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Original Article

A validated and densitometric HPTLC method for the simultaneous quantification of reserpine and ajmalicine in *Rauvolfia serpentina* and *Rauvolfia tetraphylla*

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ABSTRACT

High performance thin layer chromatographic method (HPTLC) has been developed for the quantification of reserpine and ajmalicine in root part of two different population of *Rauvolfia serpentina* (L.) Benth. ex Kurz and *Rauvolfia tetraphylla* L., Apocynaceae, collected from Punjab and Uttarakhand. HPTLC of methanolic extract of root containing indole alkaloids, *i.e.*, reserpine and ajmalicine, was performed on TLC Silicagel 60 F_{254} (10 cm × 10 cm) plates with toluene:ethyl acetate:formic acid (7:2:1), as mobile phase. Quantification of the reserpine and ajmalicine was performed in the absorption–reflection mode at 268 nm. The recovery of reserpine and ajmalicine were 99.3 and 98.7% respectively. The calibration curves were linear for both the reserpine and ajmalicine, in the range of 200–1200 ng. HPTLC densitometry has been performed for the estimation of reserpine and ajmalicine in root part of *R. serpentina* and *R. tetraphylla* for the first time. The method is simple, rapid and cost effective and can be used for routine analysis of ajmalicine and reserpine in different *Rauvolfia* species as well as for quality control of herbal drugs containing *Rauvolfia* species.

Introduction

Indole alkaloids are the bioactive compounds derived from plants which possess an array of pharmacological properties such as anticancer (Wang et al., 2015; Zhu et al., 2015), antimalarial (Chierrito et al., 2014), antimicrobial (Cheenpracha et al., 2014) and cerebroprotective (Biradar et al., 2013) properties. The major bioactive alkaloids reported from various *Rauvolfia* species are reserpine, reserpiline, ajmaline, ajamalacine, rauvolfinine, serpinine, serpentine, serpentinine, yohimbine, vomilenine, picrinine, vinorine, norseredamine, seredamine (Stöckigt et al., 1981; Batista et al., 1996; Duez et al., 1986; De Bruyn et al., 1989; Kato et al., 2002; Bindu et al., 2014). Reserpine and ajmalicine are considered as two major indole alkaloids from various *Rauvolfia* species. Reserpine is a common alkaloid known to depress the central nervous system and to lower blood pressure (Faisal et al., 2005). Reserpine has been reported for antihypertensive (Shamon and Perez, 2009), neuroprotective (Arya et al., 2009) and anticancer (Abdelfatah and Efferth, 2015) properties whereas ajmalicine has been assessed for central depressant and adrenergic blocking (Bhargava and Borison, 1957), antihypertensive and cytotoxic (Fernández-Pérez et al., 2013) activities.

Genus *Rauvolfia*, belonging to the family Apocynaceae, comprises around 80 species which are distributed in tropical climatic conditions. Traditionally, *R. serpentina* (L.) Benth. ex Kurz, commonly known as *Sarpagandha*, was reported against snakebite, insomnia, melancholia, schizophrenia or more violent mental disorders, diarrhea, dysentery, cholera and colic, scabies, malaria, eye inflammation, etc. (Dey and De, 2010, 2012). *R. serpentina* has an economical importance and the root part of the plant is used in many Ayurvedic polyherbal formations i.r sarpagandha vati. *Rauvolfia tetraphylla* L. popularly known as “devil pepper” or “be still tree” is an endangered woody shrub native in tropical Americas (Faisal et al., 2013). Ethnomedicinal importance of *R. tetraphylla* was found in terms of its use against snakebite, to stimulate uterine contraction and to facilitate difficult childbirth cases (Sarma et al., 1999; Dey and De, 2012). Moreover, *R. serpentina* has been reported for pharmacological properties such as antbacterial, anti-inflammatory and cytotoxicity (Dey and De, 2010). *R. tetraphylla* has also been reported to possess antipsychotic (Gupta et al., 2012a), antibacterial activity and anti-inflammatory (Ganga Rao et al., 2012) properties.

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Earlier *Rauvolfia* alkaloids were analyzed by quantitative thin layer chromatography (Habib and Court, 1971; Nguyen and Nikolova, 1989). HPLC and TLC identification, estimation and separation of indole alkaloid from *R. serpentina* and *R. vomitoria* was also achieved (Cieri, 1983; Klyushnichenko et al., 1994). Reserpine and rescinnamine in *R. serpentina* preparations was detected by liquid chromatography with fluorescence (Cieri, 1987). Recently, HPLC-UV and GC–MS were applied to determine indole alkaloids and related compounds in *R. verticillata* (Hong et al., 2013). In addition reserpine, ajmaline, and ajmalicine in *R. serpentina* were determined by reversed-phase HPLC (Srivastava et al., 2006). HPTLC, HPLC, and densitometry were used for the separation of different indole alkaloids from *R. serpentina* roots (Gupta et al., 2006). Quantitative densitometric determination of reserpine and ajmaline by HPTLC was performed in *R. vomitoria* (Katič et al., 1980). However, there is no HPTLC report on simultaneous quantification of reserpine and ajmalicine in different populations of *Rauvolfia* species. Altitudinal and seasonal modulation of stigmasterol was determined in *R. serpentina* following HPTLC methods (Dey and Pandey, 2014a,b). In continuation to our studies on HPTLC profiling of medicinal plants (Pandey et al., 2015, 2016), here we report the simultaneous HPTLC determination of two indole alkaloids, ajmalicine and reserpine in two wild population of *R. serpentina* and *R. tetraphylla*.

Material and methods

Chemicals and plant materials

The analytical grade chemicals used in experiment were purchased from E. Merck, India. The HPTLC plate Silica gel $60F_{254}$ (10 cm × 10 cm) used in the experiment were purchased from E. Merck (Darmstadt, Germany). The standards, reserpine (A) and ajmalicine (B) were purchased from Hi media, India.

The plant samples of *Rauvolfia* species was collected from different areas of Phagwara (Punjab) and Dehradun (Uttarakhand) in the months of September–October 2014. The plant material collected from different places was authenticated on the basis of morphological characters by Taxonomist in the Department of Botany, Lovely Professional University, Phagwara (Punjab). A voucher specimens (Voucher No. 121214 and 151214 of *R. serpentina* (L.) Benth. ex Kurz and *R. tetraphylla* L., respectively) were deposited at the Department of Biotechnology, Lovely Professional University for future reference.

Preparation of sample solution

The air dried (25–30 °C) root parts of *Rauvolfia* species (1 g) were extracted thrice with 20 ml of methanol for 45 min by reflux method at 70 °C in temperature controlled water bath. The methanolic extracts were transferred in conical flask and were concentrated and re-dissolved in 1 ml of methanol.

Preparation of standard solutions

Stock solutions of reserpine and ajmalicine (1 mg ml^{-1}) were prepared by dissolving the ajmalicine and reserpine in methanol respectively, and different amounts (2, 4, 6, 8, 10 and 12 μl) of these were loaded to a TLC plate, using an Linomat applicator V for preparing calibration curves.

Chromatography

A CAMAG HPTLC scanner equipped with an automatic Linomat-V automatic sample applicator, TLC scanner III, and integrated software WINCATS version: 1.4.4.6337 was used for the analysis of indole alkaloids in different samples. The stationary phase was pre-coated silica gel HPTLC $60F_{254}$ (10 cm × 10 cm) plate of 0.20 mm layer thickness used for the quantification of reserpine and ajmalicine in *Rauvolfia* species. The plant samples and the standards were loaded with the help of Linomat applicator V on the TLC plate at 8 mm wide bands with constant application rate of 100 nl s^{-1} under a flow of N_2 gas. The loading of the samples on the TLC plate was done by keeping space of 15 mm from the bottom and 15 mm from the side, and the space between two spots was maintained 14.4 mm of the plate.

Detection and estimation of reserpine and ajmalicine

The TLC plate was kept in a Camag twin trough chamber (10 cm × 10 cm), which was pre-saturated with 25 ml mobile phase with toluene:ethylacetate:formic acid (7:2:1) for 30 min, at room temperature (28 ± 2 °C) and 55 ± 5% relative humidity. The length of the chromatogram run was 80 mm from the base and the TLC plate was dried by using an air dryer, in a wooden chamber. Quantitative evaluation of the plate was performed in the absorption–reflection mode at 268 nm, slit width 4 mm × 0.30 mm, data resolution 100 μm $step^{-1}$ and scanning speed 20 mm s^{-1}. The radiation used in the analysis was deuterium and tungsten lamp. Each analysis was carried out in triplicate.

Validation of HPTLC densitometry method

Linearity

Stock solutions of reserpine and ajmalicine were prepared in methanol and different amounts (2, 4, 6, 8, 10 and 12 μl) of these were loaded onto a TLC plate, using Linomat applicator V for preparing six points calibration curves. The regression equation and correlation coefficient were from calibration curves, for reserpine, $Y = 538 + 431.15\,X$ and 0.991 and for ajmalicine, $Y = -108.53 + 370\,X$ and 0.994 (Table 1 and Fig. 1).

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) was determined by loading the blank methanol on the TLC plate following the method as explained before. The signal-to-noise ratio was determined as 3:1 and 10:1 considered for LOD and LOQ, respectively. The LOD and LOQ for the reserpine were 60 ng and 180 ng respectively. The LOD and LOQ for the ajmalicine were 50 ng and 150 ng respectively.

Accuracy

To the pre-analyzed sample, 50 and 100 μg each of reserpine and ajmalicine were added in the 100 mg root powder of high yielding *R. serpentina* root samples (reserpine 180 μg and ajmalicine 170 μg) and the mixture was analyzed by the proposed method. The experiment was conducted in triplicate to check recovery and accuracy

Table 1
Reserpine and ajmalicine content (ng μl^{-1}) found in different population of plant *Rauvolfia serpentina* and *R. tetraphylla* by HPTLC method ($n = 3$) collected from Uttarakhand (RS-1 and RT-1) and Punjab (RS-2 and RT-2).

Sample	Average reserpine (% dry wt.)	%CV	Average ajmalicine	(%dry wt.)CV
RS-1	0.18	1.46	0.17	1.55
RS-2	0.17	1.83	0.16	1.68
RT-1	0.16	2.42	0.15	2.67
RT-2	0.15	2.01	0.14	3.87

RS-1, *Rauvolfia serpentina* root 1; RS-2, *Rauvolfia serpentina* root 2; RT-1, *Rauvolfia tetraphylla* root 1; RT-2, *Rauvolfia tetraphylla* root 2.

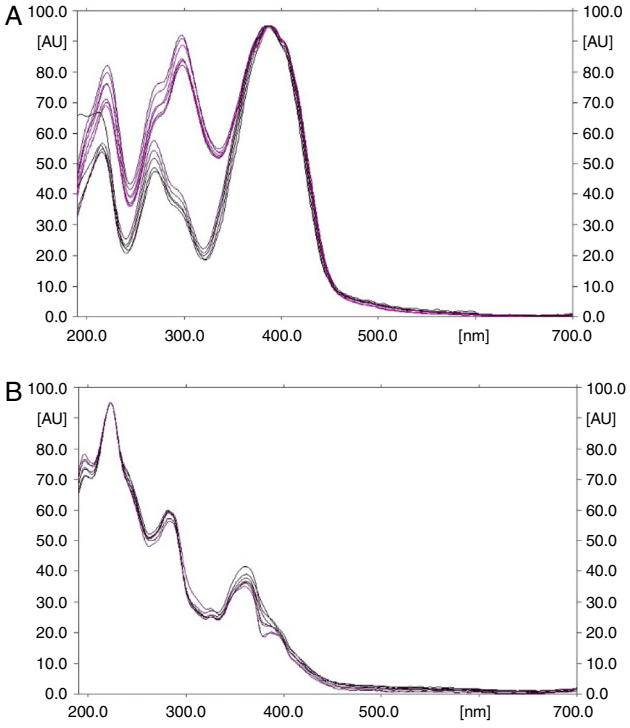

Fig. 1. (A) Overlay spectra of Reserpine in all the track. (B) Overlay spectra of ajmalicine in all the track.

of the system. The recovery for reserpine was found to be 99.3% and 99.6% and ajmalicine were 99.2% and 99.5%, respectively.

Precision

Six samples of same concentrations each from the stock solutions of reserpine and ajmalicine and spotted on the HPTLC silica gel $60F_{254}$ plate and analyzed with the proposed method and were expressed in % CV for the system precision. For method precision six samples of same concentrations were applied on a HPTLC plate and analyzed by the proposed method to determine variation expressed in % CV. The results for reserpine and ajmalicine were found to be 1.28% and 1.56%, respectively.

Results and discussion

Various compositions of mobile phases were tested to get better resolution of reserpine and ajmalicine. The resolution of reserpine

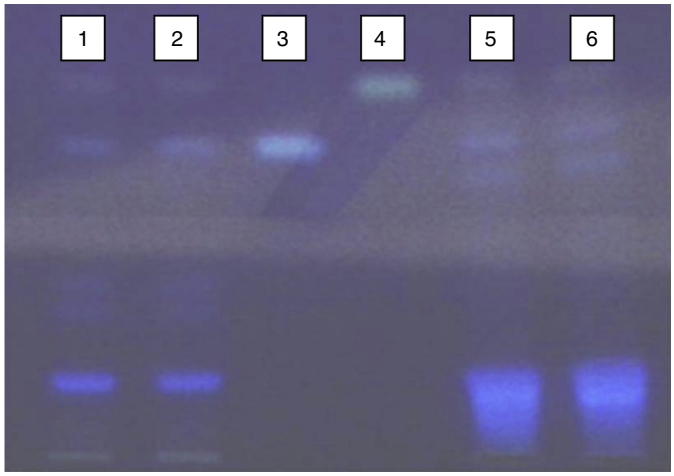

Fig. 3. Track: 1–6 represents the fingerprinting chromatogram: first two are *Rauvolfia serpentina*, root samples collected from (1) Dehradun (Uttrakhand) and (2) (Punjab), third and fourth represents the standard of reserpine (3) and ajmalicine (4), Fifth and sixth showing *R. tetraphylla* root samples collected from two states Dehradun (5) and Jalandhar (6).

and ajmalicine, with symmetrical and reproducible peaks, was achieved by using mobile phase consisting of toluene:ethyl acetate:formic acid (7:2:1). Peaks corresponding to reserpine and ajmalicine were recorded at R_f 0.69 and 0.85, respectively (Figs. 2 and 3). The methanolic extract of *Rauvolfia* species, when subjected to HPTLC, showed the presence of reserpine and ajmalicine peaks in all the samples. Comparison of the UV spectral characteristic of the peaks for standards of reserpine and ajmalicine, revealed the identity of reserpine and ajmalicine present in all samples. The calibration curves were linear in the range of 200, 400, 600, 800, 1000, 1200 ng for reserpine and ajmalicine. Peak purity tests of reserpine and ajmalicine were also conducted by comparing spectra of standard with reserpine and ajmalicine in *Rauvolfia* species sample track (Figs. 1, 3 and 4). The higher contents (0.17% and 0.18% of ajmalicine and reserpine) in roots of *R. serpentina* and *R. tetraphylla* (0.16% and 0.15%) were recorded in roots of plant samples collected from Dehradun (Uttarakhand) than compared with the plant samples collected from Jalandhar (Punjab) as compared to roots (Table 1).

Earlier, hexane, chloroform, methanol, and water extracts of *R. serpentina* roots were reportedly contained marker indole alkaloids, ajmaline, ajmalicine, and reserpine. Chloroform was cited as the most potent solvent for extraction of these alkaloids whereas defatting with hexane was indicated for the loss of the alkaloids (Gupta

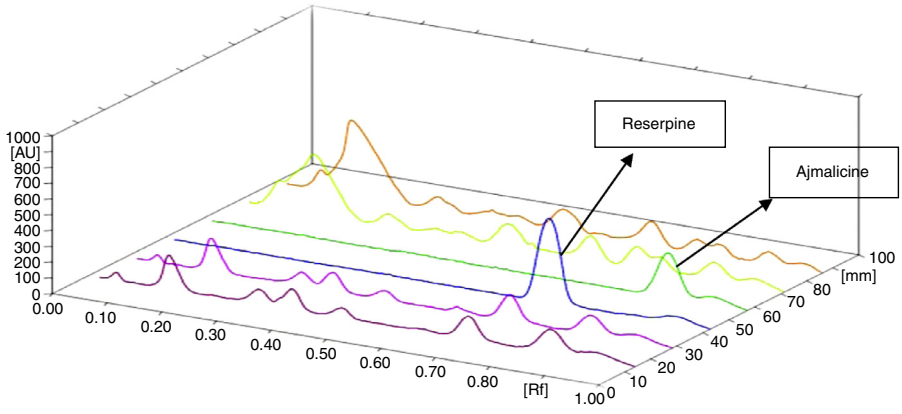

Fig. 2. HPTLC 3D overlay densitogram of standards reserpine and ajmalicine and resolution of reserpine and ajmalicine in different samples of *Rauvolfia species*.

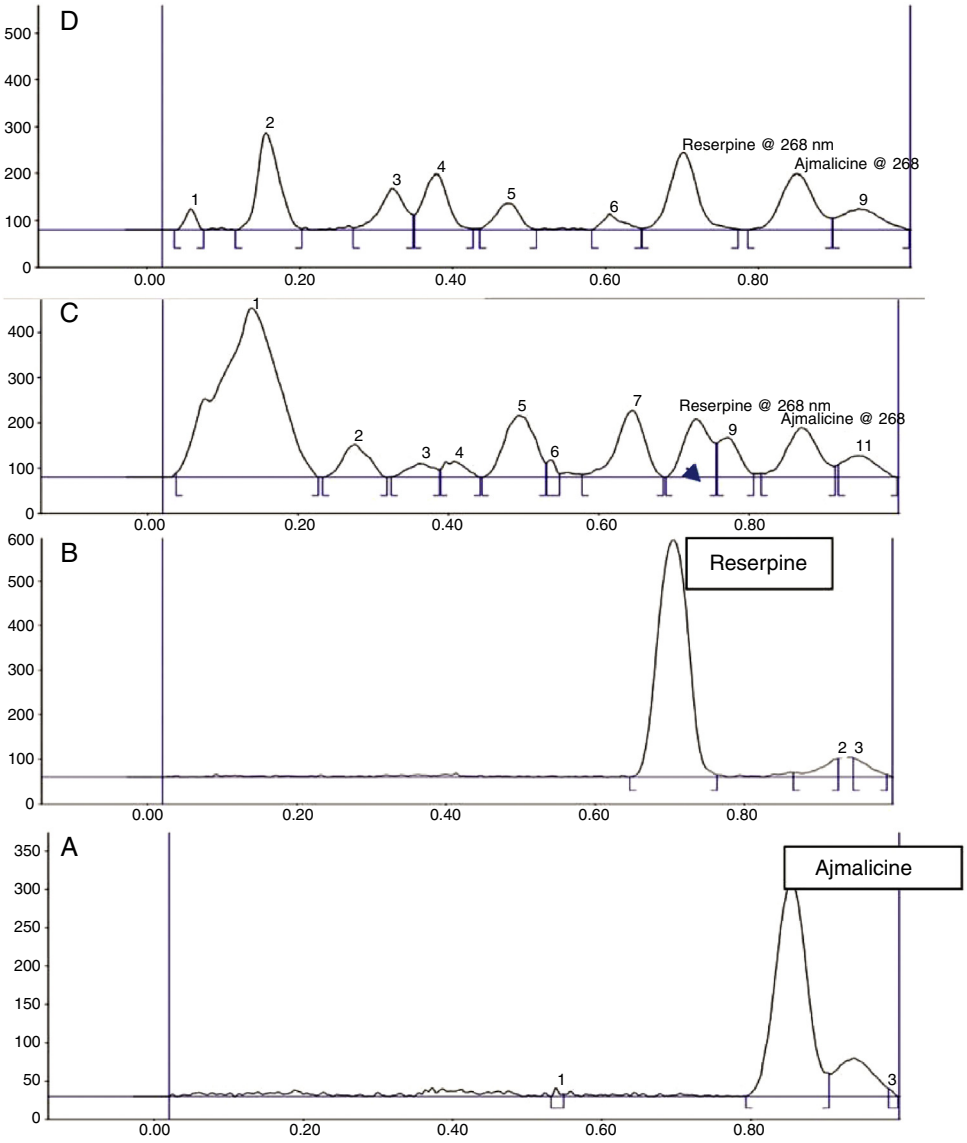

Fig. 4. (A) Standard Ajmalicine, (B) Standard Reserpine, (C) Reserpine and Ajmalicine in root part of *Rauvolfia tetraphylla* collected from Dehradun, (D) Reserpine and Ajmalicine in root part of *Rauvolfia serpentina* collected from Dehradun.

et al., 2006). Extraction efficiency of the targeted indole alakaloids with "green" solvents was studied using conventional, ultrasonication and microwave techniques (Gupta et al., 2012b). In the present study we could observe the maximum contents of ajmalicine and reserpine in roots. Tissue specific modulation of secondary metabolites has been reported in various botanicals (Williams and Ellis, 1989; Hartmann and Dierich, 1998; Baranska et al., 2006; Lim et al., 2010; Dey et al., 2016) which might be implicated to the biosynthesis and accumulation of specific metabolites depending on presence of specific enzymes (Facchini, 2001).

Conclusion

HPTLC densitometry is a rapid, reproducible, accurate, and selective alternative to HPLC for the separation of the ajmalicine and reserpine in *R. serpentina* and *R. tetraphylla.* Further, roots of *R. serpentina* are enriched in the desired constituents and canbe used for preparations of polyherbal formulations. It was found that the quantity of indole alkaloids in *Rauvolfia tetraphylla* was at par to *R. serpentina* and can be used in the preparation of herbal formulation. The advantage of HPTLC is the high sample throughput which results from the small amount of sample preparation and lesser use of solvent in separation of the compounds and the simultaneous quantification of several samples.

Authors' contributions

The collection and cultivation of the plant samples were done by Radha while the identification and HPTLC quantification was done by DKP and AD.

Conflicts of interest

The authors declare no conflicts of interest.

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