CHAPTER 3

MATERIALS AND METHODS

3.1. Study Area

Assam is the easternmost part of India, also the second largest state of the North Eastern region of India, it lies between 24°.07' N to 28°00' N Latitude and 89°.42' E to 96°.02' E Longitude. The state extends over a geographical area of 78,438 sq. km. and constitutes 2.39% of the country's total area. The state is divided into 35 administrative districts. Assam is bounded by Bhutan and Arunachal Pradesh to the north, Nagaland, Manipur, and Arunachal Pradesh to the east, Mizoram, Tripura, Meghalaya, and Bangladesh to the south, and West Bengal to the west. Brahmaputra Valley, Barak Valley, and the hilly areas of Karbi Anglong and North Cachar Hill districts divide Assam into unique physiographic domains that affect the overall distribution of rainfall. The annual average rainfall varies from place to place while maximum rainfall occurs during June and July month, with an average rainfall ranging from 200 cm to 290 cm. The state has a diverse landscape with a variety of mixed dense forests, which include different varieties of grasses, herbaceous, and shrubby vegetation along with tall trees, bamboo, and cane grooves. The protected areas like National Parks and Wildlife Sanctuaries comprise 3925 sq. km. constituting about 5% of the total topographical area of Assam. The state comprises 7 national parks, 19 wildlife sanctuaries, 5 elephant reserves, 2 biosphere reserves, 3 tiger reserves, 1 Ramsar site, and 2 world heritage sites.

3.1.1. Map of the study area

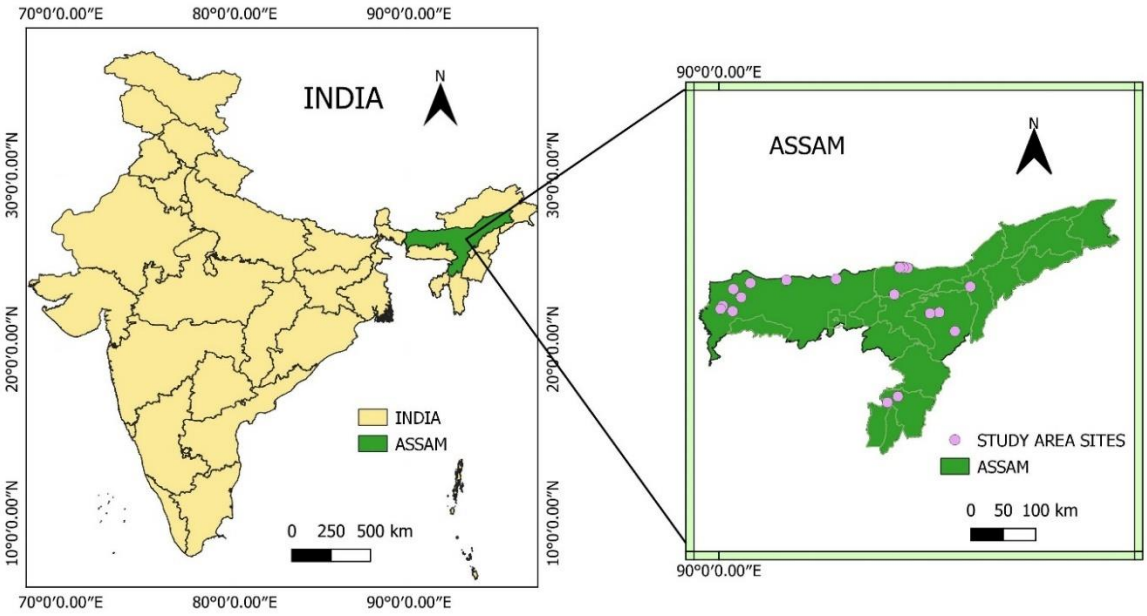

Figure 1. Map of the study area showing the collection sites of specimens

3.2. Field survey

A comprehensive field study was conducted in different seasons in different parts such as national parks, wildlife sanctuaries, and reserve forest areas of Assam from the year 2019 to 2023. Before the field trips, the appropriate authorization for gathering voucher specimens from protected and forested regions was secured from the corresponding state forest departments and municipal authorities. For conservation or protected areas i.e., national parks, wildlife sanctuaries, and reserve forests, permission has been granted from the Assam State Biodiversity Board (ASBB), Aranya Bhawan, Panjabari, Guwahati, Assam (letter no. ABB/Permission/2012/215) and Principal Chief Conservator of Forests (PCCF), Wildlife Warden, Aranya Bhawan, Panjabari, Guwahati, Assam (letter no. WL/FG/31/Research. T.C./32nd T.C. 2022, B/W/2022/ Research & Permission/7671-77).

3.3. Collection of specimens

The majority of specimens were collected in their reproductive stages. A twig with flowers and fruits was collected and kept carefully in an airtight container or plastic bag. GPS coordinates with the model GPS Garmin etrex 10 have been taken to determine the exact locality of the specimen. During collection, the specimen's collection number, habit characters, habitat, locality name, local name or vernacular name of the specimen, colour,

height, etc. were noted down in a field notebook. Photographs were taken with a high-quality mobile camera phone and a Canon EOS 1300D camera. Some primary ethnobotanical information was also gathered by interrogating the local people of different communities in the study area.

3.3.1. Plant data collection format

HERBARIUM FIELD NOTE BOOK

BODOLAND UNIVERSITY, KOKRAJHAR

ASSAM (INDIA)

Flora of ……. **Collection No. ……**

Family: ……. **Date: ……...**

Genus: ……...

Species: …….

Description:

…….

…….

…….

Locality: …….

G.P.S. Coordinates: ……..

Local Name: ……..

Local Uses, if any: …….

Identified by: …….

Collected by: …….

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3.4. Herbarium preparation and deposition of specimen

The standard methods have been followed for the preparation of herbarium specimens and preservation for anatomical studies (Jain & Rao, 1977). For the making of herbarium specimens, the specimens were poisoned using 4% mercuric chloride with ethanolic solution (Clark, 1986; Rana et al., 2014), and all the prepared herbarium specimens were deposited at BSI, Shillong, Meghalaya, India. Furthermore, each collected specimen has been preserved in a 70% alcohol or 4% formaldehyde solution for anatomical studies.

3.5. Identification of specimens

All the collected specimens were identified concerning some floras and books like '*The Flora of British India*', '*Flora of Assam*', '*Flora of China*', '*Flora of India*', '*Indo-Burmese Phyllanthaceae A Taxonomic Revision*' (Hooker, 1890; Kanjilal et al., 1940; Li & Gilbert, 2008; Balakrishnan et al., 2012; Chakrabarty & Balakrishnan, 2018). Protologues were checked through authentic literature and libraries like the Biodiversity Heritage Library (BHL). Again, Authentic or correct names were checked in online taxonomic tools (IPNI, 2023; POWO, 2023; WFO, 2023). The specimens were countered checked and confirmed by consulting herbarium specimens physically or online housed at ASSAM, ARUN, BM, CAL, K, L, LWG, NY, and RRLH.

3.6. Nomenclature

The up-to-date and accepted scientific name was provided based on the original publications in journals, floras, manuals, etc. Additionally, the scientific name was also verified by online sources such as IPNI, GBIF, and WFO.

3.7. Herbarium consultation and study of type specimen

Consultation of herbarium specimen and type specimen study has been done in BSI, Shillong, Meghalaya, CSIR IIIM, Jammu, NBRI, Lucknow (**Plate 41**), and other online herbaria (Acronyms: ARUN, ASSAM, BM, CAL, K, L, LWG, NY, RRLH).

3.8. Foliar epidermal study

3.8.1. Light Microscopy (LM) study

Leaves of some members of *Glochidion* have thick and hard leaf surfaces. Therefore, multiple techniques were used with slight modifications to study different characteristics of leaf epidermal cells.

After treating the leaves with a 10% nitric acid (HNO_3) solution, both adaxial and abaxial epidermal peelings were made by scrapping with a sharp blade. 1% aqueous safranine solution was used to stain and then the characteristics were observed under the light microscope (Boulos & Beakbane, 1971; Shahzad et al., 2022).

First leaf samples were treated with a solution containing 5% sodium hydroxide (NaOH) at room temperature until they lost their colour. Once the leaf samples were completely devoid of colour, the epidermal layers were removed using a forcep and needle, and stained with 1% of safranin solution. Then finally the characteristics were studied under the light microscope (model: LEICA DM750), also proper measurements were taken during the study (Radford et al., 1974; Lersten & Curtis, 2001).

For 2-24 hours, the leaves were treated with a 5% sodium hypochlorite (NaOCl) solution. Sodium hypochlorite is used for easy scrapping of both adaxial and abaxial leaf surfaces (Kong & Hong, 2019). Leaf samples were stored in an oven at 50ºC-60ºC in a Petri dish filled with Franklin's solution making with an equal volume of 35% hydrogen peroxide (H_2O_2) and glacial acetic acid (CH_3COOH) until they lost all colour. When they became transparent and white colour it was cleaned with distilled water and the layer of epidermis was carefully removed using a needle and forceps. Staining was done with safranin solution and one or two drops of glycerine were put on the specimen (da Silva et al., 2017; Garcia-Gutierrez et al., 2020). After covering the slide with a coverslip, it was studied using the light microscope (Model: LEICA DM750). Photographs and all the measurements of different characteristics were taken from LAS EZ Version 3.4.0.

Terminology for the study of epidermal cells, stomata, and trichomes was done by standard methods (Metcalfe & Chalk, 1950; Stace, 1965; Van Cotthem, 1970; Ayodele & Olowokudejo, 2005; Zhu et al., 2021).

3.8.2. Field Emission Scanning Electron Microscopy (FESEM) study

Both adaxial and abaxial surfaces of dried leaf specimens were examined using a field emission scanning electron microscope (Model: SIGMA VP FESEM, ZEISS). A small portion of leaf was cut and mounted with double adhesive tape on stubs, coated with gold-palladium, and the characteristics of leaf surfaces were studied using FESEM (Pathan et al., 2010; Ensikat et al., 2011; Traiperm et al., 2017).

3.9. Quantitative Analysis

The following formula was used to determine the stomatal index

Stomata Index (S.I.) in % = $\frac{S}{S+E} \times 100$ (Metcalfe & Chalk, 1979)

Where, S = The number of stomata per vision

E = Number of epidermal cells per vision.

Stomatal area was calculated using the following formula

Stomatal Area (S.A.) = $\pi \times (SW/2) \times (SL/2)$ (Eberly, 2008)

Where, SW = Width of stomata, SL= Length of stomata

The values of stomata length, stomatal width, stomatal density, epidermal cell density, and trichome length were calculated as Mean±SD.

3.10. Petiole anatomical study

For petiole anatomical studies, plant materials were taken from preserved in 70% alcohol, and freehand sections were employed for micromorphological studies (Radford et al., 1974). The hand section was stained with safranine and fast green and dehydrated through a graded ethanol series (30% to 90%). Then the sample was treated with 100% ethanol (C_2H_5OH). Finally, xylol was applied to the section and mounted with DPX. The section was observed with the help of a light microscope (Model: Labomed). Realme XT mobile camera phone was used to capture photographs.

The cross-section was made and treated with sodium hypochlorite (NaOCl) solution. If the section became colourless then stained with 1% of safranine solution. Then the slide was examined using the light microscope (Model: Labomed) (Filartiga et al., 2022).

3.11. Leaf architecture study

For the leaf architecture study mature leaves were taken from the herbarium or fresh materials or preservatives followed by the techniques of Vasco et al. (2014), García-Gutiérrez et al. (2020) and terminology were done by the methods of Melville (1976), Ellis et al. (2009). Materials were immersed in a 5% solution of Sodium hydroxide (NaOH) at 37˚C-40˚C in a hot air oven for one to several days until the leaf gets transparent, changes were made if the solution becomes dark. After the leaves became transparent let them be cooled down for 10 min. The leaves were then rinsed with tap water and kept in a water bath at room temperature for 10 min. The leaves were then bleached in 4% Sodium Hypochlorite (NaOCl) solution for 10 min. Once the cleared leaf turns white the process was stopped by rinsing it three times with tap water and kept in a water bath for 10 min. The materials were dehydrated for 30 min. in a graded ethanol series (50%, 70%, and 95%). Then staining was done by using safranine. The material was stained in 95% ethanol in safranine overnight. Then the leaves were rinsed in 95% ethanol for 10 min. Again, destained with 3-6 drops of HCl for 10-30 min. until it becomes clear white. Then the material was immersed in 100% ethanol for 10 min. and observed under the microscope. The Realme XT smartphone camera was utilised to snap microphotographs and leaf characters, venation patterns, vein termination number, vein islet number, etc. were studied under the light microscope (Model: Labomed).

3.12. Taxonomic keys

For easy identification of the taxa, bracketed dichotomous keys were arranged. Artificial taxonomic keys were made based on morphological, petiole anatomical, and leaf architecture study of the taxa.

3.13. Illustrations, Maps, and Photo plates

Illustrations or line drawings for the taxa were done by using hand drawing. The map of the study area of specimen collection sites was prepared using QGIS 3.26.3 version and the photo plates were made using Adobe Photoshop CC 2019.

3.14. Chart, Graph, and Statistical Analysis

Comparative column charts, graphs, and statistical data were analysed by using Microsoft Office Excel 2021. A significant test was done by one-way ANOVA and

unpaired t-test at $p \leq 0.05$ level in the software GraphPad Prism 9 and 10 versions respectively.

3.15. Phytochemical Study

Based on the primary and secondary ethnobotanical data provided by local villagers and traditional healers in Assam further phytochemical analyses were carried out.

3.15.1. Qualitative phytochemical analysis

3.15.2. Preparation of extracts

The preparation of extracts was followed by the methods of Harborne (1973), and Trease & Evans (2009).

The collected sample i.e. leaves, bark, and roots of the selected species (*G. ellipticum*, *G. multiloculare,* and *G. sphaerogynum*) were first rinsed with distilled water and then shade-dried for a half month until they became completely moisture free at room temperature. Then the dried sample was ground to a coarse powder with an electric grinder and the powdered material was kept in an airtight container or stored in a disinfected glass bottle. 20 g of the powdered sample was extracted by using the solvent (200 mL) for 3 days and filtered through Whatman filter paper no. 1. Subsequently, the filtrate was evaporated by setting the temperature based on the boiling point of the solvent on a vacuum rotary evaporator. After evaporation, the final residue was stored at 4°C for the analysis of different parameters of the sample.

3.15.3. Phytochemical screening

Preliminary phytochemical study for methanolic and ethanolic extract was tested to detect the occurrence of phytoconstituents such as alkaloid, reducing sugar, steroid, phlobatannin, tannin, flavonoid, terpenoid, triterpenoid, saponin, glycoside, and phenol in the plant extract according to standard methods (Harborne, 1983; Raaman, 2006; Trease & Evans, 2009; Mishra & Tripathi, 2015).

Test for alkaloid: In 0.1 g of the extract, 6 mL of concentrated HCl was added and heated. After boiling, it was cooled down and filtered with Whatman filter paper no. 1. Then the filtrate was divided into three portions in a test tube-

a. 1st test tube: 2-3 droplets of Dragendorff's reagent were mixed to the 1st portion. Yellow precipitation indicated the existence of alkaloids.

b. 2nd test tube: 2-3 drops of Mayer's reagent were applied to the 2nd portion. A creamy white precipitation indicated the occurrence of alkaloids.
c. 3rd test tube: 2-3 drops of Wagner's reagent were mixed to the 3rd portion. The appearance of a reddish-brown precipitation exhibited alkaloids.

Test for reducing sugar: After dissolving 0.1 g of extract in 2 mL of methanol, the extract was filtered. 2 mL of equal volumes of Fehling's solutions A and B were added to the filtrate and heated in the water bath for 5 minutes. The appearance of brick red precipitate showed reducing sugar.

Test for steroid:

Salkowski test: In 0.05 g of extract, chloroform was added and shaken, then H_2SO_4 was added carefully. The outcome of red colour designated the existence of steroids.

Test for phlobatannins: In 0.05 g of extract, 2 mL of HCl was added and heated. The result of red colour precipitation designated the occurrence of phlobatannins.

Test for tannins: In 0.05 g of extract, four to five drops of $FeCl_3$ solutions were added. The greenish-black colour indication showed the existence of tannins.

Test for flavonoids:

$FeCl_3$ test: A few drops of $FeCl_3$ solutions were added to 0.05 g of extract. The result of the intense green colour designated the occurrence of flavonoids.

H_2SO_4 test: In 0.05 g of extract, 3 mL of concentrated H_2SO_4 was mixed. The result of the orange colour indicated the occurrence of flavonoids.

Test for terpenoids:

Salkowski test: In 2 mL of chloroform, 0.05 g of extract was mixed with 3 mL of concentrated H_2SO_4 to get a layer forming. If a reddish-brown colour of the edge was observed then the result indicated the existence of terpenoids.

Test for triterpenoids:

H_2SO_4 test: In 0.05 g of extract, 3 mL of concentrated H_2SO_4 was added. The result of the yellow colour indicated the existence of triterpenoids.

Test for saponin:

Foam test: 2 mL of the extract was shaken with distilled water and the occurrence of foam specified the existence of saponin.

Test for glycosides:

Keller-killiani test: In 0.05 g of extract, four to six drops of $FeCl_3$ solutions were added along with concentrated H_2SO_4. The presence of a reddish-brown lower layer and bluish-green upper layer specified the occurrence of glycosides.

Borntrager's test: In 2 mL of extract, 3 mL of chloroform and 10% ammonia solution were added. The result of the pink colour specified the presence of glycosides.

Test for phenol:

$FeCl_3$ test: In 0.05 g of extract, five to ten drops of $FeCl_3$ solutions were added. The intense bluish-black colour indicated the existence of phenol.

3.15.4. Quantitative estimation of phytochemical constituents

Total alkaloid content (TAC)

TAC was done following the methods of Harborne (1973) and Ogunjobi et al. (2020). 2.50 g of each powdered sample was taken in a glass beaker, and 200 mL of 10% acetic acid (CH_3COOH) in ethanol (C_2H_5OH) solution was added, then allowed to sit for 4 hours and filtered. Then the filtrate was heated in a water bath and concentrated ammonium hydroxide (NH_4OH) was applied until the precipitation was observed. The precipitation was cooled down and washed using 20 mL of 0.1M NH_4OH and filtered through Whatman filter paper no. 1. The residue was dried in an oven and the final residue was calculated. Mathematical calculations for total alkaloid content are as follows-

$$\text{Total alkaloid content (\%)} = \frac{\text{Final weight of the residue}}{\text{Initial weight of sample}} \times 100$$

Total flavonoid content (TFC)

2.50 g of each powdered sample was placed in a 250 mL of glass beaker and added 50 mL of 80 % aqueous methanol, was covered and stood for 24 hours at 20°C in a hot water bath. The supernatant was removed and the extraction was done again with an equal volume of ethanol (C_2H_5OH). Then, Whatman filter paper no. 1 was used for the filtration of the whole mixture. The filtrate was evaporated in a water bath, and the final dry residue

was weighed in an electronic balance (Harborne, 1973; Senguttuvan et al., 2014; Ogunjobi et al., 2020). The mathematical formula for calculating total flavonoid content is-

$$\text{Total flavonoid content (\%)} = \frac{\text{Final weight of the residue}}{\text{Initial weight of sample}} \times 100$$

Total saponin content (TSC)

In 5 g of sample, 100 mL of 20% aqueous ethanol (C_2H_5OH) was added and heated at 55°C in a hot water bath for 4 hours. The same technique was repeated twice or thrice. The mixed extract was then diluted to 40 mL in a water bath heated to 90°C. The mixture was taken in a separating funnel and rapidly agitated, and then 20 mL of diethyl ether $\{(C_2H_5)_2O\}$ was added. The diethyl ether layer was then discarded. There were two purifying processes used. Following with two washes with 10 mL of 5% sodium chloride (NaCl), 60 mL of n-butanol ($C_4H_{10}O$) was added. The NaCl layer was discarded, then heated in a water bath and dried in an oven at 50°C (Harborne, 1973; Ogunjobi et al., 2020). Mathematical calculations for total saponin content are as follows-

$$\text{Total saponin content (\%)} = \frac{\text{Final weight of the residue}}{\text{Initial weight of sample}} \times 100$$

Total terpenoid content (TTC)

Estimation of TTC was examined by the procedures of Ferguson (1956) and Kong et al. (2019). 2 g of each powdered sample was dissolved in 50 mL of ethanol (C_2H_5OH) overnight. Filtration was done using Whatman filter paper no. 1. Then petroleum ether (C_6H_{14}) was applied for extraction and dried at 65°C in a water bath. The final volume was determined as-

$$\text{Total terpenoid content (\%)} = \frac{\text{Final weight of the residue}}{\text{Initial weight of sample}} \times 100$$

Total phenolic content (TPC)

TPC of each methanolic extract was analysed by using the FC (Folin Ciocalteu) method at UV-Vis Spectrophotometer (Shimadzu A 125358). TPC was made as designated by the standard method with the minute modification (Senguttuvan et al., 2014). 0.01 g of the sample was taken and made up of 1800 µL of distilled water followed by the addition of 150 µL FC reagent and 1 mL of 10% sodium carbonate (Na_2CO_3) to make 3 mL of volume. Incubation was carried out in the dark for 40 minutes and measurement was done at 765 nm against a blank. The standard curve was developed using the standard gallic acid in the range of 10–50 µg/mL. The quantification of the total phenolic content of each plant

extract was determined by mg of gallic acid equivalents of dried extract (mg GAE/g dry extract).

Total tannin content (TTC)

The total tannin content (TTC) of each methanolic extract was quantified using the Folin Denis (FD) method in UV-Vis Spectrophotometer (Shimadzu A 125358) with slight modification (Padma et al., 2013; Saxena et al., 2013; Soni et al., 2018). 0.01 g of the sample was weighed and made up of 1800 µL of distilled water. 150 µL FD reagent and 1 mL sodium carbonate (Na_2CO_3) were added to make a total volume of 3 mL and incubated in the absence of light for 40 minutes. The calibration curve was determined using standard tannic acid (10–50 µg/mL) and calculated at the absorbance of 760 nm versus a blank. The total tannin content (TTC) was quantified as mg of tannic acid equivalents of dried extract (mg TAE/g dry extract).

3.16. In vitro Antioxidant activity

3.16.1. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging assay

The antioxidant activity of each methanolic sample extract was tested using the DPPH assay with slight modifications (Shukla et al., 2014; Narzary et al., 2016; Islary et al., 2017a, 2017b). 1 mL methanolic extract solutions (10–60 µg/mL) were dissolved in 3 mL DPPH solution and incubated in the dark. 3 mL methanol was taken as blank, and the mixture of 2 mL methanol and 1 mL DPPH solution was taken as the control. After 30 minutes, the absorbance of extracts was read at 517 nm using a UV-Vis spectrophotometer, and compared to that of normal ascorbic acid at equal amounts. The IC_{50} value was obtained using linear regression analysis. A low concentration of IC_{50} value indicates strong antioxidant activity. The following formula was applied to calculate the percentage of DPPH scavenging effect-

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of the sample extract or standard}}{\text{Absorbance of control}} \times 100$$

3.16.2. 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The antioxidant activities of each methanolic extract of the sample were tested using the ABTS assay with slight modification (Shukla et al., 2014; Narzary et al., 2016; Islary et al., 2017a, 2017b).

ABTS radical cation (ABTS•+) was made by combining 5 mL of potassium persulfate (2.45 mM) and ABTS (7 mM) solutions. This reaction was allowed to proceed at room temperature for 12 to 16 hours in the absence of light. The original absorbance was then adjusted to 0.706±0.01 at 734 nm by diluting the ABTS radical solution in methanol. Then, different concentrations of sample and standard (10–60 µg/mL) were made, and 2 mL of diluted ABTS solution was added and mixed well. Using a UV-vis spectrophotometer, the solution's absorbance was determined at 734 nm after 6 minutes. The concentration of the extract was presented against a graph of % inhibition. Methanol was taken as a blank and added to the 2 mL ABTS solution was used as a control. IC_{50} value was computed using the linear regression equation deduced from the graph of % inhibition. The percentage of the ABTS scavenging effect was measured using the following formula-

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of the sample extract or standard}}{\text{Absorbance of control}} \times 100$$

3.17. GC-MS Analysis

GC-MS analysis of each methanol extract of the sample was performed by using Perkin Elmer (USA) (Model: Clarus 680 GC and amp.) with Turbo Mass Version 6.1.2 software. The system was run using a stationary phase of 5% diphenyl 95% dimethyl polysiloxane and the carrier gas i.e. helium gas (99.99 %) at the flow rate of 1 mL/min. Splitless mode was employed with an injection volume of 2 µL. The temperature difference between the ion source and the injector is 180°C and 280°C respectively. The mass spectrum at 70 eV was obtained using the electron impact positive (EI+) mode. NIST-2014 software and a library search were used to calculate the mass spectrum of corresponding peaks and compounds (Casuga et al., 2016).

3.18. Assessment of mineral contents

Atomic Absorption Spectroscopy (AAS), Model: Shimadzu AA-7000 was used to determine mineral elements such as Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), Copper (Cu), Zinc (Zn), Manganese (Mn), Chromium (Cr), Lead (Pb), and Cadmium (Cd). Sample was digested using concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) employing the wet ashing method. In 0.50 g of sample, 8 mL of nitric acid (HNO_3) was mixed and left to stand overnight. The next day, it was heated on a hot plate at the temperature of 120°C for 1 hour. Then 4 mL of H_2O_2 was added drop by drop until the digestion became colourless. The residue was again dried at 80°C on a

hot plate and diluted with HCl. The final concentration of the elements determined from Atomic Absorption Spectroscopy (AAS) was analysed in ppm (Pequerul et al., 1993).

3.19. Statistical Analysis

All the quantitative data were analysed in triplicates and were determined as Mean and Standard Deviation (Mean±SD) and the graph was determined using Microsoft Office Excel 2021. The significant test was performed by one-way ANOVA at $p \leq 0.05$ level in GraphPad Prism 9 and 10. IC_{50} value and graph plotted of antioxidant activity were analysed in GraphPad Prism 9 and 10 respectively.