# A validated high-performance thin-layer chromatography method for the simultaneous estimation of Jatrorrhizine, Palmatine and Magnoflorine from *Stephania glabra*.

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***Abstract***

*Stephania glabra* (Roxb.) Miers, a climbing shrub belonging to the family Menispermaceae, is well-recognized in India and distributed across tropical and subtropical regions, particularly in the Himalayan foothills at altitudes up to 2200 m. Traditionally, this plant is highly valued in Ayurveda and other medicinal systems for treating a wide range of ailments, including diarrhea, pyrexia, tuberculosis, dyspepsia, urinary tract infections, abdominal disorders, asthma, dysmenorrhea, wound healing, and headaches. Pharmacological studies reveal that its activity is primarily attributed to its alkaloids, such as stepharine, gindarine, magnoflorine, jatrorrhizine, palmatine, and others, which exhibit antimicrobial, antiplasmodial, antifungal, and neuroprotective properties.

This study aimed to validate a high-performance thin-layer chromatography (HPTLC) method for the simultaneous estimation of three major alkaloids—Palmatine, Magnoflorine, and Jatrorrhizine—in *Stephania glabra* tuber Ethanol extracts of the dried tubers were analyzed on pre-coated silica gel plates using ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:26, v/v) as the mobile phase. The plates were scanned at 254 nm and 366 nm using a TLC Scanner with vision CATS software. The method was validated for linearity, precision, accuracy, robustness, and specificity per ICH guidelines. Calibration plots for Palmatine (1000–6000 ng/spot), Magnoflorine (500–3000 ng/spot), and Jatrorrhizine (280–1680 ng/spot) demonstrated high linearity ($r \geq 0.99$). The method showed excellent reproducibility with intra- and inter-day precision, and peak purity confirmed its specificity. The developed method offers a sensitive, accurate, and economical approach for the quantitative and qualitative analysis of *Stephania glabra* tuber.

**Key words**: *Stephania glabra*, Alkaloids, Palmatine, Magnoflorine, and Jatrorrhizine

## 1. Introduction

*Stephania glabra* (Roxb.) Miers (Family: Menispermaceae) Previously, this plant was known as *Cissampelos glabra* Roxb but now it is also known as *Stephania rotunda* Lour and other vernecular names are "Purha and Paahraa" [1] It is a climbing shrub with peltate and membranous leaves. *S. glabra* is a well-recognized plant in India it is distributed tropical and sub-tropical regions of the various countries. Mainly this Plant grows on the lower parts of Himalayan region specifically in tropical and temperate regions at an altitude of 2200 m from Sindh eastwards to Khasia hills, Pegu.[2][3] *Stephania glabra* is highly valued in Ayurveda and other traditional medicinal systems in India. In traditional medicine, this plant has a good place which is known to treat many ailments such as, Diarrhea, in the treatment Pyrexia and Tuberculosis. To cure Dyspepsia and urinary tract infection, Abdominal ills, asthma and in Ascariasis, Dysmenorrhoea, Wound healing, as remedy of head-ache and It showed anti-malarial, anti-Bacterial, antiplasmodial and calcium antagonist activities. [4][5] The pharmacological activity of *Stephania glabra* mainly depends on the alkaloids gindarine and stepharine (stephaglabrine). Gindarine in an experimental model of animals showed inhibition of both spontaneous and phentermine induced locomotor activities.[6] Stepharine inhibited the activity of true and false cholinesterases and increased the sensitivity of organs to ach, and stimulated animal smooth muscles.[7][8] Eleven major alkaloid were found like stepharine, magnoflorine, menisperine, roemerine, palmatine, corydalmine, N-methylcorydalmine, columbamine, tetrahydropalmatine, jatrorrhizine and tetrandrine. These are the most important constituents of this plant and genus. [9][10] From that Jatrorrhizine, a protoberberine alkaloid is shown to have antifungal potential. Magnoflorine is a quaternary aporphine alkaloid widely distributed among different representatives of Magnoliaceae, Menispermaceae, Berberidaceae or Papaveraceae species [11] and Palmatine an isoquinoline alkaloid from the class of proto-ber-berines, is shown to mitigate the development

of Alzheimer's disease, [12][13] suppress the growth of Helicobacter pylori, to possess antibacterial potential and also to be a promising therapeutic agent in the treatment of heart hypertrophy.[14] Here validated method for the simultaneous estimation of Palmatine, magnoforine and jatrorrhizine in *Stephania glabra* tuber using high-performance thin-layer chromatography (HPTLC) was performed. The proposed method would not only serve as a useful tool for the quantitative analysis of the aforementioned biomarkers but also would signify the identity of *Stephania glabra* tuber since under the specified conditions a distinct chemo profile is generated that facilitates the qualitative analysis. The validation results have confirmed that the developed method is specific, sensitive, accurate, rapid and economical.

## 2. Experimental

### 2.1 Plant materials, chemicals, reagents and reference standards

Ethyl acetate, glacial acetic acid, formic acid and methanol (E. Merck, Mumbai, India) used were of analytical grade. Reference standard palmatine (ANJ Biomedicals, Mumbai, India) were purchased. Magnoforine and jatrorrhizine were obtained as gift samples from Pharmanza (India) Pvt. Ltd. (Gujarat, India). Dried tuber was collected and authenticated by Senior Scientist Dr. Vijay Bhatt [HRDI, Gopeshwar, Chamoli, Uttarakhand] in January 2022.The tuber were dried properly, powdered to 60 mesh, and stored in an airtight container at room temperature. Palmatine (1000µg/ml). Jatrorrhizine (140µg/ml) Magnoflorine (100µg/ml). standard stock solutions were prepared separately; 2, 4, 6, 8, 10 and 12 µl for Jatrorrhizine, 1, 2, 3, 4, 5 and 6µl for Palmatine and for Magnoflorine 5, 10, 15, 20, 25, 30 and 6µl solutions were applied on pre-coated TLC plates.

### 2.2 Sample preparation

Accurately weighed tuber powder (5.0 g) of sample *Stephania glabra* of was extracted exhaustively with ethanol by using different concentration and different acidic pH under reflux. Ethanolic extracts were concentrated to dryness under vacuum to yield 1.42 gm extract which was dark-brown and semisolid. 10 mg of extract was dissolved in 10 mL methanol and an aliquot of 30 µL of methanolic solution was applied to a TLC plate.

### 2.3 HPTLC analysis

HPTLC analysis was performed on 20 cm × 10 cm aluminum-backed HPTLC plates coated with a 0.2 mm layer of silica gel G 60 $F_{254}$ (Merck, Mumbai, India) prewashed with methanol and activated in an oven at 110 °C for 15 min prior to the analysis. The reference standards and samples were applied onto the plates in the form of 6 mm long bands, 8 mm from bottom edge of the plate and 14 mm from side edges, by means of a CAMAG (Muttenz, Switzerland) automatic TLC applicator Linomat 5. The application speed was 150 nL s –1. The plates were developed to a distance of 80 mm with ethyl acetate–formic acid–glacial acetic acid– water (100:11:11:26, V/V) (Wanger et al. 1984) in a CAMAG twin-trough chamber saturated with mobile phase vapor for 30 min prior to the development. After development, the plate was air-dried and scanned at 254 nm in absorbance–reflectance mode and 366 nm by means of TLC Scanner 4 (CAMAG) with vision CATS software, version 2.5.18262.1, using a deuterium lamp.

### 2.4 Validation

#### 2.4.1 Method validation

The HPTLC method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guideline for linearity, precision, accuracy and recovery, limit of detection, limit of quantification, specificity, robustness and ruggedness. All measurements were performed in triplicates.[15][16]

#### 2.4.2 Linearity and limits of quantification and detection

Calibration plots of peak area against concentration were linear in the rang 1000-6000 ng/spot of Palmatine which was accordingly 1,2,3,4,5 and 6µl Volume Spotted, Megnoflorine 500-3000ng/spot which was accordingly 5, 10, 15, 20, 25, 30µl Volume Spotted and for Jatrorrhizine concentration range 280, 560, 840, 1120, 1400 and 1680ng/spot which was accordingly 2, 4, 6, 8, 10 and 12µl Volume Spotted in application of linear regression analysis and the intercept values were not significantly different from zero. The correlation coefficient (r) was 0.99 for each equation. The limits of quantification (LOQ) and detection (LOD) were calculated using regression analysis tool in Excel (Microsoft Corporation, Redmond, WA, USA).

#### 2.4.3 Accuracy and precision

The standard addition method was used to determine the accuracy of the method. Known amounts of Palmatine, Magnoforine and Jatrorrhizine standard solutions (80%, 100% and 120%) were added at three different levels and analysis was done as described above. The accuracy determination was repeated three times. Method precision was performed using six different plates developed from different extraction processes for Palmatine, Magnoforine and Jatrorrhizine. Repeatability was assessed by applying three concentrations of the standards in triplicate on the plates separately, on the same day, and analyzing by the above procedure. The Inter-day precision of the method was determined in the same way as repeatability. Intra-Day Reproducibility variation in results within same day is called intraday variation. It was determined by repeating calibration curve 3 times on same day at 3 different concentrations.

#### 2.4.4 Robustness

Robustness is a measure of the capacity of a method to remain unaffected by small but deliberate variations in the method conditions and is an indication of the reliability of the method.

#### 2.4.5 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately in the presence of other component like synthetic precursor, excipients, degrades or matrix component that may be expected to be present in the sample. Purity of spectra was determined at three different levels, at starting, middle and end. The correlation between them was considered for determination of peak purity. It was determined by spotting working standard solution on pre-coated TLC plate and the plate was developed, dried, reprivatized and analyzed.

**2.5 Quantification of Palmatine, Magnoforine and Jatrorrhizine**

Test solution of 30 µL (1 mg mL–1 ) was spotted along with Palmatine 1000-6000 ng/spot which was accordingly 1,2,3,4,5 and 6 µl Volume Spotted for Megnoflorine500-3000ng/spot which was accordingliy5, 10, 15, 20, 25, 30µl and for jatrorrhizine 280, 560, 840, 1120, 1400 and 1680ng/spot from that 2, 4, 6, 8, 10 and 12µl Volume Spotted in application of linear regression analysis on the HPTLC plate. The peak areas were noted and quantifications of palmatine, magnoforine and jatrorrhizine were performed using linear regression equations of the respective compounds.


*Figure 1 Calibration curve of standard at 254 nm*

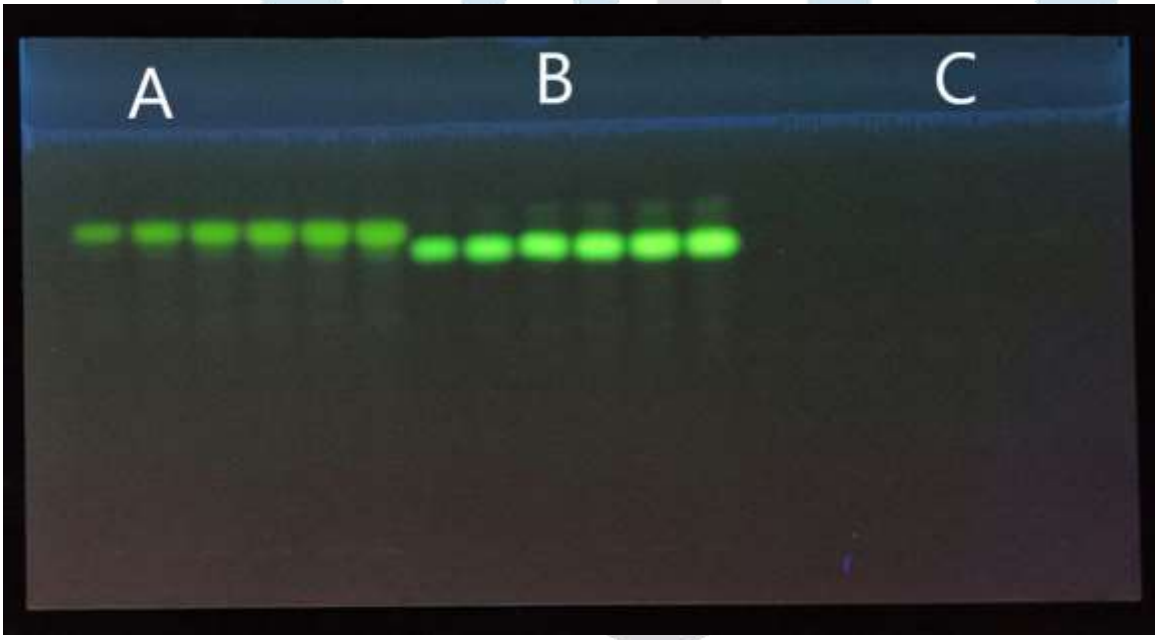


*Figure 2 Calibration curve of standard at 366 nm*

**3 Results and discussion**

**3.1 Developing solvent system**

HPTLC fingerprint patterns have been therefore evolved for the methanolic extracts of the tuber of *Stephania glabra* palmatine, magnoforine and jatrorrhizine standards were quantitated accurately using silica gel F254 HPTLC pre-coated plates with the mobile phase using ethyl acetate– formic acid–glacial acetic acid–water (100:11:11:26, V/V); the RF values were 0.49±0.02 for palmatine, 0.27±0.02 for magnoforine and 0.56±0.02 for jatrorrhizine. The chromatograms of palmatine, magnoforine and jatrorrhizine and the methanolic extracts of the tubers of *Stephania glabra* are shown in Fig. The present study is the first report on the use of the aforementioned mobile phase that otherwise is commonly used for the separation of polar compounds like flavonoid glycosides, procyanidins, etc. from botanical extracts. The chromatograms were obtained at two wavelengths (254 nm and 366 nm) but as magnoforine is a non-fluorescent compound and since we aim to simultaneously estimate the aforementioned bioactives, scanning at 254 nm is proposed.

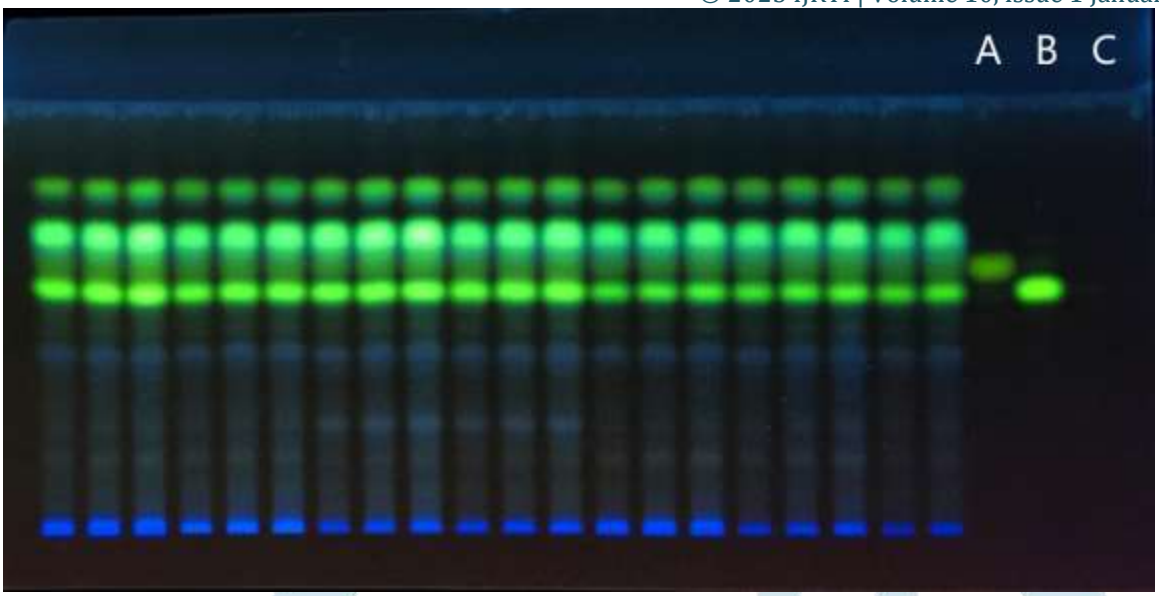


*Figure 3 HPTLC Plate along with standard at 366 nm*

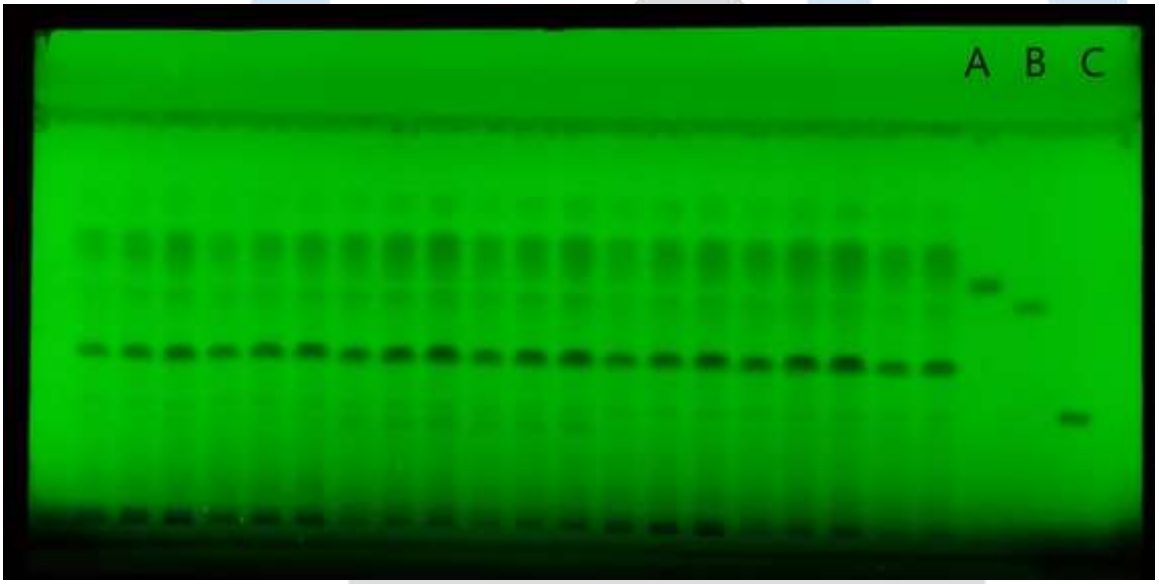


*Figure 4 HPTLC Plate along with standard at 254 nm*

**3.2 Method validation**

**3.2.1 Linearity and limits of quantification and detection**

The calibration plots indicate that the peak area response was a linear function of the amounts of standards (palmatine, magnoflorine and jatrorrhizine). The linear regression equations, derived by plotting concentration against peak area, were Y = 0.474x+391.5, 0.3218x + 155.36, 0.4539x+98.86, respectively, for palmatine, magnoforine and jatrorrhizine. It has been noted in linearity studies that the peak area is directly proportional to concentration ($r^2$=0.99) in the case of each standard. Quantification was achieved with linear calibration curves at concentration ranges of 1000-6000 ng/spot for palmatine, while 500–3000 ng/spot for magnoflorine and 280, 560, 840, 1120, 1400 and 1680ng/spot for jatrorrhizine, indicating that the method is sensitive.

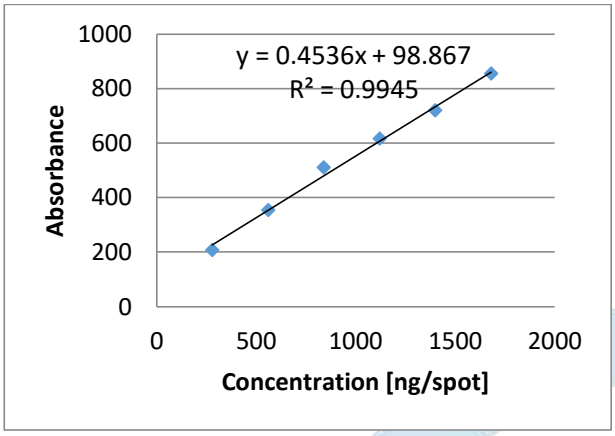


*Figure 5 Calibration curve of Jatrorrhizine*

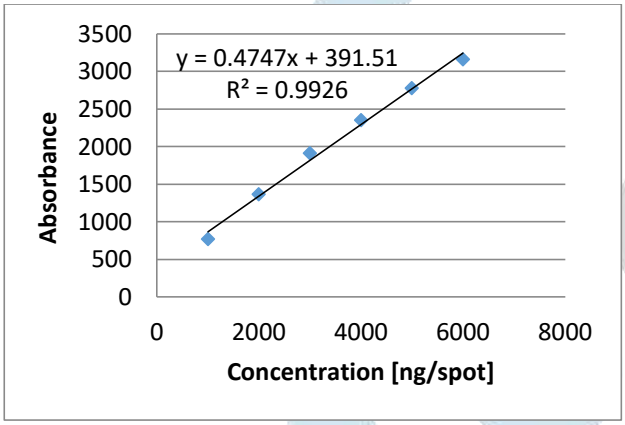


*Figure 6 Calibration of curve Palmatine*

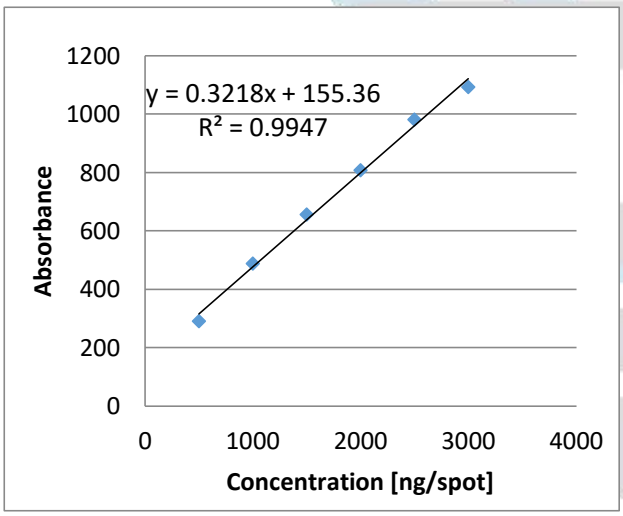


*Figure 7 calibration curve of Magnoflorine*

**3.2.1.1 Limit of detection**

The minimum detectable limit was found to be 34.301, 40.001 and 45.248 ng/band for Jatrorhizine, Palmatine and Mengoflorine respectively.

**3.2.1.2 Limit of Quantification**

The minimum quantifiable limit was found to be 103.9426, 121.2152 and 137.1153 ng/band for Jatrorhizine, Palmatine and Mengoflorine respectively.

**3.2.1.3 Accuracy**

Accuracy was performed by spiking standards at three levels (80, 100 and 120%) to the extract solution. Study for n=3 were performed and average of recovery at all the three different levels was calculated and the obtained data have been shown in the table. Recovery study

reflected that method is accurately measuring the standards Jatrorhizine, Palmatine and Mengoflorine with average % Recovery 96.88, 95.06 and 98.29 respectively.

**3.2.1.4 Precision**

The intraday coefficient of variation for jatrorrhizine is 0.21 to 1.14 for palmatine it is from 0.1 to 0.6 and megnoflorine 0.36 to 0.92 respectively and the interday for jatrorrhizine is 0.28 to 2.28 for palmatine 0.21 to 1.06 and for megnoflorine 0.5 to 2.25.

**3.2.1.5 Specificity**

It was observed that the other herbal constituents present in the extract did not interfere with the peaks of palmatine, magnoforine and jatrorrhizine. Therefore, the method is specific. Good correlations, r=0.99 all the 3 standards, were obtained between the standard and sample overlaid spectra of palmatine, magnoforine and jatrorrhizine.

**3.2.1.6 Robustness**

Parameters are saturation time (30 min ± 5 min) of mobile phase and quantity of solvent system volume (20+1 ml) and Saturation time were done Result for saturation time1.426 to 2.410, 0.363 to 0.671, 0.363 to 0.679 for jatrorrhizine, palmatine and megnoflorine respectively. Result of solvent system (mobile phase) are 0.474 to 1.035, 0.437 to 1.059 and 0.437 to 1.059 for jatrorrhizine, palmatine and megnoflorine respectively.

All three phyto-constituents showed quantifiable content in test extracts, The compounds show clear resolution from other compounds with minimum 0.5 cm distance. All bands show complete symmetry with no tailing.

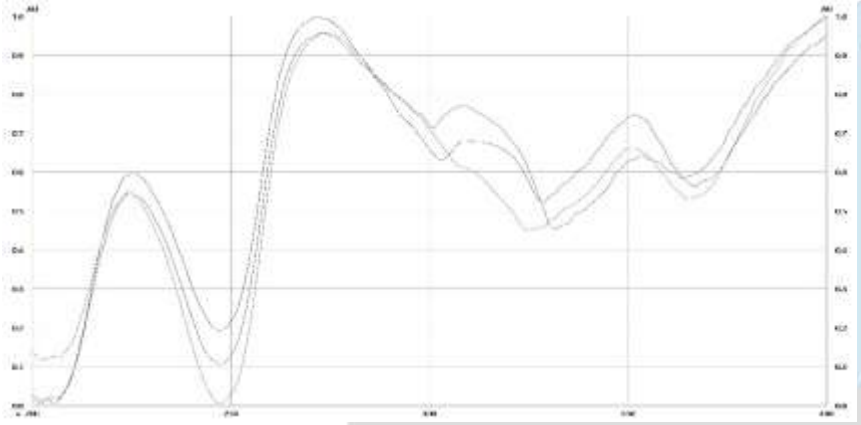

*Figure 8 Jatrorrhizine standard with two test sample*

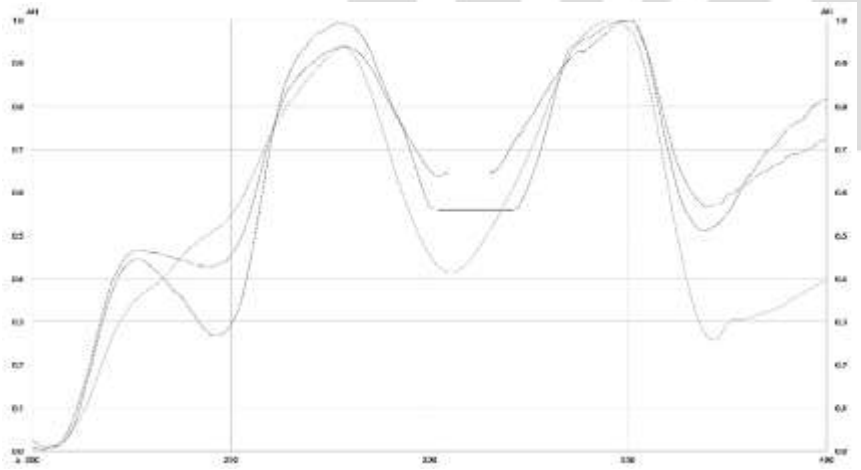

*Figure 9 Palmatine standard with two test sample*

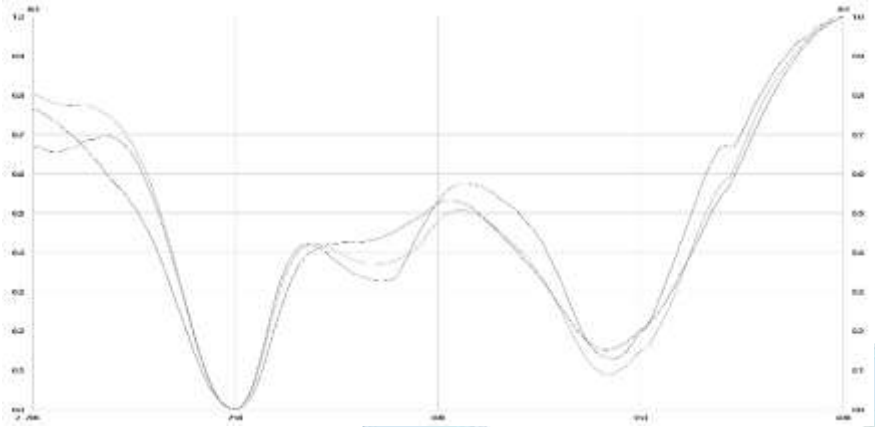

*Figure 10 Magnoflorine standard with two test sample*

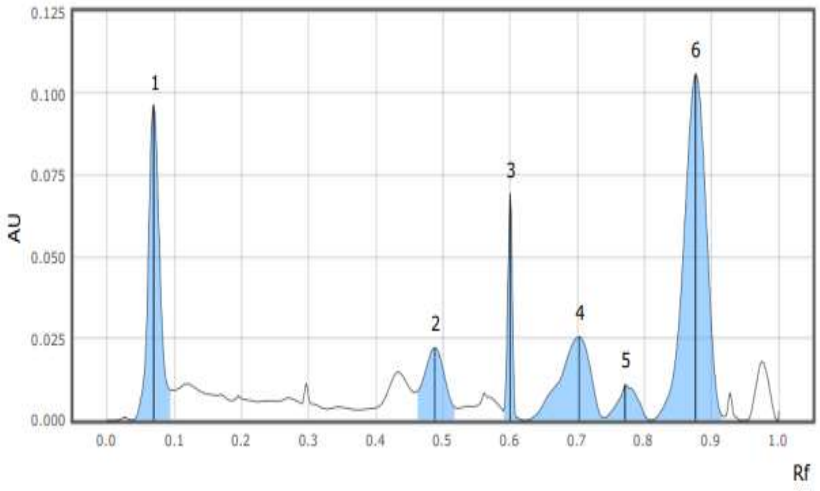


*Figure 11 Densitometric chromatogram*

A- Jatrorrhizine
B- Palmatine
C- Magnoflorine

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