Section: Research Paper

Phyto-metabolomics and mineral profile analysis of methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.: Application of LC-MS/MS, GC-MS/MS and AAS

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ABSTRACT

Background: Phyto-metabolomics deals with identification and quantification of primary and secondary metabolites. Demand qualitative and quantitative analysis of medicinal herbs is tremendously increasing pertaining to their medical value. Phytochemical profile for methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb. has remained unexplored yet.

Objective: Determination of phyto constituents using hyphenated chromatographic techniques like LC-MS/MS, GC-MS/MS, to perform minerals and proximate analysis for methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Methods: Proximate analysis was performed for crude extract. Minerals estimation was done by atomic absorption spectroscopy (AAS). Extract was analyzed both in positive and negative mode by LC- MS/MS and GC-MS/MS for methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Results & Discussion: 58 compounds were identified by LC-MS/MS, 39 compounds were identified by GC-MS/MS. Sodium (Na) concentrations were found high in minerals estimation. Proximate analysis results depicts Moisture content of 12.34 g/100g, Total ash of 7.44 g/100g, Crude fiber of 15.30 g/100g, Crude protein of 15.30 g/100g, Crude fat of 0.69 g/100g, Carbohydrate content of 66.32 g/100g. Phenolic content was found to be 0.0473 µg/ml. Flavonoid content was found to be 1.136 µg/ml.

Conclusion: Primary, Secondary metabolites were identified by LC-MS/MS, GC-MS/MS, Mineral analysis and proximate analysis was performed and reported for the first time.

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Keywords: *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb., Methanolic flower extract, LC-MS/MS, GC-MS/MS, Phyto-metabolomics, Mineral analysis, Proximate analysis, Herbal medicine.

1. Introduction

Traditional herbs are used widely as dietary supplements and source of medicine in several local communities globally (1). In recent times there is a tremendous increase in demand and commercial value for herbal medicine, thereby, increase in efforts to establish scientific evidence for their claimed medicinal properties by complete characterization of phytoconstituents and toxicological studies (2). Due to the presence of multi components there lies a challenge in analysis of herbal drugs in comparison with synthetic drugs (3). Hyphenated techniques like LC-MS/MS, GC-MS/MS have helped in prolific rise in advancement of complete characterization of herbal samples by identifying the multi components in an herbal sample by predicting compound names with the aid of inbuilt NIST spectral libraries (4). Phyto-metabolomics is an area focusing on quantitative and qualitative analysis of primary and secondary metabolites (5).

Phlogacanthus thyrsiformis (Roxb. ex Hardw.) Mabb. Is a medicinal plant with common names of Lal Vasak, Teetaphul (6) from Acanthaceae family widely distributed in north-eastern part of India, Bangladesh and China (7). Its flowers are long orange-red tubular flowers and upright spikes at the end of branches as shown in **Figure 1**. (8). It has many healing properties like anti-diabetic (9), in treatment of gout and rheumatism (10)in treatment of whooping cough and menorrhagia (11) cough, chronic bronchitis, asthma (12, 13) , to treat small pox (14), to treat skin diseases (15), treatment of cancer (15)neuro protective (7) and immune boosting properties (7).

Aqueous, chloroform extracts of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb. Has been studied for phytochemical profile (5) but methanolic extract of flower has not been studied and reported till date.

In this study, proximate analysis (Moisture content total ash, fiber content,crude protein carbohydrate content), Mineral analysis was carried out. Several primary and secondary metabolites were identified by LC-MS/MS and GC-MS/MS in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Figure 1: **Flowers of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.**

2. Materials and Methods

Plant material: Collection and identification

The flowers of *Phlogacanthus thyrsiformis* were collected from Hahara gaon, Sonapur, Kamrup Metropolitan District, Assam during February, 2021. The collected flowers were identified by Dr. Souravjyoti Borah, Curator, GUBH, Department of Botany, Gauhati University, Assam.

A herbarium was made ready after poisoning with mercuric chloride and the voucher specimen bearing accession number 19763 was deposited in the Department of Botany, Gauhati University, Assam for future reference. The fresh flowers collected were utterly cleaned and washed with fresh water, shade dried at room temperature for 14 days, coarsely powdered in mixture grinder (Philips). The powdered sample was stored in an airtight and light- resistant container, properly labeled and sealed and was used in the current investigation.

Preparation of flower extract

Flowers were thoroughly cleaned and washed to eliminate dirt and contamination. They were then shade dried, powdered coarsely. The powdered plant material was macerated with absolute methanol for 72 hours at room temperature while stirring occasionally (16). The extract was filtered using Whatman No. 1 filter paper. The filtrate that was obtained was air dried and was stored in 4^{o} C refrigerator for its further use as PTFME (*Phlogacanthus thyrsiformis* flower methanolic extract) (17). The yield obtained from the extract was 8.41 % (w/w).

Proximate analysis

Flowers of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb was analyzed for moisture content, total ash, crude fiber, crude protein, crude fat, and carbohydrate content (18).

Determination of Minerals. Concentrations of potassium, calcium, magnesium, zinc, and iron were quantified after nitric acid plus hydrogen peroxide digestion followed by flame atomic absorption spectroscopy (AAS) using a PerkinElmer model AAnalyst 200 (USA). Parameters of the instrument

were chosen in accordance with the manufacturer's instructions. Mineral content was expressed as parts per million concentration (ppm).

Preliminary phytochemical screening of crude methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Total Phenolic Content

The amount of total phenol content, in solvent extract of flower was determined by Folin- Ciocalteu's reagent method (19). 13.0.5mL of extract and 0.1 mL (0.5N) Folin-Ciocalteu's reagent was mixed and the mixture was incubated at room temperature for 15 min. Then 2.5 mL saturated sodium carbonate solution was added and further incubated for 30 min at room temperature and the absorbance was measured at 760 nm. Gallic acid was used as a positive control. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of extracted compounds).The assay was carried out in triplicate and the mean values with ± SEM is presented.

Total Flavonoid Content

The total flavonoid content was determined spectrophotometrically according to Lamaison and Carnat (20). Briefly, 0.5mL of 2% aluminum chloride ($AlCl_3$) ethanol was mixed with the same volume of vegetal extracts (0.1–1.0mg=mL). Absorption readings at 415nm were taken after 1hour against a blank (ethanol). The total flavonoid content was determined using a standard curve with quercetin (0 – 50mg=L). The mean of three readings was used and expressed as milligrams of quercetin equivalents (QE=g of dryextract).

Phyto-metabolomics profiling

LC-MS/MS analysis

Sample preparation:

1mg of sample is dissolved in 10 mL of methanol to obtain concentration of 1000 µg/mL stock solution.10µL of solution from stock solution is added into a 10 mL beaker and made up the volume

up to 10 mL with methanol to obtain a concentration of 100 ng/mL and the obtained sample solution is filtered using 0.22µm syringe filter before injecting.

Instrumentation:

The Acquity HPLC was employed for the study, which has an integrated vacuum degasser, automatic sample manager (serial #C10UPA554M, Waters Corporation, Singapore), ultra-performance binary solvent manager (serial #C10UPB081A, Waters Corporation, Singapore) and injection volume range up to 100µL with an optional extension loop. A C-18 stationary phase (Accucore C-18, 50×4.6mm, 2.6µm) was used for chromatographic separation. A photodiode array detector (DAD) was employed in conjunction with a Xevo G2-XS QToF (serial #YFA1548, Waters Corporation Wilmslow, UK) for

mass spectrometric detection.

Chromatographic method

The mobile phase is made up of 0.1% Formic acid as aqueous phase (A) and Acetonitrile as an organic modifier (B), and it is delivered at a flow rate of 0.4ml/min in the following gradient: initial to 4 minutes (10 to 95%) mobile phase B, held for 3 minutes, 7 to 9 minutes (90 to 10%) and held at 10% for 1 minute. The sample was injected in a volume of 5µL. The column oven temperature was kept at an optimal level at 22˚C throughout the chromatographic run.

For MS detection, a positive polarity electrospray ionization (ESI) source was used. The optimal instrument and acquisition parameters were as follows: Nitrogen was used as cone gas at a flow rate of 50L/hr and desolvation gas at the flow rate of 750L/hr. The temperatures for probe and source were maintained at 450˚C and 150˚C respectively. The voltages for sampling cone and source offset were maintained at 30V and 80V. The collision gas used was Argon and collision energy ramp varies from 6eV. The mass range was selected from 50 to 1500 m/z.

Sample was infused at a flow rate of 5 µL/min. To acquire and process data, Waters Corporation's Mass Lynx (V4.1, Milford, MA, USA) software was used.

GC-MS/MS analysis

Sample preparation:

1 mg of sample is dissolved in 10 mL of methanol to obtain concentration of 1000 µg/mL stock solution.10µL of solution from stock solution is added into a 10 mL beaker and made up the volume up to 10 mL with methanol to obtain a concentration of 100 ng/mL and the obtained sample solution is filtered using 0.22µm syringe filter before injecting.

Instrumentation:

Pekin Elmer Gas chromatograph Clarus 680 instrument with Pekin Elmer Elite 5MS column (30mlong×0.250 mm×1µm) and Pekin Elmer Mass spectrometer Clarus SQ 8C was used for GC-MS/MS analysis.

Chromatographic method

The oven temperatures were programmed with initial starting at 80˚C, held for 2 minutes, then increasing to 150˚C at a rate of 10˚C/min and maintaining for 1 minute, then raising the temperature to 250˚C at a rate of 15˚C/min and maintained for 10 minutes. The inlet temperature and source temperature were maintained at 250˚C and 230 ˚C respectively. The total run time was 26.6 min. The split ratio of 10:1 was used to inject at 2µL of sample. The NIST database containing over 62,000 models has been calculated for the mass range of GC-MS. The standard NIST library component collection limited the number of dark segments.

3. Results and Discussion

Proximate analysis of flowers of *Phlogacanthus thyrsiformis* (Roxb. exHardw.) Mabb.

The results of proximate analysis are presented in **Table 1.**

Table 1: **Proximate analysis of flowers of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.**

Sl. No	Test parameter	Results
01	Moisture content	12.34 g/100g
02	Total ash	7.44 g/100g
03	Crude fiber	15.30 g/100g
04	Crude protein	15.30 g/100g
05	Crude fat	0.69 g/100g
06	Carbohydrate	66.32 g/100g

Mineral analysis of methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

The results of mineral analysis are presented in **Table 2.**

Table 2: Mineral analysis of methanolic flower extracts of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Sl. No	Mineral name	Concentration (ppm)
01	Sodium (Na)	5.7351
02	Potassium (K)	0.4807
03	Calcium (Ca)	0.2236
04	Magnesium (Mg)	2.1124
05	Iron (Fe)	0.8887
06	Chromium (Cr)	0.2131
07	Copper (Cu)	0.4712
08	Nickel (Ni)	0.031
09	Zinc (Zn)	0.2543

Total Phenolic Content:

Different concentrations of Gallic acid ranging from 1-50 µg/mL were prepared and checked for absorbance in UV spectrophotometer. Calibration curve was plotted for concentration versus absorbance as shown in **Figure 2**. Phenolic content concentration can be calculated from the regression equation. Total phenolic content was found to be 0.0473µg/mL.

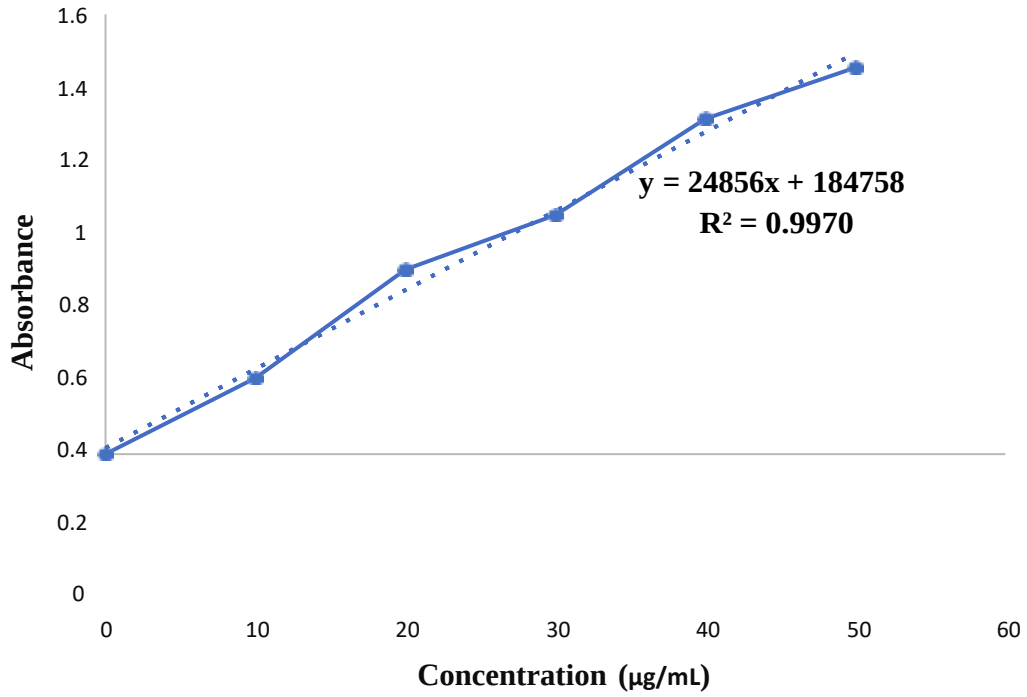

Figure 2: Calibration curve for Gallic acid (1-50 µg/mL)

Total Flavonoid Content

Different concentrations of quercetin ranging from 1-50 µg/mL were prepared and checked for absorbance in UV spectrophotometer. Calibration curve was plotted for concentration versus absorbance as shown in **Figure 3**. Flavonoid content can be calculated from the regression equation. Total flavonoid content was found to be 1.136 µg/mL.

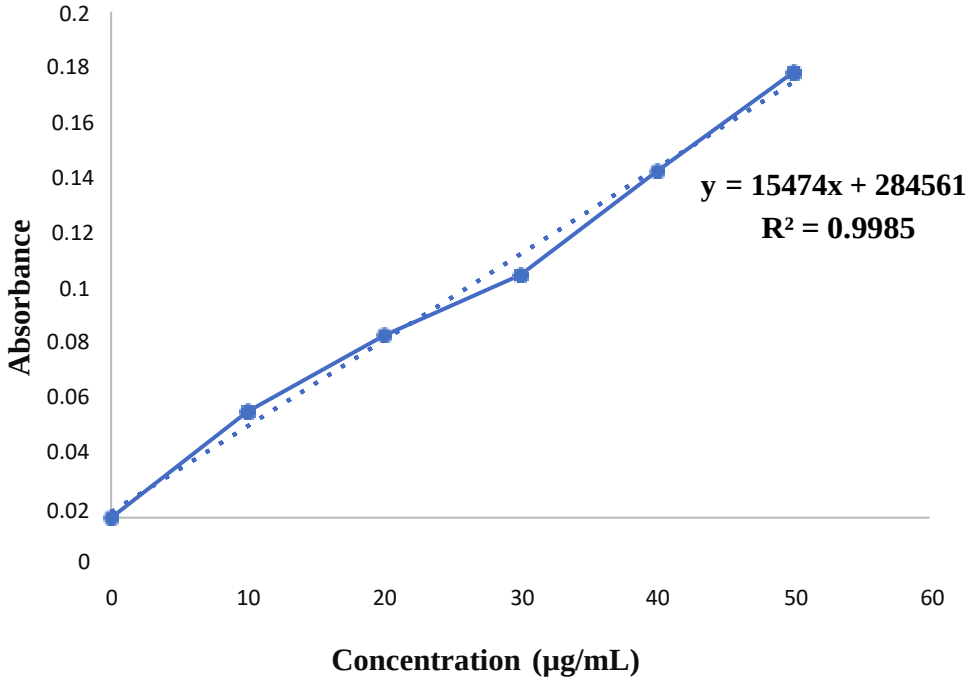

Figure 3: Calibration curve for Quercetin (1-50 µg/mL)

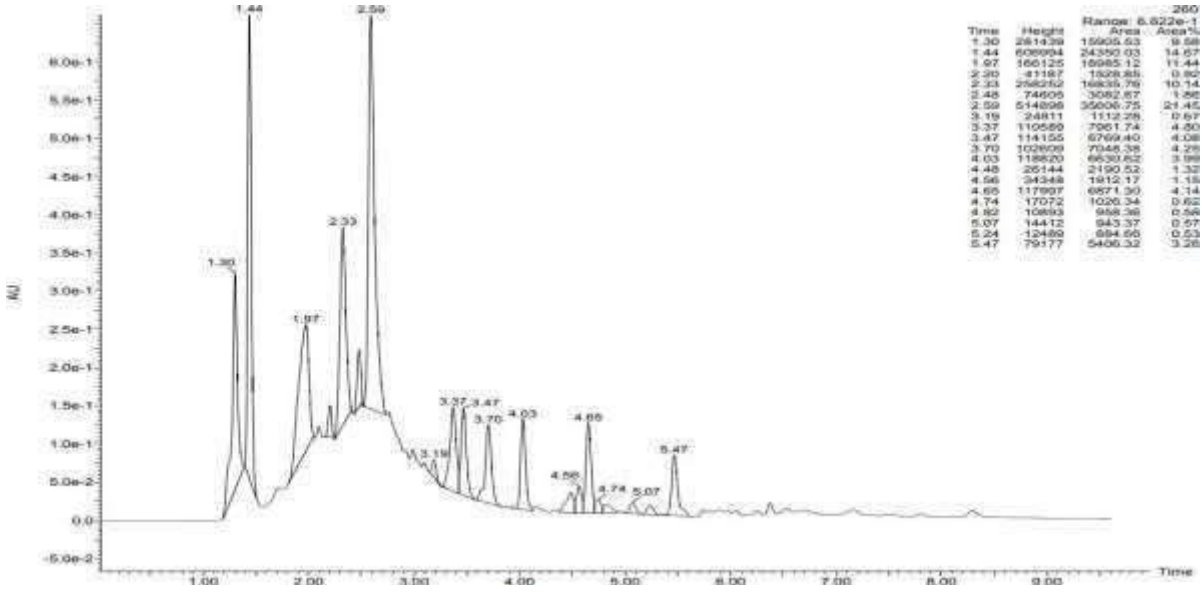

Figure 4: **LC-MS/MS chromatogram for *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.)Mabb.**

Phyto-metabolomics profiling: The LC-MS/MS chromatogram of the extract is been shown in the **Figure 4**. The phytoconstituents were analyzed using both positive and negative ionization modes. Top twenty nine phytoconstituents were found in positive ionization mode. In negative ionization mode top twenty nine phyto constituents were identified. List of compound identified in positive and negative mode of LC-MS/MS analysis are presented in **Table 3** and **Table 4** respectievely.

Table 3: List of compounds identified in positive mode of LC-MS/MS analysis

Sl. No	Compounds identified by NISTlibriary	Retention time (minutes)	Molecular formula	Molecular weight m/z	Adduct
1	Thalrugosaminine	9.914833	$C_{39}H_{44}N_2O_7$	675.3	[M+H]+
2	Sibiricaxanthone B	9.914833	$C_{24}H_{26}O_{14}$	539.14	[M+H]+
3	Taxayuntin E	9.484921	$C_{33}H_{42}O_{12}$	631	[M+H]+
4	Gomisin D	9.828918	$C_{28}H_34O_{10}$	553.2	[M+H]+
5	Deferrioxamine E	10.04379	$C_{27}H_{48}N_6O_9$	618.3821	[M+H]+
6	Pedunculoside	9.872087	$C_{36}H_{58}O_{10}$	651.41	[M+H-H_2O]+
7	Fluostatin J	10.04379	$C_{23}H_{20}O_7$	431.109	[M+H]+

8	Indaconitine	10.08655	$C_{34}H_{47}NO_{10}$	630.327	[M+H-H_2O]+
9	Neocoprogen I	9.700729	$C_{31}H_{50}N_6O_{12}$	699.35	[M+Na]+
10	Acetylportentol	10.08655	$C_{19}H_{28}O_6$	370.223	[M+H]+
11	Barbaleucamide B	9.743254	$C_{17}H_{22}Cl_6N_2O_2S$	530.958	[M+H]+
12	Salviaflaside	10.08655	$C_{24}H_{26}O_{13}$	540.1711	[M+H]+
13	Methionine_Enkephalin	9.872087	$C_{27}H_{35}N_5O_7S$	574.2336	[M+Na]+
14	Glycyrrhizate	10.00069	$C_{42}H_{62}O_{16}$	823	[M+NH_4]+
15	Vinblastine	9.743254	$C_{46}H_{58}N_4O_9$	833.4085	[M+H]+
16	Ergocristine	10.12952	$C_{35}H_{39}N_5O_5$	610.303	[M+NH_4]2+
17	Hypaconitine	10.12952	$C_{33}H_{45}NO_{10}$	616.3117	[M+H]+
18	Taxuspine D	10.00069	$C_{39}H_{48}O_{13}$	725	[M+H]+
19	Deacetylgedunin	10.17235	$C_{26}H_{32}O_6$	458.2537	[M+H]+
20	Strepin P1	10.00069	$C_{25}H_{40}N_6O_5$	505.31	[M+H-2H_2O]+
21	Hypericin	10.17235	$C_{30}H_{16}O_8$	522.1184	[M+H]+
22	Terracinolide C	10.12952	$C_{36}H_{50}O_{16}$	761.3	[M+H-H_2O]+
23	Icariin	10.21505	$C_{33}H_{40}O_{15}$	677.25	[M+H]+
24	Thermoactinoamide_J	10.04379	$C_{36}H_{66}N_6O_6$	679.512	[M+2H]2+
25	Rehmannioside A	10.30086	$C_{21}H_{32}O_{15}$	525.18	[M+H-H_2O]+
26	Legonoxamine G	10.25778	$C_{30}H_{50}N_6O_8$	623.37	[M+2H]2+
27	Neohesperidin Dihydrochalcone	10.12952	$C_{28}H_{36}O_{15}$	635.19	[M+H]+
28	Reserpic acid	10.47289	$C_{22}H_{28}N_2O_5$	401.2071	[M+H]+
29	Angoroside C	10.04379	$C_{36}H_{48}O_{19}$	807.26	[M+H]+

Table 4: List of compounds identified in negative mode of LC-MS/MS analysis

Sl. No	Compounds identified byNIST library	Retention time (minutes)	Molecular formula	Molecular weight m/z	Adduct
1	Cycloheximide	8.900969	$C_{15}H_{23}NO_4$	280.1554	[M-H]-
2	Avocadyne Acetate	8.815928	$C_{19}H_{34}O_4$	325.2384	[M-H]-
3	Arabinose 5-phosphate	8.985281	$C_5H_{11}O_8P$	229	[M-H]-
4	Methyl palmitoleate	8.942887	$C_{17}H_{32}O_2$	267.2329	[M-H]-
5	Salinomycin, Sodium	8.730547	$C_{42}H_{69}O_{11}$	771.4664	[M-H]-
6	Diclazuril	9.112474	$C_{17}H_9Cl_3N_4O_2$	404.9718	[M-H]-
7	Piperonyl butoxide	9.281565	$C_{19}H_{30}O_5$	337.202	[M-H_2O-H]-
8	Portentol	9.2392	$C_{17}H_{26}O_5$	355.174	[M-H]-
9	Chloroatranorin	9.2392	$C_{19}H_{17}ClO_8$	407.055	[M-H]-
10	3-Methylguanine	9.366631	$C_6H_7N_5O$	164	[M-H]-
11	Nilutamide	9.197131	$C_{12}H_{10}F_3N_3O_4$	316.0551	[M-H]-
12	Actinonin	9.070193	$C_{19}H_{35}N_3O_5$	384.2504	[M+Na-2H]-
13	Linoelaidic acid	9.5406	$C_{18}H_{32}O_2$	279.2326	[M-H]-
14	Linalool (+)	9.839144	$C_{10}H_{18}O$	153.1285	[M-H]-
15	Piperonyl butoxide	9.9679	$C_{19}H_{30}O_5$	337.202	[M-H]-
16	Eicosenoic acid	10.05408	$C_{20}H_{38}O_2$	309.2802	[M-H]-
17	Convallatoxin	9.71095	$C_{29}H_{42}O_{10}$	549.2705	[M-H]-

18	Glucosaminate	10.26798	$C_6H_{13}NO_6$	194	[M-H]-
19	Atenolol acid	10.01093	$C_{14}H_{21}NO_4$	266.1398	[M-H]-
20	Tolcapone	10.22524	$C_{14}H_{11}NO_5$	272.0564	[M-H]-
21	Chloralose	10.39705	$C_8H_{11}Cl_3O_6$	306.9548	[M-H]-
22	Lignoceric acid	9.9679	$C_{24}H_{48}O_2$	367.357	[M-H]-
23	Paulownin	10.52688	$C_{20}H_{18}O_7$	369.0305	[M-H]-
24	Procysteine	10.83041	$C_4H_5NO_3S$	145.9914	[M-H]-
25	4-Hexylresorcinol	10.78754	$C_{12}H_{18}O_2$	193.1237	[M-H]-
26	Arbutin	10.8731	$C_{12}H_{16}O_7$	271.0823	[M-H]-
27	Tricosanoic acid	10.78754	$C_{23}H_{46}O_2$	353.3415	[M-H]-
28	Convolidine	11.0471	$C_{15}H_{19}NO_4$	276.1241	[M-H]-
29	Glabridin	10.74413	$C_{20}H_{20}O_4$	323	[M-H]-

GC-MS/MS analysis

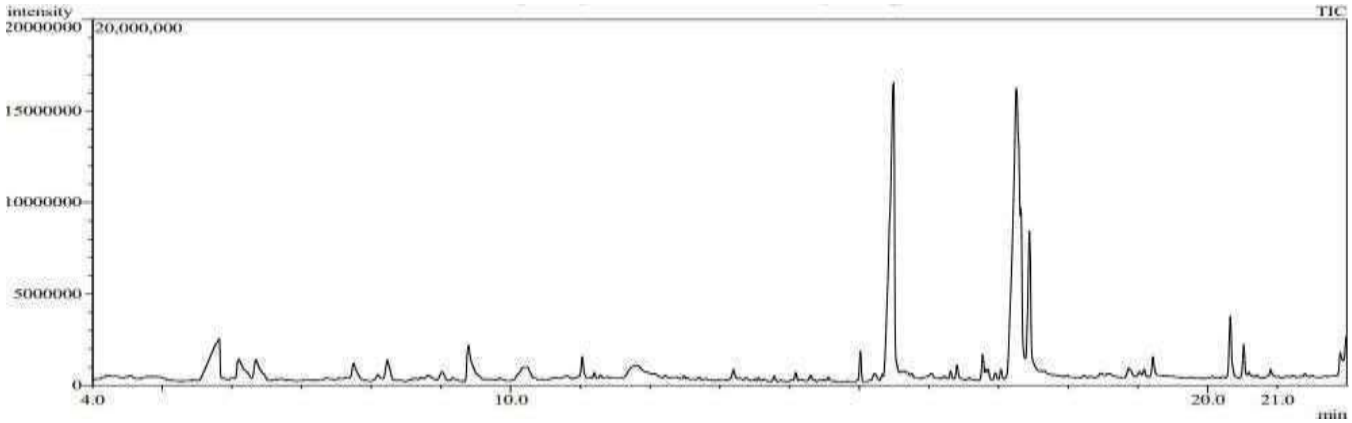

Figure 5: **GC-MS/MS chromatogram for *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.)Mabb.**

GC-MS is most suitable instrument for analyzing samples with volatile nature, and it is mostly preferred for sample resolution. The GC-MS total ion chromatogram is shown in the **Figure 5** the constituents that are identified are represented in the **Table 5**.

Table 5: Identified phytoconstituents by GC-MS/MS analysis

Sl. No	Compounds identified by NIST library	Retention time (minutes)	Similarity Index
1	Benzoic acid	6.431	97
2	Phytol	20.132	96
3	1-Nonadecene	13.024	95
4	Pentadecanal-	14.884	95
5	Hexadecanoic acid, methyl ester	17.746	95
6	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	19.913	95
7	Heptadecanoic acid, 16-methyl-, methyl ester	20.248	95
8	Vitamin E	27.782	95
9	Catechol	6.761	94
10	Heptadecane	13.133	94
11	Neophytadiene	16.593	94
12	Palmitoleic acid	17.996	94
13	n-Hexadecanoic acid	18.344	94
14	Squalene	27.603	94
15	Alpha.-Tocopherol-.beta.-D-mannoside	31.215	94
16	Gamma.-Sitosterol	31.379	94
17	Benzofuran, 2,3-dihydro-	7.059	93
18	1-Nonadecene	15.935	93
19	9-Octadecenoic acid, (E)-	19.341	93
20	2-Propenoic acid, 3-phenyl-	10.512	92
21	Beta.-D-Glucopyranose, 1,6-anhydro-	11.828	92
22	Heneicosane	16.025	92
23	Pentadecanoic acid	16.871	92
24	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	24.545	92
25	2-Methoxy-4-vinylphenol	8.739	91

26	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	16.787	91
27	Nonadecyl pentafluoropropionate	19.674	91
28	9,12-Octadecadienoic acid (Z,Z)-	20.513	91
29	9-Octadecenoic acid, (E)-	21.729	91
30	Tetracosane	26.291	91
31	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	5.981	90
32	Phenol, 2,6-dimethoxy-	9.336	90
33	DL-Proline, 5-oxo-, methyl ester	9.758	90
34	Phenol, 4-ethenyl-2,6-dimethoxy-	12.784	90
35	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	15.407	90
36	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	17.175	90
37	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	18.105	90
38	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	20.593	90
39	Octadecanoic acid	20.758	90

4. Conclusion

Here in this study, proximate and mineral analyses were performed. Phenolic and flavonoid contents were estimated. Determination of phytoconstituents present in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb was done using two different hyphenated techniques LC-MS/MS and GC-MS/MS. A total of 58 phytoconstituents were identified using LC-MS/MS technique and 39 phytoconstituents were identified using GC-MS/MS. Traditional medicines are easily available to masses but are complex mixtures of natural substances and are prone for variation and adulteration. Recent studies show that people are opting for traditional medicines because of their lower side effects. Therefore, studies regarding the safety and efficacy of the herbal medicines are to be carried out. LC-MS/MS and GC-MS/MS techniques was employed to find the major phyto constituents that are present in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb and successfully we could identify 97 phyto constituents altogether using both the methods having properties that can alleviate symptoms of diabetes.

Author Contributions

Rajashree Deka, Jogen Ch Kalita conceptualized, wrote, proofread and edited the manuscript. All the authors reviewed the final version of the manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

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