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QUANTITATION OF CAMPTOTHECIN FROM *ERVATAMIA HEYNEANA* (WALL.) T. COOKE STEM POWDER USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A sensitive and reliable quantitative high performance thin layer chromatographic method has been developed for the quantitation of camptothecin from the dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke. *Ervatamia heyneana* (Wall.) T. Cooke has anticancer activity [1]. Methanolic extract of the dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke was used for carrying out TLC on silica gel 60 F_{254} plates. Detection and quantitation was performed by densitometric scan in fluorescence, by using mercury lamp at λ = 366 nm. Accuracy of the developed HPTLC method was checked by conducting the recovery study. The average percent content of camptothecin in stem powder of the used plant, as estimated by the proposed method, was found to be 0.041mg/g. The HPTLC method developed for the quantitative determination of camptothecin in *Ervatamia heyneana (Wall.) T. Cooke* stem powder is rapid, simple and precise.

KEY WORDS;**Camptothecin, *Ervatamia heyneana* (Wall.) T. Cooke, HPTLC, Apocynaceae**

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INTRODUCTION

Ervatamia heyneana (Wall.) T. Cooke (Apocynaceae), Syn. *Tabernaeontana heyneana* Wall, is a medicinal tree, found in the Western Ghats from Konkan to Trivancore [1]. The stem of tree yielded indole alkloid camptothecin [2]. Camptothecin, a quinoline alkaloid, is used as antitumor drug, in the treatment of cancers of colon, head, breast and bladder [3, 4]. In addition to its antitumor property, camptothecin also possesses activity against retrovirus and human immunodeficiency virus 3. LD_{50} value of *Ervatamia heyneana* (Wall.) T. Cooke alcoholic extract was found to be 750 mg/kg in mice [5]. Camptothecin an important bioactive alkaloid present in *Ervatamia heyneana* (Wall.) T. Cooke, is also present in stem of *Camptotheca accuminata* Decsne. (Nyssaceae), *Nothapodytes foetida* (Wight) Sleumer. (Icacinaceae), *Ophiorrhiza pumila* Champ. (Rubiaceae) and *Ophiorrhiza mungos* Linn. (Rubiaceae)[6 to 9].

The proposed HPTLC method is thus developed to quantitate camptothecin present in the dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke. HPTLC due to its simplicity and minimum sample clean-up requirement has been widely used as a quality control tool for the phytochemical evaluation of herbal drugs.

HPLC and HPTLC methods have been reported in literature for quantitation of camptothecin from stem of *Nothapodytes foetida* (Wight) Sleumer.[10, 11]. No HPTLC method has been reported in literature for quantitation of camptothecin from *Ervatamia heyneana* (Wall.) T. Cooke. Hence, a precise and accurate HPTLC method has been developed in the present research work, which may be used as a fast and relatively cheap method for determination of camptothecin from dried stem powder of *Ervatamia heyneana* (Wall.) T. Cooke.

2 EXPERIMENTAL

2.1.1 REAGENTS AND STANDARD:

Acetonitrile, toluene, ethyl acetate and formic acid were of AR grade with 99.5%, 99.5%, 99.8% and 100.0 % purities respectively and were obtained from Qualigens Fine Chemicals, Mumbai, India. Camptothecin standard [s - (+) - camptothecin] of Lot No. 16211LI was procured from Sigma-Aldrich Chemie Gmbh (Aldrich Division; Steinheim, Federal Republic of Germany). Its reported purity is 98.3% in the Certificate of Analysis.

2.1.2 PLANT MATERIAL:

Stems of *Ervatamia heyneana* (Wall.) T. Cooke were collected from Dapoli, Maharashtra, India and its herbarium was prepared and authenticated from Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, District Ratnagiri, Maharashtra, India. The collected stems of *Ervatamia heyneana* (Wall.) T. Cooke were dried in shade, finely powdered and powder was passed through 80 mesh sieve and stored in an airtight container, at room temperature ($25^0 \pm 2^0$C).

2.2 PREPARATION OF SOLUTION:

2.2.1 PREPARATION OF STANDARD SOLUTION OF CAMPTOTHECIN (1000 µg/mL):

Accurately weighed about 100 mg camptothecin standard was transferred to a 100 mL standard volumetric flask. It was dissolved in 50mL of methanol and volume of the standard volumetric flask was made up to the mark with methanol to obtain stock solution of 1000 µg/mL.. The aliquots, 0.05 mL to 5 mL of stock solution of camptothecin (1000 µg/mL), were transferred to different 100 mL standard volumetric flasks and the volume of each of standard volumetric flask was adjusted to 100 mL with methanol, to obtain working standard solutions of camptothecin containing 0.5 µg/mL to 50 µg/mL respectively.

2.2.2 PREPARATION OF THE SAMPLE SOLUTION:

Dried stem bark powder (about 1000 mg) of *Ervatamia heyneana* (Wall.) T. Cooke was accurately weighed and added to a 20 mL standard volumetric flask. To this, 10 mL of methanol was added. The contents of the

standard volumetric flask were shaken on a mechanical shaker for 10min, followed by sonication in an ultra-sonic bath for 15min and were allowed to stand overnight, at room temperature (25^0 ± 2^0C). The contents of the standard volumetric flask were filtered through Whatmann filter paper no 41 (Merck, Mumbai, India). The clear filtrate was collected in a dry 10 mL standard volumetric flask. This solution was used for the assay experiment.

2.2.3. CHROMATOGRAPHIC CONDITIONS:

Chromatography was performed on 5.0 cm x 10.0 cm TLC plates which were cut from 20.0 cm x 20.0 cm TLC aluminum plates precoated with 200 µm layers of silica gel $60F_{254}$ (E. Merck, Mumbai, India). The plates were prewashed with methanol and activated at 105-110ºC for 15 minutes before analysis. Ten microlitre of each of these standard solutions of camptothecin and sample solution were applied, as bands, with the help of CAMAG Linomat V sample applicator with a 100µL syringe (Hamilton, Bonaduz, Switzerland)., at a distance of 10.0 mm from the bottom edge of the chromatographic plate, as bands of 7.0 mm width, at a distance of 6.0 mm from each other. Linear ascending development was carried out in a twin-trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase comprising of toluene – acetonitrile – ethyl acetate – formic acid, in the volume ratio of 6.0 + 3.0 + 1.0 + 0.1. The optimized chamber saturation time for the mobile phase was 20 minutes at room temperature (25±2ºC). The plates were developed to a distance of 80 mm from the bottom edge of the plate. After plate development, the plate was air dried and the response of the standard camptothecin was monitored using CAMAG III TLC Scanner with Win CATS software version 1.4.2., set at a wavelength, λ= 366 nm, in fluorescence under Mercury light.

2.3 METHOD VALIDATION:

2.3.1 LINEARITY:

10 µL of each of the six working standard solutions of camptothecin, in the concentration range of 0.5 µg/mL to 50 µg/mL, were applied in order of increasing concentrations in triplicates, as 7mm bands on the TLC plate by means of CAMAG Linomat V automatic sample applicator. The plates were developed under specified chromatographic conditions and scanned. The peak areas of camptothecin were recorded for each applied concentration of camptothecin. The data pairing technique was applied to determine if there is any significant variation in peak areas of camptothecin solutions of same concentration recorded in duplicate. No significant statistical difference was observed between each pair of same concentration. The calibration curve of camptothecin was obtained by plotting a graph of mean peak area vs. applied concentration of camptothecin the concentration range of 0.5 µg/mL to50 µg/mL and was found to be linear in this concentration range. The results listed in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

2.3.2 LIMIT OF DETECTION (LOD) & LIMIT OF QUANTITATION (LOQ):

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at a signal to noise ratio of 3:1 and 10:1 respectively. The LOD and LOQ values obtained for both the components are listed in Table 1.

2.3.3 PRECISION:

The method was validated in terms of instrumental precision, repeatability, and intermediate precision. Instrumental precision was studied by repetitive analysis (n = 10) of the standard solution of camptothecin (5 µg/mL), using the proposed method and the peak areas of camptothecin were recorded.

The repeatability was carried out in same laboratory, on same day, by analyzing three sample solutions of dried stem powder of *Ervatamia heyneana* (Wall.) T. Cooke*,* under the specified chromatographic conditions. The peak areas of camptothecin were recorded.

The intermediate precision of the method was evaluated by analyzing the three sample solutions of dried stem powder of *Ervatamia heyneana* (Wall.) T. Cooke*,* on three different

days, in different system, under the specified chromatographic conditions. The peak areas of camptothecin were recorded.
The results were expressed as percentage relative standard deviation of peak area of camptothecin and are listed in Table 1. The results indicate that the proposed method is precise and reproducible.

3.4 SYSTEM SUITABILITY:

System suitability was carried out to verify that resolution and reproducibility of the system were acceptable for the analysis.

System suitability test was carried out by applying 10 µL of standard solution of camptothecin, with concentration of 5.0 µg/mL, was applied as a band in five replicates on TLC plates under specified chromatographic conditions. The chromatograms were recorded. The values of percent relative standard deviation of peak area and retention factor of standards were taken as an indicator of system suitability. All the values for standard solution of camptothecin lie within the acceptable range with values of percent relative standard deviation were less than 2, indicating suitability of the system.

Table 1
Method validation data for simultaneous quantitation of camptothecin

Parameters	Camptothecin
Linear range (n=3) µg/mL	0.5 - 50.0
Correlation coefficient r	0.9999
LOD µg/mL	0.03
LOQ µg/mL	0.1
Instrumental precision mean % R.S.D. (n=10)	0.35
Repeatability mean % R.S.D. (n=3) (on the same day)	0.81
Intermediate precision mean % R.S.D (n=3) (For three successive days)	0.83

2.3.4 ESTIMATION OF CAMPTOTHECIN IN DRY POWDER OF STEM OF ERVATAMIA HEYNEANA (WALL.) T. COOKE.:

10 µL of the sample solution prepared by extracting about 1.0 g of dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke with methanol as described earlier and was applied as bands in seven replicates, on a precoated silica gel 60 F_{254} TLC plate. The plate was developed and scanned as mentioned above. The values of peak areas and mean of peak area of camptothecin were recorded. Percent relative standard deviation of peak area of camptothecin was determined. From the calibration curve, the amount of camptothecin present in the sample solution of *Ervatamia heyneana* (Wall.) T. Cooke was calculated. The purity of standard camptothecin was considered for determination of amount of camptothecin present in dry stem powder of *Ervatamia heyneana (*Wall.) T. Cooke. The assay results are listed in Table 2.

Table 2
Average content of Camptothecin in methanolic extract of dry stem powder of Ervatamia heyneana (Wall.) T. Cooke, by the proposed HPTLC method.

Sample	Weight of sample (mg)	*Mean amount of camptothecin found in sample (mg/g)	%RSD of peak area of camptothecin
Dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke	1010.5	0.0413	0.80

*** *(n=7)***

***2.3.5 ACCURACY*:**
Accuracy of the method was established by carrying out recovery experiment to study if there is any interference of other constituents present in *Ervatamia heyneana* (Wall.) T. Cooke stem powder on peak of camptothecin. About 1.00 g of dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke was accurately weighed in four different 20 mL standard volumetric flasks. Known amounts of the standard camptothecin (0.0 µg, 2.5 µg, 5.0 µg and 7.5 µg) respectively were added to each flask and extracted as described above. Each solution was analysed by developed HPTLC method, using the optimized chromatographic conditions, in seven replicates and the value of amount of camptothecin recovered from the sample for each level, was determined. The value of percent recovery was determined. The results are listed in Table 3.

Table 3
***Recovery of camptothecin from Ervatamia heyneana (Wall.) T. Cooke stem powder by proposed HPTLC method*.**

Level	Weight of sample (mg)	Amount of camptothecin added to sample (µg/mL)	Average* amount of camptothecin found in sample (µg/mL)	Percent Recovery
0	1019.6	0.0	4.13	98.65
1	1022.3	2.50	6.68	
2	1011.7	5.00	9.14	
3	1001.1	7.5	11.60	

*** (n=7)**

3. RESULTS

HPTLC method has been developed for quantitation of camptothecin from stem bark powder of *Ervatamia heyneana* (Wall.) T. Cooke. The linearity range of camptothecin was found to be 0.5 µg/mL to 50 µg/mL, with correlation coefficient as 0.9999.
When the method was validated in terms of instrumental precision, repeatability and intermediate precision, the percent relative standard deviation values for each were found

to be less than 2, indicating that the proposed method is precise and repetitive.

The mean percent content of camptothecin found from the methanolic extract of dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke, by the proposed method, was found to be 0.0413mg/g (Table 2). The accuracy of the method was established by means of recovery experiment. The percent recovery of camptothecin at three different levels was found to be 98.65 (Table 3).

The method is specific for camptothecin because it resolved the standard camptothecin (R_f=0.29) well in presence of other phytochemicals of *Ervatamia heyneana (Wall.) T. Cooke* stem.

4. DISCUSSION

The present research was carried out by using plant material collected from Dapoli, Maharashtra, India. To achieve quantitative extraction, conditions used in the extraction procedure like nature and volume of extracting solvent and time of extraction were optimized and each extract was analysed by developed HPTLC method. The extracting conditions were thus optimized to obtain maximum amount of camptothecin in the extract of stem powder of *Ervatamia heyneana* (Wall.) T. Cooke.

Various solvents systems in different proportions of organic and inorganic solvents were used as a mobile phase for the quantitation of camptothecin from the extracts of stem bark powder of *Ervatamia heyneana* (Wall.) T. Cooke, but separation was not satisfactory. In succession, a mixture of toluene: acetonitrile: ethyl acetate in different ratios was tested. Eventually, it was found that system gave a much better separation for camptothecin from its sample matrix, except slight tailing peaks were observed for the peak of camptothecin. The tailing of polar sample components can be decreased by partially deactivating the surface of silica gel, keeping the acidic and the basic centers in a molecule nonionised, by adding small amount formic acid to the mobile phase, symmetrical, sharp and well-resolved peaks were observed for camptothecin from its sample matrix. It was found that the mobile phase comprising of a mixture of toluene – acetonitrile – ethyl acetate – formic acid, in the volume ratio of (6: 3: 1: 0.1) provided a good resolution of camptothecin from the other phytoconstituents present in the dried stem bark powder of *Ervatamia heyneana* (Wall.) T. Cooke.

The plate was scanned in fluorescence, using mercury lamp at λ = 366 nm. The identity of the band of camptothecin in the sample solution was confirmed by comparing R_f value of camptothecin in sample ((R_f=0.30) with that of reference standard camptothecin (R_f=0.29). Figure 1 and Figure 2 show typical chromatograms of standard camptothecin and methanolic extract of *Ervatamia heyneana* (Wall.) T. Cooke. Figure 3 shows typical HPTLC plate illustrating the separation of camptothecin in the methanolic extract of *Ervatamia heyneana* (Wall.) T. Cooke.

No HPTLC method has been reported in literature for quantitation of camptothecin from *Ervatamia heyneana* (Wall.) T. Cooke and in the present research work, a normal phase high performance thin layer chromatographic method for the quantitative determination of camptothecin from the extract of dried stem bark powder of *Ervatamia heyneana* (Wall.) T. Cooke has been developed.

5. CONCLUSION

Camptothecin present in *Ervatamia heyneana* (Wall.) T. Cooke is one of the most impressive anticancer molecules of the recent times [10, 12]. In the present research work, HPTLC method has been developed for the quantitation of camptothecin from the methanolic extract of dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke, collected only from Dapoli, Ratnagiri, Maharashtra, India. It can be applied for the quantitation of camptothecin from the methanolic extract of the dry stem powder of *Ervatamia heyneana* (Wall.) T. Cooke, collected from any other source. The HPTLC method developed with careful validation for

the quantitation of camptothecin in the stem of the tree used in the present research work was found to be simple, precise, sensitive and accurate.

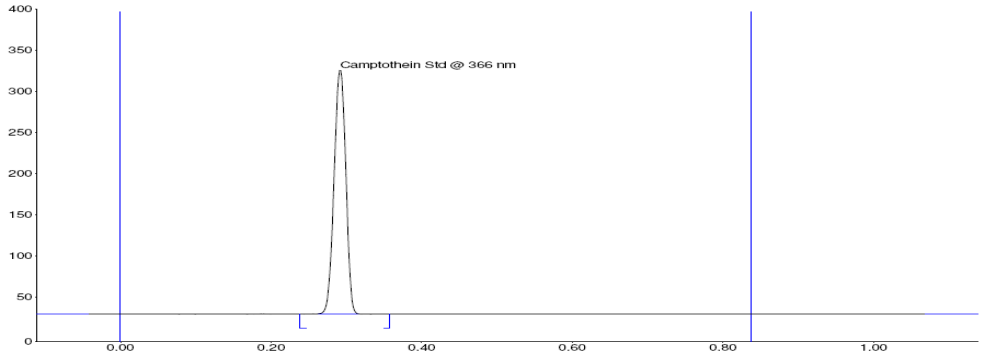


Figure 1.
TLC chromatogram of standard camptothecin scanned at λ = 366 nm.

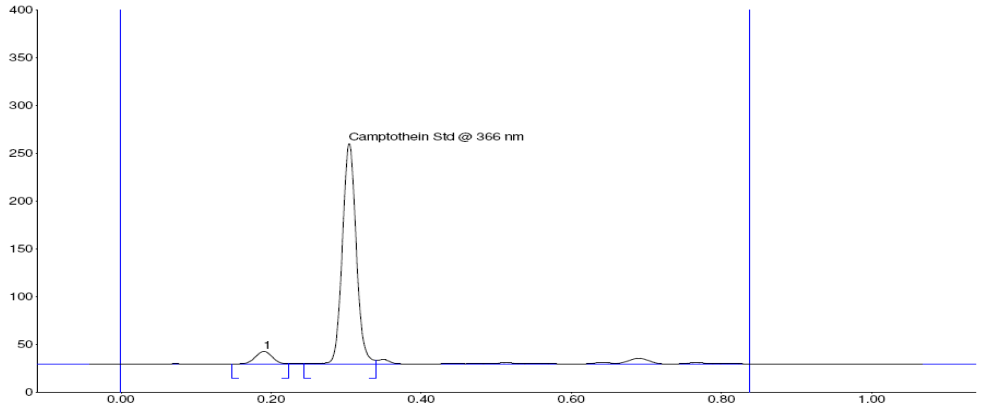
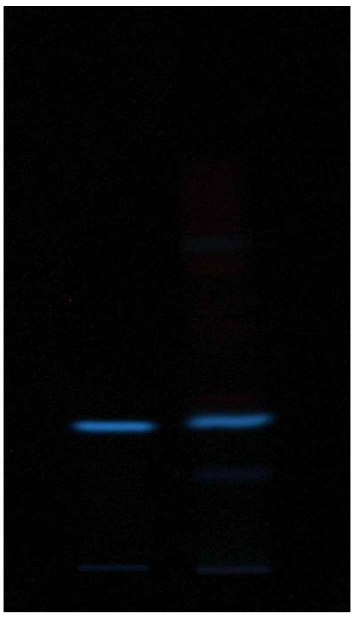


Figure 2.
TLC chromatogram of methanolic extract of dry stem powder of Ervatamia heyneana (Wall.) T. Cooke, scanned at λ = 366 nm.



A B

Figure 3.

HPTLC plate showing standard camptothecin (A) and separation of camptothecin in the methanolic extract of Ervatamia heyneana (Wall.) T. Cooke (B) at λ = 366 nm

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