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Simultaneous Quantitative Determination of Nitidine, Chelerythrine and Sanguinarine Using HPTLC from Callus Extract of *Zanthoxylum rhetsa*

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Abstract

Nitidine, Chelerythrine and Sanguinarine, all these three alkaloids are benzophenanthridine alkaloids. Nitidine was used as an anti-HIV, anti-malarial and anti-cancer. Chelerythrine had anti-cancer and anti-inflammatory activities. Sanguinarine was widely used as an anti-plaquestic and anti-cancer. High performance thin layer chromatography (HPTLC) method was used for simultaneous quantification of Nitidine, Chelerythrine and Sanguinarine in callus extract of *Zanthoxylum rhetsa* by using Silica gel 60 F_{254} as stationary phase and ethyl acetate:methanol:water:diethylamine (30:5:2:0.5 v/v) as mobile phase at 280 nm. The linearity concentration range was 5 - 160 µg/band of each alkaloid. The R_f values of Nitidine, Chelerythrine and Sanguinarine were found to be 0.28, 0.49 and 0.73. The limit of detection and limit of quantification were found to be 0.026, 0.088 µg/spot and 0.010 and 0.033 µg/spot, 0.0104 and 0.035 µg/spot respectively for Nitidine, Chelerythrine and Sanguinarine. HPTLC method was developed and validated according to ICH guidelines for simultaneous estimation of Nitidine, Chelerythrine and Sanguinarine and proved to be simple, specific, accurate, robust and rapid.

Keywords

Nitidine, Chelerythrine, Sanguinarine, HPTLC

1. Introduction

Benzophenanthridine alkaloids are one of the most important sub-classes of isoquinoline alkaloids, which are the major group of pharmacologically useful

compounds, such as nitidine, chelerythrine, sanguinarine, arborine, chelirubine, angoline, chelidonine, chelilutine, corynoline, marcapine, fagaridine, decarine, sanguilutine, sanguirubine and aricine [1] [2] and they are widely distributed among the various plant sources, which are *Macleaya cardata* [3], *Chelidonium majus* [4], *Sanguinaria canadensis*, *Dicranostigma lactucoids*, *Stylophorum lasiocarpum* [5], *Argemone mexicana* [6], *Zanthoxylum quinduense* [7], *Zanthoxylum nitidum* [8], *Zanthoxylum rhetsa* [9] and *Zanthoxylum armatum* [10].

The present paper deals with simultaneous HPTLC quantification of three benzophenanthridine alkaloids namely nitidine, chelerythrine and sanguinarine. Nitidine was reported to be used as an anti-cancer [11], anti-malarial [12] and anti-HIV [13], chelerythrine have the anti-malarial [14], anti-cancer [15], and anti-inflammatory activities [16] and sanguinarine shows the anti-inflammatory [16], anti-plaquestic [17] and anti-cancer [15] [18] properties.

Praveena and Veeresham (2014 and 2015) were reported the HPTLC quantification of nitidine from *Toddalia asiatica* roots and callus cultures [19] [20]. Bogucka-Kocka and Zalewski (2017) reported the quantification of chelerythrine and sanguinarine from *Chelidonium majus* herb and root by using HPTLC [21]. Literature reveals that the studies were carried out only on linearity, LOD and LOQ studies. Precision, robustness and system suitability studies were not done for chelerythrine and sanguinarine quantification. So we have undertaken this study for simultaneous quantification of these three benzophenanthridine alkaloids (nitidine, chelerythrine and sanguinarine) by densitometric HPTLC method. Very few reported analytical methods are available on *Z. rhetsa* which are, phytochemical screening of fruits by HPTLC [22]. Kumar *et al*., (2016) reported the cytotoxic potentiality of bioactive constituents from *Z. rhetsa* bark by GC-MS [11]. Fatema-Tuz-Zohora *et al*., (2018) reported the isolation of Quinoline alkaloids by NMR spectroscopy from *Z. rhetsa* root bark [23] and Chatterjee *et al*., (1959) reported the isolation of rhetsine, rhetsinine and chelerythrine from trunk bark by IR Spectroscopy [24]. However, there are no reports on *Z. rhetsa* whole herb/tissue culture extract nor on simultaneous HPTLC determination of nitidine, chelerythrine and sanguinarine from the callus extracts of *Z. rhetsa*. The present work illustrates the denisitometric HPTLC method establishment and validation for simultaneous quantification of nitidine, chelerythrine and sanguinarine from *Z. rhetsa* callus extract.

2. Materials and Methods

2.1. Collection of Plant Material

Z. rhetsa plants were identified and collected from Medicinal plants garden of Kerala Forest Research Institute (KFRI) of Peechi, Kerala, India and it was authenticated by Prof. T. Christopher, Taxonomist, department of Botany, Kakatiya University, Warangal, Telangana, India. Voucher specimen of the plant was deposited in the author laboratories.

2.2. Chemicals and Standards

All solvents and reagents used were purchased from Merck, Mumbai, India. Standard drugs nitidine (≥97%), chelerythrine (≥95%) and sanguinarine (≥98%) were purchased from Sigma, Mumbai, India.

2.3. Preparation of Standard Stock Solution

1 mg/ml stock solutions of nitdine, chelerythrine and sanguinarine were prepared by dissolving an accurately weighed 10 mg of each standard in 10 ml of 70% methanol in volumetric flask. Further dilutions were made from this stock.

2.4. Preparation of Sample Solutions

5 gms of dried leafy callus of *Z. rhetsa*, was taken and extracted with 10 ml of 70% v/v methanol by refluxing for 30 minutes, and then concentrated to dryness by vacuum. Dried extract was re-dissolved in 70% v/v methanol to get sample stock solution.

2.5. HPTLC Analysis

The method was developed on Camag HPTLC system, consisting of Linomat V 10 AT semi automatic applicator (Muttenz, Switzerland), Camag twin trough chamber (20 cm × 20 cm) for TLC plate development and Camag TLC scanner 3 20AT, equipped with software (version 1.4.3) win CATS and 100 µl capacity Camag Syringe. HPTLC analysis was performed by application of 10 µl of each standard drug on 10 cm × 10 cm, 0.2 mm layer thickness silica gel $60F_{254}$ (Merck, Germany) pre-coated aluminum plates as 8 mm band width with the help of semi automatic applicator under pressure of nitrogen gas. The space between each band is 6 mm, 15 mm from side and 8 mm from bottom. Development was done through twin trough chamber by linear ascending mechanism. The chamber is pre saturated with mobile phase *i.e.*, ethyl acetate: methanol: water: di ethyl amine (30:5:2:0.5 v/v) for 20 minutes at room temperature in prior to insertion of plate into solvent system. The development distance was 80 mm. After this process the plates were dried. Densitometric scanning at 280 nm was selected the maximum absorption of band, performed with Camag TLC scanner in reflection absorbance made by using a slit width 6 mm × 0.3 mm, data resolution 100 $mm{\cdot}sec^{-1}$, 20 $mm{\cdot}sec^{-1}$ scanning speed. For continuous radiation purpose deuterium lamp was used for UV-Visible region 190 - 800 nm.

3. Validation of HPTLC Method

An optimized HPTLC densitometry method was validated by following parameters.

3.1. Linearity

5, 10, 20, 40, 80, 160 µg/spot concentrations of standards were loaded on to TLC plate by using semi automatic applicator, which were prepared from standard

solutions. Each different concentration was loaded for 3 times on the plate. The plate was developed by using mobile phase and plotted the peak areas of each spot against concentration to obtain the calibration curve.

3.2. LOD and LOQ

Slope and standard deviation of the calibration curve were used for calculation of LOD and LOQ.

$$\text{LOD} = 3.3\ \sigma/S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve.

$$\text{LOQ} = 10\ \sigma/S$$

3.3. Specificity

Specificity of the method was analyzed by comparing the callus extracts and standards. The spot for nitidine, chelerythrine and sanguinarine was confirmed by comparing their R_f values with standard compounds.

3.4. Recovery

Accuracy of the method was established by performing recovery experiments using the standard addition method. To the pre analyzed samples of callus extract, standard nitidine, chelerythrine and sanguinarine solution was added by spiking at 100 µg level and the mixture was analyzed by the proposed HPTLC method.

3.5. Precision

Random errors were identified by precision. Results were expressed in relative standard deviation (% RSD). Standard solution of nitidine, chelerythrine and sanguinarine (5, 20, 80 µg/band) were applied. Inter day precision was evaluated by applying each concentration for 3 times on three different days with an interval of 24 hrs. Intraday precision was evaluated by applying each concentration three times within the day.

3.6. Robustness

To test the robustness of the method, deliberately small changes were made in the chromatographic parameters that may affect the performance of the method, *i.e.*, mobile phase composition, mobile phase value. The RSD of the peak areas was calculated for each parameter.

3.7. System Suitability

System suitability was carried out to check the reproducibility and resolution of the method. After development, the plates were scanned and peak area of each spot and their R_f values were calculated.

4. Results and Discussion

4.1. Development of HPTLC Method

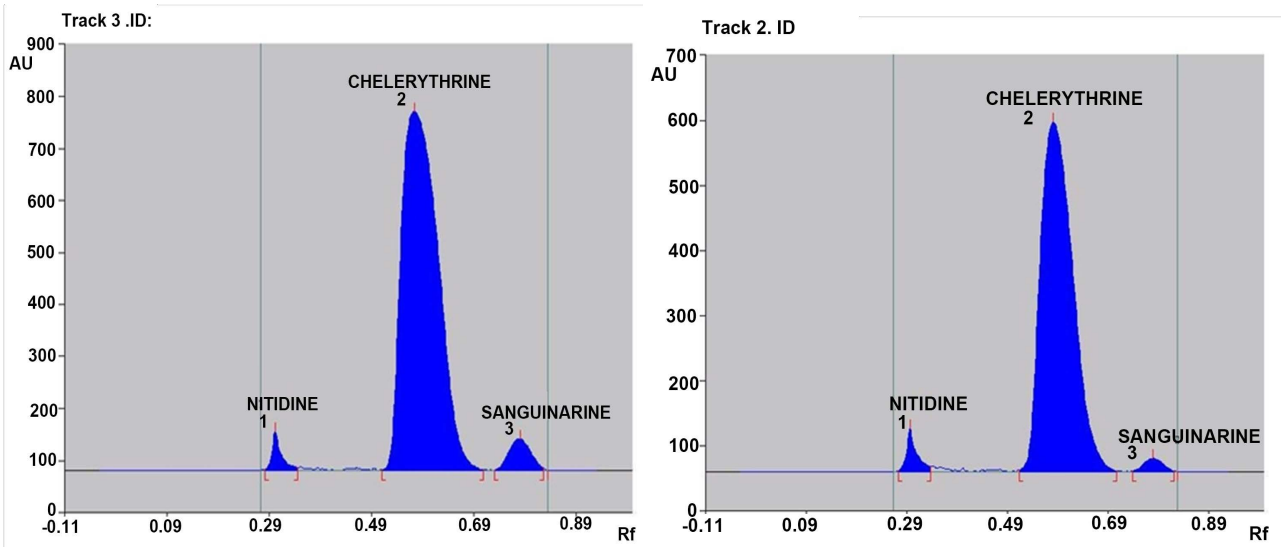
The present study deals with simultaneous quantification of three benzophenanthridine alkaloids namely nitidine, chelerythrine and sanguinarine by using densitometric HPTLC method.

The Present paper aimed to establish optimum mobile phase for TLC analysis, which would shows clear separation of nitidine, chelerythrine and sanguinarine. A number of TLC analysis as preliminary tests to separate above said alkaloids were performed by using different combinations of solvents and modifications of mobile phases. Different methods which were proposed by earlier authors for HPTLC individual quantification of nitidine (Praveena and Veeresham in 2014 and 2015 [19] [20], Baerhein *et al*., in 1983) [25], chelerythrine (Petruczynik *et al*., in 2008) [26], sanguinarine (Ghosh *et al*., in 2005, Garcia *et al*.,) ([27] [28]) for various mobile phases chloroform:methanol (7:1 v/v), n-butanol:pyridine: water (6:4:3 v/v), acetone:diisopropyl ether:diethyl amine (1:1:0.1 v/v), hexane: acetone:methanol (80:15:5 v/v), hexane:ethyl acetate:ammonia (25%) (6:4:0.1 v/v) respectively, similarly simultaneous estimation of sanguinarine and chelerythrine (Bogucka-Kocka and Zalewski in 2017 [21] and Baerhein *et al*., in 1983 [25]) for various mobile phases "toluene:ethyl acetate:methanol (83:15:2) and benzene:methanol (6:1), chloroform:ethyl acetate:methanol (2:2:1), were tried with different modifications. Because there is no report on simultaneous estimation of proposed three benzophenanthridine alkaloids, the present study was carried out with a mobile phase of ethyl acetate:methanol:water:diethylamine (30:5:2:0.5), which gave good resolution for nitidine, chelerythrine and sanguinarine with a sharp and well defined peaks at R_f = 0.28, 0.49 and 0.73 and when the chamber was saturated with mobile phase for 20 min at room temperature (25˚C ± 2˚C) during HPTLC determination of nitidine, chelrythrine and sanguinarine from plant tissue culture extracts. The plate was visualized under UV light at 280 nm without any derivatization. Identity of nitidine, chelerythrine and sanguinarine bands in sample chromatograms was confirmed by the comparison of chromatograms obtained from the sample with that obtained from the standard chromatograms (**Figure 1** and **Figure 2**) and also by comparing retention factor (Rf—0.28, 0.49 and 0.73). The peaks corresponding to nitidine, chelerythrine and sanguinarine from the sample solutions had the same retention factor as that of three standard drugs. Praveena and Veeresham in 2014 and 2015 [19] [20] reported that the nitidine has R_f value 0.28 from the roots and tissue culture extracts of *Toddalia asiatica*. Similarly Bogucka and Zalewski in 2017 [21] reported that the R_f values of chelerythine (0.35) and sanguinarine (0.45) from roots and herbs of *Chelidonium majus*. The present results are also in line with the reports of Praveena and Veeresham [20] and Bogucka and Zalewski [21].

4.2. Validation of the Proposed Method

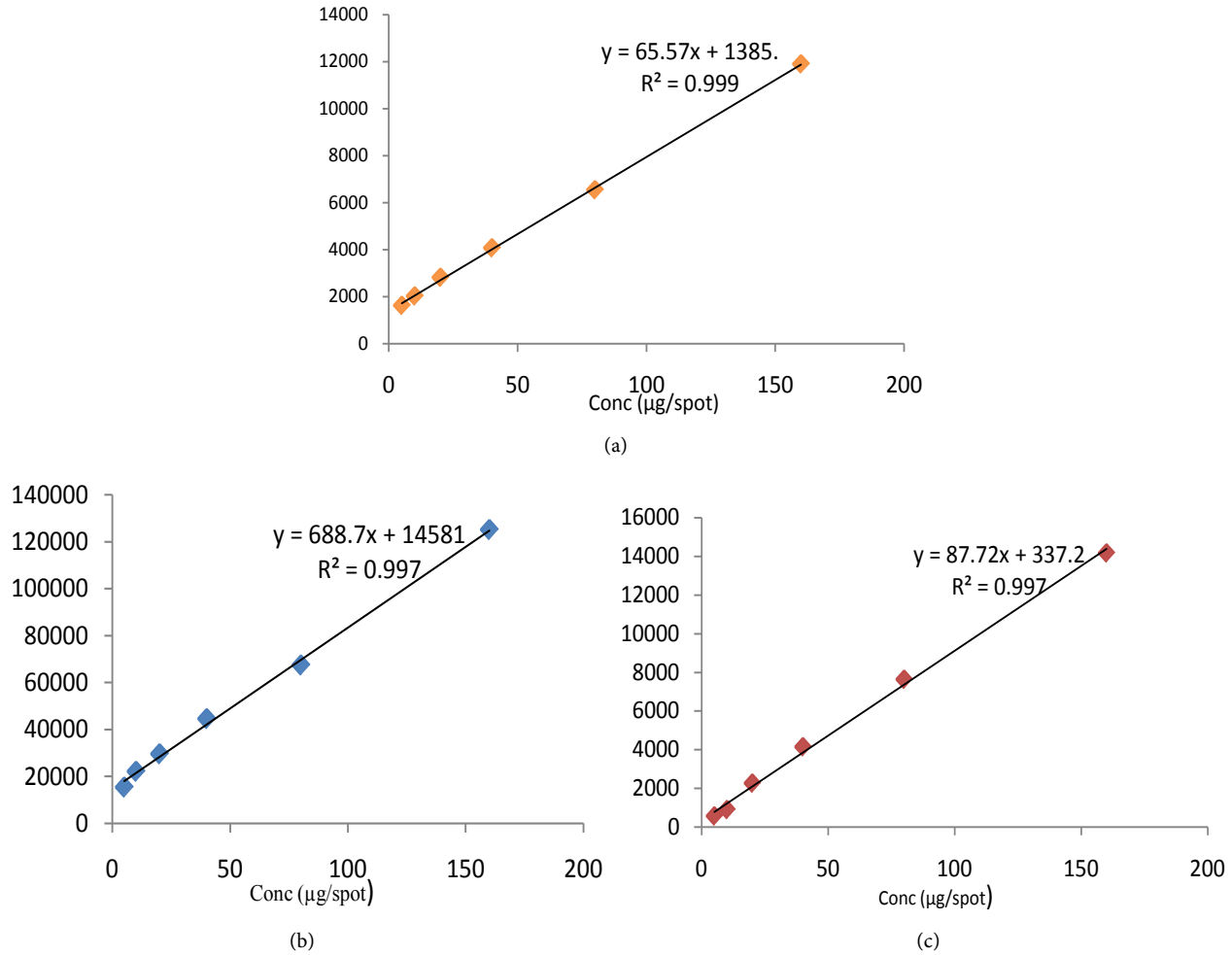
4.2.1. Linearity

Linearity was achieved with concentration range from 5 - 160 µg/band for all the



(a) (b)

Figure 1. Typical HPTLC densitogram of nitidine, chelerythrine and sanguinarine in standard (a) and sample (b).



(a) (b) (c)

Figure 2. (a) Calibration curve of standard Nitidine; (b) Calibration curve of standard Chelerythrine; (c) Calibration curve of standard Sanguinarine.

three compounds nitidine, chelerythrine and sanguinarine (**Figures 2(a)-(c)**). The Correlation coefficient, intercept and the slope were 0.998, 1447 and 65.36 for nitidine, 0.997, 14581 and 688.7 for chelerythrine and 0.997, 337.2 and 87.72 for sanguinarine respectively (**Table 1**). Praveena and Veeresham in 2014, 2015 [19] [20] reported, the linearity concentration 25 - 200 ng for nitidine, the correlation coefficient, intercept and the slope of nitidine were 0.9949, 862.9 and 34.51 respectively. Similar kind of reports were also reported by Bogucka and Zalewski 2017 [21], the linearity concentrations of chelerythrine was 10 - 100 ng and sanguinarine was 5-100 ng. correlation coefficient, intercept and the slope were 0.99996, 127.3, 51.85 and 0.9999, 149.9, 67.52. So, method is having linearity in the concentration range 5 - 160 µg/band.

4.2.2. LOD and LOQ

The values of LOD and LOQ (µg/band) of nitidine, chelerythrine and sanguinarine are 0.026, 0.088, 0.010 and 0.033 and 0.0104, 0.035 respectively and are summarized in (**Table 1**). These data shows that densitometric scanning at 280 nm is sensitive for the quantification of the tested compounds. Previous reports of LOD and LOQ values were found to be 0.026 and 0.086 for nitidine (Praveena and Veeresham 2014, 2015) [19] [20], 0.005 and 0.01 for chelerythrine, 0.002 and 0.005 for sanguinarine (Bogucka and Zalewski2017) [21].

4.2.3. Precision

From the results of repeatability and intermediate precision experiments (**Table 1**) the developed method was found to be precise as % RSD values were found to be low (<2%). So, the method was within the guidelines of ICH [29] [30].

4.2.4. Specificity

Specificity of the method was ascertained by comparing R_f values and the spectras of sample with that of standards nitidine, chelerythrine and sanguinarine (**Figure 1(a)**, **Figure 1(b)**). No interference with these peaks from other constituents of extracts was observed indicating that the proposed method is specific.

4.2.5. Accuracy

The results of recovery studies of leafy callus extracts are listed in (**Table 2**). After spiking the extract with 100 µg of each standard drugs of nitidine, chelerythrine and sanguinarine, the obtained results were within the acceptable limits demonstrating the accuracy of the method, which are 99.46% (recovery) and 0.395 (RSD%) for nitidine, 99.59% (recovery) and 0.46 (RSD%) for chelerythrine and 99.48% (recovery) and 0.565 (RSD%) for sanguinarine. Previous study of Praveena and Veeresham (2014 and 2015) the recovery of nitidine from *Toddalia asiatica* roots and tissue culture extracts (callus and shoots) were 99.67% and 99.52%, 99.11%. Similarly Bogucka and Zalewski (2017) reported the recovery of chelerythrine and sanguinarine from *Chelidonium majus* roots and herb were 98% and 96%. So, the present method is having good recovery of all these three

Table 1. Method validation data for HPTLC quantification of nitidine, chelerythrine and sanguinarine.

Parameter	Nitidine	Chelerythrine	Sanguinarine
Linearity range (μg/band)	5 - 160	5 - 160	5 - 160
Correlation coefficient (r^2)	0.998	0.997	0.997
Slope	65.36	688.7	87.72
Intercept	1447	14581	337.4
LOD [μg/band]	0.026	0.010	0.0104
LOQ [μg/band]	0.088	0.0335	0.0347
Intraday precision [%RSD, n = 3]	0.0975 - 1.906991	0.670 - 1.414	0.21 - 1.8404
Inter day precision [%RSD, n = 3]	0.460 - 1.795533	0.323 - 1.8136	0.069 - 1.79292

Table 2. Recovery studies of Nitidine, Chelerythrine and Sanguinarine in leafy callus extracts by HPTLC.

Drug name	Nitidine (μg)	Chelerythrine (μg)	Sanguinarine (μg)
Amount present	42.78	22	2.7
Amount added (n =3)	100	100	100
Amount recovered (n = 3) (mean ± sd) RSD	141.76 ± 0.778 (0.54)	121.5 ± 0.7 (0.57)	102.2 ± 0.57 (0.56)
Overall Recovery (n = 3) (%) (mean ± sd) RSD	99.46 ± 0.394 (0.395)	99.59 ± 0.69 (0.46)	99.48 ± 0.56 (0.565)

Table 3. Robustness of the HPTLC method (n = 3).

Parameter	RSD (%)
Mobile phase composition (Ethyl acetate: methanol: water: di ethylamine—28:6:3:1)	1.21
Duration of chamber saturation (30 min)	0.87
Mobile phase volume (25 ml)	0.58

alkaloids, which are much better than previous reports.

4.2.6. Robustness

The low values of the % RSD (less than 2%) for introduction of small changes in mobile phase composition, mobile phase volume and duration of mobile phase saturation time indicated the robustness of the method (Table 3).

5. Conclusion

The present study was taken into consideration for the development and validation of HPTLC densitometric method for the simultaneous quantitative estimation of nitidine, chelerythrine and sanguinarine in the callus of *Zanthoxylum rhetsa.* HPTLC method was developed and validated according to the ICH guidelines. The technique was proved to be simple, specific, accurate, robust and rapid.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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