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Quantitative analysis of stigmasterol and α-Amyrin in three antidiabetic medicinal plants using GC-MS/MS

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Traditional medicinal systems in India frequently employ *Costus igneus* N.E.Br., *Bryophyllum pinnatum* (Lam.) Oken, and *Ficus benghalensis* L. for the management of diabetes mellitus; however, systematic comparative quantitative evidence supporting their bioactive sterol content remains limited. The present investigation aimed to comparatively quantify two pharmacologically significant phytomarkers, stigmasterol and α-amyrin, in chloroform extracts of the leaves of *C. igneus* and *B. pinnatum*, and of the aerial roots of *F. benghalensis*, using a validated GC–MS/MS method operating in Multiple Reaction Monitoring (MRM) mode. Qualitative GC–MS profiling confirmed the presence of both analytes in all samples. Quantitative analysis revealed that *C. igneus* leaves contained the highest concentration of stigmasterol (4580±310 µg/kg), whereas *F. benghalensis* aerial roots were particularly rich in α-amyrin (3250±250 µg/kg). *B. pinnatum* exhibited moderate yet appreciable levels of both compounds. The distinct quantitative patterns observed suggest that although these plants share ethnomedicinal use in diabetes management, their chemical contributors may differ significantly. The findings establish a comparative phytochemical baseline that may guide future pharmacological validation and standardisation efforts.

Keywords: *Bryophyllum pinnatum*, *Costus igneus*, *Ficus benghalensis*, GC–MS/MS, Stigmasterol, α-Amyrin

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Introduction

Diabetes mellitus presents a complex and growing public health challenge worldwide, necessitating continued exploration of diverse therapeutic avenues[1,2]. Throughout history, human societies have relied heavily on medicinal plants, accumulating vast empirical knowledge of their use for various ailments, including metabolic disorders such as diabetes[3]. Investigating these traditional remedies through rigorous scientific methods is essential not only to validate their efficacy but also to identify the specific bioactive chemical constituents responsible, potentially leading to new pharmaceuticals or evidence-based phytotherapies. Increasing limitations of conventional antidiabetic therapies have encouraged the exploration of medicinal plants as alternative or complementary treatment options[2,4]. Among the many plants cited in traditional systems for their anti-diabetic potential, the leaves of *Costus igneus* N.E.Br., *Bryophyllum pinnatum* (Lam.) Oken, and *Ficus benghalensis* L. are frequently mentioned, particularly in the context of Indian traditional medicine.

C. igneus, commonly known as the 'Insulin Plant', has garnered significant attention due to its leaves, which have widespread traditional use for managing hyperglycemia across various regions[5]. This traditional acclaim is increasingly supported by preclinical studies demonstrating notable hypoglycemic, antioxidant, and lipid-lowering activities of *C. igneus* leaf extracts in diabetic models[6,7]. Phytochemical analyses have revealed a diverse array of compounds, including flavonoids, alkaloids, saponins, steroids, and triterpenoids, indicating a 006Dultifaceted chemical profile that warrants detailed quantitative investigation, especially concerning compounds relevant to its anti-diabetic effects[5].

B. pinnatum, often called 'Patharchatta', 'Miracle Plant', is another plant with a rich history in traditional medicine across the globe. Its leaves are used to treat conditions such as infections, inflammation, and kidney ailments, and are reportedly used for diabetes management[8-10]. Its complex phytochemistry features unique phytoconstituents, various flavonoids, phenolic acids, and a range of triterpenoids and phytosterols[10,11]. While many biological activities have been explored, pinpointing and quantifying the specific chemical constituents that

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may contribute to its traditional use in metabolic regulation remains important research objective[12].

F. benghalensis, the Indian Banyan, is deeply integrated into Ayurvedic tradition[13]. While different parts possess medicinal properties, the aerial roots are particularly recommended in traditional practices for managing diabetes mellitus, often cited for their cooling, astringent, and restorative actions[12]. Chemical studies have identified tannins, flavonoids, sterols such as beta-sitosterol, and other phytochemical compounds in the aerial roots[13,14]. Despite some evidence suggesting hypoglycemic effects of root extracts, comprehensive quantitative data mapping specific marker compounds related to metabolic health in the aerial roots is limited[15].

When investigating the potential chemical basis for anti-diabetic activity in these plants, the phytosterol Stigmasterol and the pentacyclic triterpene α-amyrin emerge as compelling candidates. Stigmasterol, a widely distributed plant sterol, has demonstrated significant promise in numerous experimental models, exhibiting anti-hyperglycemic effects, improving insulin action, and beneficially modulating lipid metabolism[13,16-19]. These actions suggest it may contribute significantly to the metabolic benefits of plants containing it. α-amyrin, a prevalent triterpene, exhibits strong anti-inflammatory and antioxidant activities – processes highly relevant to mitigating diabetic complications[1,20,21]. Moreover, emerging studies suggest α-amyrin may also directly impact glucose and lipid pathways favorably[16,21,22]. Consequently, comparing the quantities of both Stigmasterol and α-amyrin within these plants could provide valuable clues regarding their potential roles in the plants anti-diabetic profiles.

Despite this background, a clear knowledge gap exists: a direct, head-to-head quantitative comparison of Stigmasterol and α-amyrin across *C. igneus* leaves, *B. pinnatum* leaves, and *F. benghalensis* aerial roots, utilising a consistent and appropriately validated analytical method, has not been previously reported. Such a comparative analysis is crucial for understanding whether these potentially important markers are consistently associated with the traditional anti-diabetic uses of these diverse plants, or if their levels vary substantially, pointing towards distinct chemical contributions and possibly different primary mechanisms of action.

To effectively address this knowledge gap, a systematic analytical approach was designed. Initially, a preliminary qualitative phytochemical screening was performed on the chloroform extracts obtained from the leaves of *C. igneus* and *B. pinnatum*, as well as the aerial roots of *F. benghalensis*[12,15,23]. Subsequently, direct Gas Chromatography-Mass Spectrometry (GC-MS) analysis was planned to identify and confirm the target molecules, Stigmasterol and α-amyrin[10,24]. The primary objective, however, was to accurately quantify and compare the levels of these two key compounds across the three different plant samples. To achieve these goals with precision and reliability, a sensitive Gas Chromatography-Tandem Mass Spectrometry (GC–MS/MS) method was developed and validated—paying particular attention to the limits of detection (LOD) and quantification (LOQ)—and then applied. This method, operated in Multiple Reaction Monitoring (MRM) mode, was selected for its high selectivity and employed using external standard calibration[12,14,23]. However, a direct comparative quantification of stigmasterol and α-amyrin across these three plants using a single GC-MS/MS method has not been reported[12,15,25]. Similar GC-MS-based phytochemical characterisation has also been successfully applied in other medicinal plants for antidiabetic compound screening[26,27].

Materials and Methods

Plant material collection and authentication

Fresh, healthy leaves of *C. igneus* N.E.Br. and *B. pinnatum* (Lam.) Oken, along with young aerial roots of *F. benghalensis* L., were collected from the botanical garden of Shri D. D. Vispute College of Pharmacy and Research Centre, Navi Mumbai, Maharashtra, India. The plant materials were authenticated by the Department of Pharmaceutical Chemistry and Biotechnology, Shri D.D. Vispute College of Pharmacy and Research Centre, Navi Mumbai. Voucher specimens were prepared and deposited in the institutional herbarium for future reference under the following accession numbers: *C. igneus* – Voucher No: ASR/05/02, *B. pinnatum* – Voucher No: ASR/05/03, *F. benghalensis* – Voucher No: ASR/05/04.

Sample preparation

Collected plant materials were first thoroughly washed under running tap water to remove debris, followed by a final rinse with distilled water. The cleaned samples were then air-dried in the shade at room temperature (25-30°C) for approximately 10 days until a constant weight was achieved. The

completely dried materials were ground into a fine, consistent powder using a sterile mechanical grinder and stored in airtight, labelled containers protected from light and moisture until extraction.

Chemicals and reagents

Chloroform (HPLC grade, ≥99.8%) and Ethyl Acetate (MS grade, ≥99.9%) were sourced from Merck Life Science Pvt. Ltd. (Mumbai, India). High-purity reference standards of stigmasterol (≥95%) and α-amyrin (≥98%) were purchased from Sigma-Aldrich. All other reagents were of analytical grade. Ultra-pure water was generated using a Millipore Milli-Q system.

Soxhlet extraction

A 10 g sample of each dried plant powder was subjected to Soxhlet extraction with 100 mL of chloroform. The extraction was run continuously for 9-10 hours at 100°C. After extraction, the solution was filtered, and the chloroform was removed under reduced pressure using a rotary evaporator at 35°C. The resulting crude extract was dried completely under vacuum, weighed to determine the extraction yield, and stored at 4°C.

Preliminary phytochemical screening

Preliminary phytochemical tests were conducted to obtain a qualitative assessment of the principal compound classes in the chloroform extracts. Approximately 100 mg of each extract was dissolved in 5 mL of chloroform and tested using standard methods[23]. These tests targeted Alkaloids (Mayer's test, Dragendorff's reagent), Flavonoids (Alkaline reagent test), Steroids (Liebermann-Buchard test), Triterpenoids (Salkowski test), Saponins (Saponification test), Tannins (Ferric chloride test), and Phenolic Compounds (Lead acetate test). Positive (+) or negative (-) results were recorded based on observable reactions, and the complete findings are presented in Table 1.

Qualitative GC-MS analysis

To understand the overall chemical composition of the chloroform extracts, Gas Chromatography-Mass Spectrometry (GC-MS) was employed to create a chemical 'fingerprint' for each plant sample[22,25]. The samples were prepared by dissolving approximately 50 mg of each dried extract in 10 mL of Ethyl Acetate, followed by filtration through a 0.22 µm PTFE syringe filter to remove any small particles. These solutions were then analysed directly. The samples were injected into the GC-MS system, which consisted of an Agilent 7890B GC coupled with an Agilent 7000D Mass Spectrometer Triple Quadrupole. GC conditions were: Carrier Gas- Helium, HP-5MS capillary column (30m x 0.25mm x 0.25µm), Split liner ratio 1:50, Injection volume 0.5 µL, Inlet temperature 250°C, run time 56.0 min, column oven program: 40°C hold for 1 min, ramp 5°C/min to 210°C hold 5 min, ramp 10°C/min to 280°C hold 9 min. MS conditions were: Ion Source EI+ (Electron Ionisation), Electron voltage- 70eV, Solvent delay time- 3.5 min, Mass range 50-500 amu. The data were analysed using the Agilent Mass-Hunter Qualitative Analysis software. Matches with a quality score above 85% in the NIST library were considered as potential identifications[18,19,28].

Quantitative GC–MS/MS Analysis

For precise quantification, a targeted GC-MS/MS method was developed. *Instrumentation:* GC Model- Agilent 7890B GC System, MS Model- Agilent 7000D MS/MS Triple Quadrupole. Data Quantification: Agilent Mass-Hunter Quantitation. *Sample Preparation:* 10 mg of extract was diluted in 10 mL of ethyl acetate solvent and filtered through a 0.22 µm PTFE syringe filter. *GC conditions:* Carrier Gas- Helium, Collision Gas- Nitrogen, Column: HP-5MS capillary column (30m x 0.25mm x 0.25µm), Split-less liner, Injection volume- 1 µL, Inlet temperature- 280°C, Run time 20.75 min. Column oven program: 60°C hold for 1 min, ramp 40°C/min to 170°C hold 0 min, ramp 10°C/min to 310°C hold 3 min. *MS/MS Optimisation:* The analysis was performed in Multiple Reaction Monitoring (MRM) mode. Optimised MRM transitions for stigmasterol (Quantifier: 375.1→323.1; Qualifier:

Table 1 — Preliminary phytochemical screening of chloroform extracts

Phytochemical group	Test performed	*C. Igneus* leaves	*B. pinnatum* leaves	*F. benghalensis* aerial roots
Alkaloids	Mayer's test / Dragendorff's reagent	+	+	-
Flavonoids	Alkaline reagent test	-	-	-
Steroids	Liebermann–Burchard test	+	+	+
Triterpenoids	Salkowski test	+	+	+
Saponins	Saponification test	-	-	-
Tannins	Ferric Chloride test	-	-	-
Phenolic Compounds	Lead acetate test	+	+	-

375.1→283.1) and α-amyrin (Quantifier: 389.1→334.1; Qualifier: 389.1→255.1) were used. Quantification was based on an external standard calibration curve, with a determined Limit of Quantification (LOQ) of 10.0 µg/kg for both analytes.

Statistical analysis

All extractions and quantitative determinations were performed in triplicate (n = 3). Results are expressed as mean ± standard deviation (SD). Statistical calculations were performed using Microsoft Excel.

Results

Extraction yield

The chloroform extraction process yielded distinct amounts from each plant material, with *C. Igneus* leaves providing 1.98±0.07% (w/w, dry basis), *B. Pinnatum* leaves yielding 2.16±0.10%, and *F. benghalensis* aerial roots producing 1.56±0.06%. These yields, while varying, provided adequate material for the subsequent comprehensive chemical analyses.

Preliminary phytochemical screening

Initial qualitative phytochemical screening, detailed in Table 1, offered preliminary insights into the chemical constitution of the chloroform extracts. Significantly, positive results for steroids (Liebermann-Burchard test) and triterpenoids (Salkowski test) were observed in all three plant extracts. This finding was crucial as it supported the anticipated presence of Stigmasterol and α-amyrin, the target compounds of this study. Alkaloids identified in the extracts of *C. igneus* and *B. pinnatum* (Mayer's test/Dragendorff's reagent), and phenolic compounds were also found in these two species (Lead acetate test). As anticipated for a non-polar solvent like chloroform, tests for flavonoids, saponins, and tannins were negative across all extracts.

Qualitative phytochemical profiling by GC-MS

To further elucidate the composition of the non-polar extracts and to specifically confirm the presence of Stigmasterol and α-amyrin, direct GC-MS analysis was conducted. The resulting Total Ion Chromatograms (TICs) for the extracts of *C. igneus* leaves, *B. pinnatum* leaves, and *F. benghalensis* aerial roots are depicted in Fig. 1.

Crucially, the GC-MS analysis verified the presence of peaks corresponding to Stigmasterol (RT ~42.52 minutes) and α-amyrin (RT ~37.49 minutes) in all three plant extracts. Beyond Stigmasterol and α-amyrin, the GC-MS analysis

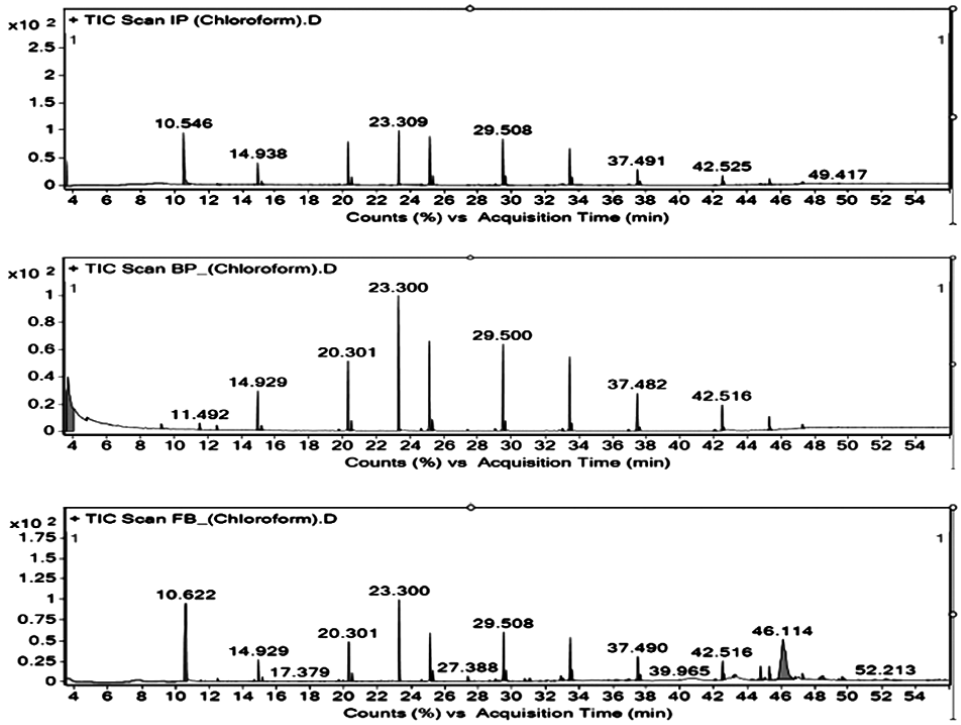

Fig. 1 — TICs of antidiabetic plants by GC-MS Scan.

revealed a diverse array of other phytochemicals within each plant extract, as catalogued in Table 2 (for *C. igneus*), Table 3 (for *B. pinnatum*), and Table 4 (for *F. benghalensis*). Notably, 2,4-Di-tert-butylphenol, an antioxidant compound, was identified as a significant component across all three extracts, with area sum percentages of 16.96% in *C. igneus*, 27.33% in *B. pinnatum*, and 18.87% in *F. benghalensis*.

Comparative quantification of stigmasterol and α-Amyrin using GC-MS/MS

A direct GC-MS/MS method employing Multiple Reaction Monitoring (MRM) was successfully developed and optimised for the sensitive and selective quantification of Stigmasterol and α-amyrin following their qualitative identification. Representative MRM chromatograms are presented in Figs. 2 and 3. The

Table 2 — List of qualitatively identified compounds from *C. Igneus* leaves by GC-MS

Peak No.	Retention time (min)	Area sum (%)	Name of compound
1	10.546	15.96	1-methyl-2-Pyrrolidinone
2	14.938	7.24	1-Dodecene
3	15.183	1.23	2-methyl- Decane
4	20.31	11.86	1-Tetradecene
5	23.309	16.96	2,4-Di-tert-butylphenol
6	25.15	10.17	Cetene
7	29.508	13.25	E-15-Heptadecenal
8	33.47	10.17	1-Docosene
9	37.491	5.08	α-Amyrin
10	42.525	6.39	Stigmasterol
11	49.417	1.69	Hexamethyl- Cyclotrisiloxane

Table 3 — List of qualitatively identified compounds from *B. Pinnatum* leaves by GC-MS

Peak No.	Retention time (min)	Area sum (%)	Name of compound
1	9.219	1.07	Cyclodecane
2	11.492	2.27	Benzothiazole
3	12.522	1.4	2-Diethoxymethyl-3-methyl-butan-1-ol
4	14.929	7.1	Oleic Acid [147]
5	20.301	16.5	Dodecyl acrylate
6	23.3	27.33	2,4-Di-tert-butylphenol
7	25.133	5.67	1-Hexadecanol
8	29.5	19.83	E-15-Heptadecenal
9	33.461	5.67	1-Eicosanol
10	37.482	7.08	α-Amyrin
11	42.516	4.25	Stigmasterol
12	45.312	1.83	Tetracosan-10-yl acetate

Table 4 — List of qualitatively identified compounds from F. Benghalensis aerial roots by GC-MS

Peak No.	Retention time (min)	Area sum (%)	Name of compound
1	10.622	16.04	1,1-diethoxy- Ethane
2	14.929	4.72	1-Dodecene
3	17.397	0.94	11-(1-ethylpropyl)- Heneicosane
4	20.301	10.38	Tetradecyl trifluoroacetate
5	23.3	18.87	2,4-Di-tert-butylphenol
6	25.141	3.77	1-Hexadecanol
7	27.388	2.83	Dodecyl acrylate
8	29.508	11.32	E-15-Heptadecenal
9	37.49	5.66	α-Amyrin
10	39.965	0.94	4β-Methylandrostane2,3-diol-1,17-dione
11	42.516	4.72	Stigmasterol
12	44.771	3.77	Bis(2-ethylhexyl) phthalate
13	45.312	0.94	Tetracosan-10-yl acetate
14	46.114	13.21	(3β)-Olean-12-en-3-ol acetate
15	52.213	1.89	Lupeol

quantitative determination of Stigmasterol and α-amyrin, derived from triplicate analyses (n=3) for each plant material, are detailed as mean ± standard deviation (SD) in Table 5 and graphically summarised in Fig. 4. The GC-MS/MS quantification (Table 5, Fig. 4) unequivocally showed that *C. igneus* leaves are exceptionally abundant in Stigmasterol, with a concentration of 4580±310 µg/kg. This level is substantially greater than that detected in *B. pinnatum* leaves (1525±110 µg/kg) and *F. benghalensis* roots (512±45 µg/kg).

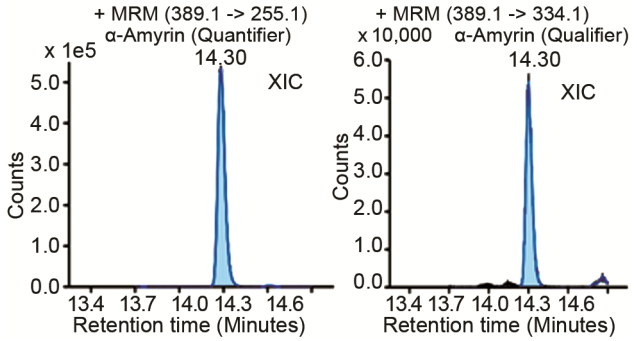

Fig. 2 — Chromatogram of α-Amyrin by GC-MS/MS.

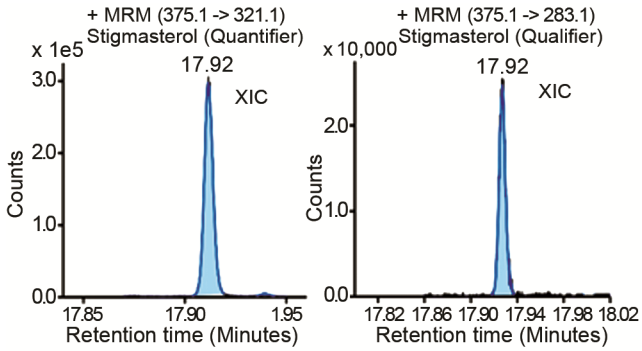

Fig. 3 — Chromatogram of stigmasterol by GC-MS/MS.

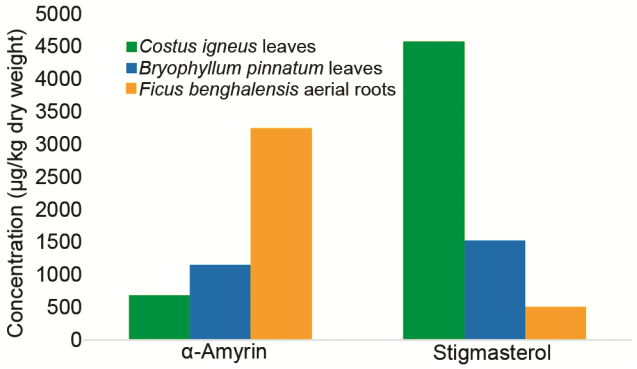

Fig. 4 — Comparative quantification of Stigmasterol and α-Amyrin.

In contrast to Stigmasterol, α-amyrin reached its highest concentration in *F. benghalensis* aerial roots, measured at 3250±250 µg/kg (Table 5, Fig. 4). *B. Pinnatum* leaves exhibited a more balanced phytochemical signature, containing substantial quantities of both marker compounds: Stigmasterol at 1525±110 µg/kg and α-amyrin at 1150±90 µg/kg (Table 5).

Discussion

This investigation, to our knowledge, represents the first direct comparative quantification of these two bioactive compounds across *C. igneus leaves, B. pinnatum* leaves, and *F. benghalensis* aerial roots using a sensitive direct GC-MS/MS MRM technique. The data reveal markedly distinct quantitative profiles for these triterpenoids and sterols[3,4]. The exceptionally high Stigmasterol content in *C. igneus* lends chemical support to its common designation as the 'Insulin Plant'. It provides a strong biochemical rationale for linking Stigmasterol, recognised for its beneficial impacts on glucose and lipid metabolism, to the potent anti-diabetic effects previously reported for *C. igneus* extracts. While Stigmasterol is also present in *B. pinnatum* and *F. benghalensis*, its comparatively reduced levels suggest it may contribute differently to their overall bioactivity, possibly through synergistic interactions. The predominance of α-amyrin in *F. benghalensis* aerial roots implies that such triterpenoids could be key contributors to the beneficial effects traditionally ascribed to this plant part, particularly in managing diabetes and its associated inflammatory complications[23,29]. The dual presence of both markers in *B. pinnatum* reflects its complex chemistry and suggests potentially multifaceted therapeutic actions, aligning with its diverse traditional uses[25,29]. Similar variability in

Table 5 — The quantitative determination summary of plants by GC-MS/MS

Plant material	Compound	Retention time (min)	Quantifier transition (m/z → m/z)	Concentration (µg/kg dry weight)
C. igneus leaves	Stigmasterol	17.92	375.1>323.1	4580±310
	α-Amyrin	14.3	389.1>334.1	680±55
B. pinnatum leaves	Stigmasterol	17.92	375.1>323.1	1525±110
	α-Amyrin	14.3	389.1>334.1	1150±90
F. benghalensis aerial roots	Stigmasterol	17.93	375.1>323.1	512±45
	α-Amyrin	14.3	389.1>334.1	3250±250

sterol and triterpenoid content has been reported in other medicinal plants analysed by chromatographic techniques[22,30,31]. Collectively, these divergent quantitative patterns clearly demonstrate that, while all three plants share an ethnobotanical association with diabetes management, the specific chemical underpinnings of their non-polar fractions appear quite distinct. This underscores the importance of conducting quantitative phytochemical comparisons and serves as a caution against presuming similar mechanisms of action based solely on overlapping traditional applications[3,10,32].

From a methodological perspective, the sequential analytical workflow adopted in this study proved effective for targeted phytochemical analysis. However, the quantitative approach relied on external standard calibration, which assumes minimal matrix effects. Although careful optimisation of chromatographic conditions helped minimise potential interferences, complete elimination of matrix effects without isotopically labelled internal standards remains challenging. Future studies should therefore incorporate internal standards and complementary analytical platforms such as LC-MS/MS to improve analytical robustness and expand phytochemical coverage.

Conclusion

The present study provides a systematic comparative quantification of two pharmacologically important phylomarkers, stigmasterol and α-amyrin, in chloroform extracts of *C. igneus* leaves, *B. pinnatum* leaves, and the aerial roots of *F. b*enghalensis using a validated GC–MS/MS method operating in Multiple Reaction Monitoring (MRM) mode. The analytical results clearly demonstrated notable interspecies variation in the distribution of these bioactive compounds. Among the investigated samples, *C. igneus* leaves exhibited the highest concentration of stigmasterol, whereas the aerial roots of *F. benghalensis* were particularly enriched in α-amyrin. In contrast, *B. pinnatum* displayed a comparatively balanced presence of both phytoconstituents.

These findings indicate that although the three medicinal plants share traditional ethnomedicinal applications in the management of diabetes mellitus, their potential therapeutic contributions may arise from different predominant phytochemical constituents. Establishing such comparative quantitative phytochemical data is important for understanding the chemical basis underlying traditional medicinal claims and for supporting future phytochemical standardisation of herbal materials.

Furthermore, the GC–MS/MS analytical approach developed in this study proved to be a reliable and sensitive method for the determination of phytosterols and triterpenoids in complex herbal matrices, thereby offering a useful analytical platform for future phytochemical investigations and quality control studies.

Future research should aim to correlate these quantitative phytochemical profiles with biological activity through systematic in vitro enzyme inhibition studies and in vivo diabetic models to further elucidate the therapeutic significance of these compounds and their potential role in plant-derived antidiabetic formulations.

Conflict of interest

The authors declare that there is no conflict of interest.

AI use disclosure

ChatGPT (OpenAI) was used solely for language editing and grammar improvement during manuscript preparation. No AI tool was used for data generation, analysis, interpretation, or scientific decision-making.

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