Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(6): 274-278

Received: 07-09-2016
Accepted: 08-10-2016

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HPTLC fingerprint profile and FTIR analysis of hypericin from *Hypericum gaitii* Haines

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Abstract
Aim: To identify the hypericin content in *Hypericum gaitii* Haines through HPTLC & FTIR, an endangered medicinal plant and potential for curing many diseases like cancer, AIDS, tumour etc.
Methods: Methanolic extract of leaves derived from *in vitro* raised plants were used in the mobile phase of Toluene: Ethyl acetate: Formic acid (10:8:3) and scanned under UV at 254nm and 366 nm and under visible light. Dried extract powered from leaves were encapsulated in 100 mg of KBr pellet in order to prepare translucent sample discs prepared through applying pressure for FTIR analysis. The data of infrared transmittance was collected over a wave number ranged from 500 to 4000 cm^{-1}.
Results: The HPTLC analysis of the methanolic extract has shown several peaks with different Rf values. Five microliter concentrations leaf extract provide 12 spots with hypericin content having Rf value range from 0.65 to 0.68.
Conclusion: Both HPTLC and FTIR methods could be used for qualitative and quantitative determination, identification and authentication purposes in order to prevent adulteration in herbal medicines.

Keywords: HPTLC fingerprints, hypericin content, FTIR analysis, *Hypericum gaitii*

1. Introduction
In spite of great advances of modern scientific medicine, traditional medicine is still the primary source of treating various diseases of majority of people in developing countries. According to the report of World Bank in 1997, it is apparent that the significance of plant based medicines has been increasing all over the world. Nearly, 50% of medicines in the market are made of natural basic materials. Interestingly, the market demands for medicinal herbs are likely to remain high because of the many active ingredients in medicinal plants cannot yet be prepared synthetically. The universal role of plants in the treatment of disease is exemplified by their employment in all major systems of medicine irrespective of the underlying philosophical premise. The World Health Organisation estimates that about 80% of the population living in the developing countries rely almost exclusively on traditional medicine for their primary healthcare needs. In almost all the traditional medical systems, the medicinal plants play a major role and constitute their backbone. Out of these drugs derived from traditional system, 400 are of mineral and animal origin while the rest are of the vegetable origin. India has a rich heritage of traditional medicine and the traditional health care system has been flourishing in many countries. Population in developing countries mainly depends on the indigenous traditional medicine for their primary healthcare needs. Traditional medicines have not however been incorporated in most national health systems and the potential of services provided by the traditional practitioners is far from being fully utilized. Herbal medicines are of great importance to the health of individuals and communities, but their quality assurance need to be developed.
During the last two decades, the use of herbal medicine has been increased. In recent years, the use of herbal medicines worldwide has provided an excellent opportunity to India to look for therapeutic lead compounds from an ancient system of therapy, which can be utilized for development of new drug. Over 50% of all modern drugs are of natural product origin and they play an important role in drug development programs of the pharmaceutical industries [1]. *Hypericum* species is a large genus of herbs and shrubs that belongs to family Hypericaceae, widely grown in Europe, Asia, Northern Africa and Australia more than 484 species in the world. On the basis of morphology and biogeography, the genus *Hypericum* is represented by 484 species with 36 taxonomic sections. *Hypericum gaitti* is widely known because of its

remarkable pharmaceutical properties [2]. Out of 484 species, about 29 species evolved in India [3]. Among these 29 species, 13 were native to the high Himalayan region and remaining 16 species were declared as non-natives. Three species namely *Hypericum assamicum*, *H. gaitti* and *H. gracilipes* were endemic. Another six species, viz., *H. cordifolium*, *H. dyeri*, *H. oblongifolium*, *H. podocarpoides*, *H. tenuicaule* and *H. williamsii* were distributed in the Himalayan region of Pakistan, Afghanistan, Bhutan and Nepal, hence have been identified as near endemic. These species have been used as traditional medicine for various purposes including wound healing, bactericidal and anti-inflammatory properties [4]. The secondary metabolite i.e hypericins primarily accumulate in the dark glands (black spheroidal formations) of both leaves and flowers of *Hypericum* species. Extracts of the crude drug are widely used in the treatment of mild and moderate depression. *Hypericum gittai* has potential for curing many diseases like cancer, AIDS, tumour etc. and also act as an antidepressant [5]. Due to commercial importance, there is great interest to identify the hypericin content associated with *Hypericum gaitti* through HPTLC and FTIR analysis.

2. Materials and methods

2.1 Plant materials

Five gm of young leaves of *Hypericum gaitii* were collected from *in vitro* grown plants and powdered very well. The dry powder leaf samples were taken into a thimble and placed in a Soxhlet apparatus. The extraction was made by using different type of solvents (i.e. methanol, hexane, acetone) (250 ml) was added and extracted according to the boiling point for 6 h and the complete extraction was effected within 24 h. The solvent was removed from crude extract at a reduced pressure with the help of rotary vacuum evaporator to yield a yellowish or brown residue. The definite quantity of residue was dissolved in the solvent and was further used for HPTLC analysis. After completion of extraction the yellowish brown extract was stored in refrigerated condition until further use.

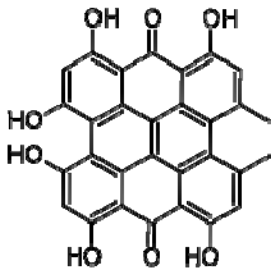

Fig 1: Structure of Hypericin: Mol. Formula: $C_{30}H_{16}O_8$ (4,5,7,4',5',7'-Hexahydroxy-2,2'-dimethylnaphthodianthrone), Molar Mass: 504.45 g·mol^{-1})

2.2 Chromatography condition

Reference standards of Hypericin (Fig.1) was procured from Sigma-Aldrich, Switzerland. Silica Gel 60 F254 aluminium plates (EMerck) were used as stationary phase. Different solvents in various compositions were prepared and used as mobile phase. Methanol and water were used as solvent. The extracts were dissolved in methanol to have final concentration 100 mg/ml each. The reference standard solution of hypercine (1.0 mg/ml) was prepared in methanol. Chromatography was performed on a pre-coated silica gel HPTLC aluminium plate pre-coated with silica gel (60 F254, 20 x 9 x 10 cm) (EMerck Limited, India). Samples (5 and 10 µl) and standards were applied on the plate as 6 mm wide bands with a Camag Linomat-V automatic TLC applicator positioned 15 mm from the lower edge of the plate and 20 mm from side of the plate. The application parameters were identical for all the analysis performed. The plates were developed with the help of mobile phase containing (toluene: ethyl acetate: formic acid at the ratio of 10:8:3) under laboratory conditions (25–30 ^{0}C and 40–50 % relative humidity). Methanol and concentrated sulphuric acid (95:1 v/v) reagent along with valilin (0.1%) is used as derivatizing agent for visualization. After development, the plate was dried at 60 ^{0}C in an oven for 5 minutes. For quantitative determination, spots corresponding to standards were scanned at 366 nm and Rf values were recorded with Camag TLC Scanner–III (Switzerland) in conjunction with win WINCATS softwares version 1.2.3. The amount of hypercine present in each extract was calculated by comparing the peak area of reference standard and respective samples. The following formula was used to quantify the active constituent

$$\% \text{ Hypericine} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{Conc. of standard}}{\text{Conc. of sample}} \times 100$$

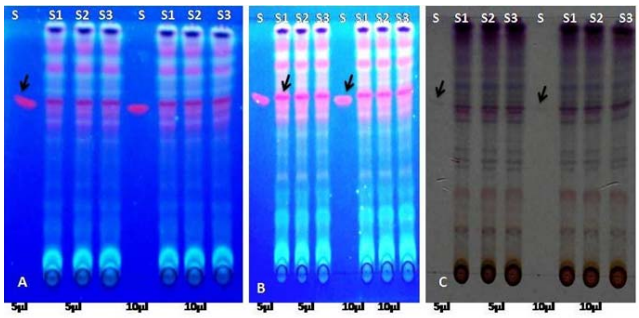

Fig 1: HPTLC profile of methanolic leaf extract of *Hypericum gaitii* on two UV wavelength (A: 366 nm; B: 254 nm) and Visible light (C), S- Standard Hypericin (Arrows), S1-S3: Methanolic leaf extract samples,

2.3 Fourier transform infrared spectroscopy (FTIR) analysis

Fourier Transform Infrared Spectrophotometer (FTIR) is the most powerful tool for identifying the types of chemical bonds/functional groups present in the phytochemicals. The spectra was recorded in FTIR instrument (Perkin Elmer Spectrum Two, USA), with PC based software controlled instrument operation and data processing. The wavelength of light absorbed is salient feature of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a compound can be determined. A small amount of dried powdered leaf and callus samples were used for FTIR analysis. Ten mg of the dried extract powered from shoots and calluses were encapsulated in 100 mg of KBr pellet in order to prepare translucent sample discs prepared through applying pressure for FTIR analysis. The data of infrared transmittance was collected over a wave number ranged from 500 to 4000 cm^{-1}. All the samples were analyzed in triplicates with plain KBr pellets as blank. The spectral data were compared with a reference to identify the functional groups existing in the sample.

3. Results and Discussion

The HPTLC profile was developed by using solvent system and individual peak scanned at a wavelength of 366 nm showed significant data to measure the retention value (Rf) and area unit (AU). The HPTLC images shown in Fig.1

indicate that all the reference substances and sample constituents were clearly separated on silica gel 60 F254 TLC plates. Constituent of the sample extracts were identified by comparison of bands in sample with reference substances on the same plate. The identification of hypericin in the leaf extract was confirmed by superimposability of UV–visible spectra of the of the standard within the same Rf value (Fig.2) The Rf values of the bands for reference standards was 0.63 to 0.68 for hypericin. Constituents of the three sample extracts (Track-1,2 &3) were recognized by comparison of bands in sample with reference to standard on the same TLC plate (Fig.2). Hypericin was found to be the major compound in all of the tested samples. It was observed that hypericin was present in all the samples. It is also evident from Table 1 (track-1) that the 5µl metabolic leaf extract of *H. gaitaii* having 12 peaks with Rf values (0.01,0.05, 0.10, 0.13, 0.25, 0.38, 0.61,0.66, 0.73, 0.83, 0.89 and 0.93 spectively) and shown in Fig.3A. Out of the 12 peaks occurrence, 6 peaks such as 0.01, 0.05, 0.13, 0.15, 0.73 and 0.93 were found to be more predominant as the percentage of area were 16.94%, 30.29%, 10.68%, 13.68%, 6.11% and 0.93% respectively. The data present in the Table-2 (track-2) reveled that the methanolic leaf extract of *Hypericum gaitii* produced 13 peaks. All the 13 peaks are shown in Fig.3B with Rf values i.e. 0.01, 0.04, 0.9, 0.12, 0.23, 0.28, 0.34, 0.59, 0.70, 0.74, 0.80, 0.87 and 0.92 respectively. Among the 13 peaks, five peaks were more predominant and percentage of aera were 18.43%, 31.18 %, 11.88 %, 12.69 % and 6.29% respectively. Table 3 (track-3) and Fig. 3C revealed that the 5 µl leaf methanolic extract produced 13 peaks having Rf values ranged from 0.01 to 0.90. Among the 13 peaks, four peaks were more predominant having the Rf values i.e. 0.01 (15.24% of area), 0.04 (26.60% area), 0.22 (14.44% area) and 0.68 (9.68% area). As per the date presented in Figure.2, the major active constituent (hypericin) occur in Rf values ranged from 0.63 to 0.68. The HPTLC technique reported here is suitable for the rapid screening of germplasm of *Hypericum gaitti* for the determination of chemical profiles and quantification of the major constituents as they have higher commercial importance. Similarly, Verma *et al* [6] used high-performance thin layer chromatography method for separation and quantification of ferulic acid in *Hemidesmus indicus* root extracts.

The FTIR spectroscopic investigation revealed different characteristic peak values with various functional compounds in the extracts. The methanol leaf extracts of *Hypericum gaitii* confirmed the presence of alcohols, phenols, alkanes, carboxylic acids, ketones, primary amines and aliphatic amines compounds, which showed major peaks. The FTIR method was performed on a spectrophotometer system, which was used to identify the characteristic peak values and their functional groups. The absorption bands, the wave number (cm^{-1}) of prominent peaks obtained from absorption spectra in Figure 4. The IR spectrum of leaf callus extracts reveals structural information about major and minor constituents. The peak at 2168.67 cm^{-1} assigned to the C = C stretching vibration, the peak appear in the range of 3304.65 - 1660.91 cm-1 mainly attributed to the stretching vibration of C=C and hydrogen bond. Those characters of peaks intensities and position at 3149.45 cm-1 in the IR spectrum display the characteristic of primary and secondary amines and amides group vibration. In addition, the peak at 3304.65 cm-1 assigned to the C-O stretching vibration means that some carbonyl compounds existed in the leaves of *H. gaitti*. So, depending on the fingerprint characters of the peaks positions, shapes and intensities, the fundamental components may be identified [7]. The peak at 3619.74 cm-1 and the peak situated at 3304.65 and 2168 cm-1 assigned to the absorption of O-H and C-O. Presence of C=O, C-H, C=C and C-O, C-C and O-H bonding structures are responsible for the formation of alkyl groups, alkyne groups, alcohols, carboxylic acid, anhydrides and deoxyribose as reported by Sohrabi *et al*. [8]. The methanol extract suggests the presence of aromatic benzene, aliphatic amine, carboxylic acid, alkanes and phenol. Peaks in the range between 3304-3619 cm-1 corresponds to stretching vibrations of OH groups as well as from amides. Many workers revealed the FTIR spectrum as an effective tool for differentiating, classifying and discriminating closely related plants and other organisms. Zavoi *et al* [9] reported hepatoprotective action using FTIR analysis of polyphenolic composition of number of medicinal herbs i.e. *Cynara scolymus*, *Taraxacum officinalis*, *Chelidonium majus*, *Hypericum perforatum*, *Silybum marianum* and *Lycopodium clavatum* from wild flora. Csernatoni *et al* [10] used FTIR screening to characterize and identify the main biomarkers of food supplement PROMEN by analysis of plant ingredients comparatively with the final product. Similarly, Ramamurthy and Kannan [11] used FTIR spectroscopy to reveal qualitative characters regarding the organic molecules in *Calotropis gigantea* collected from industrial area. Several unique bands that are pertained to functional groups represent chemical components or metabolic products in the plant.

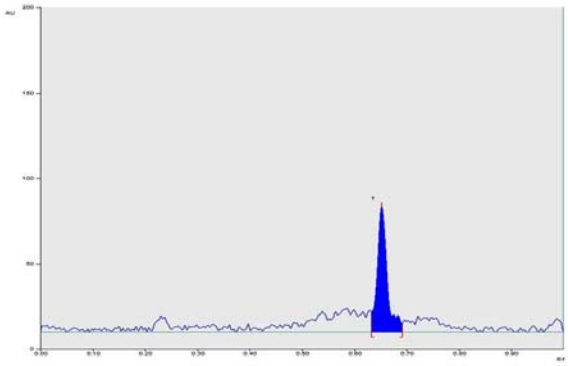

Fig 2: HPTLC chromatogram of standard Hypericin marker compound (5 µl) at 366 nm

Track	Peak	Maximum Rf	Max Height	Area %
4, Figure1	1	0.65 Rf	73.5 AU	100.00%

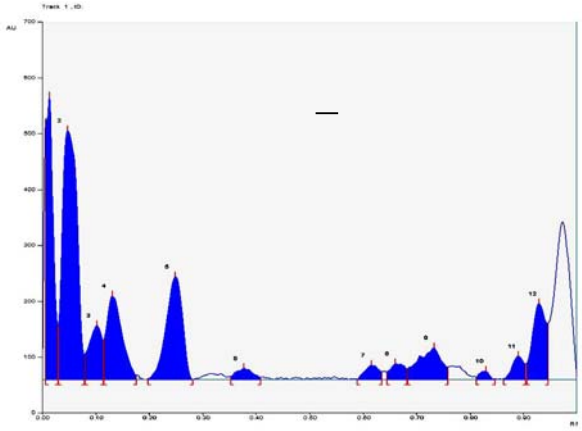

Fig 3A: HPTLC chromatogram of Leaf extract of *Hypericum gaitti* (5 µl) (Track-1) at 366 nm.

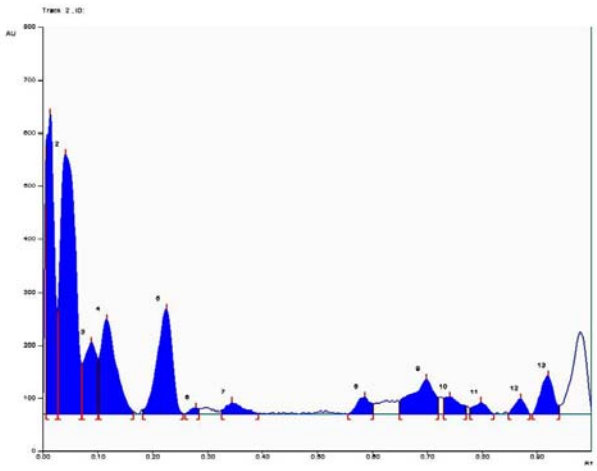

Fig 3B: HPTLC chromatogram of Leaf extract of *Hypericum gaitti* (5 µl) (Track-2) at 366 nm

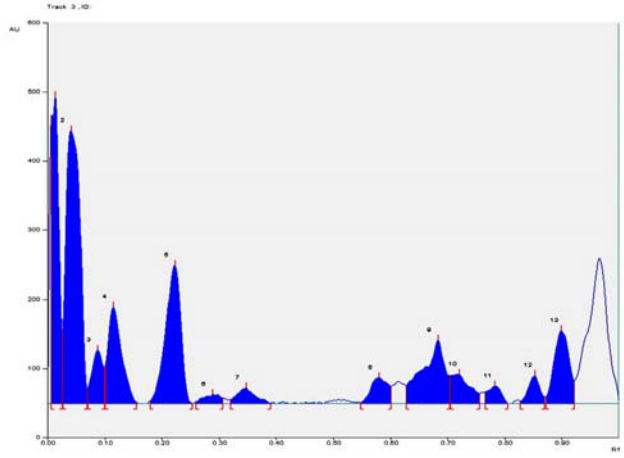

Fig 3C: HPTLC chromatogram of leaf extract of *Hypericum gaitti* (5 µl) (Track-3) at 366 nm

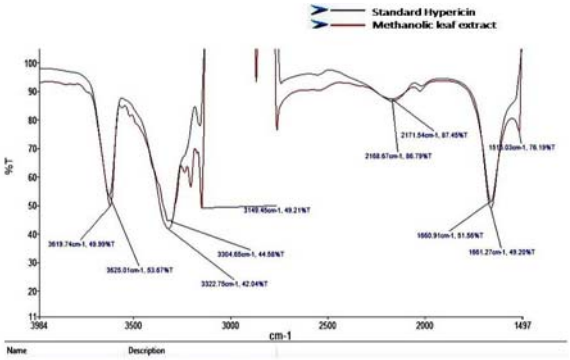

Fig 4: FTIR spectroscopic analysis of methanolic leaf extract of *Hypericum gaitii.*

Table 1: List of peak and Rf value of the HPTLC chromatogram of leaf extract of *Hypericum gaitti* (5 µl) (Track-1, Figure 3A).

Track	Peak	Maximum Rf value	Maximum height	Area %
1	1	0.01 Rf	506.6 AU	16.94%
1	2	0.05 Rf	445.6 AU	30.29%
1	3	0.10 Rf	96.5 AU	5.64%
1	4	0.13 Rf	149.8 AU	10.68%
1	5	0.25 Rf	184.0 AU	13.68%
1	6	0.38 Rf	20.2 AU	1.56%
1	7	0.61 Rf	25.7 AU	1.70%
1	8	0.66 Rf	28.2 AU	1.92%
1	9	0.73 Rf	57.0 AU	6.11%
1	10	0.83 Rf	14.8 AU	0.67%
1	11	0.89 Rf	41.8 AU	2.28%
1	12	0.93 Rf	135.7 AU	8.53%

Table 2: List of peak and Rf value of the HPTLC chromatogram of leaf extract of *Hypericum gaitti* (5 µl) (Track-2, Figure 3B).

Track	Peak	Maximum Rf value	Maximum height	Area %
2	1	0.01 Rf	564.6 AU	18.43%
2	2	0.04 Rf	488.5 AU	31.18%
2	3	0.09 Rf	134.0 AU	7.14%
2	4	0.12 Rf	177.7 AU	11.88%
2	5	0.23 Rf	197.7 AU	12.69%
2	6	0.28 Rf	11.0 AU	0.43%
2	7	0.34 Rf	21.2 AU	1.55%
2	8	0.59 Rf	31.4 AU	1.83%
2	9	0.70 Rf	65.5 AU	6.29%
2	10	0.74 Rf	31.8 AU	2.14%
2	11	0.80 Rf	21.7 AU	1.21%
2	12	0.87 Rf	28.6 AU	1.24%
2	13	0.92 Rf	71.3 AU	3.98%

Table. 3. List of peak and Rf value of the HPTLC chromatogram of leaf extract of *Hypericum gaitti* (5 µl) (Track-3, Figure 3C).

Track	Peak	Maximum Rf value	Max height	Area %
3	1	0.01 Rf	444.3 AU	15.24%
3	2	0.04 Rf	394.1 AU	26.60%
3	3	0.09 Rf	77.6 AU	3.95%
3	4	0.12 Rf	140.0 AU	9.11%
3	5	0.22 Rf	200.1 AU	14.44%
3	6	0.29 Rf	12.8 AU	1.06%
3	7	0.35 Rf	22.4 AU	1.92%
3	8	0.58 Rf	37.4 AU	3.02%
3	9	0.68 Rf	91.7 AU	9.63%
3	10	0.72 Rf	42.3 AU	3.69%
3	11	0.78 Rf	25.4 AU	1.61%
3	12	0.85 Rf	40.3 AU	2.22%
3	13	0.90 Rf	105.7 AU	7.53%

4. Conclusion

In conclusion, the present results obtained from qualitative and quantitative evaluation of hypericin content in leaf through HPTLC fingerprints was adequate for identification. On the basis of FTIR spectroscopy is valuable techniques to analyze the different biomolecules extracted with methanolic extracts of *Hypericum gattai*, rare endangered medicinal herbs with commercial application. FTIR data will be correlated in future with the detailed HPTLC analysis of the same extracts, in order to validate the FTIR method as a good tool to investigate the fingerprint and to predict the composition and evaluation of the quality and authenticity of formulating commercial potential. Both HPTLC and FTIR methods could be used for qualitative and quantitative determination, identification and

authentication purposes in order to prevent adulteration in herbal medicines.

5. Acknowledgement
The authors wish to acknowledge to Department of Biotechnology, Govt. of India for providing the financial assistance under R & D project No. BT/Env/BC/01/2010. The authors also thankful to Officer-in-Charge, Central Instrumentation facility, OUAT, Bhubaneswar for providing the instrumental facility to carry out the experiment.

6. Conflict of interest Statement: We declare that we have no conflict of interest.

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