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PHYTOCHEMICAL ANALYSIS IN METHANOLIC EXTRACT OF *INDIGOFERA LONGERACEMOSA* BAILL.

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ABSTRACT

High Performance Thin Liquid Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC) and Fourier Transform Infra Red (FT-IR) spectroscopic techniques were done for quantification of methanolic extract of leaf and stem of *Indigofera longeracemosa*. Chromatographic technique was used for separation of components from methanolic extract. This study was planned to develop a HPLC, HPTLC fingerprint profile of methanol extract. A HPTLC method for the separation of the active constituents has been developed. HPLC and HPTLC profiling of the methanol extract confirmed the presence of various phytochemicals. HPLC and HPTLC fingerprint scanned at 400nm revealed 12 peaks with Rf values in the range of 0.23 to 0.88 respectively. The functional groups were identified by FT-IR spectrum. Overall, FTIR fingerprinting has the potential to be a fast and reliable analytical methodology for the discrimination of the plant and hence chemically similar samples.

Keywords: Phytochemical, HPLC, HPTLC, FT-IR, *Indigofera longeracemosa*

INTRODUCTION

Plants have limitless ability to synthesize aromatic substances mainly secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total and their contents vary depending on climate, regions of cultivation and seasons of harvesting which make it difficult to ensure batch to batch uniformity. The quality control of standardized herb extracts is essential for the therapeutic reproducibility, efficacy and safe application of extract [1]. Conventional quality control mainly focuses on the analysis of the active constituents of herbal medicines [2].

Recently the chromatographic fingerprint technique was introduced as a tool to evaluate the quality of herbal samples or their derived products [3-5]. A quick, sensitive and accurate analytical method is required for the analysis of a large number of plants samples. In the past decade, the chromatographic fingerprints established by TLC, HPLC, HPTLC and FTIR have been recognized as rapid and reliable means for the identification and qualification of herbal medicines [6-7]. HPLC method is gaining importance for qualitative and quantitative analysis of plant extracts, being useful for quality control of phytochemical compounds [8]. This method is able to quantify the marker compounds in plants. The identity, consistency and authenticity of samples can be determined by comparison of their chromatographic fingerprints (Chromatogram) using similarity analysis and chemometric methods [9]. This method enables the simultaneous identification of the major bioactive constituents present in medicinal herb [10].

Indigo is an important blue dyestuff, extracted from *Indigofera* species and used in the treatment of epilepsy, bronchitis, liver disease and psychiatric illness [11-14]. The aim of this study was to investigate the phytochemical analysis of methanol extract of stem and leaf of *Indigofera longeracemosa.*

MATERIALS AND METHODS

Plant Material

The wild, fully grown *Indigofera longeracemosa* was collected from Thlaianai Puliyenkudi, Western Ghats of India.

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Preparation of Plant Material

In the present study the plant powder of *Indigofera longeracemosa* defatted with methanol. 100g was packed in a Soxhlet apparatus and extracted successively. The extraction was carried out until the extractive becomes colorless. The extract was filtered through a cotton plug, followed by Whatman filter paper. The extract was evaporated under reduced pressure using evaporator.

HPTLC

Sample preparation

The methanol extract of each sample was used for HPTLC analysis. The extracts were filtered through a Whatman No.1 filter paper. 25 ml filtered extract was evaporated by a rotatory vaccum evaporator. 10 ml evaporated extracts were used for analysis.

Procedure

HPTLC analysis was performed on a CAMAG semi automated HPTLC system equipped with an automatic TLC sampler (ATS4); TLC scanner 3 integrated with documentation device Reprostar 3 with win CATS version 1.4.4 planer chromatography manager software. UV cabinet and automatic developing chamber ADC2 with humidity control facility was used for the analysis. 2 ml of substance of each sample added with 2 ml of ethanol. The solution was filtered and 10 µl of filtrate was applied on merck aluminum plate60 F254 Precoated with silica gel of 0.2mm thickness. The samples were loaded in 10 mm bands at 10 mm from the bottom, 15 mm from the sides and with 8 mm space between the two bands. The plates were developed to a a height of about 8 cm from the base in toluene: ethylacetate (5:2). Plates were developed in software controlled CAMAG automatic developing solvent phase for 30 min at room temperature (25 ± 2^0C) and relative humidity was maintained at 45 ± 1 % with the mobile phase (25 ml) consisting of Toluene : Ethylacetate (5:2) after development , the plates were removed, dried and the spots were visualized under UV light. The mobile phase and chamber conditions resulted in plate was performed in the reflectance /absorbance mode at 254 nm using deuterium lamp in CAMAG HPTLC instrument with following conditions: slit width 6mm × 0.3 mm, scanning speed 20 mm/s and resolution 100 µ/step. To check the identity of the bands, UV absorption spectrum of each standard was overlaid with the corresponding band in the track (Sethi, 1996; WHO, 1998). The Rf value, area of peak percentage of area were calculated from the calibration graph.

HPLC

Preparation of sample solutions

The methanol extract of each sample was used for HPLC analysis. All extracts were filtered through a Whatman No.1 filter paper. 30ml filtered extract was evaporated by a rotatory vaccum evaporated residues with constant weight were stored prior to analysis in dark at 4^0C. 200mg of extract was dissolved with 4ml methanol, sonicated at 35^0C for 15 minutes and filtered through 0.45 µm filter and applied (50µl) on to HPLC column.

Apparatus and chromatographic conditions

The HPLC analysis was carried out using Shimadzu, LC-10 AT VP, Consisting of SCL -10 Avp system controller, degassing unit DGU-14 A, low-pressure gradient flow control value FCV-10 ADvp, auto injector SIL-10 ADvp with 500 µl loop, column oven CTO-10 AC, a UV detector SPD-10 Avp with 500µl loop, column oven CTO-10AC, a UV detector SPD-10Avp using a 254 (5µm). The temperature was maintained at 25°C with injection volume of 200µl and flow rate of 1ml/min. Active compounds were separated using reverse-phase LiChrosorb C-18 column with the methanol: water mobile phase and detected at 210nm. HPLC conditions for analysis of samples were achieved by varying mobile phase composition 80% methanol in a gradient with 100% methanol. The chromatography system was equilibrated by the mobile phase. When same retention times and peak areas for repetitive injections of standard were observed, separation of sample could then be carried out. Calibration data calculated from peak area and height at different retention time were compared with standard. The peak area of each sample was plotted against the concentration to obtain the calibration graph.

FTIR analysis

FTIR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks and their functional groups. The peak values of the FTIR were recorded. Each and every analysis was repeated twice and confirmed the spectrum.

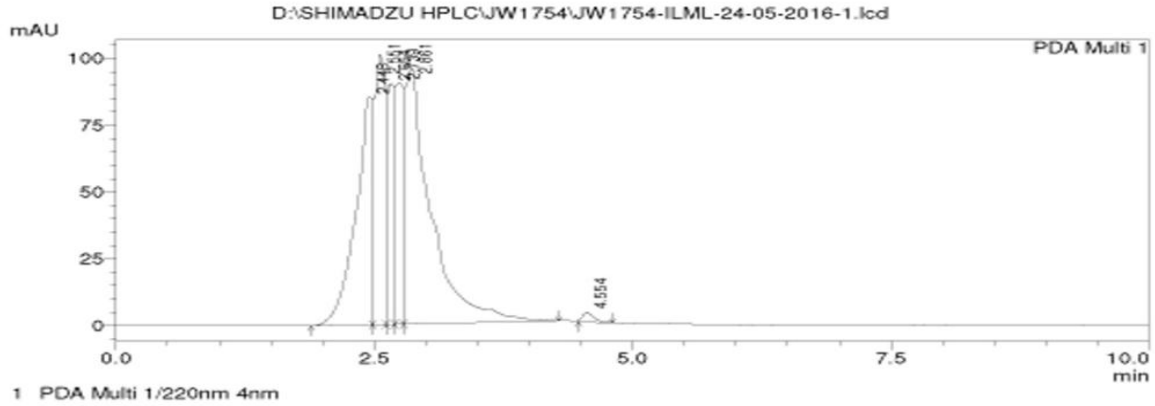
RESULTS AND DISCUSSION

HPLC Analysis

The qualitative HPLC fingerprint profile of methanol extract of different plant parts (leaf and stem) of *Indigofera longeracemosa*. was selected at a wavelength of 210nm due to the sharpness of the peaks and proper baseline.

Figure 1. HPLC chromatogram methanol leaf extract of *Indigofera longeracemosa*

<Chromatogram>



PeakTable

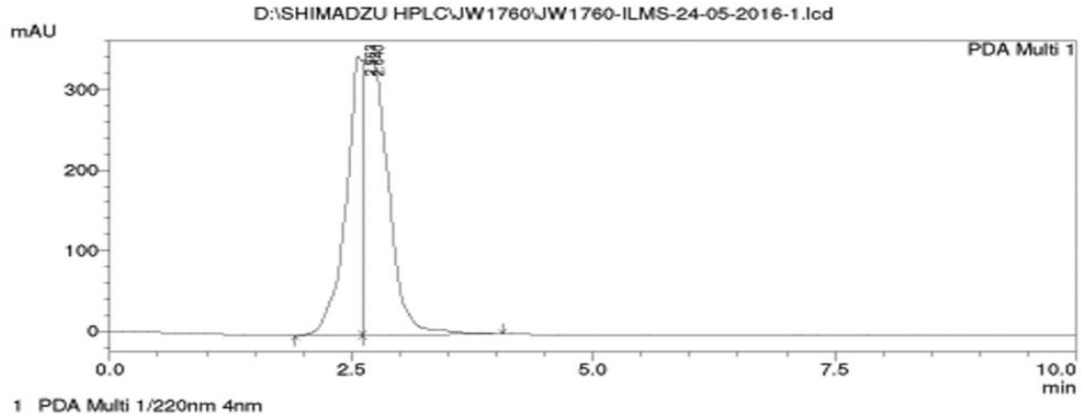
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.448	967685	85266	22.649	18.285
2	2.551	702339	101039	16.439	21.668
3	2.654	455884	90032	10.670	19.307
4	2.739	458100	90273	10.722	19.359
5	2.861	1659806	95936	38.849	20.573
6	4.554	28652	3766	0.671	0.808
Total		4272467	466313	100.000	100.000

The methanol leaf extract prepared by hot extraction was subjected to HPLC for the separation and identification of constituents present in the *Indigofera longeracemosa.* Six compounds were separated at different retention times of 2.448min, 2.551min, 2.654min, 2.739min, 2.861min and 4.554min respectively. The profile displayed five prominent peaks at the retention times of 2.448min, 2.551min, 2.654min, 2.739min, 2.861min followed by one moderate peak was also observed at the retention times of 4.554min as shown in figure 1.

Figure 2. HPLC chromatogram methanol stem extract of *Indigofera longeracemosa*

<Chromatogram>



PeakTable

PDA Ch1 220nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.563	4025247	346058	39.735	50.263
2	2.640	6104982	342435	60.265	49.737
Total		10130229	688494	100.000	100.000

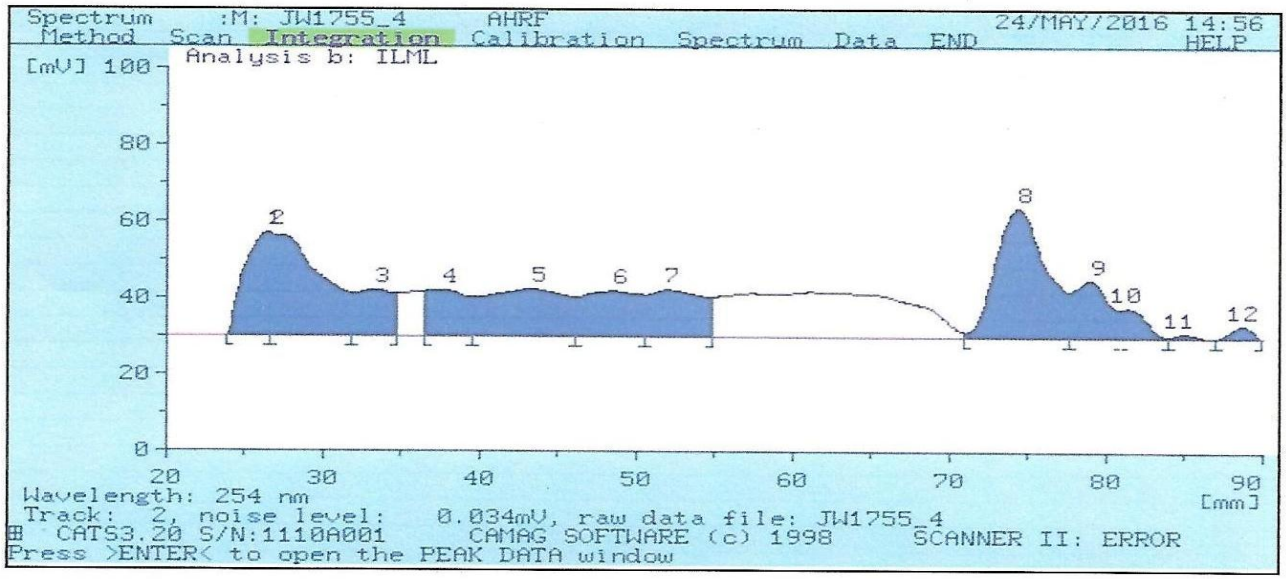
The methanol stem extract prepared by hot extraction was subjected to HPLC for the separation and identification of constituents present in the *Indigofera longeracemosa*. Two compounds were separated at different retention times of 2.563mis and 2.640min respectively. The profile displayed two prominent peaks at the retention times of 2.563mis and 2.640min. The spectrum did not show any moderate peaks (Figure 2).

HPTLC fingerprint

The qualitative HPTLC fingerprint profile of methanol extracts of different plant parts (leaf and stem) of *Indigofera longeracemosa* were obtained and the chromatogram were observed under 200-400nm. The densitogram data was analyzed.

12 peaks were eluted in the methanol leaf extract of *Indigofera longeracemosa*, the maximum height 33.7AU with Rf value 0.74 was observed in 8th peak and maximum height and a maximum percentage of area (1261.3%). The 11th peak had the minimum height 1.2 AU with Rf value (0.85) and a minimum percentage of area (17.9%) as shown in figure 3.

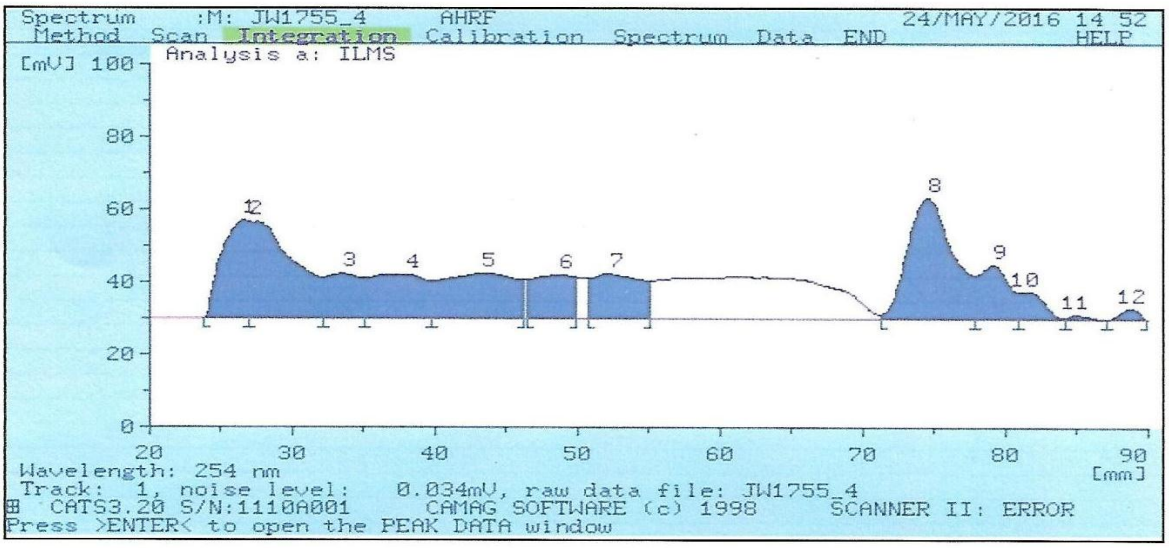
Figure 3. HPTLC densitogram methanol leaf extract of *Indigofera longeracemosa.*



S/NO	Rf	HEIGHT	AREA	LAMDAMAX
1	0.23	22.7	480.2	254
2	0.29	27.0	1008.8	254
3	0.33	12.2	337.9	254
4	0.37	12.1	356.7	254
5	0.43	12.5	751.2	254
6	0.48	12.0	502.2	254
7	0.52	12.3	493.1	254
8	0.74	33.7	1261.3	254
9	0.79	15.1	386.5	254
10	0.82	7.7	136.6	254
11	0.85	1.2	17.9	254
12	0.88	3.3	57.7	254

In methanol stem extract, 12 peaks were predicted, the maximum height 33.3 AU with Rf value 0.53 was observed in 8th peak and maximum height and a maximum percentage of area (1241.5%). The 11th peak had the minimum height 1.2 AU with Rf value (0.82) and a minimum percentage of area (17.6%) as shown in Figure 4.

Figure 4. HPTLC densitogram methanol stem extract of *Indigofera longeracemosa*



S/NO	Rf	HEIGHT	AREA	LAMDAMAX(nm)
1	0.26	27.1	569.1	254
2	0.30	26.6	976.1	254
3	0.33	12.4	343.0	254
4	0.36	12.3	548.9	254
5	0.38	12.6	750.4	254
6	0.42	12.1	395.1	254
7	0.48	12.4	496.6	254
8	0.53	33.3	1241.5	254
9	0.74	14.9	372.2	254
10	0.79	7.6	147.2	254
11	0.82	1.2	17.6	254
12	0.85	3.1	45.2	254

FT-IR analysis

The FT-IR spectrum was used to identify the functional group of the active components based on the peak value in the region infra-red radiation. The results of FTIR analysis peaks values and functional groups were represented in Table-1-2.

Table 1. FT-IR spectral values and functional groups obtained for the methanol leaf extract of *Indigofera longeracemosa* Baill.

S. No	Peak value	Functional groups
1.	3450.99	Alcohols, phenols
2.	3251.40	1°2°amines, amides
3.	2925.48	Alkenes
4.	1726.94	α, β unsaturated esters
5.	1610.27	1°amines
6.	1512.88	Nitro compounds
7.	1384.64	Unknown
8.	1243.86	Aliphatic amines
9.	1071.26	Aliphatic amines

10.	753.06	Aromatics
11.	668.214	Alkynes

Table 2. FT-IR spectral values and functional groups obtained for the methanol stem extract of *Indigofera longeracemosa* Baill.

S. No	Peak value	Functional groups
1.	3381.57	Alcohol, phenols
2.	1724.05	α,β-unsaturated esters
3.	1613.16	1°amines
4.	1512.88	Nitro compounds
5.	1446.35	Aromatics
6.	1383.68	Unknown
7.	1206.26	Aliphatic amines
8.	1034.62	Aliphatic amines
9.	758.852	Alkyl halides
10.	426.191	Unknown
11.	413.656	Unknown

The FTIR spectrum profile was illustrated in Figure 5 & 6. The FTIR spectrum confirmed the presence of functional groups such as alcohols, phenols, 1° 2° amines, amides, alkenes, α, β unsaturated esters, 1°amines, nitro compounds, aliphatic amines, aromatics, alkynes, alkyl halides.

Figure 5. FT-IR Spectrum analysis of methanol stem extract of *Indigofera longeracemosa* Baill.

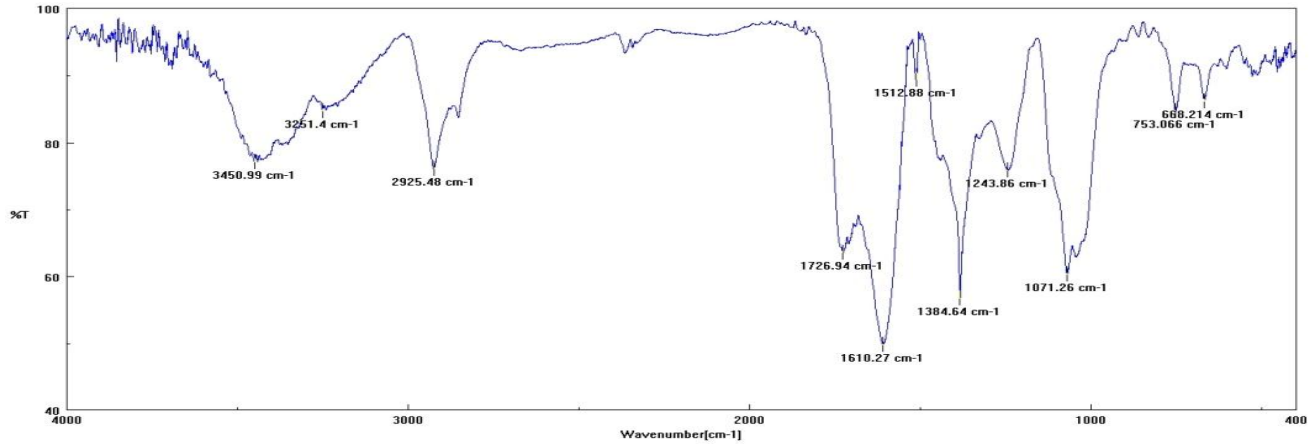
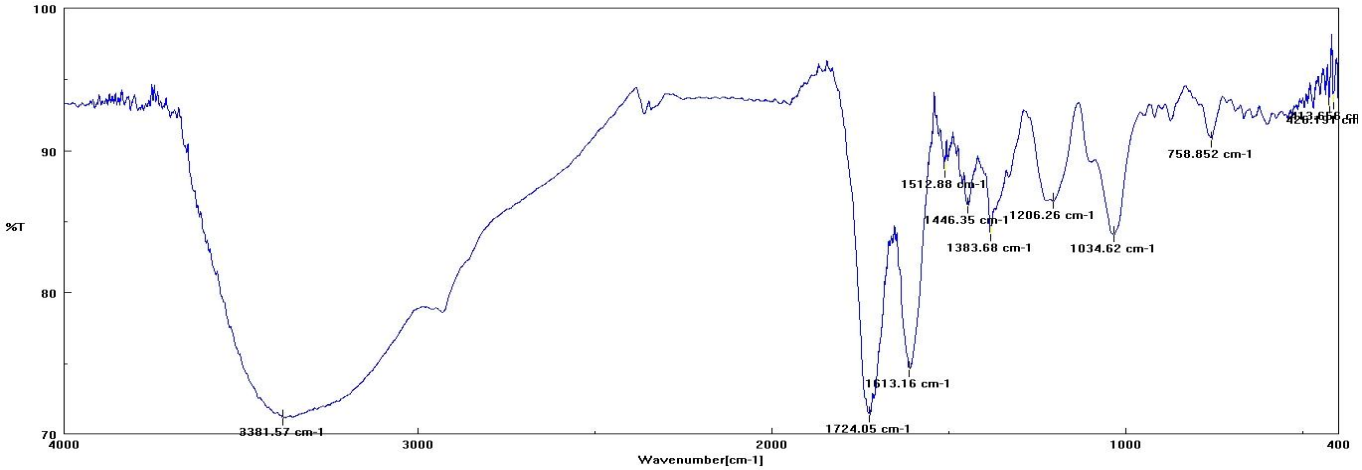


Figure 6. FT-IR Spectrum analysis of methanol stem extract of *Indigofera longeracemosa* Baill.



CONCLUSION

In conclusion, the results obtained that HPTLC, HPLC and FT-IR fingerprint analysis can be used as a diagnostic tool for the correct identification of the plant. Though further work to characterize the other chemical constituents and perform quantitative estimation with marker compounds is also necessary these data can also be considered along with the values of *Indigofera longeracemosa* plant. The methanol extract of this plant were used for phytochemical analysis of HPTLC separation of phytochemicals and quantification of active compounds by HPLC method. HPTLC analysis had different number of compounds and in HPLC analysis the active compounds were quantified which varied from methanol extract of leaf and stem sample. It can be concluded that HPTLC, HPLC and FT-IR fingerprint analysis of methanol extracts of leaf and stem parts of *Indigofera longeracemosa* can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also a good estimator of genetic variability in plant population.

CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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