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Investigation of phytochemical constituents, GC-MS, DPPH free radical scavenging assay, and mineral contents of *Glochidion sphaerogynum* (Mull. Arg.) Kurz bark extract

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Abstract

The aim of the present study was to assess phytochemical constituents, chemical composition, DPPH free radical scavenging assay and mineral contents of *Glochidion sphaerogynum* (Mull.Arg.) Kurz bark extract. Standard procedures were used to test preliminary phytochemical constituents as well as quantitative analysis for total alkaloid, flavonoid, saponin, tannin, phenolic and terpenoid content. The extract was examined using gas chromatography-mass spectrometry (GC-MS) to know the biologically active compound. *In-vitro* antioxidant potential was investigated using DPPH free radical scavenging assay and the IC_{50} value for the antioxidant activity of bark extract was 37.4479 µg/mL. The qualitative phytochemical investigation revealed the presence of important phytochemical constituents as well as considerable amounts of total alkaloid, flavonoid, saponin, tannin, phenolic and terpenoid content. GC-MS revealed the presence of biologically active compounds like 1,1,6-Trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethenyl-pentadec-4-enyl)cyclohexane; Benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl) -4-hydroxy-, methyl ester; Neophytadiene etc which would be important for medicinal industries. Mineral contents were determined by using Atomic Absorption Spectrometry (AAS). The result revealed the presence of a good concentration of Na (10.552±0.343 ppm) and Ca (8.973±0.310 ppm) elements followed by K, Fe, Mg and Mn and very less concentrations of heavy metals such as Cd, Cr and Pb indicated the species was devoid of harmful metals.

Keywords

Glochidion sphaerogynum, phytochemical, GC-MS, DPPH, mineral contents

Introduction

The Plant produces many phytochemical constituents that protect them against insects, pathogens, and herbivores (1). Many of these phytochemical constituents also have different biological activities and protect human beings against various diseases (2, 3). According to WHO (World Health Organization), 80% of people rely on indigenous plant-based medicines for curing different diseases (4). Plant produces antioxidants as secondary metabolites, which are substances that act in the cell to counteract free radicals and reactive oxygen species (ROS) and control oxidative damage in the body that become scientifically compelling compounds as a consequence of many pharmacological activities (5, 6). One of the reliable analytical methods for identifying the different chemical components in a plant sample is gas chromatography-mass spectrometry (GC-MS) which plays a significant role in the analysis of phytochemical compounds (7-9).

Glochidion sphaerogynum is a small tree belonging to the genus *Glochidion* of the family Phyllanthaceae which mainly grows in primary evergreen forests, secondary forests, hilly, or roadside areas, distributed in Bhutan, Nepal, Bangladesh, Myanmar, China, Vietnam, Thailand and states of India (10). This species can be identified by its glabrous plant body, long acuminate leaves, axillary inflorescence and distinguishable lobed capsules from other members of the genus *Glochidion*. The genus *Glochidion* contains various chemical constituents that include flavonoid, terpenoid, glycoside, steroid, phenolic compounds, triterpenoid, tannin, saponin, alkaloid, resin, anthraquinone, reducing sugar, phlobatannin and carbohydrate (11). This proves the importance of the genus as a source of medicinal value. The leaves and branches of *G. sphaerogynum* have been used mainly for the treatment of influenza, skin problems, common cold, fever, ulcers and inflammation in some countries (12-14). However, no detailed examination of the phytochemical composition, GC-MS analysis, antioxidant potential and assessment of mineral contents of this plant has been published so far. So, the present study desired to evaluate phytochemical analysis, identification of biologically active compounds using GC-MS, antioxidant potential using DPPH free radical scavenging assay and assessment of mineral contents in the bark extract of *G. sphaerogynum* based on their medicinal importance. Hence, the present study was also conducted to know the biologically active components, their mineral composition and the antioxidant capacity present in the bark extract.

Materials and Methods

Collection and identification of the plant material

The field survey was conducted from January to April 2021 and fresh plant specimens were collected in the Kokrajhar district of Assam. The plant material was identified following the literature i.e., Flora of Assam and a voucher specimen (008 BUBH) was deposited in the Bodoland University Botanical Herbarium (BUBH), Kokrajhar, Assam (15, 16).

Preparation of the plant extracts

After identification of the specimen fresh bark material was collected. The collected bark was properly washed with distilled water and dried in the shade for one and half months until it becomes completely moisture free. After drying, it was ground to a coarse powder and kept in an airtight container. Twenty gms of the powdered sample was taken and soaked in 200 mL of methanol for 72 hrs and filtered through Whatman filter paper no. 1. Then the filtrate extract was evaporated with a vacuum rotary evaporator (Ikon instruments) and set the temperature according to the boiling point of the solvent. After evaporation residue was taken and stored at 4 °C for future analysis of the sample (17, 18).

Phytochemical screening

Alkaloid, reducing sugar, steroid, phlobatannin, tannin, flavonoid, terpenoid, triterpenoid, saponin, glycoside and phenol were tested according to standard methods (18-21).

Quantitative estimation of phytochemical constituents

Total alkaloid content

2.50 g of powder bark sample was measured, and 200 mL of 10% acetic acid (CH_3COOH) in ethanol (C_2H_5OH) was added, followed by 4 hrs of rest. The filtrate was then heated in a water bath after filtration. Until the precipitation was complete, concentrated ammonium hydroxide (NH_4OH) was applied. It was then washed with 20 mL of 0.1M ammonium hydroxide (NH_4OH) and filtered using Whatman filter paper no. 1. The residue was dried in an oven before being weighed on an electronic balance. The total alkaloid content can be calculated using the following formula-

Alkaloid (%) = Final weight of the residue/Initial weight of sample ×100 (18, 19).

Total flavonoid content

2.50 g of powder bark sample was mixed with 50 mL of 80% aqueous methanol (CH_3OH) in a 250 mL beaker, it was covered and kept at 20 °C for 24 hrs. The supernatant was discarded, and the residue was extracted 3 times more, each time with the same volume of ethanol (C_2H_5OH). Then, using Whatman filter paper no. 1, the entire solution was filtered. The filtrate was dried and weighed in an electronic balance after being evaporated over a water bath. Total flavonoid content can be done as-

Flavonoid (%) = Final weight of the residue/Initial weight of sample ×100 (18, 19).

Total saponin content

5 g of powdered bark material was put into a 250 mL conical flask, followed by 100 mL of 20% aqueous ethanol (C_2H_5OH), and heated in a hot water bath for 4 hrs at 55 °C. The procedure was carried out twice more. The mixed extract was then reduced to 40 mL in a water bath at 90 °C. The mixed extract was placed in a separating funnel and rapidly shaken with 20 mL diethyl ether {$(C_2H_5)_2O$}. The ether layer was then removed. The purifying process was repeated 2 times. After that, 60 mL of n-butanol ($C_4H_{10}O$) was added, followed by 2 times washing with 10 mL of 5% sodium chloride (NaCl). To obtain a final concentration of saponin, the sodium chloride layer was removed and the residual solution was heated in a water bath and dried at 50 °C in an oven. Total saponin content can be done as-

Saponin (%) = Final weight of the residue/Initial weight of sample ×100 (18, 19).

Total phenolic content

The total phenolic content of the methanolic bark extract was determined following the Folin Ciocalteu's (FC) method using UV-Vis Spectrophotometer (Shimadzu A 125358) with slight modification (22, 23). 0.01 g sample was weighed and made up to 1800 µL of distilled water. Then 150 µL of Folin Ciocalteu reagent and 1 mL of 10% sodium carbonate (Na_2CO_3) were added and made up to 3 mL of volume and incubated in the absence of light for 40 min. Absorbance was measured at 765 nm against a blank and the calibration curve was created using the standard gallic acid in the range of 10-50 µg/mL. Based on the average of three experiments, the phenolic content of bark extract

was quantified as mg of gallic acid equivalents of dried extract (mg GAE/g dry extract).

Total tannin content

The tannin content of the bark extract was quantified using the Folin Denis (FD) method in UV-Vis Spectrophotometer (Shimadzu A 125358) with slight modification (24-27). 0.01 g of the sample was weighed and made up of 1800 µL of distilled water. After that, 150 mL FD reagent and 1 mL sodium carbonate (Na_2CO_3) were added to make a total volume of 3 mL, which was then incubated in the absence of light for 40 min. The calibration curve was built using tannic acid as a standard (10-50 g/mL) and calculated at the absorbance of 760 nm versus a blank. Based on the average of 3 experiments the total tannin content was quantified as mg of tannic acid equivalents of dried extract (mg TAE/g dry extract).

Total Terpenoid content

Total terpenoid content was determined as described by the given method (28, 29). For 24 hrs, 2 g of the material was dissolved with 50 ml of ethanol (C_2H_5OH). The mixture was then filtered using Whatman No. 1 filter paper. The filtrate was collected and extracted with petroleum ether (C_6H_{14}) before being dried at 65 °C in a water bath. Then the residue was collected and calculated the final volume. Total terpenoid content can be determined as-

$$\text{Terpenoid (\%)} = \frac{\text{Final weight of residue}}{\text{Initial weight of sample}} \times 100$$

GC-MS Analysis

GC-MS analysis of *G. sphaerogynum* (Mull.Arg.) Kurz bark extract was performed with a Perkin Elmer (USA) Clarus 680 GC and amp. Turbo Mass Ver. 6.1.2 instrument. The system was programmed using the software, and the peaks were examined with the NIST-2014 software. The stationary phase was 5% diphenyl 95% dimethyl polysiloxane, while the carrier gas was helium gas (99.99 %) at a flow rate of 1mL/min. A 2 µL injection volume was used in splitless mode. The injector has a temperature of 280 °C, whereas the ion source has a temperature of 180 °C. Electron Impact positive (EI+) mode was used to obtain Mass Spectra at 70 eV. The mass spectrum of peaks and compounds was determined using NIST-2014 software and a library search (30).

DPPH free radical scavenging assay

The antioxidant activity of a methanolic extract of *G. sphaerogynum* bark was tested using the 2,2-Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay with slight modification (31-34). In the absence of light, 1 mL methanolic extract solutions (10-60 µg/mL) were dissolved in 3 mL DPPH solution and incubated. 3 mL methanol was used to make the blank, and 2 mL methanol and 1 mL DPPH solution were used to make the control. With a UV-Vis spectrophotometer, the absorbance of extracts was measured at 517 nm after 30 min and compared to that of normal ascorbic acid at equal amounts. The ability of the DPPH radical scavenging assay was calculated using the following formula-

% of inhibition = (Absorbance of control - Absorbance of the sample extract or standard)/Absorbance of control × 100

The IC_{50} value was obtained using linear regression analysis. If the IC_{50} value is lower, the antioxidant activity is stronger.

Assessment of mineral contents

Atomic Absorption Spectrometry (AAS), Model: Shimadzu AA-7000, was used to determine Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), Manganese (Mn), Cadmium (Cd), Chromium (Cr) and Lead (Pb) at CIF IASST, Guwahati, Assam. The material was digested with concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) using the wet ashing process. 0.50 g of sample was mixed with 8 mL of HNO_3 and let to stand overnight. The following day, it was heated for 1 hr at 120 °C on a hot plate with 4 mL H_2O_2 until the digestion was colorless. At 80 °C, the residue was dried and diluted with hydrochloric acid (HCl) and filtered. The results of the AAS were determined as ppm concentration based on the average of 3 readings in AAS (35).

Statistical Analysis

All the tests were done in 3 replicates. The data was determined as an average of 3 experiments (n=3) and calculated mean ± standard deviation (SD) using Microsoft Excel. The statistical analysis was performed by ANOVA single factor at $p \leq 0.05$ level in Microsoft Excel.

Results

Phytochemical screening

The presence of significant secondary metabolites such as alkaloid, steroid, phlobatannin, tannin, flavonoid, terpenoid, triterpenoid, saponin, glycoside and phenol was confirmed in the methanolic bark extract of *G. sphaerogynum* (Table 1).

Table 1. Preliminary phytochemical screening of methanolic bark extract of *G. sphaerogynum*

Phytochemical constituents	Test/Reagent	Result
Alkaloid	Dragendorff's reagent	+
	Mayer's reagent	+
	Wagner's reagent	+
Reducing sugar	Fehling's test	-
Steroid	Salkowski test	+
Phlobatannin	HCl test	+
Tannin	$FeCl_3$ test	+
Flavonoid	$FeCl_3$ test	+
	H_2SO_4 test	-
Terpenoid	Salkowski test	+
Triterpenoid	H_2SO_4 test	+
Saponin	Foam test	+
Glycoside	Keller-Killiani test	-
	Borntrager's test	+
Phenol	$FeCl_3$ test	+

Positive sign (+) = Present; Negative sign (-) = Absent.

Quantitative estimation of phytochemical constituents

According to the result of quantitative phytochemical analysis, total alkaloid and flavonoid contents obtained 3.73% (0.093±0.025) and 1.24% (0.031±0.019) yield respectively. The total saponin content exhibited a 12.94% (0.646±0.473) yield. Total tannin and phenolic contents were 6.92112 mg TAE/g dry extract (0.744±0.062) and 0.1666 mg GAE/g dry extract (0.328±0.045) respectively. The total terpenoid content obtained was 8.66% (0.173±0.066) yield (Table 2).

Table 2. The total alkaloid, flavonoid, saponin, phenolic, tannin and terpenoid contents in methanolic bark extract of *G. sphaerogynum*

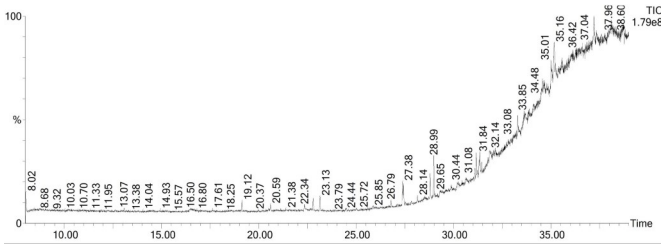
Parameters	Result
Total alkaloid content (% yield)	0.093±0.025
Total flavonoid content (% yield)	0.031±0.019
Total saponin content (% yield)	0.646±0.473
Total phenolic content (mg GAE/g)	0.328±0.045
Total tannin content (mg TAE/g)	0.744±0.062
Total terpenoid content (% yield)	0.173±0.066

Sample was analyzed in three replicates and data were indicated as an average of three tests (n=3), mean ± standard deviation (SD), and they were significantly different from each other at $p \leq 0.05$ level (ANOVA single test).

GC-MS Analysis

The bioactive compounds present in methanolic bark extracts of the *G. sphaerogynum* were shown in Fig. 1. The retention time, molecular weight, molecular formula and % of peak area were identified using the NIST library and presented in Table 3. The important bioactive compound present in the methanolic bark extract were Hentriacontane, Xanthosine, Fluorene, Phenanthrene, Neophytadiene, Benzenepropanoic acid,3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-,methyl ester, 2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene)pentadecane, Z,Z-6,28-Heptatriactontadien-2-one, 1,1,6-Trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethenyl-pentadec-4-enyl)cyclohexane, Methyl 2-hydroxy-eicosanoate.

Fig. 1. GC-MS chromatogram of methanolic bark extract of *G. sphaerogynum*



DPPH free radical scavenging assay

The antioxidant potential of bark extract was evaluated using the DPPH assay. DPPH free radical scavenging assay of concentrations (10-60 µg/mL) and percentage inhibition of bark extract and the standard ascorbic acid were presented in Table 4. Radical scavenging assay at the concentration of 60 µg/mL, bark extract and ascorbic acid showed inhibition of 58.503±0.095% and 71.055±0.064% respectively. The IC_{50} value of bark extract and standard ascorbic acid were 37.4479 µg/mL and 20.9167 µg/mL respectively that indicating a good amount of antioxidant potential.

Assessment of mineral contents

The result of the mineral analysis of *G. sphaerogynum* bark extract was enlisted (Table 5). The result showed that sodium (Na) had the highest mineral contents of 10.552±0.343 ppm followed by calcium (Ca) and potassium (K) contents of 8.972±0.310 ppm and 7.230±0.086 ppm respectively. Sodium, calcium and potassium are one of the most important minerals in the body. K and Na help to maintain ionic balance, which helps to prevent hypertension and improve blood pressure (36, 37). Ca helps to maintain teeth, bone, blood clotting and muscle to contract and regulate normal heart rhythms and nerve function (38).

Discussion

The results of preliminary qualitative phytochemical screening of the methanolic extract of *G. sphaerogynum* bark showed the presence of important phytochemical constituents such as alkaloid, flavonoid, steroid, phlobatannin, tannin, terpenoid, triterpenoid, saponin, glycoside and phenol that would be implied on the identification of new sources of plants as pharmaceutical applications. The quantitative phytochemical estimation revealed a high % yield of saponin content (12.94%) followed by terpenoid content (8.66%) and a good concentration of tannin content of 6.92112 mg TAE/g dry extract (0.744±0.062) and phenolic content of 0.1666 mg GAE/g dry extract (0.328±0.045) respectively. In the present study, a good amount of alkaloids and flavonoids also have been recorded. Important biological activities such as antioxidant, anti-inflammatory, antibacterial and antimicrobial activities are known to exist in terpenoids, phenols, flavonoids, saponins, tannins and alkaloids (39).

The molecule 1,1,6-Trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethenyl-pentamethyl-3-ethenyl-pentadec-4-enyl) cyclohexane which is the primary compound identified as having pharmacological activities such as antimicrobial, anti-inflammatory, anticancer, antiarthritic, antiviral with the highest peak area of 2.263% followed by the compound Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester with the peak area of 1.091% that has antioxidant and antifungal properties found during the GC-MS analysis. From the GC-MS data, all the identified compounds possess important biologically active compounds such as antioxidants, antitumor, antimicrobial, anti-inflammatory, antifungal, cytotoxic and various pharmacological activities which creates a basis for defining the plant's potential benefits in medicinal industries (Table 3).

The DPPH free radical scavenging assay was used to examine antioxidant activity. The result showed that the concentrations of methanolic bark extracts had significant DPPH free radical scavenging effects. The highest % inhibition (59.512±0.557) showed in 50 µg/mL followed by 58.503±0.095 % inhibition in 60 µg/mL with a potent antioxidant amount of IC_{50} value. Further assays are required to know the significance of the antioxidant capacities of the extract.

Table 3. Identified chemical composition of methanolic bark extract of *G. sphaerogynum* using GC-MS and their biological activity

Sl. No.	Retention time	Compound name	Peak area %	Molecular weight	Molecular formula	Biological activity
1	19.123	Hentriacontane	0.249	436	$C_{31}H_{64}$	Anti-inflammatory, antitumor and antimicrobial activities (44)
2	20.588	Xanthosine	0.247	284	$C_{10}H_{12}N_4O_6$	Therapeutic and pharmacological property (45)
3	23.139	Fluorene	0.473	166	$C_{13}H_{10}$	Drug design (46)
4	27.381	Phenanthrene	0.608	178	$C_{14}H_{10}$	Analgesic, antitussive, antimalarial, cytotoxic, anti-constipation, antioxidant, anti-inflammatory activity (47)
5	28.141	Neophytadiene	0.229	530	$C_{20}H_{38}$	Anti-inflammatory, antipyretic, analgesic, antioxidant, antimicrobial, antifungal, antibacterial activity (48-52)
6	28.987	Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester	1.091	292	$C_{18}H_{28}O_3$	Antioxidant, antifungal (53-56)
7	31.848	2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane	0.468	348	$C_{25}H_{48}$	Anti-inflammatory, Antioxidant (57)
8	33.849	Z,Z-6,28-Heptatriactontadien-2-one	0.751	530	$C_{37}H_{70}O$	Vasodilatory, carcinogenic, antioxidant activity (58, 59)
9	35.174	1,1,6-Trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethenyl-pentadec-4-enyl) cyclohexane	2.263	442	$C_{32}H_{58}$	Pharmacological activity, antimicrobial, anticancer, antiarthritic, anti-inflammatory and antiviral properties (60, 61)
10	37.955	Methyl 2-hydroxy-eicosanoate	0.275	342	$C_{21}H_{42}O_3$	Pharmaceutical property, antioxidant, anti-inflammatory (62, 63)

Table 4. DPPH assay of methanolic bark extract of *G. sphaerogynum*

Concentration (µg/mL)	% inhibition	
	Bark extract	Ascorbic acid
10	38.272±1.165	41.767±0.641
20	44.080±0.586	59.143±0.109
30	42.013±1.190	56.091±0.461
40	51.070±0.794	62.072±0.346
50	59.512±0.557	65.985±0.031
60	58.503±0.095	71.055±0.064
IC_{50}(µg/mL)	37.4479	20.9167

The sample was analyzed in three replicates and data were indicated as an average of three tests (n=3), mean ± standard deviation (SD) and they were significantly different from each other at $p \leq 0.05$ level (ANOVA single test)

Table 5. Mineral assessment of bark of *G. sphaerogynum*

Minerals	Result (ppm)
Sodium (Na)	10.552±0.343
Potassium (K)	7.230±0.086
Calcium (Ca)	8.973±0.310
Magnesium (Mg)	3.379±0.167
Iron (Fe)	4.026±0.076
Manganese (Mn)	3.367±0.179
Chromium (Cr)	0.162±0.011
Lead (Pb)	0.162±0.016
Cadmium (Cd)	0.009±0.002

The sample was analyzed in three replicates and data were indicated as an average of three tests (n=3), mean ± standard deviation (SD), and they were significantly different from each other at $p \leq 0.05$ level (ANOVA single test)

In order to be healthy, the human body requires a variety of minerals found in different varieties of plants. Apart from this, plants possess minerals that are beneficial for humans and some toxic metals which are harmful to human health. The presence of heavy metal toxicity in plants may create severe problems. The determination of mineral composition showed the highest concentration in sodium (Na) elements followed by calcium (Ca), potassium (K), iron (Fe), magnesium (Mg), manganese (Mn), and less concentration of chromium (Cr), lead (Pb) and cadmium (Cd). Na maintains the osmotic pressure in the body and activates nerves and muscle functions (40). K and Na maintain the ionic equilibrium that helps to prevent hypertension and enhances blood pressure (36, 37). Mg is important for enzyme activation and bone formation and Ca plays an important role as a constituent of bones and teeth formations, regulation of nerves and muscles functions in living cells and helps in membrane formulating (40). Fe is linked to haemoglobin and oxygen transmission from the lungs to tissue cells. Mn activates enzymes and is involved in urea formation, haemoglobin formation, helping in the nervous system and normal bone growth (37, 41, 42). Cd, Pb and Cr are non-essential elements in both plants and humans if it consists of high concentration. They are responsible for toxic substances (43). Plants are a good source of phytochemicals and minerals that can be useful for the nutraceutical industry. Several investigations have found elemental content in plant extracts that we consume as herbal health supplements or medicine and biochemical processes in the human body are affected by macro and trace elements (36, 37, 41). Therefore, it was evident from the results that the plants would be free of harmful materials because of the very low concentration of Cr, Pb, and Cd and could be important mineral consuming plants due to the presence of the important mineral elements.

Conclusion

The study revealed that plant has a potential source of phytochemical constituents that would be beneficial to humankind. The GC-MS analysis justifies the presence of important biological and pharmacological properties such as antifungal, antibacterial, antioxidant, antimicrobial, anti-inflammatory, antimalarial, analgesic and cytotoxic activities). This study can serve as a basis for additional research on the biological activities of the plant. The DPPH assay of bark extract exhibited a good amount of antioxidant compound which could be useful in the pharmaceutical and medicinal industry. Mineral determination indicated the presence of a significant number of mineral factors such as sodium (Na), calcium (Ca), potassium (K), iron (Fe), magnesium (Mg), manganese (Mn) and the plant could be free from toxic substances due to less concentration of heavy metal such as Cadmium (Cd), Chromium (Cr) and Lead (Pb). This is the first report citing mineral contents and detailed phytochemical evaluation, GC-MS and antioxidant analysis of the *G. sphaerogynum*. Further studies are needed to isolate biologically active compounds as important in pharmacological industries.

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Authors contributions

PB collected and identified the specimen, analyzed the data, performed the statistical analysis and drafted the manuscript. SB participated in the design of the study, supervised the work and revised the manuscript. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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