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Alkaloid Profiling and Estimation of Reserpine in *Rauwolfia serpentina* Plant by TLC, HP-TLC and HPLC

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Abstract: In the present investigation, alkaloid profiling of *Rauwolfia serpentina* plant samples, including roots, leaves and callus, were performed which were collected from different localities. The CAF of samples suggest that roots are rich in alkaloid content as compared to leaves and callus. The maximum CAF was obtained from the *in vitro* regenerated roots (496 mg g^{-1}). The TLC analysis of all the samples, including roots, leaves and callus, showed that roots are rich in reserpine and they also contain other alkaloids besides reserpine. Callus tissues also contain reserpine, but in the leave samples alkaloids could not be estimated. However, reserpine was detected in all the root and leaf samples by HP-TLC. The alkaloid profiling through HPLC detected a number of alkaloids from all the plant samples. The highest reserpine content was obtained from the *in vitro* regenerated roots (33 mg g^{-1}) and the least from the leaves.

Key words: Thin layer chromatography, high performance-thin layer chromatography, high performance liquid chromatography, *Rauwoolfia serpemtina*

INTRODUCTION

Tropical plant *Rauwolfia serpentina* L. (Apocyanaceae) is a source of various principal bioactive Terpenoid Indole Alkaloids (TIA) such as reserpine, ajmaline, ajmalicine, ajmalidine, sarpagine, serpentine, raucaffricine, recinnamine, yohimbine, etc. These alkaloids are employed for the treatment of several diseases such as cardiovascular disorder, hypertension, snake bite, rheumatism, insanity, epilepsy and eczema (Joshi and Kumar, 2000; Manuchair, 2002). Recent advances in the metabolic engineering offer unprecedented opportunities to exploit the biochemical potential of plants to produce and design novel compounds. It becomes possible to isolate these medicinally important metabolites apart from their natural sources and metabolomics will ultimately allow us to produce these high-value compounds biotechnologically in cell or organ cultures.

HPTLC and HPLC were used for separation of *R. serpentina* extracts. Different chromatograms of *R. serpentina* root extracts revealed the presence of three marker indole alkaloids, ajmaline, ajmalicine and reserpine, in all four extracts. Use of chloroform resulted in most efficient extraction of these three alkaloids (Gupta *et al*., 2006).

Reserpine is an indole alkaloid chemically it is (3β, 16β, 17α, 18β, 20α)-11, 17-dimethoxy-18 [(3,4,5-trimethoxybenzoyl)-oxyl] yohimbine-16-carboxylic acid methyl ester or 3,4,5-trimethoxybenzoyl methyl resperate, used in lowering blood pressure and as a tranquilizer (Adegoke *et al*., 2007).

Understanding how environmental factors affect the production of secondary metabolites will be of great importance for the conservation of medicinal plants and optimizing field growth conditions for maximal recovery of phytomedicinal chemicals (Cai *et al*., 2009). Kumar *et al*. (2010a) studied the effect of geographical conditions on the reserpine content through TLC, HP-TLC and HPLC. Significant variation in the content of reserpine (TIA) has been reported in wild, cultivated and *in vitro* regenerated plants/plant parts of *R. serpentina.*

Quantification of reserpine through HPTLC from *Rauwolfia serpentina* was performed and the method was found as very precise, sensitive, specific and reproducible with an average recovery of 98% (Kumar *et al*., 2010b). The aim of this was to investigate reserpine in *Rouwolfia serpentina*.

MATERIALS AND METHODS

Rauwolfia serpentina plants were regenerated *in vitro* from shoot tip and excised shoot tip explants by multiple shoot induction and subsequent rooting and hardening (Singh and Guru, 2007).

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Extraction and estimation of alkaloids: Total alkaloid contents were estimated from different plant parts of *R. serpentina,* (e.g., callus, leaves and roots) collected from different habitats as well as *in vitro* regenerated plants. Hundred gram of powdered dry samples of *R. serpentina* were soaked in 10.0 mL methanol and left for 30 min. After 30 min, the soaked plant material was filtered. The residue obtained after filtration is further dissolved in 5.0 mL methanol and filtered after 10 min, the same step is repeated once again and the final filtrate is collected in 50 mL conical flask. The extract was evaporated to dryness in the soxhlet evaporator. The crude extract was dissolved in 100 mL of 0.01 M HCl. The pH of filtered solutions were adjusted to 6.0 with 0.01 M NaOH. The crude extracts obtained were used for TLC, HP-TLC and HPLC analysis (Klyushnichenko *et al*., 1995). The crude extract obtained, was concentrated to dryness to yield Crude Alkaloid Fraction (CAF).

The purified samples were spotted on TLC-pre-coated silica gel plate. Each plate contains five samples and two standards of reserpine and recinnamine (purchased from Hi-media Laboratories) dissolved in methanol (1.0 g mL^{-1}). The mobile phase was chloroform ($CHCl_3$) and methanol (CH_3OH) in 97:3 ratio (*v/v*) or chloroform, methanol and aqueous ammonia (NH_3) in 95:4.5:0.5 (*v/v/v)* ratio. Spots were visualized by the spray of Dragendorff's reagent. The sprayed plates develop orange spots. Spots intensify if the plates further sprayed with HCl, or 50% water-phosphoric acid and finally the R_f value was calculated.

HP-TLC plates (Silica 60, F_{254}, 20×20 cm) were visualized in UV chamber prior to use for ensuring the clearness of plates. The sample extracts from different plant parts were loaded (1.2 μL $spot^{-1}$) on the plates with the help of a sample loader (Model: CAMAG-5). Six samples were loaded on each plate. After loading the sample, plates were placed into saturated TLC chamber containing the mobile phase $CHCl_3$: CH_3OH (97:3 *v/v*). Thereafter, plates were taken out and solvent front was marked and then plates were dried in the oven. After drying, plates were visualized in UV-chamber and scanned by CMAG TLC scanner at 200-700 nm and different spectral pattern and peaks were observed.

HPLC analysis was performed with the help of Shimadzu HPLC system (Model: SCL-10 vp) fitted with a diode array detector (Model: SPD-MDA vp). A stainless steel columns (Luna 5μ, C_{18} (2), 250×4.60 mM-Make Phenomenax, USA) reversed phase C_{18} column packed with spherisorb S5 ODS-2 was used for the separation. The mobile phase, methanol (CH_3OH): acetonitrile (CH_3CN) (60:40 *v/v*), was prepared carefully. The flow rate was maintained at 1.0 mL min^{-1} and the wavelength was fixed at 254 nm. Colored samples were passed through the activated charcoal matrix for decolourization. All the standard as well as samples were filtered through 0.2 μm fluoropore filters prior to injection and 20 μL was injected into the column using a 25 μL Hamilton sample injector. The reserpine standard was purchased from Hi-media Laboratories. All experiments were performed in triplicates and the results expressed as mean values.

RESULTS

Crude alkaloid fraction (CAF): Table 1 illustrate that the CAF differed significantly among the different plant parts of wild, cultivated and *in vitro* regenerated plants. CAF were higher in the roots than in the leaves. Roots of *in vitro* grown plants and cultivated plants had higher CAF than roots of wild plants. CAF of leaves were highest in the plants regenerated *in vitro*. CAF of callus, regenerated from leaves in both MS and B_5 medium was similar to that of the leaves. CAF of callus regenerated from stem was significantly lower than the leaf callus.

Alkaloid profiling by thin layer chromatography (TLC): The qualitative estimation of alkaloids from different plant parts of *R. serpentina* were performed by comparing the R_f (retardation factor) values of samples with the reserpine standards. The alkaloid profile of different plant parts of *R. serpentina* revealed that all the root and callus samples contains reserpine while, reserpine was not detected in all the leave samples. All the three root samples also contains alkaloids other than reserpine whose R_f values range between 0.46-0.48 (Table 2).

Alkaloid profiling by HP-TLC: Table 3 illustrate that the root samples contains reserpine as a major alkaloid component, while leaf and callus samples contains reserpine at a very low concentration but contains other alkaloids in greater proportion (Fig. 1).

Table 1: Crude alkaloid fraction (CAF) of different plant parts

[a]CAF (mg g^{-1} dry wt.)		Root	Leaves
Wild plant		416[b]	217[b]
Cultivated plant		487[a]	232[b]
In vitro grown plant		496[a]	265[a]
[b]CAF (mg g^{-1} dry wt.)			Callus 2-month old
Leaf	MS		272[a]
	B_5		287[a]
Stem	MS		232[b]

*Mean values followed by similar letters are non-significant at p = 0.05. [a]CAF in roots and leaves of wild cultivated and *in vitro* grown plants [b]CAF in callus derived from leaf and stem explants in MS and B_5 medium

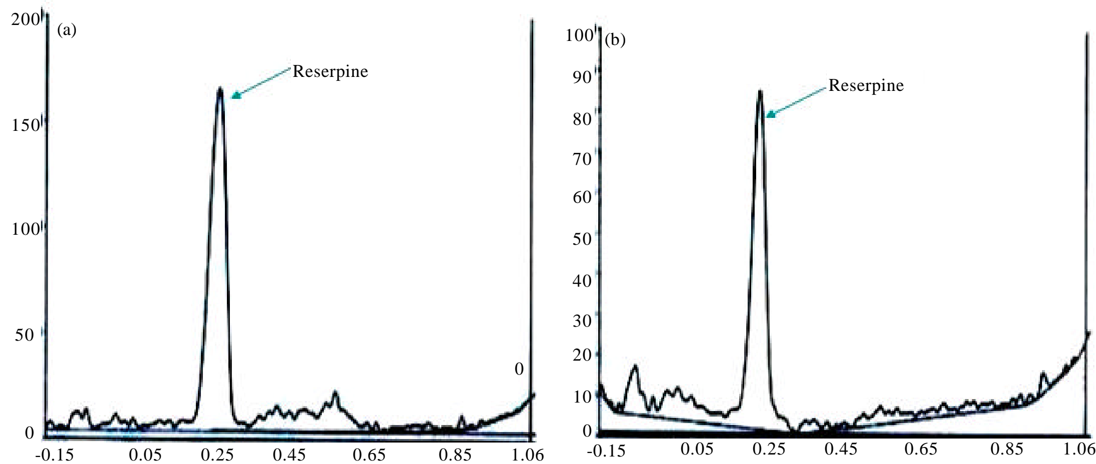

Fig. 1(a-b): HP-TLC ananlysis showing alkaloid profile of roots of *Rauwolfia serpentina* plants: (a) *In vitro* regenerated plant and (b) wild plant

Table 2: Qualitative estimation of reserpine in the different plant parts of *R. serpentina* L. by TLC. The R_f values for Reserpine (0.96)

Plant samples	Reserpine	Others
Roots		
Wild plant	+	+
Cultivated plant	+	+
In vitro regenerated plant	+	+
Callus		
Leaf callus B_5 medium	+	-
Leaf callus MS medium	+	-
Stem callus MS medium	+	-
Leaves		
Wild plant	-	-
Cultivated plant	-	-
In vitro regenerated plant	-	-

Table 3: Qualitative analysis of reserpine in different plant samples of *R. serpentina* collected from different localities as well as *in vitro* regenerated plants by HP-TLC

Plant sample	R_f	Compound detected
Wild root	0.25	Reserpine
Cultivated root	0.25	Reserpine
In vitro root	0.25	Reserpine
Wild leaf	0.25	Reserpine
Cultivated leaf	0.25	Reserpine
Tissue culture plant	0.25	Reserpine

Table 4: Reserpine content in different plant samples of *R. serpentina* L. quantified through HPLC. (DW: dry weight)

Plant sample	Reserpine content (mg g^{-1} DW)
Wild root	26.74
Cultivated root	25.16
In vitro root	33.00
Stem callus (MS)	6.81
Leaf callus (B5)	8.98
Leaf callus (MS)	7.34
Cultivated leaf	2.35
Wild leaf	1.67

Alkaloid profiling by HPLC: HPLC analysis of plant parts, collected from different localities as well as *in vitro* regenerated callus and plants, revealed that a large number of alkaloids were detected including reserpine. Roots of wild plants contains seven alkaloids while cultivated root contains six alkaloids. Root of *in vitro* regenerated plants contains three alkaloids. Among all the three-root samples, reserpine was detected as a major alkaloid component (Fig. 2). HPLC analysis of callus tissues regenerated from leave and stem explants in MS and B_5 medium showed that six alkaloids including reserpine were detected in two-month-old leaf and stem callus regenerated in MS medium. Five alkaloids including reserpine were detected in two-month-old leaf callus regenerated in B_5 medium. In all the three callus samples, reserpine was the primary component (Fig. 3). Maximum number of alkaloids including, reserpine were detected in cultivated leaves (4-alkaloids) and three alkaloids in *in vitro* regenerated leaves while, two alkaloids were detected in the wild leaves (Fig. 4).

The reserpine content were estimated in all the samples with the help of reserpine standard. As shown in Table 4 maximum reserpine contents were present in the roots, callus and leaves, respectively. Among root samples, highest reserpine content (33 mg g^{-1}), were obtained from *in vitro* regenerated roots while the least reserpine content was observed in the cultivated roots. Callus regenerated in MS and B_5 medium also contains a significant amount of reserpine and B_5 medium was found as more productive for reserpine biosynthesis.

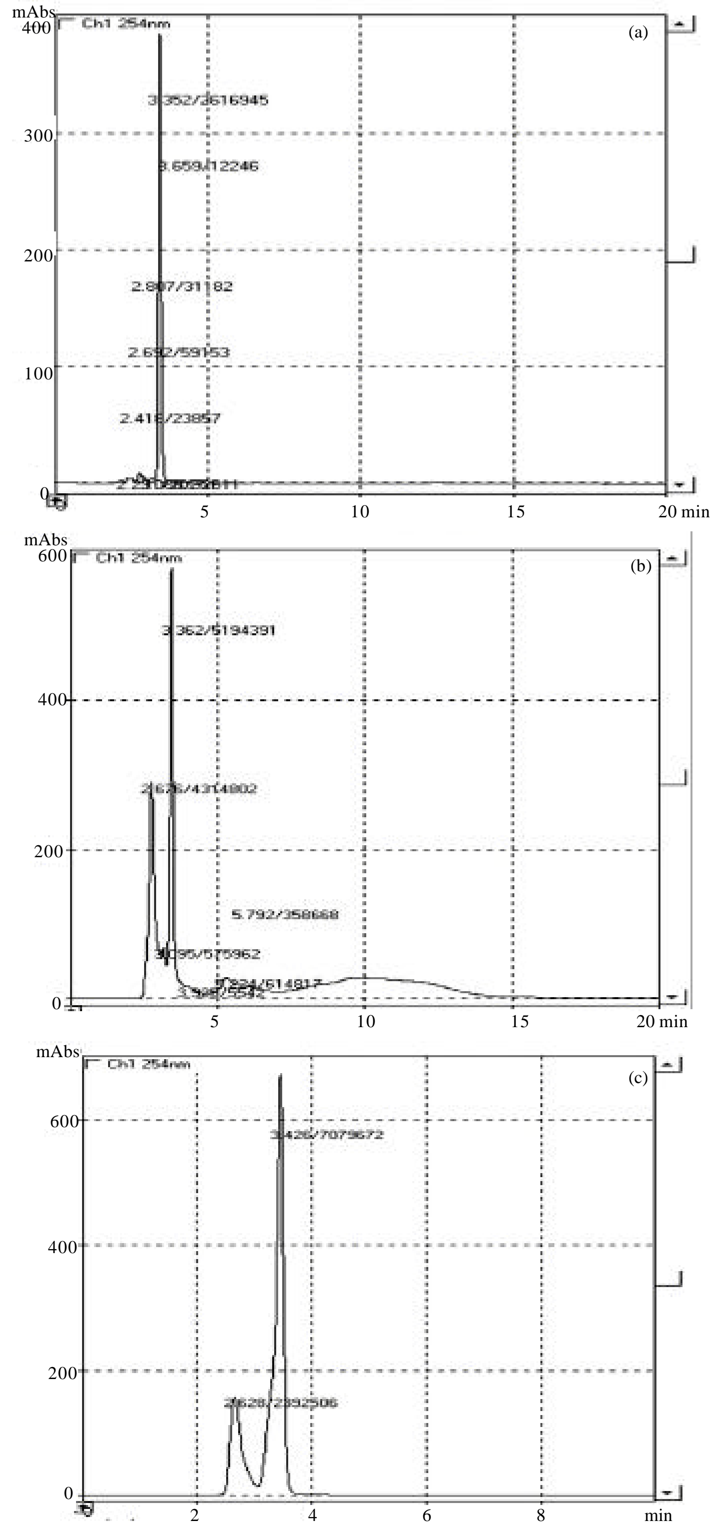

Fig. 2(a-c): Chromatogram showing alkaloids profile of (a) *Rauwolfia serpentina* roots of wild, (b) cultivated and (c) *in vitro* regenerated plants

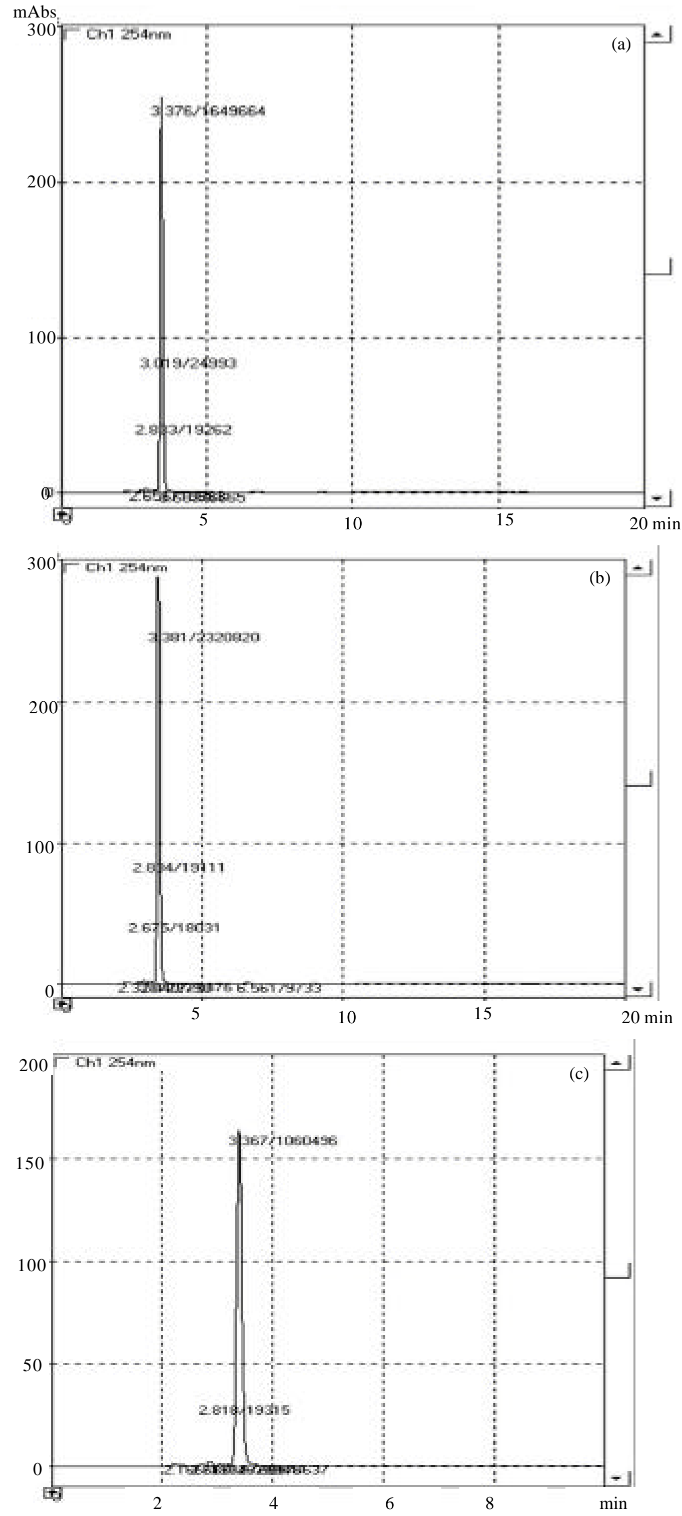

Fig. 3(a-c): Chromatogram showing alkaloids profile of 2-month old callus of (a) *R. serpentina* regenerated from leaf explant in B_5, (b) MS medium and (c) from stem explants in MS medium

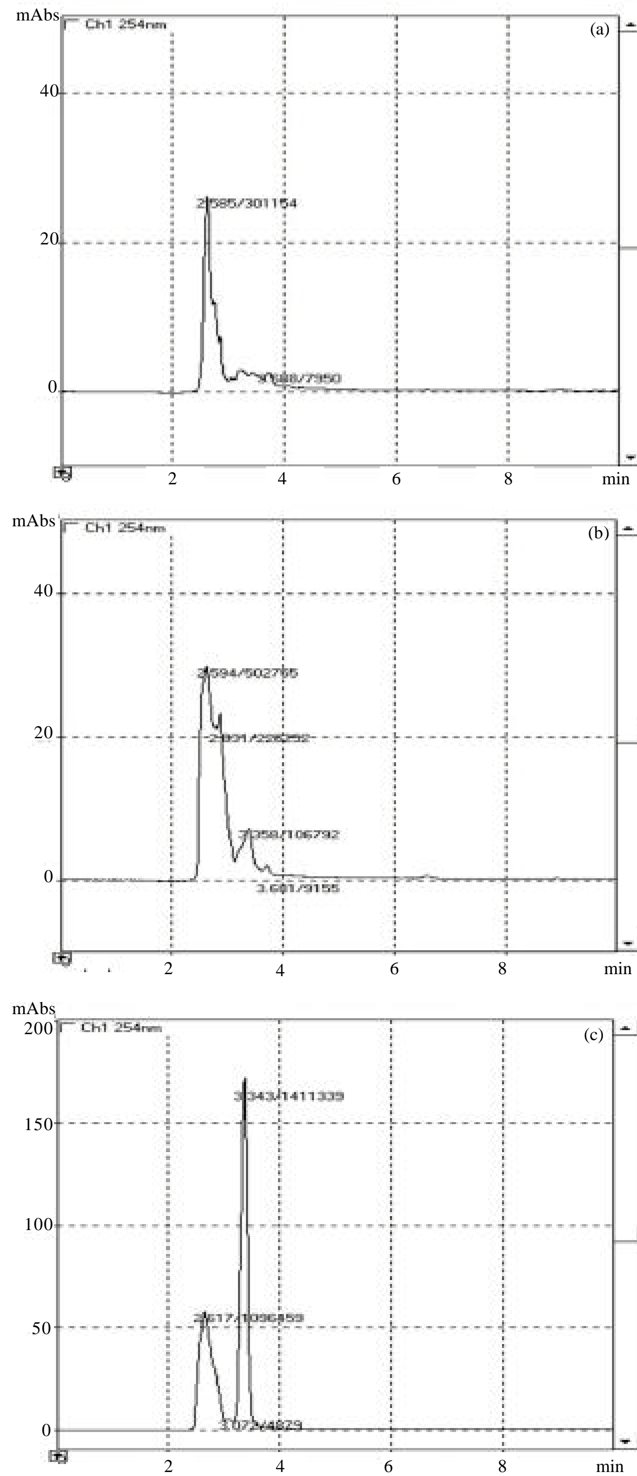

Fig. 4(a-c): Chromatograms showing alkaloid profile of leaves of *R. serpentina* plants (a) wild plant (b) cultivated plant and (c) tissue culture regenerated plants

DISCUSSION

The CAF were reported to be higher in the roots as compared to the callus, regenerated in MS medium which is in accordance to the earlier findings (Sehrawat *et al*., 2001, 2002).

Alkaloid analysis by TLC showed that reserpine is the major alkaloid in the roots and present in all the three root samples as well as in the callus tissues. Leaves of all the plants were found to be devoid of alkaloids or present in very low amount, which is not detectable by TLC. The earlier reports also suggested that reserpine is the major component of roots in *R. serpentina* (Habib and Court, 1974; Court and Timmins, 1975; Roja *et al*., 1984; Roja *et al*., 1987; Roja and Heble, 1996; Sehrawat *et al*., 2002).

HP-TLC analysis also illustrated that all the plant samples, including roots, leaves and callus, contains reserpine but the root samples are containing major proportion of reserpine content. HP-TLC analysis also detected other alkaloids in all the samples, which could not be detected by TLC, with more precision.

There are several reports on HPLC analysis of *R. serpentina* plant samples in which higher alkaloid contents were reported in the root tissues (Cieri, 1983; Roja *et al*., 1987; Roja and Heble, 1996; Sehrawat *et al*., 2001). HPLC analysis of leaf and callus tissues also detected those alkaloids, which were present in very low amount and were not detectable through TLC or even HP-TLC.

The alkaloid profiling of all the plant samples, including roots, leaves and callus of *R. serpentina* exhibit a great deal of variation in the number of alkaloids in each plant samples and 2-7 alkaloids were detected in all tissues analyzed. Highest number of seven alkaloids were detected in wild roots followed by six alkaloids in cultivated plant roots and callus regenerated from leaf and stem explants in MS medium. Five alkaloids were detected in callus regenerated from leaf explants in B_5 medium. While four alkaloids were detected in cultivated leaves followed by three in *in vitro* regenerated roots and leaves of *in vitro* regenerated plants. The least number of alkaloids were detected in the leaves of wild plants.

The alkaloid profile of root samples shows that roots are rich in reserpine content. In addition they also contain other alkaloids but at lower concentration. The roots of *in vitro* regenerated plants contain higher amounts of reserpine than the wild and cultivated plants, similar to the earlier findings (Ruyter *et al*., 1991).

Alkaloid profile of the calli regenerated in MS and B_5 medium from leaf and stem explants showed that the two-month-old callus regenerated from leaf explants in B_5 medium contains higher reserpine content than the callus from MS medium and callus regenerated from stem explants in MS medium. It were also observed that the reserpine contents are higher in the leaf callus in comparison to the stem callus.

The alkaloid profile of leaf samples of wild and cultivated plants showed that the reserpine content was very low in the leaves. Leaf samples are containing other alkaloids (R_t 2.6 min) at higher concentration than the reserpine (R_t 3.3 min).

The alkaloid profiling of different plant samples envisage that the production of secondary metabolites by plants depends greatly on the physiological and developmental stages of the plants. The synthetic capacity of de-differentiated tissue often differs substantially from that of fully differentiated tissues, both qualitatively and quantitatively, because of differences in the enzyme profiles which regulate the organ-specific expression of the biosynthetic genes. The differentiated cultures often show biochemical and genetic stability and hence, offers a predictable and high-productivity system which does not require extensive optimization (Dixon, 2001). The biosynthesis usually occurs in an organ in a tissue -specific manner and is often temporally restricted during the development (Hartmann, 1996; Kurz and Constabel, 1998; Caldentey and Inze, 2004).

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