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HPTLC FINGERPRINTINGS OF ETHYL ACETATE EXTRACT OF *CYNOGLOSSUM ZEYLANICUM* (BORAGINACEAE)

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ABSTRACT

Background: Qualitative and Quantitative assessment is a fundamental requirement for standatization of herbal drugs. High performance thin layer chromatography (HPTLC) is the best method for the screening of herbal drugs with respect to different secondary metabolite with their quality and quantity. **Aim:** To study the HPTLC fingerprinting profile of ethyl acetate extract of *Cynoglossum zeylanicum* (Boraginaceae). **Methods:** A CAMAG HPTLC system equipped with LINOMAT 5 applicator, a TLC scanner 3, REPROSTAR 3 and WIN CATS-4 software were used. **Results:** The study revealed the presence of coumarin glycosides (12 peaks), terpenoids (11 peaks) and steroids (6 peaks) in ethyl acetate extract of *Cynoglossum zeylanicum* **Conclusion:** The HPTLC fingerprinting profile developed for ethyl acetate extract would help in proper identification and quantification of lead compounds. By isolating and identifying lead compounds, new lead molecule can be isolated to treat the diseases.

KEYWORDS: HPTLC; Ethyl acetate; *Cynoglossum zeylanicum*; Coumarin glycosides; Terpenoids; Steroids.

1. INTRODUCTION

Identification and quality assessment of crude herbal extracts is a fundamental requirement. It is an accepted fact that the qualitative analysis of crude extracts constitutes an important and reliable part of quality control protocol. The HPTLC fingerprint profile of *Cynoglossum zeylanicum* (CZ) as any change in the quality of extract directly affects the constituents. Standardization and quality control of herbal drugs is very complicated because herbal products contain a group of phytoconstituents and are very capable of variation. There is the variability within the same plant material or between the different parts of the same plant. The variability may be from grower to grower, plant to plant and also depends on the harvest and post-harvest handling. On the other hand herbal drugs have multiple phytoconstituents including active, inactive, unknown which are dietary rather than therapeutic.[1] Hence, methodologies that can generate a fingerprint of each extract in large collections would be useful to detect stability of the same extract over time. Preferably, the method should be based on electronic storage, retrieval and analysis of the data.[2] Various extraction methods and analytical methods such as spectrophotometry has been developed for the study about plant active compounds.[3] HPTLC based methods could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. The major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces the time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing the possibilities of environment pollution. It also facilitates repeated detection of chromatogram with same or different parameters.[4] CZ (Fig.1) belongs to Boraginaceae family. This plant has been used to treat various ailments and reported to possess hepatoprotective, antioxidant, anti-cancer, anti-diabetic, and anti-fertility properties.[7-12] The present research deals with the development of HPTLC fingerprintings of ethyl acetate extract of CZ which can be used for identification, authentication and characterization of that plant.

Figure 1: *Cynoglossum zeylanicum*.

2. MATERIALS AND METHOD

2.1 Plant MaterialS

The whole plant of CZ was collected from Nilagiri Biosphere Reserve, Western Ghats, Tamilnadu. It was identified by the Survey of Medicinal Plants and Collection Unit, Ministry of AYUSH, Government of India and voucher specimen was deposited.

2.2 Plant Sample Extraction

The whole plant was cleaned, shaded dried and pulverized to powder in a mechanical grinder. Dried plant powder was exhaustively extracted by Soxhalation method with different solvents of increasing order of polarity, starting with a highly non polar solvents viz., hexane followed by Chloroform, ethyl acetate, Ethanol and Water. To ensure the complete extraction process, extraction was applied with each solvent for 24 h. Extracts of different organic solvents were collected separately into dry clean beakers, after that they were recovered from the solvents by evaporation in a rotary evaporator, then were dried in desiccators for 1 hour. The ethyl acetate residue was then subjected to preliminary photochemical analysis and HPTLC analysis.

2.3 Preliminary Phytochemical Analysis

The chemical tests for various phytoconstituents present in the ethyl acetate extract were carried out. The result of preliminary phyto-chemical scanning of the ethyl acetate extract of CZ is tabulated in Table 1.

Table 1: The Preliminary Phytochemical Screening of Extract of CZ.

Phytoconstituents	Test	Ethyl acetate extract of CZ
Alkaloid	Mayer's test	+
	Hager's test	+
	Wagner's test	+
Carbohydrate	Molish test	-
	Fehling's test	-
Saponin	Foam test	-
Terpenoids	Salkowski's test	++
Phenols	Ferric chloride test	-
	Lead acetate test	-
Tannins	Ferric chloride test	-
Steroids	Libermann burchard test	++
Proteins	Biuret test	-
	Million's test	-
	Ninhydrin test	-
Coumarin Glycoside	Alkaline test	++
Cardiac Glycoside	Keller killani test	-

2.4 HPTLC analysis

50 mg of ethyl acetate extract of *Cynoglossum zeylanicum* was dissolved in 5 mL of ethyl acetate by using sonicator. This solution was used as a test solution for HPTLC analysis. The sample solution (5µL and 10µL) was spotted (6mm band width) on a pre-coated silica gel 60 F_{254} aluminum sheets (5 x 10 cm) HPTLC plate using a Hamilton syringe and LINOMAT 5 applicators attached to CAMAG HPTLC (CAMAG, Muttenz, Switzerland). The plates loaded with samples were kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phases (Coumarin, Steroids and Terpenoids) and the plate was developed in the respective mobile phase up to 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in a photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured in white light at 254 nm and 366 nm. The peak table, peak display and peak densitogram were recorded.

2.4.1 Coumarin profile

- Mobile phase - Toluene: Diethyl ether (1:1 saturated with 10% acetic acid)
- Spray reagent - 10 % Ethanolic KOH
- Detection - Intense blue or Blue-green fluorescence (simple coumarins) yellow, brown, blue or blue-green fluoresces (furano and pyranocoumarins), yellow-green (non substituted coumarin) colored zone in 366 nm present in the given sample track

observed in the chromatogram after derivatization, which confirmed the presence of coumarin glycoside in the given in the sample.

2.4.2 Terpenoids profile

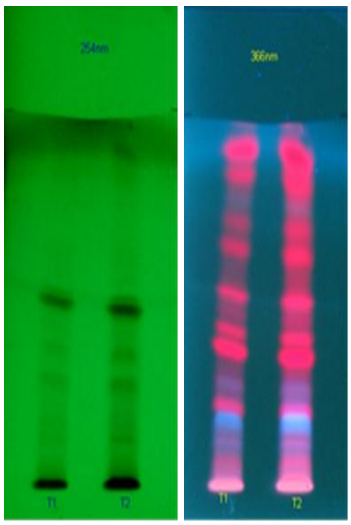
- Mobile phase - Toluene: Ethyl acetate (93:7).
- Spray reagent - Anisaldehyde sulfuric acid reagent.
- Detection - Blue-violet, pinkish violet colored zone at 366nm present in the given sample track observed in the chromatogram after derivatization, which confirmed the presence of terpenoid in the given in the sample.

2.4.3 Steroids profile

- Mobile phase: Toluene-methanol (9:1).
- Spray reagent: Anisaldehyde sulfuric acid reagent.
- Detection: blue, pink and blue-violet colored zone in 366 nm present in the given sample track observed in the chromatogram after derivatization, which confirmed the presence of steroid in the given in the sample.

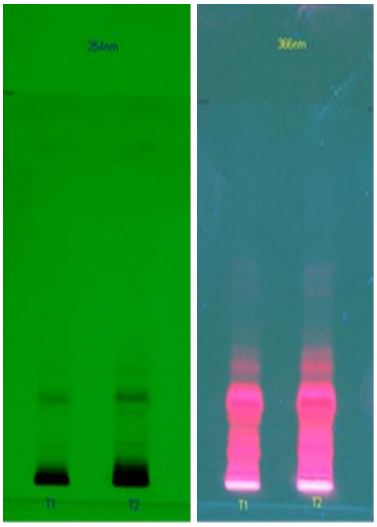
3. RESULTS

Preliminary phytochemical analysis of ethyl acetate extract of *Cynoglossum zeylanicum* revealed the presence of alkaloid, tepenoids, steroids and coumarin glycosides. The TLC chromatogram was run for *Cynoglossum zeylanicum* along with a standard for various profiles such as Coumarin, Terpenoids and Steroids. Table 2 has shown the presence of various Coumarin and its related compounds (12 peaks) with retention factor (R_f) values of 0.05, 0.09, 0.12, 0.21, 0.30, 0.36, 0.43, 0.58, 0.74, 0.82, 0.90, 0.97 were found to be more prominent. As the percentage area was more with 3.50%, 2.13%, 0.91%, 6.63%, 5.71%, 1.51%, 34.15%, 2.12%, 0.36%, 4.01%, 21.11%, and 17.87%. The highest peak area (%) of the Coumarin compound was found to be 34.15% and its corresponding R_f value was 0.43. Table 3 shows the presence of various Terpenoids and its related compounds (11 peaks) with retention factor (R_f) values of 0.04, 0.10, 0.13, 0.17, 0.24, 0.40, 0.58, 0.70, 0.73, 0.85, and 0.98 were found to be more prominent. As the percentage area was more with 2.5%, 1.69%, 10.08%, 43.61%, 7.27%, 2.11%, 4.31%, 2.07%, 4.27%, 18.22% and 3.86%. The highest peak area (%) of the Terpenoids compound was found to be 18.22% and its corresponding R_f value was 0.85. Table 4 shows the presence of various Steroids and and its related compounds (6 peaks) with retention factor (R_f) values of 0.06, 0.11, 0.15, 0.19, 0.24, and 0.94 were found to be more prominent. As the percentage area was more with 53.84%, 28.42%, 7.07%, 7.42%, 1.87%, 1.37%, The highest peak area (%) of the Terpenoids compound was found to be 83.84% and its corresponding R_f value was 0.06. Exposure of the spotted and developed HPTLC plate to UV 254 nm showed the presence of numerous organic compounds as dark and light bands in a green background. HPTLC slides developed at UV 366 nm exposure revealed multi-colored bands with varying intensities, which showed purple, light and dark blue, bluish purple and fluorescent intense blue or Blue-green fluorescence purple color bands, respectively. In the present study,dark blue color bands were observed, which confirmed the presence of Coumarin compounds in the plant extract (Fig.2). Light pink zones were detected from the chromatogram after derivatization which confirmed the presence of Terpnoids (Fig 3) and Bluish purple color indicated the presence of Steroids (Fig 4). This confirms the presence of Coumarin, Terpeniods and Steroids in this ethylacetate extract.



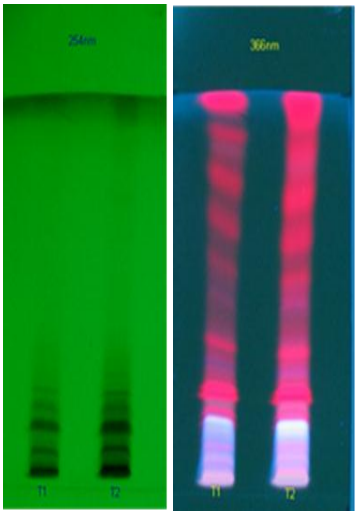
5 µL **10 µL**

Fig.2 HPTLC Chromatogram of Coumarin profile of EA extract of CZ



5 µL **10 µL**

Fig.3 HPTLC Chromatogram of Terpenoid profile of EA extract of CZ



5 µL **10 µL**

Fig.4 HPTLC Chromatogram of Steroid profile of EA extract of CZ

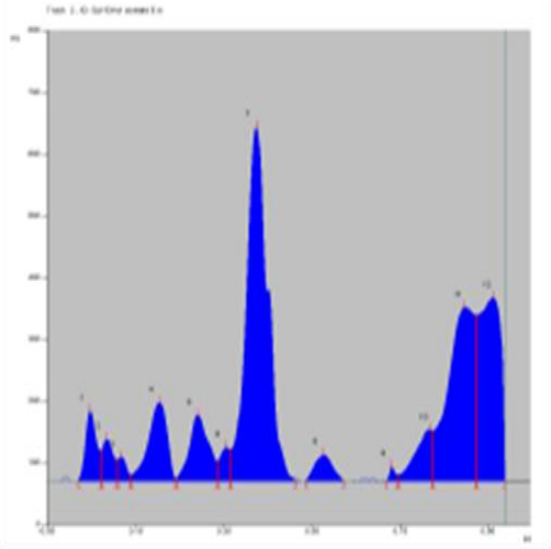


Fig. 2a HPTLC finger print of Coumarin profile of CZ ethyl acetate extract.

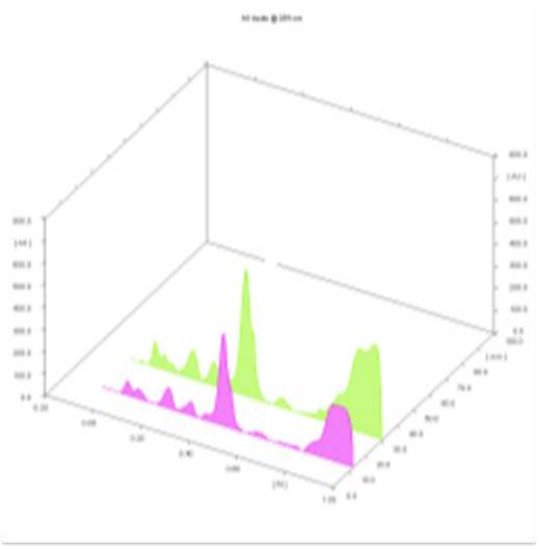


Fig. 2b 3D HPTLC finger print of Coumarin profile of ethyl acetate extract of CZ showing different peaks.

Table 2: Finger print data of Coumarin profile of CZ at 254 nm.

Peak	R_f value	% area
1	0.05	3.50
2	0.09	2.13
3	0.12	0.91
4	0.21	6.63
5	0.30	5.71
6	0.36	1.51
7	**0.43**	**34.15**
8	0.58	2.12
9	0.74	0.38
10	0.82	4.01
11	0.90	21.11
12	0.97	17.87

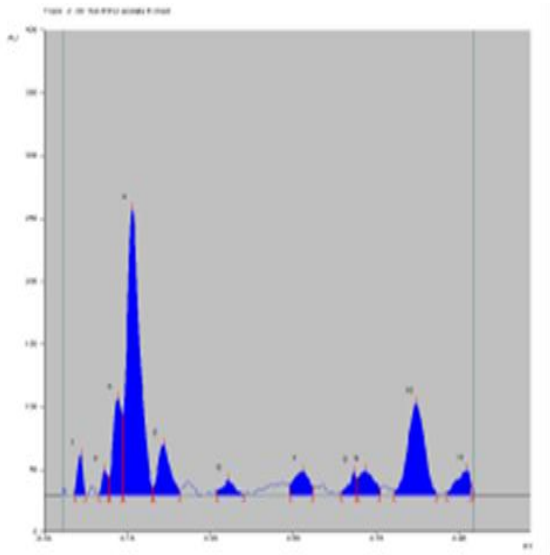


Fig. 3a HPTLC finger print of Terpenoid profile of CZ ethyl acetate extract

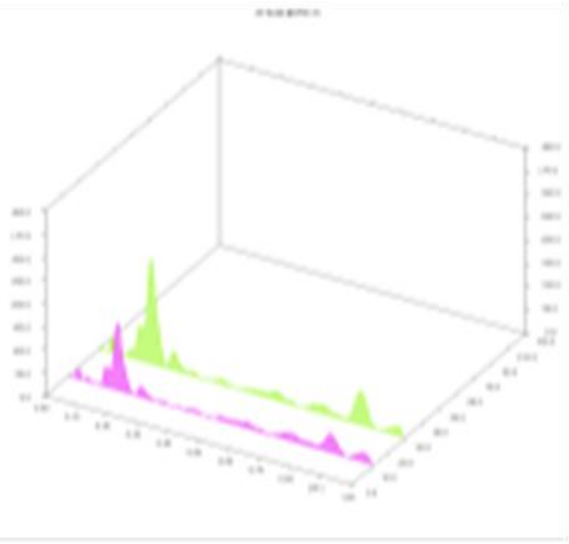


Fig. 3b 3D HPTLC finger print of Terpenoids profile of ethyl acetate extract of CZ showing different peak

Table 3 Finger print data of Terpenoid profile of CZ at 254 nm.

Peak	R_f value	% area
1	0.04	2.51
2	0.10	1.69
3	0.13	10.08
4	0.17	43.61
5	0.24	7.27
6	0.40	2.11
7	0.58	4.31
8	0.70	2.07
9	0.73	4.27
10	0.85	18.22
11	0.98	3.86

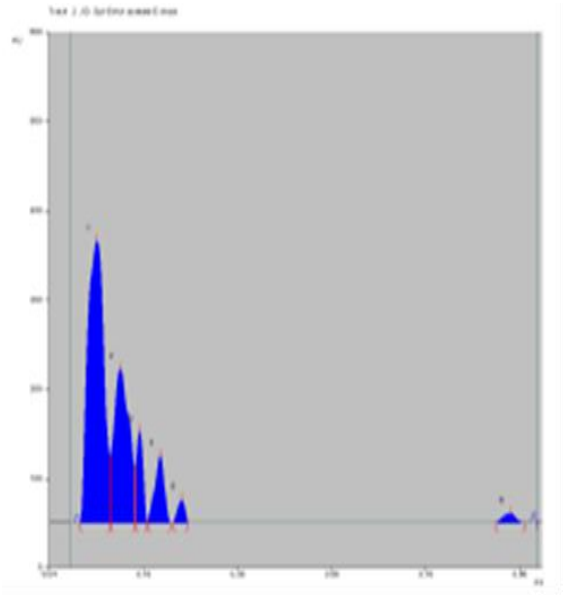


Fig. 4a HPTLC fingerprint of steroid profile of CZ ethyl acetate extract

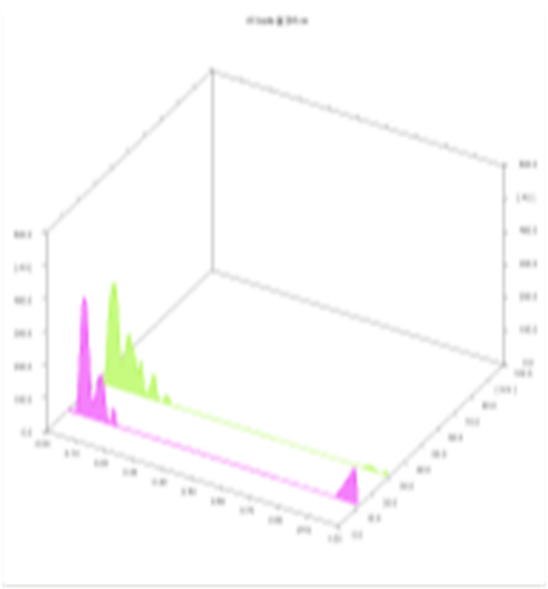


Fig. 4b 3D HPTLC fingerprint of steroid profile of ethyl acetate extract of CZ showing different peaks

Table 4 Finger print data of steroid profile of CZ at 254 nm.

Peak	Rf value	% area
1	0.06	53.84
2	0.11	28.42
3	0.15	7.07
4	0.19	7.42
5	0.24	1.87
6	0.94	1.37

5. DISCUSSION

Nowadays, the interest in the study of natural products is growing rapidly, especially as a part of drug discovery programs. This preliminary study was carried out with HPTLC and the results showed that there are many compounds in *C.zeylanicum*. The present study helps to detect and identify phytoconstituents which can be used to characterize the ethyl acetate extract of *Cynoglossum zeylanicum* for further therapeutic use. Characteristic HPTLC Coumarin finger printing of particular plant species will not only help in the identification and quality control of a particular species, but also provide basic information useful for the isolation, purification, characterization and identification of marker chemical compounds of the same plant. HPTLC profile differentiation is such an important and powerful procedure which has often been employed for this purpose. The development of chromatogram will be specific with selected solvent systems. R_f values are serving a better tool for standardization of the drug. HPTLC is feasible for development of chromatographic fingerprints to determine major active constituents of *Cynoglossum zeylanicum*. The separation and resolution are much better, and the results are much more reliable and reproducible than TLC. Combined with digital scanning profiling, it has the main advantage of in situ quantitative measurement by scanning densitometry.

5. CONCLUSION

The present study clearly gives evidence of the bioactive quantitative of phytochemical in ethyl acetate extract. Further, this method can be effectively used for routine quality control of algal materials as well as for formulations containing any or both of these compounds.

The present study establishes the fact that HPTLC fingerprinting profile can be used as the diagnostic tool to identify and determine the quality and purity of experimental plant *C. zeylanicum* in future studies.

6. ACKNOWLEDGEMENT

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