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The diversity of the phytochemical array in unexplored high altitude medicinal plant *Gonostegia hirta* (Blume. ex Hassk.) Miq.

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Nature can be considered as vast and diverse laboratory with an intricate mechanism that allows it to exhibit its remarkable qualities in unique and fascinating ways. One example is medicinal plants that grow in nature and have been used for centuries to cure various ailments. This study focuses on one of the unexplored plants, *Gonostegia hirta*, a medicinal herb grown in three regions of high altitude across the Indian subcontinent. The study aims to understand the phytochemical array in this plant and how it varies in different regions. To achieve this goal, we used LC-MS/MS (QTOF) to analyse the methanolic extracts of the plant samples from the three locations. The findings of the study revealed that the plants grown in Itanagar were highly populated with diverse phytochemicals, with a rich presence of some of the most potent phytocompounds like retronecine, heliotrine, zapotin, and moxestrol. This indicates that natural variations, such as climate and soil characteristics, significantly influence the phytochemical diversity of medicinal herbs. Overall, this study sheds new light on the incredible diversity of nature and its potential to provide us with valuable resources to improve our health and well-being.

Keywords: Diversity, *Gonostegia hirta*, LC-MS/MS, Phytochemicals

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Introduction

Natural diversification of plant phytocompounds refers to the wide array of chemical compounds synthesised by plants for various purposes, including defence against predators, attraction of pollinators, and interaction with their environment[1,2]. These compounds, also known as secondary metabolites those are not directly involved in plant growth, development, or reproduction but play crucial roles in their interactions with other organisms and their ecosystems. Various secondary metabolites, such as alkaloids, flavonoids, glycosides, phenolics, phytosterols, terpenoids, etc., show the diversifications. The diversification of these compounds within plant species is driven by evolutionary processes such as natural selection, genetic drift, and gene duplication, as well as environmental factors such as climate, soil composition, and biotic interactions[3-6]. In addition to that, seasonal variations in phytocompounds are noted[7]. This diversity of phytocompounds contributes to the adaptability and resilience of plants in various ecological niches and has significant implications for human health and agriculture.

Gonostegia hirta (Blume. ex Hassk.) Miq. is a perennial plant that grows up to 1 meter tall. It blooms from June to July, and its seeds mature from August to September[8]. This plant thrives in sandy loam and clay soil, and it prefers moist soil with a pH ranging from neutral to mildly alkaline. It can grow in areas with partial shade, such as light woodlands, or in full sunlight[9]. This plant is commonly found in high altitudes of northern India, including Assam, Meghalaya, Uttarakhand, and Sikkim[10,11]. This species is consumed as a healthy edible, mostly as a leafy vegetable, by the Galo tribe of the Indian Eastern Himalayan state of Arunachal[12,13]. With its profound use as an edible plant, the nutritional value of this species has been studied by a group of researchers[14-17].

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According to some researchers, the plant was found to have good antioxidant capacities, presented by DPPH, FRAP, ABTS, and hydroxyl radical scavenging capacity[14,18]. A study reported the characterisation of phenolic compounds, carotenoids and vitamins present in the leaves[19]. Phytochemical analysis revealed the presence of different classes of secondary metabolites like flavonoids, terpenoids, saponins, etc.[15,20]. Paste and decoction of the plant leaves show good response against the gastrointestinal disorder[21,22].

The literature available to date does not have detailed information on the phytochemicals present in the plant leaves; thus, it appears that *G. hirta* is an unexplored medicinal herb and very little is known about the secondary metabolites present therein. Authors of the present work have taken that opportunity to explore the phytochemicals treasure produced by this plant. In addition, authors have discovered variations in the phytochemical array due to the regional biodiversity.

Materials and Methods

Chemicals and reagents

All the HPLC grade solvents used during the experiments were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

Plant material collection, authentication, and processing

The process of collecting plant samples for the study of biodiversity is a crucial stage of experimental work. The following issues were strictly followed. Selecting an appropriate location, recording adjacent vegetation and soil textures, noting the time and date of collection, and ensuring proper storage, transportation, and authentication of the collected samples[23]. To identify locations where the plant of interest is highly abundant, an exhaustive literature and herbarium specimen study was conducted, followed by a field survey at three locations. Field notebooks and images of natural habitats were recorded for future references[24]. There are five major methods for sampling plant communities, like, transect, bisect, trisect, ring counts, and quadrat method[25]. As our investigation aimed to study an environmental gradient or eco-tone and vegetation is widespread, we preferred to use the quadrat sampling method. The size of the quadrat in a given community was determined by constructing a species curve[26]. We defined a quadrat of 3 x 3 m² and noted the names of the different species growing in the quadrat. This was done using the nested quadrat method, which involves laying a series of quadrats one over the other with gradually increasing size[27]. A total of 10 healthy specimens were collected from the defined nested quadrat area. The specimens of *G. hirta* were collected from the natural habitats of three different locations in mid-September 2021. The strains were authenticated and kept for future reference with designated reference numbers in the Department of Pharmacognosy, Central Ayurveda Research Institute, Kolkata. Details of the locations, reference numbers and plant sample designations are mentioned in Table 1. The leaves of the collected specimens were separated from the collected plant. Authenticated leaf samples were washed with water and subsequently with ethanol and pressed under filter paper to remove excess solvent adhered to the plant materials. Finally, the leaves were shade-dried. The plant material was dried until the moisture content was reduced to 2.40%. Moisture content was checked by Aczet MB 50 IR moisture balance[28]. The dried material was then pulverised, which was qualified through a 2000 sieve number with an aperture size of 2 mm conforming to ISO standard 565 (1990) to obtain coarse powder[29,30]. Live records (images) and field data during plant material collection were preserved.

Optimisation of extraction solvents

The extraction of medicinal herb is a crucial step in accurately determining the quality and quantity of phytocompounds present in the plant[31]. Selecting an incorrect solvent for extraction can lead to an inaccurate phytocompound profile of the plant, resulting in either the absence of certain phytochemicals or only partial presence of others[32,33]. To optimise the extractability, solvents with varying polarities, ranging from low polar to high polar through medium polar, were used. Hexane was used as a low polar solvent, chloroform and ethyl acetate were used as medium polar solvents, and ethanol, methanol, and water were used as high polar solvents, keeping the extraction methods identical for each case[34].

Table 1 — Detail of collection and designation of the plant samples under investigation

S. No.	Location of plant leaves collection	Reference Number	Plant sample designation
1	Galuki, Nagaland (25º67′64″N, 93º72′39″E)	CALP/NIF09G	G
2	Itanagar, Arunachal Pradesh (27º08′44″ N, 93º60′53″E)	CALP/NIF09I	I
3	Mhamizer, Uttarakhand (29º52′06″N, 79º44′16″E)	CALP/NIF09M	M

G = Galuki, Nagaland, I = Itanagar, Arunachal Pradesh, M = Mhamizer, Uttarakhand

Extraction of plant sample for LC-MS/MS

Given that methanol was found to be the best extraction media, the same has been used for extraction for LC-MS/MS purposes. An amount of 1 g of pulverised plant sample was subjected to Soxhlet extraction using 100 mL of methanol for 3 hours[35]. Finally, the extract was filtered off using Whatman filter paper (No. 41), and the filtrate was transferred to a 100 mL volumetric flask. The final volume was made up to 100 mL by adding the required amount of methanol in it.

Qualitative identification of phytocompounds by LC-MS/MS (QTOF)

As stated above, the methanol was found to be the best-extracting media based on the total extractive values and was subjected to Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) Quadrupole Time of Flight (QTOF). For this purpose, the extracted solution was used instead of the solidified extract because it was observed that a very small part of the solidified extract remained unattended during re-dissolving while preparing the sample for liquid chromatography.

Instrumentation and chromatographic condition

Agilent make LC, with HiP sampler (G4226A), Binary pump (G4220B), Column compartment (G1316C) and PDA detector (G4212B) were used for the generating chromatogram. In the whole experiment, a Syncronis-C18 100 x 2.1 mm, 1.7 microns (make Agilent) column was used.

For chromatographic separation, multiple column temperatures ranging between 30°C to 50°C were tried, while keeping the column temperature constant at 40°C was found most suitable in generating a resolute chromatogram. A three steps gradient program was adopted with mobile phase consisting of 0.1% formic acid in Milli-Q water in channel A and in channel B methanol (5%) for 30 min, 0.1% formic acid in Milli-Q water (100%) for 1 min and in last step of gradient the mobile phase consisting of 0.1% formic acid in Milli-Q water in channel A and in channel B methanol (5%) was used with constant flow rate of 0.3 mL/min$_{-1}$ by keeping the runtime 35 min.

Instrumentation and mass spectrometry condition

For mass spectrometric (QTOF-Mass spectrometer) detection and identification, a G6550A model with a dual ion source of Agilent make was used. For data acquisition and processing, 6200 and 6500 series TOF and Q-TOF B.05.01 (B5125.3) version was utilised.

The source parameters were optimised with ESI (Electron Spray Ionisation) voltage of 5500 V and 4500 V for positive and negative ionisation respectively. The variable pressure were maintained like, nebuliser gas at 60 psi, auxiliary gas at 50 psi and curtain gas pressure kept at 60 psi and turbo gas temperature was maintained at 450°C. Declustering potential was raised to 60 V both for positive and negative ionisation whereas, focusing potential were fixed to 350 V. Nitrogen was used in all cases for an operational time of 30 min. MS absorbance threshold and MS/MS absorbance threshold were set to 200 and 5, respectively. The samples were analysed using auto-acquisition mode, which was preset to the m/z ratio in the range of 126 to 1200 which can automatically select candidate ions for MS/MS study. The scan source parameters were optimised for gas temperature (250ºC), flow rate (13 L min^{-1}), nebuliser pressure (35 psi), and nozzle voltage (1000 V) for fragments scanning. The collision energy (CE) was set from 20 to 70 eV to optimise signals to obtain maximal structure information from the ions of interest. Accurate mass measurements of each peak from the total ion chromatograms (TICs) were obtained by the dynamic auto-calibration method that allows for real-time internal calibration during data acquisition.

Results and Discussion

Optimisation of extracting solvents

The standard method of extraction for optimising the best solvent revealed that the use of methanol was the best choice among all the solvents tested for all three samples (Table 2). The gross extractive values for methanol were 19.85, 21.23, and 17.34% for Galuki, Nagaland (G), Itanagar, Arunachal Pradesh (I), and Mhamizer, Uttarakhand (M), respectively, which were appreciably higher than those of any other solvent used here. Ethyl acetate, ethanol, and water were moderately effective at extraction but were not effective in extracting as good as methanol. However, hexane and chloroform were proven to be the weakest solvents in

Table 2 — Extractive values of *G. hirta*[a] in solvents of variable polarity

Extracting solvents	Weight percentage of extract obtained		
	G	I	M
Hexane	1.79±0.13	1.34±0.03	0.98±0.02
Chloroform	1.33±0.12	1.32±0.04	1.11±0.01
Ethyl acetate	12.76±0.13	11.56±0.11	12.23±0.04
Methanol	19.85±0.09	21.23±0.06	17.34±0.11
Ethanol	8.27±0.07	11.34±0.07	12.11±0.09
Water	11.17±0.11	12.12±0.09	11.98±0.09

[a]values are expressed as the mean±SD for triplicate analysis

this case. Hence, methanol was considered as most efficient solvent for extraction and was eventually used in phytochemical detection and profiling.

Qualitative identification of secondary metabolites

It is worth noting that no single method can detect all the compounds present in herbs. On the other hand, LC-MS/MS QTOF may not detect all the compounds, and it is still a rapid and effective method with high selectivity and sensitivity. To ensure maximum identification, we took into account sample preparation and the appropriate ionisation mode (positive and negative) to detect the highest number of compounds using this method.

To screen and qualitatively identify secondary metabolites in different solvent extracts, we compared the reported data using mz Cloud software. We also included images of positive and negative ESI chromatograms of all the extracts for reference (Fig. 1–3).

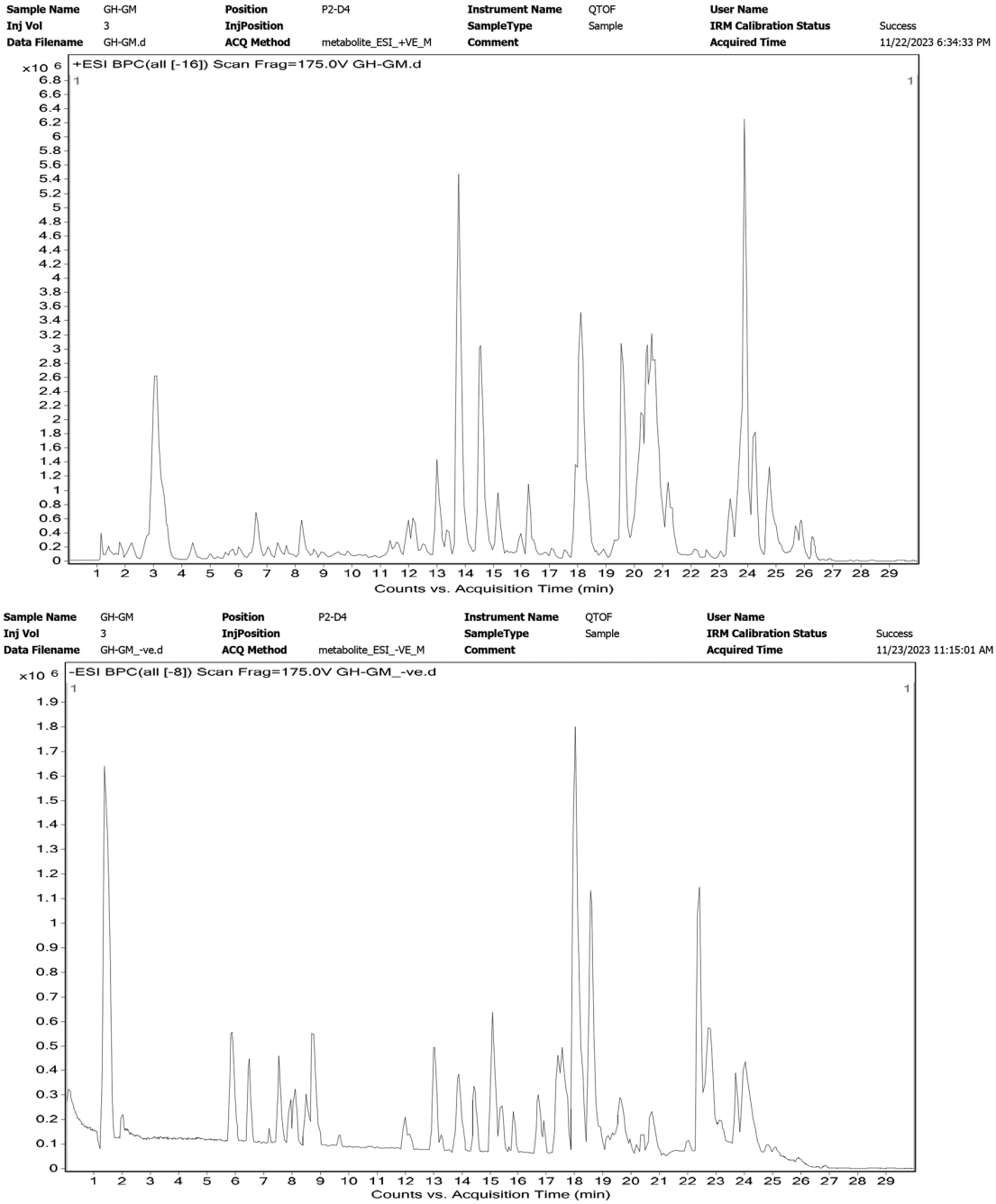

Fig. 1 — Representative LC-MS chromatograms of the extract G (Galuki, Nagaland) in dual ionisation.

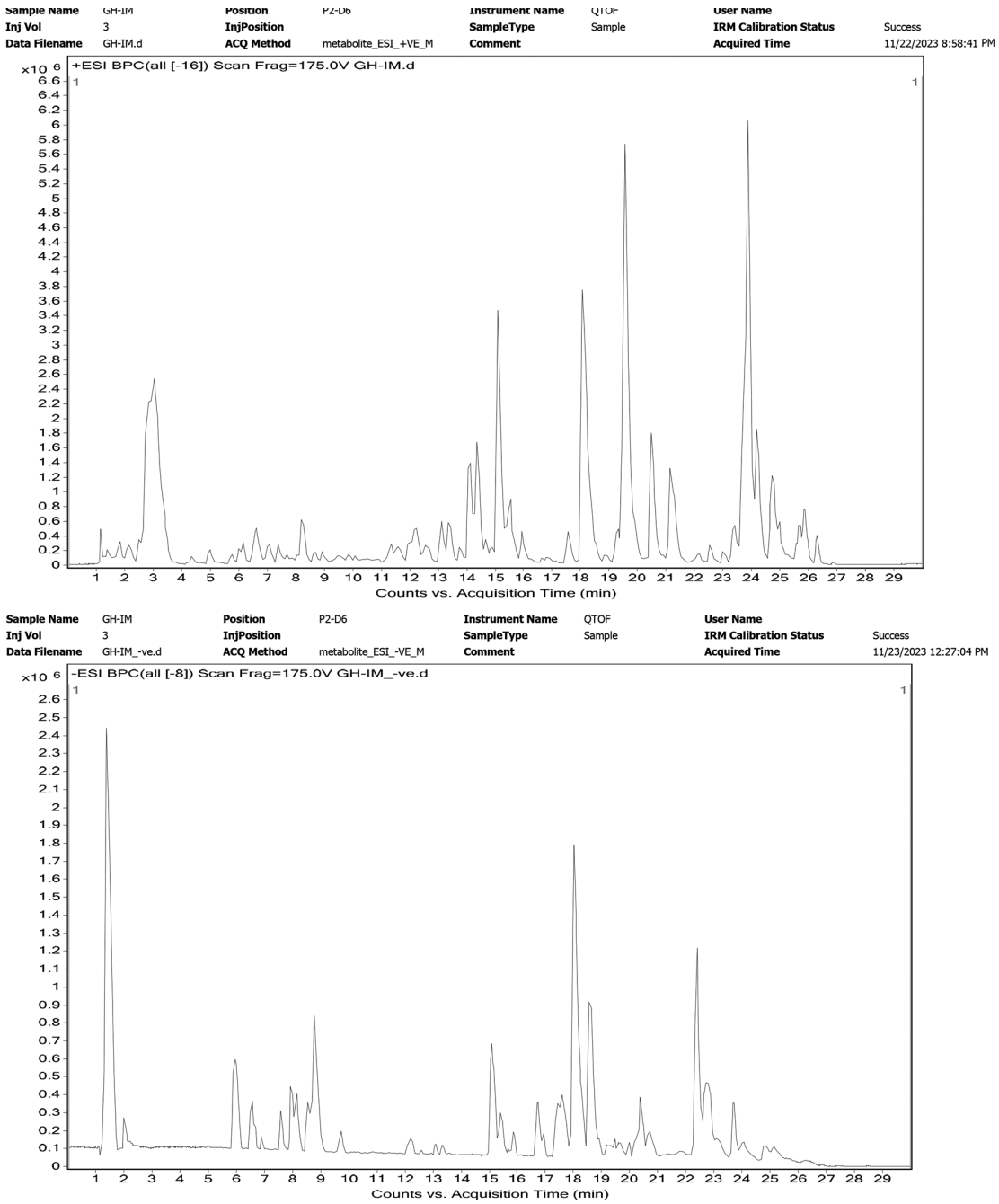

Fig. 2 — Representative LC-MS chromatograms of the extract I (Itanagr, Arunachal Pradesh) in dual ionisation.

The methanolic extracts of the plant analysed through LC-MS/MS have been found to be a rich source of diverse phytocompounds. The analysis revealed the presence of several classes of compounds, with secondary metabolites like alkaloids, flavonoids, and terpenoids being the major ones. Additionally, the plant's methanolic extracts contain anthocyanins, phenolic glycosides, polyphenols, benzoquinone derivatives, and tetrahydrofuran derivatives (Table 3). The plant extracts are rich in carboxylic acids and their derivatives, lactones, indoles, cyclic and acyclic acetates, and many more cyclic and acyclic derivatives. LC-MS/MS has also identified several unclassified compounds.

The minor phytocompounds that were found in all three methanolic extracts are Corchori fatty acid A ($C_{18}H_{28}O_4$), Cucurbic acid ($C_{12}H_{20}O_3$), Isolimonic acid ($C_{26}H_{34}O_{10}$), Limonoate (C_{26} $H_{34}O_{10}$), Quinic acid ($C_7H_{12}O_6$), Ureidoglycine ($C_3H_7N_3O_3$), Intermedine

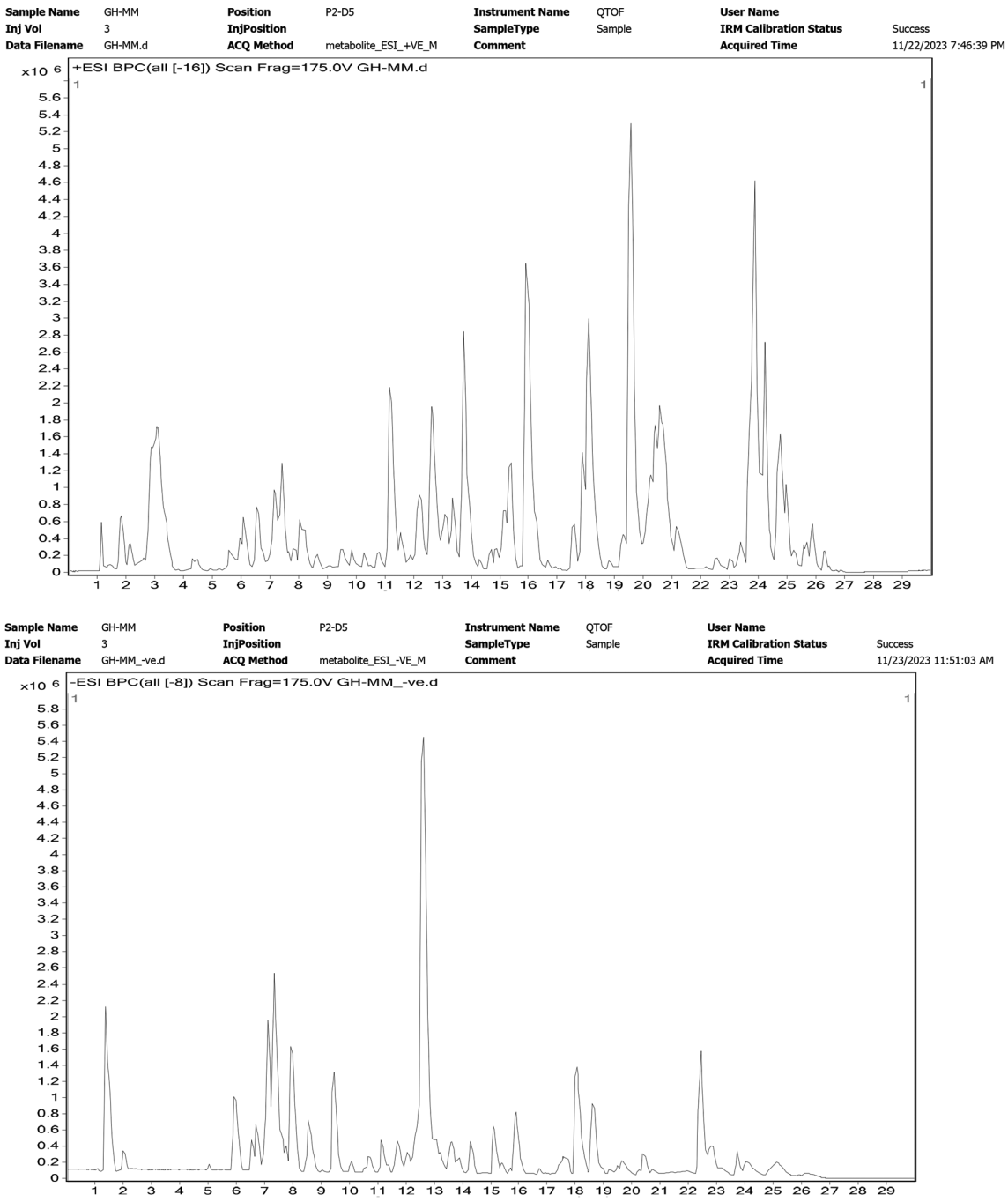

Fig. 3 — Representative LC-MS chromatograms of the extract M (Mhamizer, Uttarakhand) in dual ionisation.

($C_{15}H_{25}NO_5$), Neramic acid ($C_9H_{17}NO_8$), Azaadenosine ($C_9H_{12}N_6O_4$), 11-amino-undecanoic acid ($C_{14}H_{21}NO$), Acalyphin ($C_{14}H_{20}N_2O_9$), Bis-N-butyl phthalate ($C_{22}H_{29}NO_{10}$, Bis-N-butyl phthalate ($C_{22}H_{29}NO_{10}$), Blasticidin S ($C_{17}H_{26}N_8O_5$), Bopindolol ($C_{23}H_{28}N_2O_3$), Perillyl acetate ($C_{12}H_{18}O_2$), Petasinine ($C_{13}H_{21}NO_3$), Piperidolate ($C_{21}H_{25}NO_2$), Retronecine ($C_8H_{13}NO_2$), Sphinganine($C_{18}H_{39}NO_2$).

The compounds whose presence was detected only in extract of plant from Itanagar, Arunachal Pradesh (I) are (7'R,8'R)-4,7'-Epoxy-3',5-dimethoxy-4',9,9'-lignanetriol 9'-glucoside ($C_{26}H_{34}O_{11}$),14-Dihydroxycornestin ($C_{16}H_{20}O_6$), 16-Hydroxy hexadecanoic acid ($C_{16}H_{32}O_3$), Butoctamide hydrogen succinate ($C_{16}H_{29}NO_5$), Butyl 3-O-caffeoylquinate ($C_{20}H_{26}O_9$), Canavalmine ($C_{11}H_{28}N_4$), Convoline ($C_{16}H_{21}NO_5$), Cyclopassifloside II

Table 3 — Presence of secondary metabolites identified in methanolic extracts of *G. hirta* leaves of different locations

Class of phytocompounds	Name of phytocompound	Available in locations	Activities
Alkaloid	Allosamidine	G, I, M	Antifungal[36]
	Europine	G, I	Antimicrobial[37]
	Fabianine	G, I, M	Diuretic[38]
	Haplophytine	I	Insecticides[39]
	Heliotrine	I	Cytotoxicity[40]
	Retronecine	I	Hepatotoxicity[41]
	Cuauchichicine	G, I, M	Cytotoxicity[42]
	Capsiate	I, M	Antiobesity[43]
	Petasinine	I	Toxicity[44]
	Lyngbyatoxin	I	Carcinogenic[45]
Flavonoid	Morusin	M	Antitumor[46]
	Lespenefril	G, I, M	Diuretic[47]
	Apigenin	I, M	Antialzheimer[48]
	Kuwanon Z	I, M	Antibacterial[49]
	Quercetin	I, M	Anticancer[50]
	Zapotin	I	Anticancer[51]
	3R-Sophorol	G	-
	Santin	G, M	-
	Auriculoside	M	Anticancer[52]
Terpenoid	Jatrophone	G, I	Antiproliferative[53]
	Schidigeragenin B	I, M	Food preservative[54]
	Ganoderic acid F	G, I, M	Antitumor[55]
	Ganosporelactone A	I	Antioxidant[56]
	Cyclopassifloside II	G	Antiinflamatory[57]
	7,8-Dehydroastaxanthianthin	G	-
	Polyporusterone D	I	Cell regrowth[58]
Phytosterol	Moxestrol	G, I, M	Antidopaminergic[59]
	Ampeloside Bs1	M	-
Phenolic glycoside	Leonuriside A	G, I, M	-
	Aloesol 7-glucoside	I	-
Anthocyanin	cis-3,4-Leucopelargonidin	I	-
Benzoquinone derivative	Embelin	G, I	Antihelmintic[60]
THF derivative	Apotrichodiol	I, M	-

(C_{37} $H_{62}O_{11}$), Dehydroastaxanthianthin ($C_{40}H_{50}O_4$), N-Acetyl-b-glucosaminylamine ($C_8H_{16}N_2O_5$), N-tert-Butyloxycarbonyl-deacetyl-leupeptin ($C_{23}H_{44}N_6O_5$), Oxidised dinoflagellateluciferin ($C_{33}H_{38}N_4O_7$).

Following compounds have been detected in the extracts of Itanagar, Arunachal Pradesh (I) and Galuki, Nagaland (G), 6-C-Galactosylluteolin ($C_{21}H_{20}O_{11}$), 7alpha-Hydroxy-5beta-cholan-24-oic Acid ($C_{24}H_{40}O_3$), 7-beta-D-Glucopyranosyloxybutylidenephthalide ($C_{18}H_{22}O_8$), 7-Dehydrologanin tetraacetate ($C_{25}H_{32}O_{14}$), Demethyl-desacetyl-rifamycin S ($C_{34}H_{41}NO_{11}$), Epothilone D ($C_{27}H_{41}NO_5S$), Ganosporelactone A ($C_{30}H_{40}O_7$), Glucosyloxyanthraquinone ($C_{20}H_{18}O_8$), Hygromycin B (C_{20} $H_{37}N_3O_{13}$), Irinotecan ($C_{33}H_{38}N_4O_6$), Lenacil ($C_{13}H_{18}N_2O_2$), Methyl 2-(10-heptadecenyl)-6-hydroxybenzoate ($C_{25}H_{40}O_3$).

The compounds like, Demethyl-desacetyl-rifamycin S ($C_{34}H_{41}NO_{11}$), Dihydrocapsaicin ($C_{18}H_{29}NO_3$), Dinoflagellateluciferin ($C_{33}H_{38}N_4O_7$), Dolichyl phosphate ($C_{25}H_{45}O_4P$), epi-Tulipinolidediepoxide ($C_{17}H_{22}O_6$), Methyl-7-epi-12-hydroxyjasmonate glucoside ($C_{19}H_{30}O_9$), Mitoxantrone ($C_{22}H_{28}N_4O_6$), Mycophenolic acid($C_{17}H_{20}O_6$), Taurodeoxycholate ($C_{26}H_{45}NO_6S$), Tetracenomycin F2 ($C_{20}H_{16}O_8$), Trihexosylceramide (C_{54} $H_{101}NO_{18}$) have been found in plant extracts of Galuki, Nagaland (G) and Mhamizer, Uttarakhand (M) but absent in the extract of Itanagar, Arunachal Pradesh (I) sample.

The biosynthesis of phytomolecules results from plant metabolism, which is influenced by natural factors such as soil composition, temperature, rainfall, and more. Each of these factors, either individually or collectively, affects plant growth and metabolism, leading to varying outcomes in metabolic paths. The phytochemical array is an example of such an output, which is diversified in many ways.

Soil composition plays a crucial role in determining the phytochemical profile of plants. Nutrient availability, soil pH, organic matter content, and the presence of specific minerals can all influence the production of phytochemicals such as antioxidants, flavonoids, and alkaloids in plants. The micronutrients present in soil also have some influence in generating phytochemical profiling of a plant. Additionally, soil microbiota can interact with plant roots and affect phytochemical production through symbiotic relationships or by influencing plant stress responses. Rainfall patterns can indeed impact plant phytochemical production in various ways. Adequate rainfall can ensure sufficient soil moisture, which is essential for nutrient uptake and overall plant growth. This can indirectly influence phytochemical production by providing the necessary resources for plant metabolism and biosynthesis of secondary metabolites. Temperature plays a crucial role in influencing plant physiology and the production of phytochemicals. Different phytochemicals respond differently to temperature variations, and optimal temperature for their synthesis can vary among plant species.

Overall, variations in soil texture and nature, as well as fluctuations in rainfall and temperature, can have complex effects on plant metabolism and phytochemical synthesis, with both optimal and stress-induced responses contributing to the chemical composition of plants in different environmental conditions.

The present study reveals that the plant grown in Itanagar, Arunachal Pradesh (I), possesses the highest number of phytochemicals, and that may be due to the favourable climatic and soil conditions of Itanagar. The reported annual rainfall in this region is 3000 mm, which is on the higher side of the Indian subcontinent[61]. This heavy rainfall can probably leach nutrients from the soil, potentially affecting the availability of minerals and other compounds necessary for phytochemical production.

According to a group of researchers, clay soil and slightly alkaline pH in the range of 7.1 to 7.7 are the characteristics of this region[62]. Possibly, this feebly alkaline pH influences the solubility and mobility of minerals in the soil, which are essential for this plant metabolism and phytochemical synthesis. Moreover, this pH range possibly affects the activity of enzymes involved in phytochemical biosynthesis by converting the precursor molecules into specific phytochemicals. The clay soil of this region facilitates water retention and moisture content, which is possibly a favourable condition for the phytochemical synthesis within the plant. Additionally, clay soil bears a good amount of organic matter and is normally rich with nitrogen content due to huge vegetation and dead masses, which normally add organic content and enhance the available nitrogen. This may be the reason for the higher number of alkaloids in the plant leaves of this region. Overall, based on the above discussion, it may be stated that the plant phytochemical profile hugely varies due to natural diversity, and no two plants are identical in phytochemical array either qualitatively or quantitatively.

Conclusion

The above dataset and discussion indicate that nature is the most diversified lab with an inimitable mechanism through which it manifests itself. Diversification expressed in the phytochemical array of a medicinal plant results in potency because that potency depends on the phytochemicals present in the plant. These diversifications are caused by several factors, like soil, rainfall, temperature, humidity, and biotic presence, which are crucial in making a medicinal plant less potent or more potent. India is an example of a biodiversity hotspot. Hence, in a country like India, where medicinal plants are used abruptly, ailments cannot be healed with the same degree of efficacy. Therefore, it is prudent to obtain a very clear picture of the phytocompounds in a medicinal plant before stepping into any drug development or phytopharmaceutical development. The present work is based on this logic. It is a boon to mankind that the greater the diversification in nature, the greater the opportunities for better living and sustenance of healthy lives.

Conflict of interest

The authors individually and jointly declare that no conflicts of interest are associated with this article.

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