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Quantitative analysis of total phenolic, flavonoid contents and HPTLC fingerprinting of flower extracts of *Phlogacanthus thyrsiflorus* Nees

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Abstract
Objective: To quantify total phenolic, flavonoid contents in *Phlogacanthus thyrsiflorus* to evaluate the antioxidant potential along with HPTLC fingerprinting for identification of phytocompounds present in this medicinal plant.
Methods: Phytochemical screening, content of total phenolics and flavonoids were determined. The HPTLC fingerprinting of methanolic flower extract (MFE) of the *Phlogacanthus thyrsiflorus* was also carried out using CAMAG-HPTLC system.
Results: Phytochemical investigations revealed the presence of alkaloids, terpenes, tannins, saponins, glycosides and flavonoids. Total phenolic and flavonoid contents were found to be 65 µg/mg and 46 µg/mg, respectively. HPTLC analysis of MFE revealed numerous bands, indicating the presence of diverse groups of phytocompounds and also confirmed naringenin and ascorbic acid in the MFE sample.
Conclusions: It can be concluded that HPTLC fingerprint analysis of MFE of *Phlogacanthus thyrsiflorus* can be used as a diagnostic tool for the correct identification of the plant and it is also useful as a phytochemical marker.

Keywords: *Phlogacanthus thyrsiflorus*, phytochemical screening, phenolic and flavonoid contents, HPTLC fingerprinting

Introduction
India is one of the biodiversity centre over world having around 45,000 plant species. India also has equivalent to 3/4th of its land exclusive economic zone in the ocean harbouring a large variety of flora and fauna, many of them with therapeutic properties [1]. Therefore, an exponential growth in the field of herbal medicines is seen and these drugs are gaining much popularity in developing as well as in developed countries because of their natural origin and very less side effects [2]. 21,000 plants are listed by World Health Organization (WHO) used for medicinal purposes all over the world. Out of these, 2500 species are from India [3]. India is called as botanical garden of the world because of largest production of medicinal herbs [2].
Many medicinal plants, traditionally being used for many years, are present in the Indian traditional health care system, (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used, some have been thoroughly investigated and some of are still need to be explored [4]. Phytocompounds are chemicals produced by plants through primary or secondary metabolism. They generally have biological activity in the plant host. Some phytochemicals have been used as poisons and others as traditional medicine. Phytochemical screening assay is a simple, quick, and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and it is an important tool in bioactive compound analyses [5]. With an increasing demand for herbal products as medicines, there is an urgent need for standardization of plant products. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment of the traditional system of medicine throughout the world [6]. High-performance thin-layer chromatography (HPTLC) fingerprinting is proved to be a precise and accurate method for herbal identification and can be used further in authentication and characterization of the active constituents present in the medicinal plant [7]. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants. Main advantage of using this HPTLC method is its ability to examine several samples at the same time using a single small quantity of mobile phase. In addition, it also minimizes exposure risks and reduces disposal problems of toxic organic effluents [1].
Phlogacanthus thyrsiflorus Nees, known as titaaphul in Assam and dieng-soh kajut in Meghalaya, is mostly found in the sub-tropical Himalayas, upper Gangetic plain, Bihar, North Bengal, Assam and Meghalaya [8]. It is also a common medicinal herb which belongs to the

family of Acanthaceae [9]. It is famous as Vasaka in Hindi. Whole plant is used in whooping cough and menorrhagia. Fruits and leaves are burnt and it is given for fever. The leaves are informed to contain diterpene lactone, phlogantholide A. Leaves are also beneficial in liver and spleen diseases [8]. Jaintia tribe of Meghalaya uses the fruit and leaf ash of *Phlogacanthus thyrsiflorus* Nees to treat fever [10]. It was found that ethanolic extract of *Phlogacanthus thyrsiflorus* Nees have analgesic activity on experimental mice [11]. It is also reported that *Phlogacanthus thyrsiflorus* Nees has antimicrobial activity [12]. Free radicals generation has been implicated in the causation of several diseases of known and unknown etiologies such as Rheumatoid Arthritis, Cancer, Diabetes etc., and compounds that can scavenge free radicals have great potential in ameliorating these disease processes. *Phlogacanthus thyrsiflorus* Nees has prominent free radical scavenging property so it may prove as a very good medicinal herb [13]. These therapeutic uses of *Phlogacanthus thyrsiflorus* are expected to be due to some phtocomponents present in the plant.

2. Materials and methods

2.1 Chemicals

All the chemicals were used were of analytical grade and used without further purification. Naringenin, gallic acid, tannic acid, quercetin were purchased from Sigma-Aldrich Co. USA. L-ascorbic acid, methanol, sulphuric acid were from Merck, India. The other chemicals used were of analytical grade procured from Merck Co. (Mumbai, India) and Sisco Research laboratory.

2.2 Plant Material

Flowers of *Phlogacanthus thyrsiflorus* were collected during the month of January from Assam, India (Voucher No: 12055). The specimen was submitted and authenticated by Dr. P.B. Gurung Curator herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

2.3 Aqueous and methanolic extracts preparation:

The flowers were separated, weighed, washed and subjected to the shade-dried. It was then powered and homogenized. AFE (Aqueous Flower Extract) of *Phlogacanthus thyrsiflorus* was prepared by macerating with distilled water (10 x volumes) for 24 hours at room temperature with continuous stirring. It was then filtered through Whatman paper number 1. The filtrate was transferred into lyophilized tube and freeze dried (lyophilisation) for 1-2 days and stored in -20 ˚C freezer for further use [14]. Similarly, methanolic flower extract (MFE) of *Phlogacanthus thyrsiflorus* was prepared by extracting it with 10 x volume of methanol: aqueous solution (4:1). After overnight stirring (extracting), the mixture was filtered and the filtrate evaporated to dryness at 40 ˚C in a rotary evaporator [14]. The dried mass was stored and used for further investigations.

2.4 Phytochemical Screening

The phytochemical investigation of *Phlogacanthus thyrsiflorus* was carried out with standard protocol [15]. The phytochemical tests were carried out with water and methanol. The results are presented in Table 1.

2.5 Total phenolic content

The total phenolic content of aqueous and methanolic flower extract were determined by Folin-Ciocalteu method [16]. AFE and MFE solution in the concentration of 1 mg/ml were used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of extracts solution, 2.5 ml of Folin-Ciocalteu reagent dissolved in water and 2.5 ml 7.5 % sodium bicarbonate. The samples were thereafter incubated at 45 ºC for 45 minutes. The absorbance was read at λ 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the solution of gallic acid (standard) and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolic was read (µg/ml) from the calibration line; then the content of phenolic in extracts were expressed in terms of gallic acid equivalent (µg of gallic acid/mg of extract).

2.6 Total flavonoid content

Total flavonoid content of aqueous and methanolic flower extract were determined by aluminum chloride colorimetric method [16]. The reaction mixture contained 1 ml of solution of extracts in the concentration of 1 mg/ml and 1 ml of 2 % aluminum chloride solution dissolved in water. The samples were incubated for an hour at room temperature. The absorbance was read at λ 415 nm. The same procedure was repeated for the solution of quercetin (standard) and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoids was read (µg/ml) from the calibration line; then the content of flavonoids in extracts was expressed in terms of quercetin equivalent (µg of quercetin/mg of extract).

2.7 HPTLC Fingerprinting

HPTLC studies were carried out following the method of Syed *et al.* [17].

- **Sample preparation**: The standards and the MFE were dissolved in 1 ml of chromatographic grade methanol, which was used for sample application, on HPTLC plates pre-coated silica gel 60F 254 aluminium sheets.
- **Developing solvent system**: A number of solvent systems were tried, for extract, but the satisfactory resolution was obtained in the solvent ethyl acetate : ethyl methyl ketone : formic acid : water (5:3:2:1v/v/v/v)
- **Sample application**: Samples were applied (2 µl and 4 µl) on pre-coated silica gel 60F 254 aluminium sheets (100.0 X 100.0 mm) with the help of Linomat 5 applicator attached to CAMAG make HPTLC system, which was programmed through VisionCATS-Server-PH, version 2.5.18053.1.
- **Development of chromatogram**: After the application of sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with the solvent ethyl acetate: ethyl methyl ketone: formic acid: water (5:3:2:1v/v/v/v) for 20 min.
- **Detection of spots**: The air-dried plates were viewed in white light, UV λ 254 nm and UV λ 366 nm with and without staining with anisaldehyde-sulfuric acid stains. The chromatograms were scanned by Densito-metry TLC Scanner 4. The Rf values and finger print data were recorded by Vision CATS-Server-PH, version 2.5.18053.1 software.

3. Results

3.1 Phytochemical screening

All results of phytochemical analysis are showed in the table 1. In the study, tannins, glycosides, flavonoids and terpenes were determined to be present in higher amount (++) in methanolic flower extract (MFE) but alkaloids and saponins

were adequately present (+) in MFE. Similarly, aqueous flower extract (AFE) was showing maximum presence (++) of saponins but tannins, glycosides, flavonoids and terpenes were adequately present (+) and at the same time alkaloids are not found to be present in the AFE.

Table 1: Phytochemical screening results of *Phlogacanthus thyrsiflorus* flower extract fractions.

Metabolites	Aqueous flower extract (AFE)	Methanolic flower extract (MFE)
Alkaloids	-	+
Tannins	+	++
Glycosides	+	++
Flavonoids	+	++
Terpenes	+	++
Saponins	++	+

++: highly present, +: low, -: absent

3.2 Phenolic and flavonoid contents

Quantitative evaluation for presence of total phenolic and flavonoids are illustrated in table 2. Total phenolics were found to be present in more amounts in MFE than AFE. Similar result was obtained for flavonoids wherein higher amount were obtained in MFE than AFE.

Table 2: Total phenolic and flavonoids content of AFE and MFE of *Phlogacanthus thyrsiflorus* Nees.

Extract	Total phenolic content (µg Gallic acid/mg extract)	Total flavonoid content (µg Quercetin/mg extract)
AFE	29	13
MFE	65	46

3.3 HPTLC fingerprinting

HPTLC analysis of MFE revealed numerous bands, indicating the presence of diverse groups of phytocompounds. The study revealed that MFE showed best results in ethyl acetate: ethyl methyl ketone: formic acid: water (5:3:2:1v/v/v/v) solvent system. The plates were visualized at λ 254 nm, λ 366 nm and visible light range (λ 400-λ 600 nm after spraying with anisaldehyde sulphuric acid reagent). HPTLC images shown in figure 1 and 2 indicate that the MFE sample constituents were clearly separated without any tailing and diffuseness.

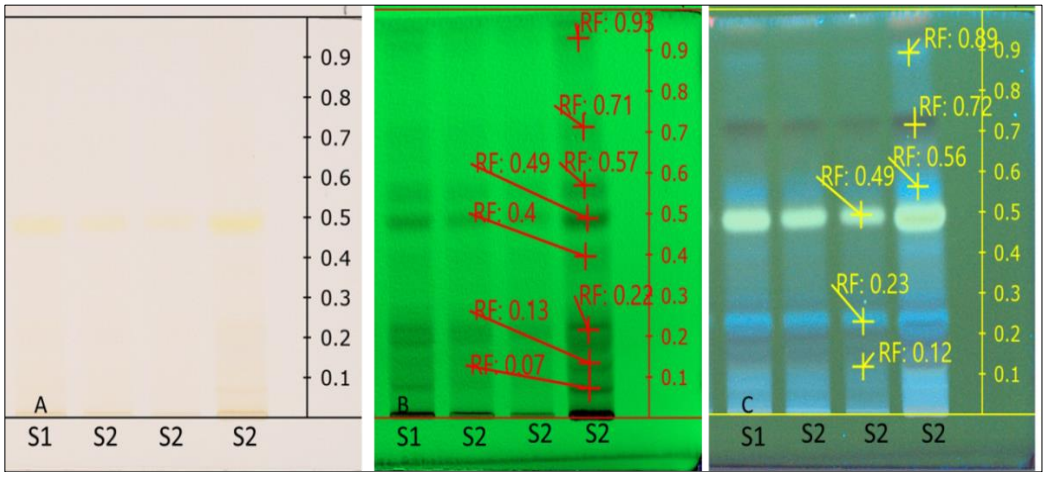

Fig 1: HPTLC fingerprinting images of MFE of *Phlogacanthus thyrsiflorus* observed at white light (A), UV λ 254 nm (B) and UV λ 366 nm (C), S1: 2 µl of MFE sample, S2: 4 µl of MFE sample.

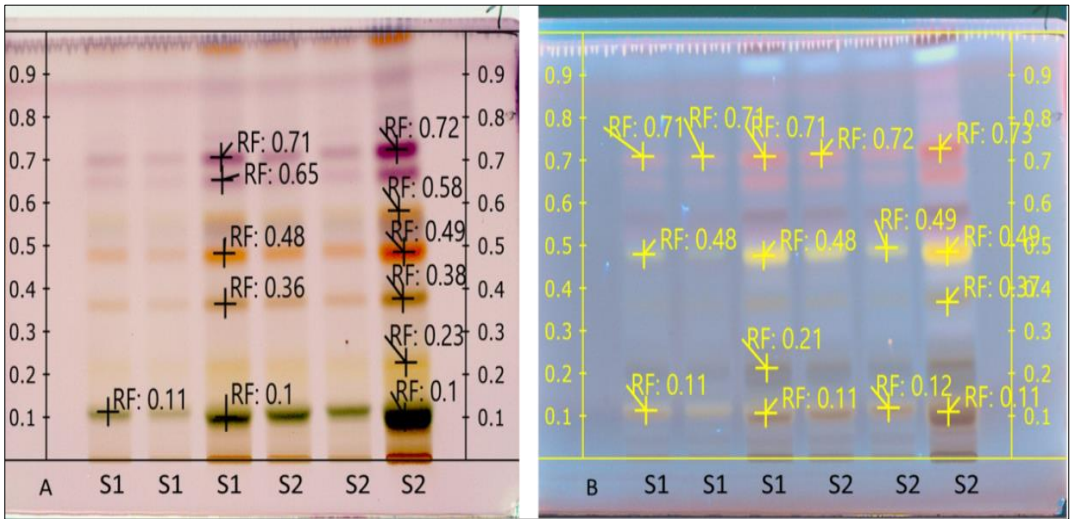

Fig 2: HPTLC fingerprinting images of MFE of *Phlogacanthus thyrsiflorus* observed at white light (A) and UV λ 366 nm (B) after derivatization, S1: 2 µl of MFE sample, S2: 4 µl of MFE sample.

HPTLC chromatogram was run for MFE along with some reference compounds (Gallic acid, Tannic acid, Quercetin, Ascorbic acid and Naringenin) to confirm their presence in the MFE sample. All the standards and the MFE sample were spotted on the TLC plate and different individual solvents as well as a combination of solvents were tired to get a good

separation. The mobile phase n-butanol: glacial acetic acid: water (4:1:1 v/v/v) gave a good resolution with Rf 0.73, Rf 0.71, Rf 0.79, Rf 0.42, Rf 0.81 for gallic acid, tannic acid, quercetin, ascorbic acid and naringenin. Well defined spots were obtained when the chamber was saturated with the mobile phase for 30 minute, at room temperature. The Rf values of the MFE sample were compared with the standards shown in figure 3.

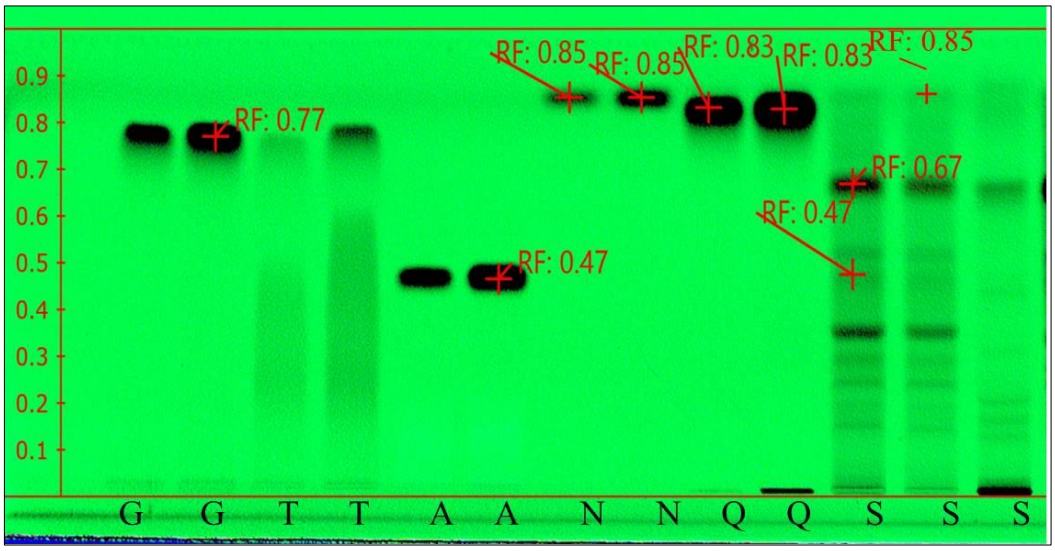

Fig 3: HPTLC fingerprinting images of Gallic acid (G), Tannic acid (T), Ascorbic acid (A), Naringenin (N), Quercetin (Q), and MFE Sample (S) observed at UV λ 254 nm.

As shown in figure 4, the MFE sample revealed 9 peaks. The Rf value of standard ascorbic acid was found to be 0.42 and peak area 2575. The fifth peak Rf value of MFE (0.42) was coinciding with standard Rf value and its peak area was 1538. The Rf value of standard naringenin was found to be 0.81 and peak area 1906. The eighth peak Rf value (0.82) of MFE was coinciding with standard Rf value and its peak area was 1455. Using this analytical method, ascorbic acid and naringenin found to be present in MFE sample. There was no detectable gallic acid, tannic acid and quercetin compounds in MFE of *Phlogacanthus thyrsiflorus* from the study.

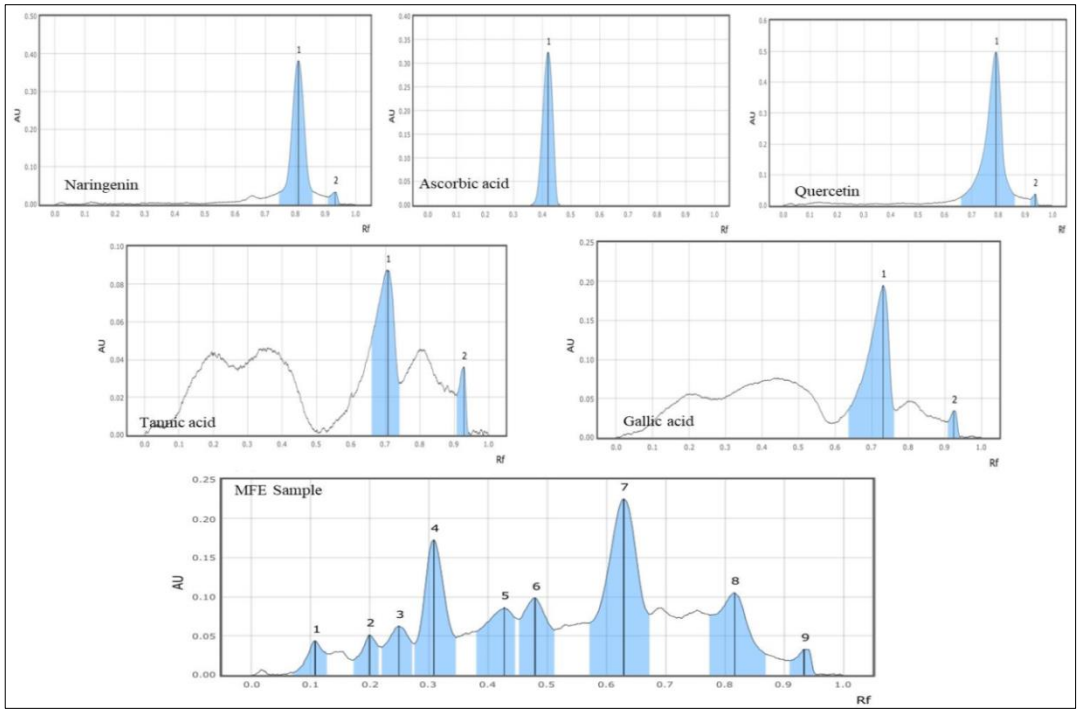

Fig 4: HPTLC scanning chromatograms of Naringenin, Ascorbic acid, Quercetin, Tannic acid, Gallic acid and MFE sample of *Phlogacanthus thyrsiflorus* Nees.

4. Discussions

The phytochemical screening revealed that the methanolic flower extract (MFE) of *Phlogacanthus thyrsiflorus* showed presence of tannins, glycosides, flavonoids and terpenes but present in lower amount in the aqueous flower extract (AFE) of *Phlogacanthus thyrsiflorus*. Alkaloids were completely absent in AFE and moderately present in MFE. Saponins on the other hand were strongly present in AFE but in lesser amount in MFE.

Phenolics rich plant materials are progressively being used in the food industry because they hinder oxidative degradation of lipids and improve the quality and nutritional value [18].

Phenolic compounds are considered as secondary metabolites and these are derived from phenylalanine and tyrosine which occurs universally in plants [19]. The MFE exhibited higher total phenolics content compared to the AFE fraction. Phenolic compounds of plants are also important because their hydroxyl groups confer scavenging ability. Phenolic compounds of plants fall under several categories; major class among these are the flavonoids which have potent antioxidant activities [20]. Flavonoids occur naturally in plants and are believed to have positive effects on human health. Studies on flavonoid derivatives have showed that they have a wide range of antibacterial, antiviral, antiinflammatory, anticancer, and antiallergic activities [21, 22]. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [20] implicated in several diseases. Our findings shows the presence of higher total phenolic and flavonoid contents in MFE compared to AFE, suggesting that phenolic acids and flavonoids of MFE may be the major contributors for the antioxidant activity and thus, MFE was taken up for further *in vivo* studies.

Herbal medicines are composed of many constituents. Hence it is a much need to obtain reliable chromatographic fingerprints that signify pharmacologically active and chemically characteristic components of the herbal medicine. High-performance thin-layer chromatography (HPTLC) fingerprinting is proved to be a precise and accurate method for herbal identification and can be used further in authentication and characterization of the important medicinal plant [6]. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants. HPTLC of MFE revealed numerous bands, indicating the presence of diverse groups of phytocompounds. This preliminary study may help to detect many unidentified phytocompounds, which can be used to characterize MFE. The identification and characterization of the phytocompounds can further help in finding out molecular targets/mechanism of action of the constituents of this shrub that are responsible for its biological activities. In the study, the presence of ascorbic acid and naringenin in MFE was observed.

5. Conclusion

Evaluation of all physical constants established shown satisfactory results. HPTLC chromatogram of methanolic and aqueous extract results showed that there are many compounds in *Phlogacanthus thyrsiflorus*. From the HPTLC studies, it has been found that methanol extract contain not a single compound but a mixture of compounds and so it is established that the pharmacological activity shown by them are due to the cumulative effect of all the compounds present in the extract.

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