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ORIGINAL RESEARCH PAPER

High performance liquid chromatography based quantification of reserpine in *Rauwolfia tetraphylla* L. and enhanced production through precursor feeding

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ABSTRACT

Rauwolfia tetraphylla L., is an important medicinal plant in Apocynaceae family and is recognized as an alternative source to *Rauwolfia serpentina* L., in terms of anti-hypertensive alkaloid production *i.e.* reserpine. In view of this, the present study is conducted to estimate the reserpine content in different parts (leaf, stem and root) of field grown plants (2 years old), tissue cultured plantlets (R_1) (two months old) and cell suspensions cultures (two months old with and without precursor feeding) of *R. tetraphylla* by using high performance liquid chromatography (HPLC) technique. Overall maximum content of reserpine (in %) was estimated from the root samples. Roots of field grown plants has recorded high percent of reserpine (0.39%) followed by roots of tissue cultured plantlets (0.35%) and root callus based cell suspension cultures (0.38 %) which was fed with precursor amino acid (100 mg/L of tryptophan). In control type of root callus based cell suspension cultures, reserpine content was quantified as 0.14%; by precursor feeding (100 mg/L of tryptophan) it was enhanced to 0.38%. In conclusion, the reserpine content (0.35 and 0.38%) produced by the roots of tissue cultured plantlets (R_1) and 100 mg/L tryptophan fed root callus based cell suspensions was comparable to that of the reserpine content (0.39%) of root parts of field grown plants. The present study demonstrates the reserpine production by *in vitro* cell suspension cultures throughout the year without sacrificing the medicinal plants.

KEYWORDS

anti-hypertensive, cell suspension, high performance liquid chromatography (HPLC), *Rauwolfia tetraphylla*, reserpine, tryptophan

INTRODUCTION

Rauwolfia tetraphylla L., is an important medicinal plant belonging to class: Magnoliopsida, order: Gentianales and family: Apocynaceae. The Apocynaceae family is well known for several medicinally important plant species such as *Catharanthus roseus*, *Rauwolfia* spp., *Allamanda cathartica*, *Wrightia tinctoria*, *Cryptolepis buchananii*, *Tabernathei boga*; *Nerium oleander*; *Pleiocarpa* spp. etc [1–3]. Among the different genus of Apocynaceae; the *Rauwolfia* genus is popularly known as the major source of pharmaceutical alkaloids [4, 5]. Among the *Rauwolfia* species; initially *Rauwolfia serpentina* L. Benth. Ex Kurz has gained lot of importance as it was exploited initially as a source of medicinally important indole alkaloids such as reserpine, ajmalicine and ajmaline [6, 7]. Due to its high medicinal values;

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R. serpentina plants were collected indiscriminately from the wild by medicinal practitioners as well as by pharmaceutical industries which has resulted in endangered status of this medicinal plant [8–10].

After *R. serpentina*, the species *R. tetraphylla* has identified as an alternative source of medicinally important alkaloids [11]. *R. tetraphylla* is a small woody shrub plant commonly known as ‘four leaf-devil pepper’ with great demand for its pharmacological compounds [12]. The major phytochemicals found in *R. tetraphylla* L. are alkaloids, phenolic compounds, saponins and resins [13]. Most of the pharmacological activities of *R. tetraphylla* L. is due to the presence of alkaloids such as reserpine, serpentine, ajmaline, ajmalicine, yohimbine, deserpidine, recinnamine and tetraphylline [14]. Among the above mentioned alkaloids, the major alkaloid with more pharmaceutical applications is reserpine, which is used mostly for the treatment of hypertension [15], treatment of insomnia, cardiovascular diseases, anxiety and other mental disorders [16].

An alkaloid known as canescine was also reported and isolated from its roots, which has reported to show the same activity as that of reserpine in treatment of hypertension [17]. Similarly, raувotetraphylline alkaloid was reported from this plant species in eight analog forms by spectroscopic methods [18]. Other recently identified medicinal compounds from *R. tetraphylla* L., are quercitin, reserpiline, isoreserpiline, methoxy tetrahydroalstonine, 10-demethoxyreserpiline and 11-demethoxyreserpiline [19–21]. Even though many types of alkaloids were reported and isolated by several researchers from *R. tetraphylla* L., till to date reserpine is the most economically and pharmaceutically important alkaloid.

Due to over exploitation of *R. tetraphylla* L. by the local people, by pharmaceutical industries for pharmacological applications and due to the poor seed germination abilities, this plant has resulted in endangered status [22, 16]. Moreover, the government of India has restricted the export of *Rauwolfia* plant based drugs in order to reduce its over exploitation and to protect the plants from extinction [14]. This has resulted in short supply of reserpine alkaloids in world market, due to which a concern is raised to produce the reserpine in an alternate way without sacrificing the medicinal plants of *Rauwolfia* spp.

Commercial production of reserpine alkaloid from *in vivo* *R. tetraphylla* L. plants (wild or cultivated) is also not suitable because the amount of alkaloid production from the wild or field cultivated *Rauwolfia* plants is variable based on the *Rauwolfia* species, duration of cultivation, geographical location of plants cultivated, agricultural practices and harvesting period [23, 24]. So an alternate strategy like *in vitro* synthesis of reserpine through callus and suspension cultures is needed. Further the enhancement of reserpine production can also be achieved in presence of tryptophan; an aromatic amino acid used as a precursor for indole alkaloids synthesis [25].

In vitro culture based synthesis of reserpine is continuous in nature, free from environmental effect and more importantly this process yields reserpine without uprooting of

Rauwolfia plants. So, in view of the above limitations and requirement; the present research work is carried to initially estimate the reserpine content present in leaf, stem and root parts of field grown plants (2 years old), tissue culture plantlets (R_1) (2 months old) of *R. tetraphylla* L. by using HPLC technique. This research study is also aimed to carry out the *in vitro* synthesis and enhancement of reserpine production in cell suspension cultures through precursor feeding by using different concentrations of tryptophan amino acid.

EXPERIMENTAL

Field grown plants

Two years old *R. tetraphylla* L. plants which were maintained in the medicinal arboretum of the Department of Biotechnology (18° 01' 35.2" N, 79° 33' 31.8" E), Kakatiya University, Warangal (District), Telangana (State) India was used as experimental material in this study. The plants were grown in tropical conditions at 30–37 °C with regular interval of irrigation twice a week. The plant was identified and preserved as a herbarium specimen (No. 1866) in the Department of Botany, Kakatiya University. Dry powder of leaf, stem and roots parts (1 gm of each) obtained from two years old field grown plants was used for extraction and quantification of reserpine (Fig. 1A–F).

Tissue cultured plantlets (R_1)

The large scale production of *R. tetraphylla* L. through tissue culture technique was developed earlier by our research group [26] in the Department of Botany, Kakatiya University, Warangal (District), Telangana (State) India. Leaf (1 gm), stem (1 gm) and root (1 gm) parts of two months old *in vitro* growing tissue cultured plantlets (R_1) was used for extraction and quantification of reserpine.

Chemicals and standards

Murashige and Skoog medium in readymade form without sucrose and agar was purchased from Himedia, Mumbai. The standard reserpine, extra pure (RM 1149-1G; C33H40N2O9; Mol. Wt: 608.69) was purchased from Himedia, Mumbai. Silica gel-GF254 coated plates of 20 × 10 cm were obtained from Merck, Mumbai. The chemicals of chloroform, diethyl amine, ethyl acetate, methanol, potassium hydrogen phosphate, toluene were purchased from Himedia, Mumbai and Loba Chemie, Mumbai.

Cell suspension cultures

The protocol for establishment of cell suspensions of *R. tetraphylla* L. was developed in this study. Initially, friable callus was induced from root, stem and leaf explants obtained from tissue cultured R_1 plantlets of *R. tetraphylla* L. on MS agar solidified medium supplemented with 2, 4-D (2.0 mg/L), and cultured for 1 month duration [27]. About one gram of callus induced from leaf, stem or root explants

Fig. 1. Extraction of Reserpine from leaf, stem and root parts of *R. tetraphylla* L. (2-Years-Old). (A), (B) & (C) Leaf, stem and root parts of *R. tetraphylla* L. (D), (E) & (F) Powdered leaf, stem and root parts of *R. tetraphylla* L. (G), (H) & (I) Leaf, stem and root powder extracts of *R. tetraphylla* L. with liquid ammonia (J), (K) & (L) Methanolic extracts of leaf, stem and root parts of *R. tetraphylla* L.

was transferred to MS liquid medium amended with 2,4-D (2.0 mg/L) and Tryptophan (50, 75, 100 & 200 mg/L) and incubated on orbital shaker (100 RPM) for one month, until a fine cell suspension was formed (Fig. 2A–C). A control (without tryptophan) cell suspension of each type (leaf, stem and root callus based) was also maintained. Two months old cell suspensions established from root, stem and leaf callus was used for extraction and quantification of reserpine.

Sample preparation

The leaf, root and stem parts of field grown plants (2 years old) and tissue cultured R_1 plantlets (2 months old) of *R. tetraphylla* L. was separated, washed under running tap water, dried in a shaded place and coarsely powdered. Similarly, cell suspensions established from root, stem and leaf explants based callus was dried in an oven at 60 °C for

20 min and macerated. One hundred (100) mg of powdered leaf, stem, root samples and 100 mg of dried cell suspensions was treated with 1 mL of ammonia solution and incubated for 10 min (Fig. 1G–I). Methanol (90%-10 mL) was added to the mixture, incubated in water bath for 10 min and filtered. The methanolic extracts of root, stem and leaf of 2 years old field grown plants (Fig. 1J–L), tissue cultured R_1 plantlets (2 months old) and 100 mg of cells were concentrated separately using rotary evaporator and finally dried in a vacuum oven. The concentrated extracts was dissolved in 1 mL of methanol, filtered and used for HPLC analysis.

The standard reserpine, extra pure (RM 1149-1G; C33H40N2O9; Mol. Wt: 608.69) was purchased from Himedia. Silica gel-GF254 (Merck) coated plates of 20 × 10 cm is used for separation of standard reserpine alkaloid and to assess the presence of reserpine in leaf, stem and root extracts by thin layer chromatography (TLC) technique. The

Fig. 2. Enhanced production of reserpine through cell suspension cultures of *R. tetraphylla* by precursor (tryptophan) treatment (A) Orbital shaker & (B) Suspension cultures incubated on orbital shaker (100 RPM) (C) Cell suspension cultures fed with different concentrations of tryptophan. (50, 75, 100 & 200 mg/L)

presence of reserpine in leaf, stem and root parts was confirmed by exposing TLC analyzed silica gel plates to UV rays, where a corresponding band was visualized in each sample with that of standard reserpine (Fig. 3). After separation, the R_f (rate of flow) value of standard reserpine was calculated as 0.47 by TLC analysis.

HPLC conditions

The quantification of reserpine present in leaf, stem and root samples of field grown plants (2 years old), tissue cultured R_1 plantlets (2 months old) and cell suspensions (2 months old) of *R. tetraphylla* L., was carried by HPLC technique. The HPLC instrument (HPLC: Shimadzu, Model: Prominane) was used with experimental conditions of Pump: LC-20 AT, Detector: SPD-20 A; Injector: Manual; Buffer: 6.8 g of potassium hydrogen phosphate monobasic in 1,000 mL water, P^H of 3.0; Column: C18 type, 250 × 4.6 mm size; mobile phase: toluene, chloroform, ethyl acetate, diethyl amine in 7:7:4:1; Sample Preparation:

20 μ L of sample is taken and it is made upto 1 mL with mobile phase.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The samples prepared from leaf, stem and root parts were injected into injector port of Shimadzu LC-20 HPLC, equipped with auto sampler and LC solutions software. The samples were eluted with mobile phase into the column and the pump was adjusted with steady flow rate of 1.0 mL/min. The detector generated the amount of sample allowing for quantification of the reserpine. The reserpine compound is detected at a wavelength of 268 nm. The digital microprocessor and user software has controlled the HPLC instrument and provided the data which consists of retention time, height of peak, area of peak etc. When initially standard reserpine (1 mg/mL) was subjected to HPLC analysis with

Fig. 3. Separation of reserpine from leaf, stem and root extracts using Thin Layer Chromatography. Lane 1: Standard reserpine (Rf Value = 0.47); Lane 2: Leaf extract; Lane 3: Stem extract and Lane 4: Root Extract

mobile phase of toluene, chloroform, ethyl acetate, diethyl amine in 7:7:4:1, a peak was eluted at retention time of 23.347 min (Fig. 4A). Reserpine content from each sample was estimated and the data is given in percentage. All the

experiments were repeated three times and the results were expressed as mean values.

Quantification of reserpine from different parts of *in vivo* plants

In this study quantitative estimation of reserpine was carried in leaf, stem and root parts of 2 years old field grown plants, 2 months old tissue cultured plantlets (R_1) and 2 months old cell suspension cultures of *R. tetraphylla* L., using HPLC. Initially the standard reserpine was separated by TLC and the Rf value of standard reserpine was found to be 0.47. These results are in accordance with earlier reports, where the Rf value of reserpine separated by TLC technique was reported as 0.45 [28]. Literature survey reveals that UV spectroscopy [28, 29], HPTLC [30–32], GC [33] and HPLC [34–36] methods were reported for the determination of reserpine in pharmaceutical formulations as well as from the leaf, stem and root parts of different species of *Rauwolfia* plants. As HPLC technique is more reliable and as per the above cited literature as most of the authors has carried the HPLC based quantification of reserpine, the present study of quantification of reserpine from different parts and cell suspensions of *R. tetraphylla* L., was carried by using HPLC technique.

After estimation by HPLC analysis of all the samples, it was observed that the maximum amount of reserpine of 0.39 and 0.38% was estimated in root extracts of 2 years old field grown plants and in 2 months old cell suspensions fed with tryptophan (100 mg/L), respectively (Table 1). High (0.39%), moderate (0.21%) and low percent (0.15%) of reserpine was estimated in root, stem and leaves of 2 years

Fig. 4. Quantification of reserpine through HPLC analysis from leaf, stem and root extracts of *in vivo* plants (2 years old) of *R. tetraphylla* L. at 268 nm. A) Chromatogram of standard reserpine, B) Chromatogram of leaf extract, C) Chromatogram of stem extract and D) Chromatogram of root extracts

Table 1. Quantification of reserpine content by HPLC analysis in different parts of *in vivo* and *in vitro* plants and cell suspension cultures of *Rauwolfia tetraphylla* L.

#	<i>In vivo</i> plants of <i>R. tetraphylla</i> (2 years old)		<i>In vitro</i> plants of <i>R. tetraphylla</i> (2 months old)		Cell Suspension Cultures (Control) (2 months old)		Cell Suspension Cultures (Tryptophan Precursor feeded) (2 months old)		
	Plant Part (100 mg Dry Weight)	Reserpine Content (%)	Plant Part (100 mg Dry Weight)	Reserpine Content (%)	Suspension culture established from (callus type)	Reserpine Content (in % per 100 mg of cell suspension)	Suspension culture established from (callus type)	Conc. of Tryptophan (mg/L)	Reserpine Content (in % per 100 mg of cell suspension)
1	Leaf:	0.15%	Leaf:	0.07%	Leaf callus:	0.04%	Leaf callus:	50	0.04
								75	0.06
								100	0.07
								200	0.05
2	Stem:	0.21%	Stem:	0.11%	Stem callus:	0.12%	Stem callus:	50	0.12
								75	0.12
								100	0.14
								200	0.10
3	Root:	0.39%	Root:	0.35%	Root callus:	0.14%	Root callus:	50	0.22
								75	0.28
								100	0.38
								200	0.27

old field grown plants (Fig. 4B–D). Separation of indole alkaloids (reserpine, ajmalicine, ajmaline and yohimbine) from leaf, stem and root parts of *in vivo* plants of *R. serpentina* by HPLC, HPTLC and TLC methods were reported [31, 34, 35, 37–39].

All of the above cited reports are of *R. serpentina* and there is a single report on reserpine quantification from various parts of *R. tetraphylla* by UV spectroscopy method where 0.205, 0.102 and 0.016 w/w of the root, stem and leaf tissues was reported [28]. About 0.2 % of reserpine content was reported from 1 g of root sample of *R. tetraphylla* [28], whereas in the present study HPLC technique was used and more amount of reserpine (0.39%) was estimated from 1 g of dried root of *R. tetraphylla* L. plants (*in vivo*), this variation in content of reserpine might be due to variation in geographical location, field capacity of soils and agronomical practices.

Quantification of reserpine from different parts of tissue cultured plantlets

When reserpine quantification was carried from different parts of tissue cultured plantlets of *R. tetraphylla* (2 months old), a similar pattern of reserpine profile was displayed with that of *in vivo* plants. The reserpine content present in leaf, stem and roots of 2 months old tissue cultured plantlets (R_1) was determined as 0.07, 0.11 and 0.35%, respectively (Fig. 5A–C). Quantification of reserpine was performed earlier from *in vitro* regenerated *R. serpentina* plantlets using HPLC technique and reported highest reserpine in roots (0.3% dry weight) of *in vitro* plantlets [40]. Similarly, in the present study from *in vitro* raised plantlets (2 months old) of *R. tetraphylla* maximum amounts of reserpine were observed in roots (0.35%) followed by stem (0.11%) and leaf (0.07%) samples.

Enhancement of reserpine production by precursor treated cell suspension cultures

The present study is also aimed to establish the cell suspension cultures and carry the quantification of reserpine in control as well as precursor (tryptophan) treated suspensions. In previous studies percentage of reserpine and ajmaline in callus of *R. serpentina* varies and high levels of reserpine (0.096%) and ajmaline (0.092%) was recorded in callus [41]. Stimulation of reserpine production through elicitors (abscisic acid, salicylic acid and dimethyl sulphoxide) and precursor (tryptamine) treatment in the *in vitro* plant cultures of *R. serpentina* was reported earlier [42]. Similarly there are some reports about the *in vitro* synthesis of reserpine using callus cultures or cell suspension cultures supplemented with either stress inducing agents like NaCl [43], influence of auxins [44], different concentrations of sucrose and culture medium amended with a precursor amino acid for reserpine biosynthesis *i.e.* tryptophan [25].

So, in the present study cell suspensions were established initially from leaf, stem and root explants based callus. In cell suspension cultures of control type (without tryptophan), the reserpine content was determined as 0.04, 0.12 and 0.14% from the leaf, stem and root callus based suspension cultures (Fig. 6A–C). These values are less when compared to the reserpine contents estimated from both *in vivo* and *in vitro* plants. As the estimated amounts of reserpine were low in control cell suspension cultures, tryptophan amino acid is feeded as a precursor in different concentrations (50, 75, 100, 200 mg/L) to the broth medium to enhance the reserpine production (Table 1). After HPLC analysis of different concentrations of tryptophan precursor feeded cell suspensions, significant enhancement of reserpine content (0.07, 0.14 and 0.38%) was observed from the

Fig. 5. Quantification of reserpine through HPLC analysis from leaf, stem and root extracts of *in vitro* plants (2 months old) of *R. tetraphylla* L. at 268 nm. A) Chromatogram of leaf extract, B) Chromatogram of stem extract and C) Chromatogram of root extracts

leaf, stem and root callus based cell suspension cultures (2 months old) amended with 100 mg/L concentration of tryptophan (Figs 7–9).

In the previous studies, *in vitro* synthesis and enhancement of reserpine production up to 2 fold increase was achieved from leaf callus based cultures in *R. tetraphylla* by using 50 mg/L of tryptophan as a precursor [25]. Whereas in the present study 3 fold increase was achieved through the cell suspension cultures of root based callus treated with 100 mg/L of tryptophan. As roots are the major source of reserpine synthesis and as cell suspensions are better than callus cultures for long term culture and production, the developed protocol is an improvement over the existing protocols.

Overall as per the previous studies conducted on estimation of reserpine, the maximum amount of reserpine content was recorded in the range of 0.01–0.33% from the root parts of different *Rauwolfia* species. Whereas, in the present study maximum amount of reserpine production of

Fig. 6. Quantification of reserpine through HPLC analysis from cell suspension cultures (control) of *R. tetraphylla* L. at 268 nm. A) Chromatogram of leaf callus based cell suspension cultures (Control-without tryptophan), B) Chromatogram of stem callus based cell suspension cultures (Control-without tryptophan), C) Chromatogram of root callus based cell suspension cultures (Control-without tryptophan)

0.39, 0.35 and 0.38% was recorded from the root samples of 2 years old *in vivo* plants, root samples of 2 months old *in vitro* plants and root callus based cell suspensions of *R. tetraphylla* fed with 100 mg/L tryptophan, respectively. This study clearly demonstrates that the percent of reserpine produced by root callus based cell suspensions (0.38%) cultured with 100 mg/L of tryptophan was comparable to that of the *in vivo* roots (0.39%) obtained from field grown plants (2 years old).

CONCLUSION

The content of reserpine estimated from root samples of tissue culture plantlets (0.35%) is comparable to that of root samples of 2 years old *in vivo* plants (0.39%). In cell suspension cultures, the reserpine content was enhanced from 0.14 to 0.38% in root callus based cell suspensions by precursor feeding with 100 mg/L of tryptophan. So there is good scope for utilization of *in vitro* plantlets and cell suspension cultures for continuous production of reserpine

Fig. 7. Quantification of reserpine through HPLC analysis from leaf callus based cell suspension cultures (2 months old) of *R. tetraphylla* L. fed with A) 50 mg/L of tryptophan B) 75 mg/L of tryptophan C) 100 mg/L of tryptophan and D) 200 mg/L of tryptophan at 268 nm

in *in vitro* conditions without sacrificing field grown or wild plants. Further this kind of approach will reduce the over exploitation of the valuable medicinal plants

(*Rauwolfia* spp.) from the wild, and fulfills the industrial requirement constantly for anti-hypertensive reserpine alkaloid.

Fig. 8. Quantification of reserpine through HPLC analysis from stem callus based cell suspension cultures (2 months old) of *R. tetraphylla* L. fed with A) 50 mg/L of tryptophan B) 75 mg/L of tryptophan C) 100 mg/L of tryptophan and D) 200 mg/L of tryptophan at 268 nm

Fig. 9. Quantification of reserpine through HPLC analysis from root callus based cell suspension cultures (2 months old) of *R. tetraphylla* L. fed with A) 50 mg/L of tryptophan B) 75 mg/L of tryptophan C) 100 mg/L of tryptophan and D) 200 mg/L of tryptophan at 268 nm

Conflict of interest: There are no conflicts to declare.

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