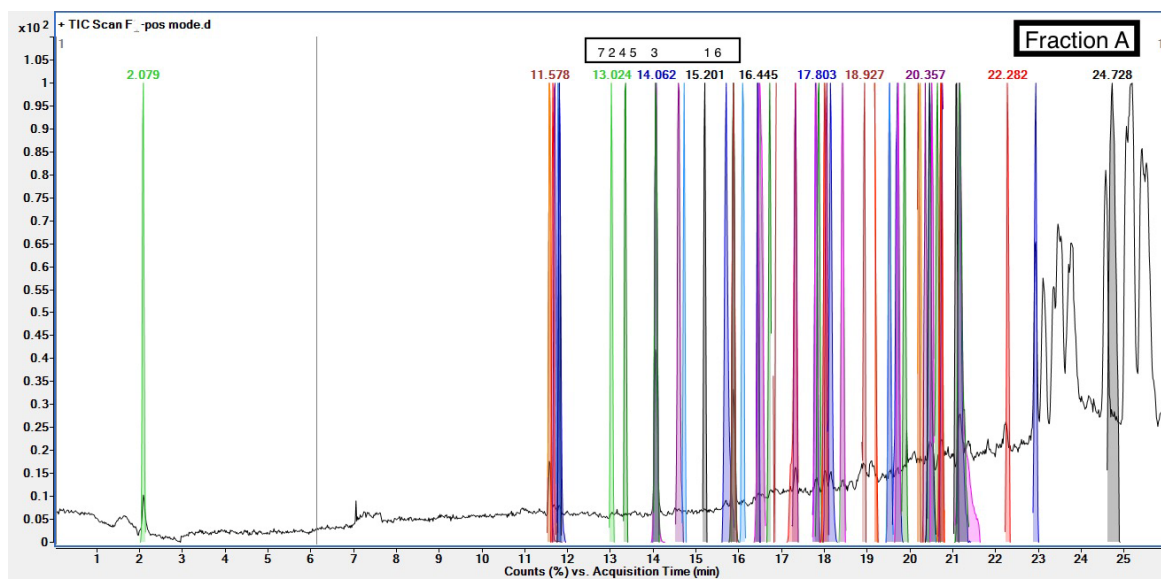
The fraction B of *G. inodorum* leaf reflux extract contained seven phenolic compounds, including Erysothiopine, Schisanhenol B, Kanzonol P, Fluocinolone, Bufotalinin, Chamuvaritin and Tomentolide A among the

active compounds. The 6 H Reflux extract of *G. inodorum* leaf, fraction C, screened for four potential phenolic compounds, namely 2,2-Dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate, Clausarinol, 8-Deoxy-11,13-dihydroxygrosheimin, and Eudesmin. The catalog of these phenolic compounds is mentioned in Table 3. The Phenolic compounds in a fraction of *G. inodorum* leaf identified by LC-QTOF/MS, and Figure 3 shows chromatograms for LC-QTOF/MS of phenolic compounds. Compounds with a database mass difference greater than 10 ppm or a matching score below 85% are flagged here as lower-confidence annotations pending confirmation with authentic reference standards or MS/MS fragmentation, consistent with accepted metabolomics reporting standards; several Table 3 entries (mass differences of 18–23 ppm) fall into this range.

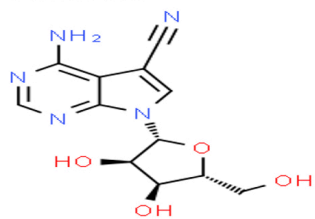
Table 3. The Phenolic compounds in a fraction of *G. inodorum* leaf identified by LC-QTOF/MS

Sample	Peak No	Retention Time	Mass	[M+H] ⁺	Mass Diff (db/ppm)	Matching Score (%)	Formula	Tentative Phenolic Compound
Fraction A	1	15.48	414.20	415.2123	1.01	97.57%	C ₂₄ H ₃₀ O ₆	Bufotalinin
	2	13.65	407.10	425.1368	18.03	96.67%	C ₁₉ H ₂₁ NO ₇ S	Erysothiopine
	3	14.11	386.17	387.1805	1.01	95.54%	C ₂₂ H ₂₆ O ₆	Schisanhenol B
	4	13.65	280.09	281.1021	1.01	85.66%	C ₁₄ H ₁₆ O ₆	Zhebeiresinol
	5	13.66	368.16	369.1698	1.00	83.60%	C ₂₂ H ₂₄ O ₅	Kanzonol P
	6	15.48	396.19	397.2010	1.01	82.43%	C ₂₄ H ₂₈ O ₅	8-[(2Z)-3,6-Dimethyl-2-hepten-1-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one
	7	13.47	291.09	309.1315	18.04	82.22%	C ₁₂ H ₁₃ N ₅ O ₄	Toyocamycin
Fraction B	1	13.65	407.10	425.1368	18.03	96.67%	C ₁₉ H ₂₁ NO ₇ S	Erysothiopine
	2	14.03	386.17	387.1812	1.01	94.48%	C ₂₂ H ₂₆ O ₆	Schisanhenol B
	3	13.66	368.16	369.1698	1.00	83.60%	C ₂₂ H ₂₄ O ₅	Kanzonol P
	4	14.03	412.17	413.1777	1.00	82.57%	C ₂₁ H ₂₆ F ₂ O ₆	Fluocinolone
	5	15.48	414.20	415.2123	1.01	81.92%	C ₂₄ H ₃₀ O ₆	Bufotalinin
	6	15.85	452.16	453.1682	1.00	80.18%	C ₂₉ H ₂₄ O ₅	Chamuvaritin

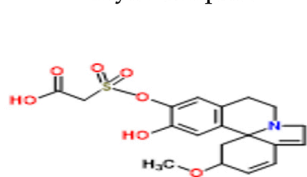
	7	14.49	402.14	425.1369	22.99	79.90%	C ₂₅ H ₂₂ O ₅	Tomentolide A
Fraction C	1	15.87	414.20	437.1931	22.99	98.00%	C ₂₄ H ₃₀ O ₆	Clausarinol
	2	14.06	386.17	409.1627	22.99	91.51%	C ₂₂ H ₂₆ O ₆	Eudesmin
	3	15.87	430.17	453.1669	22.99	87.85%	C ₂₇ H ₂₆ O ₅	2,2-Dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate
	4	15.48	280.13	281.1388	1.00	85.47%	C ₁₅ H ₂₀ O ₅	8-Deoxy-11,13-dihydroxygrosheimin



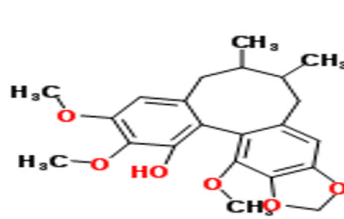
Bufotalinin



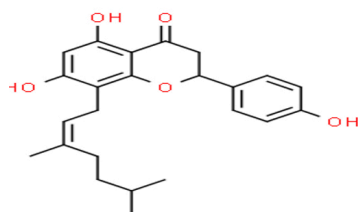
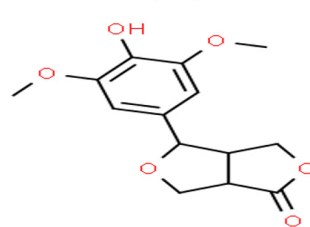
Erysothiopine



Schisanhenol B

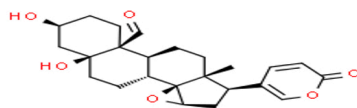


Zhebeiresinol

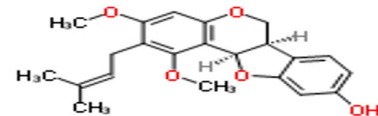


Kanzonol P

(a)



(2Z)-3,6-Dimethyl-2-hepten-1-yl



Toyocamycin

CC1=C(C(=O)O)C(=C2C(=C1)C(=C3C(=C2)C(=C4C(=C3)C(=C5C(=C4)C(=C6C(=C5)C(=C7C(=C6)C(=C8C(=C7)C(=C9C(=C8)C(=C10C(=C9)C(=C11C(=C10)C(=C12C(=C11)C(=C13C(=C12)C(=C14C(=C13)C(=C15C(=C14)C(=C16C(=C15)C(=C17C(=C16)C(=C18C(=C17)C(=C19C(=C18)C(=C20C(=C19)C(=C21C(=C20)C(=C22C(=C21)C(=C23C(=C22)C(=C24C(=C23)C(=C25C(=C24)C(=C26C(=C25)C(=C27C(=C26)C(=C28C(=C27)C(=C29C(=C28)C(=C30C(=C29)C(=C31C(=C30)C(=C32C(=C31)C(=C33C(=C32)C(=C34C(=C33)C(=C35C(=C34)C(=C36C(=C35)C(=C37C(=C36)C(=C38C(=C37)C(=C39C(=C38)C(=C40C(=C39)C(=C41C(=C40)C(=C42C(=C41)C(=C43C(=C42)C(=C44C(=C43)C(=C45C(=C44)C(=C46C(=C45)C(=C47C(=C46)C(=C48C(=C47)C(=C49C(=C48)C(=C50C(=C49)C(=C51C(=C50)C(=C52C(=C51)C(=C53C(=C52)C(=C54C(=C53)C(=C55C(=C54)C(=C56C(=C55)C(=C57C(=C56)C(=C58C(=C57)C(=C59C(=C58)C(=C60C(=C59)C(=C61C(=C60)C(=C62C(=C61)C(=C63C(=C62)C(=C64C(=C63)C(=C65C(=C64)C(=C66C(=C65)C(=C67C(=C66)C(=C68C(=C67)C(=C69C(=C68)C(=C70C(=C69)C(=C71C(=C70)C(=C72C(=C71)C(=C73C(=C72)C(=C74C(=C73)C(=C75C(=C74)C(=C76C(=C75)C(=C77C(=C76)C(=C78C(=C77)C(=C79C(=C78)C(=C80C(=C79)C(=C81C(=C80)C(=C82C(=C81)C(=C83C(=C82)C(=C84C(=C83)C(=C85C(=C84)C(=C86C(=C85)C(=C87C(=C86)C(=C88C(=C87)C(=C89C(=C88)C(=C90C(=C89)C(=C91C(=C90)C(=C92C(=C91)C(=C93C(=C92)C(=C94C(=C93)C(=C95C(=C94)C(=C96C(=C95)C(=C97C(=C96)C(=C98C(=C97)C(=C99C(=C98)C(=C100C(=C99)C(=C101C(=C100)C(=C102C(=C101)C(=C103C(=C102)C(=C104C(=C103)C(=C105C(=C104)C(=C106C(=C105)C(=C107C(=C106)C(=C108C(=C107)C(=C109C(=C108)C(=C110C(=C109)C(=C111C(=C110)C(=C112C(=C111)C(=C113C(=C112)C(=C114C(=C113)C(=C115C(=C114)C(=C116C(=C115)C(=C117C(=C116)C(=C118C(=C117)C(=C119C(=C118)C(=C120C(=C119)C(=C121C(=C120)C(=C122C(=C121)C(=C123C(=C122)C(=C124C(=C123)C(=C125C(=C124)C(=C126C(=C125)C(=C127C(=C126)C(=C128C(=C127)C(=C129C(=C128)C(=C130C(=C129)C(=C131C(=C130)C(=C132C(=C131)C(=C133C(=C132)C(=C134C(=C133)C(=C135C(=C134)C(=C136C(=C135)C(=C137C(=C136)C(=C138C(=C137)C(=C139C(=C138)C(=C140C(=C139)C(=C141C(=C140)C(=C142C(=C141)C(=C143C(=C142)C(=C144C(=C143)C(=C145C(=C144)C(=C146C(=C145)C(=C147C(=C146)C(=C148C(=C147)C(=C149C(=C148)C(=C150C(=C149)C(=C151C(=C150)C(=C152C(=C151)C(=C153C(=C152)C(=C154C(=C153)C(=C155C(=C154)C(=C156C(=C155)C(=C157C(=C156)C(=C158C(=C157)C(=C159C(=C158)C(=C160C(=C159)C(=C161C(=C160)C(=C162C(=C161)C(=C163C(=C162)C(=C164C(=C163)C(=C165C(=C164)C(=C166C(=C165)C(=C167C(=C166)C(=C168C(=C167)C(=C169C(=C168)C(=C170C(=C169)C(=C171C(=C170)C(=C172C(=C171)C(=C173C(=C172)C(=C174C(=C173)C(=C175C(=C174)C(=C176C(=C175)C(=C177C(=C176)C(=C178C(=C177)C(=C179C(=C178)C(=C180C(=C179)C(=C181C(=C180)C(=C182C(=C181)C(=C183C(=C182)C(=C184C(=C183)C(=C185C(=C184)C(=C186C(=C185)C(=C187C(=C186)C(=C188C(=C187)C(=C189C(=C188)C(=C190C(=C189)C(=C191C(=C190)C(=C192C(=C191)C(=C193C(=C192)C(=C194C(=C193)C(=C195C(=C194)C(=C196C(=C195)C(=C197C(=C196)C(=C198C(=C197)C(=C199C(=C198)C(=C200C(=C199)C(=C201C(=C200)C(=C202C(=C201)C(=C203C(=C202)C(=C204C(=C203)C(=C205C(=C204)C(=C206C(=C205)C(=C207C(=C206)C(=C208C(=C207)C(=C209C(=C208)C(=C210C(=C209)C(=C211C(=C210)C(=C212C(=C211)C(=C213C(=C212)C(=C214C(=C213)C(=C215C(=C214)C(=C216C(=C215)C(=C217C(=C216)C(=C218C(=C217)C(=C219C(=C218)C(=C220C(=C219)C(=C221C(=C220)C(=C222C(=C221)C(=C223C(=C222)C(=C224C(=C223)C(=C225C(=C224)C(=C226C(=C225)C(=C227C(=C226)C(=C228C(=C227)C(=C229C(=C228)C(=C230C(=C229)C(=C231C(=C230)C(=C232C(=C231)C(=C233C(=C232)C(=C234C(=C233)C(=C235C(=C234)C(=C236C(=C235)C(=C237C(=C236)C(=C238C(=C237)C(=C239C(=C238)C(=C240C(=C239)C(=C241C(=C240)C(=C242C(=C241)C(=C243C(=C242)C(=C244C(=C243)C(=C245C(=C244)C(=C246C(=C245)C(=C247C(=C246)C(=C248C(=C247)C(=C249C(=C248)C(=C250C(=C249)C(=C251C(=C250)C(=C252C(=C251)C(=C253C(=C252)C(=C254C(=C253)C(=C255C(=C254)C(=C256C(=C255)C(=C257C(=C256)C(=C258C(=C257)C(=C259C(=C258)C(=C260C(=C259)C(=C261C(=C260)C(=C262C(=C261)C(=C263C(=C262)C(=C264C(=C263)C(=C265C(=C264)C(=C266C(=C265)C(=C267C(=C266)C(=C268C(=C267)C(=C269C(=C268)C(=C270C(=C269)C(=C271C(=C270)C(=C272C(=C271)C(=C273C(=C272)C(=C274C(=C273)C(=C275C(=C274)C(=C276C(=C275)C(=C277C(=C276)C(=C278C(=C277)C(=C279C(=C278)C(=C280C(=C279)C(=C281C(=C280)C(=C282C(=C281)C(=C283C(=C282)C(=C284C(=C283)C(=C285C(=C284)C(=C286C(=C285)C(=C287C(=C286)C(=C288CC1(C)CC2=C(C1)C(=C(C=C2)OC(=O)C3C(C(C3)OC)OC)C4=C(C=C5C(=C(C=C5)OC)OC)C(=C4)OC

The chemical structure is a complex steroid derivative. It features a four-ring steroid nucleus. Key functional groups include:

- A ketone group (C=O) at the C3 position.
- Hydroxyl groups (OH) at the C11, C14, and C15 positions.
- A fluorine atom (F) at the C13 position.
- A side chain at the C17 position containing a ketone group (C=O) and a hydroxyl group (OH).
- Methyl groups at the C10 and C13 positions.
- A double bond between C4 and C5.



Chemical structure of 2-(3-phenyl-3-oxoprop-1-en-1-yl)-3,4-dihydro-2H-chromene-6-carboxylic acid. The structure features a chromene core with a carboxylic acid group at position 6, a 3-phenyl-3-oxoprop-1-en-1-yl group at position 2, and hydroxyl groups at positions 3 and 4.

Tomentolide A

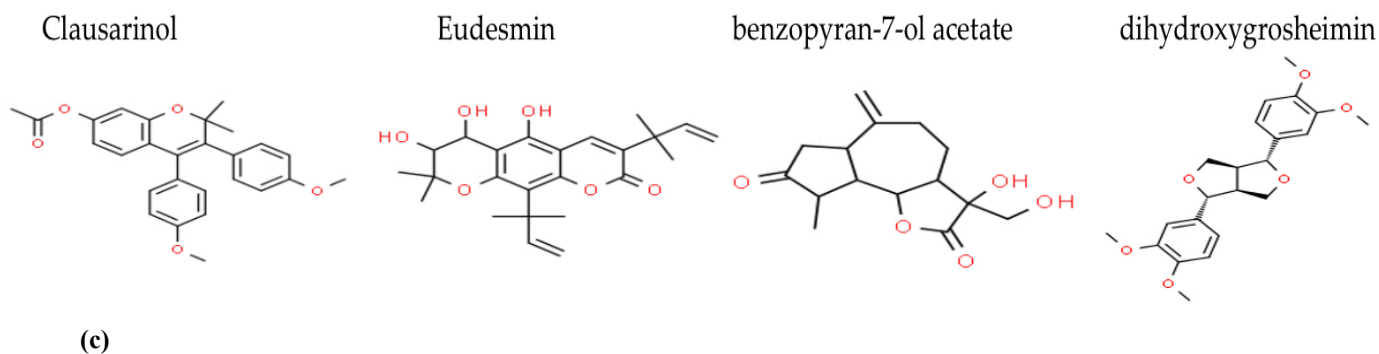
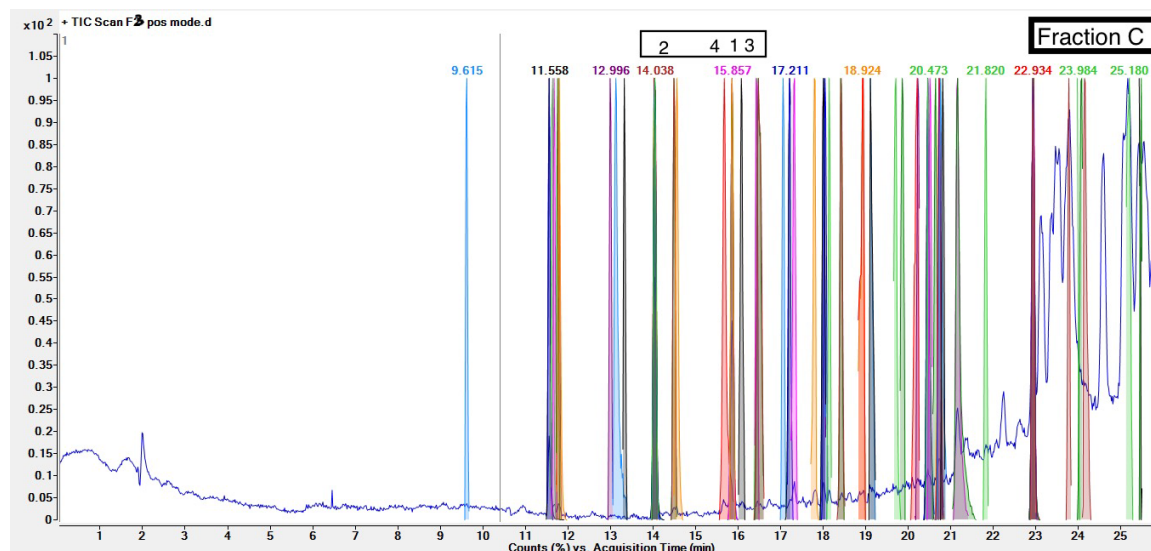


Figure 3. Phenolic compounds LC-QTOF/MS analysis in fractions of *G.inodorum* extract, operated under the following parameters: ionization mode [positive], ionization source [Dual AJS ESI], and collision energy [0]. Mass measurements were conducted with high resolution and accuracy 1.3 Da, 3ppm respectively, ensuring precise determination of molecular masses (a) LC-QTOF/MS analysis of fraction A phenolic compounds; (b) LC-QTOF/MS analysis of fraction B phenolic compounds; (c) LC-QTOF/MS analysis of fraction C phenolic compounds.

3.2 Discussion

This investigation studied different methods for determining the antioxidant potential, screening phenolic compounds, and analyzing the gymnemic acid level using other compound extraction laboratory techniques. Column chromatography and thin-layer chromatography have been used to detect and separate active compounds in the extract (Abeyrathne et al., 2022). Our results suggest that column separation is a good technique for obtaining the active compound in the extract. Interestingly, preliminary thin-layer chromatography results were similar for column separation, which

facilitated selecting the solvents for the best separation of fractions.

Various phenolic classes of Apocynaceae family have attracted significant interest due to their physiological roles, encompassing activities such as scavenging free radicals, preventing mutations, inhibiting carcinogenesis, and reducing inflammation (Brand-Williams et al., 1995). The antioxidative efficacy of phenolic compounds is primarily due to their redox properties, enabling them to function as reducing agents, hydrogen donors, quenchers of singlet oxygen, and potential metal chelators (L. Yu et al., 2002).

The column chromatographic fractionation of *G. inodorum* leaf extract has successfully resolved the critical limitations associated with raw, unfractionated crude extract. Standard crude extractions, such as ethanol reflux, yield highly complex mixtures where pigments and lipids remain co-extracted (Wu et al., 2006), (Cianciosi et al., 2018). These compounds form a dense matrix that physically encapsulates active phenolics, thereby limiting their kinetic rate of free radical scavenging (Nuchuchua et al., 2024). Further, based on the results, a notably elevated concentration of phenolic compounds was detected in the fractions of column separation reflux extract obtained from the leaves of *G. inodorum*. This finding could provide a scientific basis for the extensive historical utilization of this plant in traditional folk medicine practices as well as evidence for high antioxidant activity.

The antioxidant activities of the phytochemical compounds are essential to scavenging free radicals and have a beneficial effect against oxidative-related disorders (Benzie & Strain, 1996). Many studies have reported that plant extracts' bioactive compound possesses a strong antioxidative capacity, possibly enhancing human health (Huang et al., 2005). The DPPH assay has been employed for radical scavenging activity that revealed the antioxidant activity of each fraction. This assay revealed that the separation of fractions via column chromatography showed increased radical scavenging activity of the DPPH radical, which may indicate an increased ability of purified compounds in fractions to donate hydrogen ions (Nwozo et al., 2023). This kinetic pathway is highly favored by less polar, highly mobile phenolic acids and lipophilic flavonoids, which were concentrated in Fraction A. Therefore, it may be postulated that fraction A of *G. inodorum* leaf extract contains higher potent DPPH and ABTS radical scavenging activity due to abundant purified phytochemicals that reduce the radical due to their hydrogen-reducing ability. However, fractions B and C have lower scavenging of DPPH and ABTS radicals, which may be due to the compound's light affinity, temperature, and

solubility of extracts in the solvent system, which were reported factors for affecting the capacity of compounds to quench the radical (Srinuanchai et al., 2019).

The antioxidant capacity test by ABTS assay (Haideri et al., 2024) revealed that fraction A has high ABTS+ radical scavenging, the same as the standard at the highest 1 mg/mL concentration. This indicated the capability of active compounds in our extract to scavenge free radicals, indicating the potential presence of specific phenolic compounds within our extract with in vitro antioxidant activity relevant to oxidative damage associated with free radicals, pending confirmation of therapeutic efficacy in vivo.

The FRAP assay measures the antioxidative capacity of an antioxidant by its reaction with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex, resulting in the formation of a colored ferrous tripyridyltriazine (Fe^{2+} -TPTZ) product (L.-H. Yu et al., 2004). FRAP assay does not involve free radicals but measures the reduction of a ferric tripyridyltriazine complex to its ferrous form via single electron transfer mechanism. Based on the FRAP assay results, conversely, fraction C has reported the highest antioxidant activity than fractions A and B, opposite to DPPH and ABTS results. This divergence likely reflects the different reaction chemistries of the assays: DPPH and ABTS are radical-scavenging assays that respond preferentially to the lipophilic, hydrogen-donating phenolics and flavonoids concentrated in the less polar Fraction A, whereas FRAP measures single-electron-transfer reducing power, which favors the more polar, hydrophilic constituents enriched in Fraction C - consistent with Fraction C also showing the highest TFC and gymnemic acid content in this study (Table 1 and Table 2). FRAP involves the reduction of a ferric ion to its ferrous form by electron donation from these reducing agents, rather than the hydrogen-atom or electron transfer to a stable free radical measured by DPPH/ABTS (L.-H. Yu et al., 2004). Our results are supported by a previous study on *G. inodorum* for high antioxidant activity in crude extract (Wang et al., 2020).

The gymnemic acid levels quantified across the three fractions (Table 2) were markedly higher than the 0.0194 mg/g reported in a previous study, irrespective of extraction method (Srinuanchai et al., 2019), supporting the applied method for separation of active compounds in *G. inodorum* leaf extract. Furthermore, the extraction method's efficiency is bolstered by the determination of gymnemic acid levels.

The current study has shown that the fractions of *G. inodorum* leaf extract contains high concentrations of phenols and flavonoids as active compounds; thus, it is inferred that these active compounds are responsible for high antioxidant activity (Montoro et al., 2018). The correlation between antioxidant activity and total phenolic contents of *G. inodorum* extract is consistent in several reports. The antioxidant activity of fractions revealed that the fractions have higher activity as compared to crude extract. The previous study (Barker, 2019) has published 0.13506 mg TE/g as the highest DPPH antioxidant activity for *Gymnema inodorum* leaf extract, which is lower in comparison to the activity of our fractionated extracts. Furthermore, the fractionation showed better activity for FRAP and ABTS as compared to crude extract. Moreover the results showed the connection between the total phenolic contents of the fractions of *G. inodorum* leaf extract and the antioxidant activity as assessed by the DPPH or ABTS assay. The findings demonstrated a direct relationship between total phenolic levels and either DPPH or ABTS radical scavenging activity. Both the DPPH and ABTS tests indicated the high antioxidant capacity of the fractions with high total phenolic contents. Therefore, the fractions of *G. inodorum* leaf extract have considerable phenolic compounds with antioxidant properties. Compared with previously reported crude *G. inodorum* extracts, the chromatographic fractions demonstrated enhanced antioxidant activity, suggesting enrichment of active phenolic constituents through fractionation.

Interestingly, LC-QTOF/MS analysis results supported that fractions with higher phenolic

compound contents showed greater antioxidant activity. There may be a relationship between phenolic compounds and bioactivity, particularly in terms of scavenging free radicals, as evidenced by the higher quantities of these compounds found in fractions with increased antioxidant activity. Nonetheless, a thorough statistical analysis that definitively establishes this association can support the study's conclusions even more and call for further investigation in the future. The difference in concentration of phenolics and flavonoids was reported by other *Gymnema* studies (Haideri et al., 2024). Moreover, the screened active phenolic compound, Schisanhenol B was found in *Schisandra chinensis* and magnolia vine or five-flavor berry, which were the new active phenolic compounds in *G. inodorum* leaf extract, an aspect not reported in the previous study. These active compounds have been shown to have an antioxidative effect in human low-density lipoprotein (Montoro et al., 2018). Among the identified phenolic compounds, Zhebeiresinol a phenolic lignan has potential health benefits, including antioxidant and anti-inflammatory properties (Barker, 2019). The compound Fluocinolone has been studied for treating localized psoriasis (L.-H. Yu et al., 2004). Furthermore Eudesmin is found in LC-QTOF/MS result, which is natural phenolic lignan having specific antioxidant and anti-inflammatory potential (Barker, 2019), while Clausarinol, Chamuvaritin and Tomentolide A seemed to be a new compounds with limited or no prior study. The identification of Tomentolide A in fraction B is considered tentative because the matching score (79.90%) is comparatively lower than other compounds. The consumption of this plant may play a vital role due to active phenolic compounds for various disease treatments such as diabetes, hyperglycemia, and premature aging. Nevertheless, additional antioxidant potential in vivo models and various antioxidant mechanisms should be comprehensively studied.

Pearson and Spearman correlations were computed across the three fraction-level means (TPC, TFC, DPPH, ABTS, FRAP, and

gymnemic acid; Figure 4). With only $n = 3$ fractions, none of these correlations reach statistical significance (all $p > 0.1$) and the coefficients should be read as exploratory only. The strongest associations were between TFC

and gymnemic acid (Pearson $r = 0.97$) and between ABTS and gymnemic acid ($r = -0.98$), while TPC and DPPH showed a moderate positive association ($r = 0.69$), consistent with the positive association reported in the Abstract.

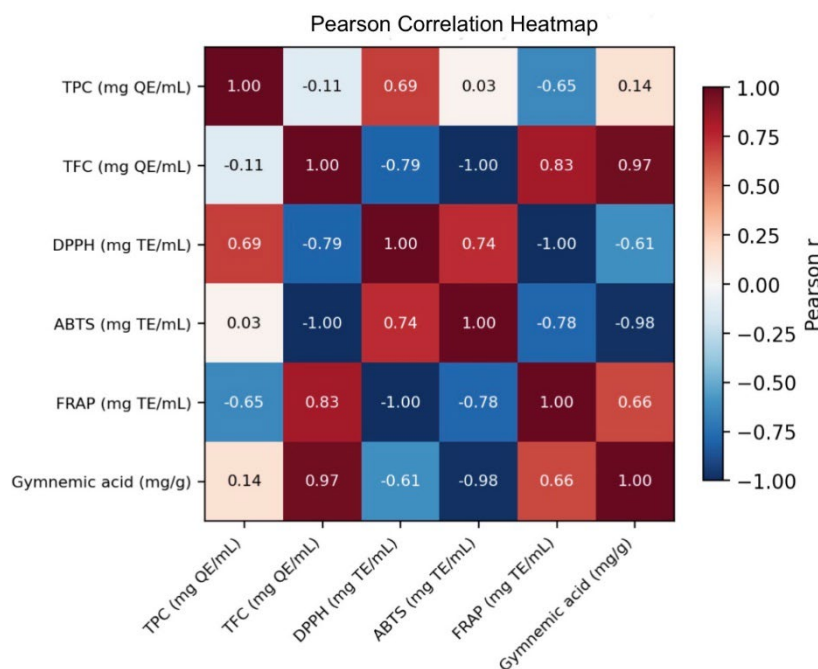


Figure 4. Pearson correlation heatmap across TPC, TFC, DPPH, ABTS, FRAP, and gymnemic acid, computed from the three fraction replicates level means ($n = 9$). using true Trolox/quercetin-equivalent concentrations.

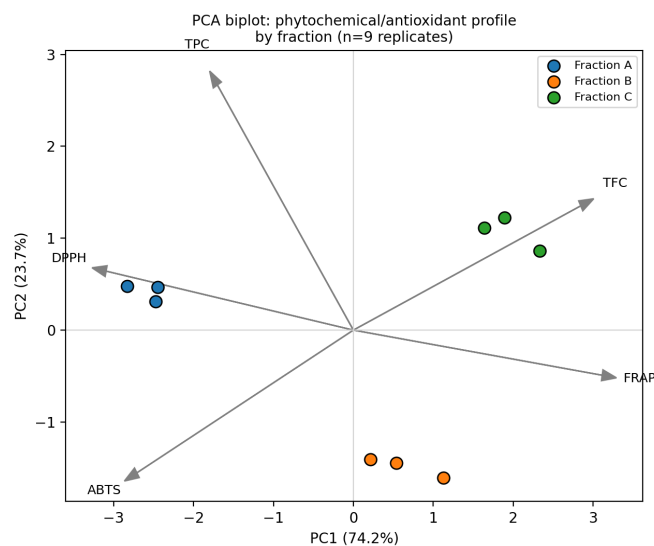


Figure 5. PCA biplot of TPC, TFC, DPPH, ABTS, and FRAP (standardized, $n=9$ replicate readings). Points are individual replicates colored by fraction; arrows show variable loadings

Principal Component Analysis (PCA) on the same five standardized variables (Figure 5) shows the three fractions separating cleanly along two components explaining 98% of total variance (PC1 = 74.2%, PC2 = 23.7%): PC1 is driven by TFC/FRAP loading positively against DPPH/ABTS/TPC loading negatively, separating Fraction C (high TFC/FRAP) from Fractions A and B (high TPC/DPPH/ABTS).

4. Conclusion

In conclusion, the fractions of extract of the plant *G. inodorum* showed significant antioxidant activity and phenolic compounds. This suggests the presence of additional beneficial active phenolic compounds within the plant extract, indicating further potential effects that could be beneficial from its use. The different level of antioxidant activity observed from each fraction are probably due to variation of active phytochemicals. Fraction A exerted the highest radical scavenging capacity as well as highest total phenolic content. The study supported that phenolic compounds may be responsible to antioxidant capacity of *G. inodorum* leaf extract. Our findings further indicate that the column chromatography technique is capable of providing effective purification for these active compounds. Moreover, the results of this study have promising implications across various fields.

Primarily, the endorsement of conventional medical approaches is substantiated by the identification of numerous phenolic components in the leaf extract of *G. inodorum*, coupled with their correlation to potent antioxidant activity. This implies that *G. inodorum* may have therapeutic relevance for oxidative stress-related disorders, pending confirmation in vivo. Moreover, this investigation illuminates neglected phytochemical compounds, prompting additional re-search to unveil the concealed therapeutic potential of this locally edible plant. Utilizing column chromatography for their purification offers promise in isolating and studying these valuable compounds. Nonetheless, future research focusing on characterization and identification of active compounds, and its health effect especially

through in-vivo models and clinical studies, is imperative to translate these findings into practical applications for human health.

5. References

- Abeyrathne, E. D. N. S., Nam, K., Huang, X., & Ahn, D. U. (2022). Plant- and Animal-Based Antioxidants' Structure, Efficacy, Mechanisms, and Applications: A Review. *Antioxidants*, 11(5), 1025.
- Abu, F., Mat Taib, C. N., Mohd Moklas, M. A., & Mohd Akhir, S. (2017). Antioxidant Properties of Crude Extract, Partition Extract, and Fermented Medium of *Dendrobium sabin* Flower. *Evidence-Based Complementary and Alternative Medicine*, 2017(1), 2907219.
- Arnao, M. B., Cano, A., & Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chemistry*, 73(2), 239–244.
- Baba, S. A., & Malik, S. A. (2015). Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *Journal of Taibah University for Science*, 9(4), 449–454.
- Bajpai, V. K., Majumder, R., & Park, J. G. (2016). Isolation and purification of plant secondary metabolites using column-chromatographic technique. *Bangladesh Journal of Pharmacology*, 11(4), 844–848.
- Barker, D. (2019). Lignans. In *Molecules* (Vol. 24, Number 7). MDPI AG.
- Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry*, 239(1), 70–76.
- Boonyapranai, K., Sarakul, O., Somsak, V., & Sukati, S. (2022). Effects of *Gymnema inodorum* Leaf Extract on the Alteration of Blood Coagulation Parameters and Platelet Count in *Plasmodium berghei*-Infected Mice. *Journal of Parasitology Research*, 2022.
- Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to

- evaluate antioxidant activity. *LWT - Food Science and Technology*, 28(1), 25–30.
- Chaves, N., Santiago, A., & Alías, J. C. (2020). Quantification of the Antioxidant Activity of Plant Extracts: Analysis of Sensitivity and Hierarchization Based on the Method Used. *Antioxidants*, 9(1), 76.
- Chiabchalard, A., Tencomnao, T., & Santiyanont, R. (2010). Effect of *Gymnema inodorum* on postprandial peak plasma glucose levels in healthy human. *African Journal of Biotechnology*, 9, 1079–1085.
- Cianciosi, D., Forbes-Hernández, T. Y., Afrin, S., Gasparri, M., Reboredo-Rodríguez, P., Manna, P. P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., Quiles, J. L., Giampieri, F., & Battino, M. (2018). Phenolic Compounds in Honey and Their Associated Health Benefits: A Review. *Molecules*, 23(9).
- Dai, J., & Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, 15(10), 7313–7352.
- Devi, K., & Jain, N. (2014). A validated HPLC method for estimation of Gymnemic acids as Deacyl gymnemic acid in various extracts and formulations of *Gymnema sylvestre*. *International Journal of Phytomedicine*.
- Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal Processing Enhances the Nutritional Value of Tomatoes by Increasing Total Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, 50(10), 3010–3014.
- Dunkhunthod, B., Talabnin, C., Murphy, M., Thumanu, K., Sittisart, P., & Eumkeb, G. (2021). *Gymnema inodorum* (Lour.) Decne. Extract Alleviates Oxidative Stress and Inflammatory Mediators Produced by RAW264.7 Macrophages. *Oxidative Medicine and Cellular Longevity*, 2021, 1–20.
- Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., & Kader, A. A. (2002). Antioxidant Capacities, Phenolic Compounds, Carotenoids, and Vitamin C Contents of Nectarine, Peach, and Plum Cultivars from California. *Journal of Agricultural and Food Chemistry*, 50(17), 4976–4982.
- Gini, T. G., & Jeya Jothi, G. (2018). Column chromatography and HPLC analysis of phenolic compounds in the fractions of *Salvinia molesta mitchell*. *Egyptian Journal of Basic and Applied Sciences*, 5(3), 197–203.
- Plants of the world online. (2022, October 24). *Gymnema — The Plant List*.
- Haideri, M. H., Phanjaroen, T., Khiaolaongam, W., Boonchalaem, T., Laoung-on, J., Chaipoot, S., Hongsibsong, S., Boonyapranai, K., & Ounjaijean, S. (2024). Effect of Different Extraction Techniques on Phenolic Profile and Phytochemical Potential of *Gymnema inodorum* Leaf Extract. *Molecules*, 29(22).
- Huang, D., Ou, B., & Prior, R. L. (2005). The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry*, 53(6), 1841–1856.
- Islam, M., Prottay, A. A. S., Sultana, I., Al Faruq, A., Bappi, M. H., Akbor, M. S., Asha, A. I., Hossen, M. M., Machado, P. E. M., Secundo Junior, I. J., Coutinho, H. D. M., & Islam, M. T. (2023). Phytochemical screening and evaluation of antioxidant, anti-inflammatory, antimicrobial, and membrane-stabilizing activities of different fractional extracts of *Grewia nervosa* (Lour.) Panigrahi. *Food Bioscience*, 54.
- Jeeno, P., Tongban, S., Yana, P., Wongta, A., Sutan, K., Yadoung, S., & Hongsibsong, S. (2022). Tentative Identification of Phytochemicals from *Smilax glabra* and *Smilax corbularia* Extracts by LC-QTOF/MS and Their Bioactive Potential. *Plants*, 11(16), 2089.
- Jeytawan, N., Yadoung, S., Jeeno, P., Yana, P., Sutan, K., Naksen, W., Wongkaew, M., Sommano, S. R., & Hongsibsong, S. (2022). Antioxidant and Phytochemical Potential of and Phytochemicals in *Gymnema inodorum* (Lour.) Decne in Northern Thailand. *Plants*, 11(24).
- Kasote, D. M., Katyare, S. S., Hegde, M. V., & Bae, H. (2015). Significance of Antioxidant Potential of Plants and its Relevance to

- Therapeutic Applications. *International Journal of Biological Sciences*, 11(8), 982–991.
- Manthey, J. A. (2000). Biological properties of flavonoids pertaining to inflammation. *Microcirculation (New York, N.Y. : 1994)*, 7(6 Pt 2), S29-34.
- Miliauskas, G., Venskutonis, P. R., & Van Beek, T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, 85(2), 231–237.
- Montoro, V., Asensio, C., Martínez, Á., Lorente, J., Rodríguez, F. J., Montojo, J., Gavilanes, J., Sarria, P., Langdon, C., & Prades, E. (2018). Efficacy and safety of fluocinolone acetonide 0.025% otic solution in patients with otic eczema: a randomized, double-blind, placebo-controlled clinical trial. *Journal of International Medical Research*, 46(10), 4050–4060.
- Nuchuchua, O., Srinuanhai, W., Chansrinaiyom, C., Suttisansanee, U., Temviriyankul, P., Nuengchamnong, N., & Ruktanonchai, U. (2024). Relationship of phytochemicals and antioxidant activities in *Gymnema inodorum* leaf extracts. *Heliyon*, 10(1).
- Nwozo, O. S., Effiong, E. M., Aja, P. M., & Awuchi, C. G. (2023). Antioxidant, phytochemical, and therapeutic properties of medicinal plants: a review. *International Journal of Food Properties*, 26(1), 359–388.
- Ounjaijean, S., Romyasamit, C., & Somsak, V. (2021). Evaluation of Antimalarial Potential of Aqueous Crude *Gymnema Inodorum* Leaf Extract against *Plasmodium berghei* Infection in Mice. *Evidence-Based Complementary and Alternative Medicine*, 2021.
- Phanjaroen, T., Haideri, M. H., Khiaolaongam, W., Somsak, V., Boonyapranai, K., & Ounjaijean, S. (2024). Phytochemical Content, Antioxidant and α -Glucosidase Inhibitory Activities of *Gynostemma pentaphyllum* and *Gymnema inodorum* Extracts. *Journal of Current Science and Technology*, 14(3).
- Pietta, P. G. (2000). Flavonoids as antioxidants. In *Journal of Natural Products* (Vol. 63, Number 7, pp. 1035–1042).
- Srinuanhai, W., Nooin, R., Jarussophon, S., Kasemwong, K., & Nuchuchua, O. (2019). Determination of gymnemic acid level in *Gymnema inodorum* leaves using multiple reaction monitoring mass spectrometry. *Journal of Chemical Metrology*, 13(2), 75–79.
- Suwan, N., Baison, W., & Chuajedton, A. (2022). Purification of *Gymnema inodorum* leaf extract and its antifungal potential against *Colletotrichum gloeosporioides*. 92(3), 667–677.
- Tiwari, P., Mishra, B. N., & Sangwan, N. S. (2014). Phytochemical and pharmacological properties of *Gymnema sylvestre*: An important medicinal plant. *BioMed Research International*, 2014.
- Wang, X., Ma, S., Lai, F., Wang, Y., & Lou, C. (2020). Traditional Applications, Phytochemistry, and Pharmacological Activities of *Eupatorium lindleyanum* DC.: A Comprehensive Review. *Frontiers in Pharmacology, Volume 11-2020*.
- Wu, L., Hsu, H.-W., Chen, Y.-C., Chiu, C.-C., Lin, Y.-I., & Ho, J. A. (2006). Antioxidant and antiproliferative activities of red pitaya. *Food Chemistry*, 95(2), 319–327.
- Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J., & Qian, M. (2002). Free Radical Scavenging Properties of Wheat Extracts. *Journal of Agricultural and Food Chemistry*, 50(6), 1619–1624.
- Yu, L.-H., Liu, G.-T., Sun, Y.-M., & Zhang, H.-Y. (2004). Antioxidative effect of schisanhenol on human low-density lipoprotein and its quantum chemical calculation 1. *Acta Pharmacol Sin*, 25(8).
- Zhang, Y.-J., Gan, R.-Y., Li, S., Zhou, Y., Li, A.-N., Xu, D.-P., & Li, H.-B. (2015). Antioxidant Phytochemicals for the Prevention and Treatment of Chronic Diseases. *Molecules (Basel, Switzerland)*, 20(12), 21138–21156.

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