Original research article

Prospects of *Plumbago rosea* L. hairy root culture in traditional preparations: a phytochemical comparison with tuberous roots

Satheeshkumar K., Binoy Jose, Dhanya B. P. and Krishnan P. N.

Biotechnology & Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Palode, Thiruvananthapuram 695 562, India
Corresponding author: K. Satheeshkumar, E-mail: bioproduction09@gmail.com, Phone: +91 9496154183

Received on February 15, 2013; Accepted on June 14, 2014

Abstract: *Agrobacterium rhizogenes* mediated hairy root culture of *Plumbago rosea* L. is an attractive alternative for the production of plumbagin which is the major bioactive compound in *P. rosea* tuberous roots. The traditional industries form the major consumer of the tuberous roots as these are used in many ayurvedic preparations. The present work investigates the prospects of utilizing hairy roots in the place of tuberous roots based on bacterial survival test of hairy roots and comparison through phytochemical analyses (TLC, Spectrophotometry, HPLC and LC-MS). Since the traditional system of medicine follows stringent curing procedure before incorporation of the roots in medicinal preparations, cured tuberous and hairy roots were also compared. The phytochemical profile of hairy roots was remarkably similar to that of tuberous roots. Curing caused no change in the phytotchemical composition of the roots but only a reduction in the amount of plumbagin and other molecules. Plumbagin was reduced to 0.372 ± 0.026% dry weight (DW) in cured tuberous roots (1.15 ± 0.08% DW in uncured) and 0.061± 0.0043% DW in cured hairy roots (1.32 ± 0.09% DW in uncured). An 11.3 fold increase in root biomass with 1.56% DW plumbagin obtained in bioreactor as against 5.39 fold in shake-flasks (with 1% w/v inoculum over 3 weeks period), adds to the prospects of its applicability in traditional systems. The results suggest a refurbishment of conventional high quantity cured roots in traditional preparations with low quantity uncured roots, irrespective of root types.

Keywords: bioreactor, curing, eluent, plumbagin, traditional medicine

Introduction

The traditional industries that rely on the holistic approach of herbal medicine have earned the faith of 80% of the world's population by providing natural, effective, safe, and side-effects free healthcare through plant drugs (Dubey et al. 2004). The idea that multi-site mechanism of action of herbal preparations from the crude extracts may offer greater chances for success where single-site agents have been disappointing, is achieving greater acceptance in the scientific community (Wermuth 2004). But the advancements remain confined to the technologies for enhanced production and isolation of individual bioactives, which is not supportive to the traditional medicine arena.

In herbal preparations, roots of several medicinal plants form important ingredients. Medicinal properties are attributed to certain root-specific bioactive compounds, many of which are isolated and used as drugs. Considering the provision for holistic approach being incorporated in plant-root based herbal drugs, without depleting the natural resources; the hairy root culture is a prospective technology.

Over the past two decades, *Agrobacterium rhizogenes* induced "hairy root culture" or the "transformed root culture" has proven to be an efficient tool for producing root-specific secondary metabolites (Hu and Du 2006). Mass production systems for many such metabolites have already been established which include hairy roots of *Beta vulgaris* in bubble-column bioreactor for betalain (Savitha et al. 2006) *Panax ginseng* in spray reactor for ginseng production (Palazon et al. 2003), *Lithospermum erythrorhizon* in two phase bubble column reactors for shikonin production (Sim and Chang 1993) and so on. Among the numerous characteristics that have made hairy root culture a remarkable system, an important attribute is genetic stability, which ensures

Satheeshkumar K, Jose B, Dhanya BP, Krishnan PN 2014 Prospects of *Plumbago rosea* L. hairy root culture in traditional preparations: a phytochemical comparison with tuberous roots. Plant Root 8:13-23. doi:10.3117/plantroot.8.13

a stable differentiated phenotype leading to long term biochemical stability even with large scale culture and industrial production (Srivastava and Srivastava 2007). Hairy roots are advantageous over field-derived roots owing to the availability of well-studied large scale root biomass production systems, season independent availability without field cultivation besides easier and shorter harvesting time. Also, *A. rhizogenes* mediated transformants are free from legal controls of Genetically Modified Organisms (GMOs) in countries like Japan (Mishiba et al. 2006) and well established even in crops used for consumption, like groundnut (*Arachis hypogaea*) (Venkatachalam et al. 2000). Nevertheless, the use of hairy roots remains limited to the production of active principles and the traditional industries (ayurveda, siddha and others) still rely on the conventional roots for manufacturing medicinal preparations. The name "neoplastic hairy" root, possible presence of bacteria used for root induction, or the doubt of an altered chemical profile due to *in vitro* cultivation may be the aspects that raise suspicion on the reliability of hairy roots for traditional preparations. All these necessitate a scientific validation to compare the field-derived roots and hairy roots, for which *Plumbago rosea* L. is the plant of our choice.

P. rosea (synonymous *P. indica*) (Plumbaginaceae) is a rare medicinal herb (Chetia and Handique 2000) distributed in the tropical regions of India (CSIR 1989) and traditionally used in the treatment of leucoderma, secondary syphilis, leprosy (Evans 1996), diarrhoea, piles, dyspepsia, intestinal worms and skin diseases (Gangopadhyaya et al. 2008). The medicinal properties are due to plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naturally occurring yellow pigment found mainly in the plants of *Plumbago* species and accumulated mostly in the roots (Kirtikar and Basu 1975; Van der Vijver 1972). *P. rosea* is the best source of plumbagin (Mallavadhani et al. 2002) and the hairy root culture established from the plant offers great prospects to industrial level scaling-up for development as an attractive alternative system for plumbagin production (Gangopadhyaya et al. 2008, Gangopadhyaya et al. 2011). Plumbagin is endowed with a variety of significant pharmacological activities like anticancer (Parimala and Sachdanandam 1993; Ahmad et al. 2008), antibacterial (Ahmad et al. 1998), antioxidant (Tilak et al. 2004), anti-inflammatory (Dorni et al. 2006), anti-malarial (Likhitwitayawuid et al. 1998), radiosensitizing (Nair et al. 2007), cardiotonic (Itoigawa et al. 1991) etc.

A major consumer of *P. rosea* tuberous roots, the traditional medicine industry (Shastri 1994) has brought about extensive exploitation of the plant from its natural habitat. This has made the slow-propagating, seed-set lacking plant rare in several parts of India (Chetia and Handique 2000). *P. rosea* roots form an ingredient of many ayurvedic preparations including herbal mix extract, Kumaryasawaya, Pippalyasawaya, Yogaraja gugulu, Mahamasha massage oil, Hinguvachadi, Indukanta etc (Mallavadhani et al. 2002). Manufacturing procedures for traditional medicines being stringent (Shastri 1994), the tuberous roots of *P. rosea* are used only after curing (purification/detoxification). Several traditional purification (detoxification) methods have been reported such as treating it with cow dung, lime water etc (Moos 1976; Shanavaskhan et al. 1997). The reason that toxicity reduction and efficiency enhancement are required for the effective use of plumbagin (Singh et al. 1997) in medicinal preparations is not good enough to justify the curing procedure which leads to wastage of huge quantity of roots of the rare herb.

Taking all these into consideration, in an effort to increase the expanse of the merger of biotechnology and traditional medicine from a mere sharing of the bioactive principle of medicinal plants, we examine the suitability of utilizing hairy root cultures wherever the conventional tuberous roots find an application. We also analyze the phytochemical significance of the detoxification procedure carried out by the traditional industries.

Materials and Methods

Plant Material

Tuberous roots of *P. rosea* (A voucher specimen is deposited at the Institute Herbarium (TBGT), under reference number 3129) were collected from one year old plants grown in the medicinal garden of TBGRI (Fig. 1a).

A fast-growing hairy root clone induced using *A. rhizogenes* strain A4 (ATCC 43057), was established in Murashige and Skoog (MS) basal agar medium as described earlier (Satheeshkumar et al. 2009). The hairy roots grown in disposable Petri dishes (90 mm, Tarsons, India) were then inoculated into MS basal liquid media (1% w/v roots into 50 mL in 250 mL flasks), cultured in darkness at 25±2ºC on a gyratory shaker (80 rpm) and maintained by subculture at three weeks interval. The three weeks grown culture was taken for the experiments (Fig. 1b).

Bacterial survival test for hairy roots

Bacterial survival test was conducted as per the standard protocol (Mishiba et al. 2006) to confirm the absence of any systemic infection in the hairy roots.

Samples (1g approximate FW) randomly selected from hairy root culture were crushed using mortar and pestle with sterile distilled water, under sterile conditions. Serial dilution was done to obtain samples ranging from 10^{-1} to 10^{-5} dilutions. Yeast-Mannitol agar plates inoculated with the diluted samples were incubated at 25±2ºC for 5 days. Plate inoculated with sterile distilled water was taken as the control.

Curing (Detoxification / Purification) of roots

Roots (tuberous and hairy) were washed in running tap water to remove the soil particles/media remnants**.** Washed roots (10 g) were partially crushed and repeatedly treated with super-saturated lime water following the traditional methods. The process was repeated till the absence of red color development in the eluent (Moos 1976; Shanavaskhan et al. 1997). The red colored solution (eluent) was collected for further analysis.

Meantime, equal quantities (10 g) of uncured tuberous and hairy roots were taken as the control. All the root samples for analysis (tuberous, hairy, cured tuberous and cured hairy roots) were then air dried till a constant weight was observed and each root sample was taken in triplicate.

Extract Preparation

The air dried root samples were powdered for soxhlet extraction. The root samples extracted individually for 5hrs, with 150 mL of each of the solvents hexane, chloroform and methanol (AR grade of Merck, USA), giving 12 samples (extracts), each in triplicates.

Analysis of the sample

The root samples were subjected to phytochemical screening (Harborne 1973; Trease and Evans 1989; Harborne 1998). The filtered (Whatmann No.1), extracts were concentrated to 10 mL in a Rotavapour (Heidolf, Germany) then compared using TLC, HPLC and UV Spectrophotometric analysis. TLC (Si G-60) was done with the authentic plumbagin (Sigma Aldrich, USA). The mobile phases were Toluene:Hexane:Ethyl acetate (5:1:4) for hexane extracts, Chloroform:Methanol (5:5) for chloroform extracts and Chloroform:Methanol (6:4) for methanol extracts. The extracts were analysed and plumbagin quantified using HPLC (Gilson, France) at 254 nm using the mobile phase aqueous 0.2 M acetic acid (pH adjusted to 3.5 with trimethyl amine) and Methanol 80% in a C18 kromacil column (250 x 4.6mm) attached to a Gilson 321 binary gradient pump with a flow rate 0.8 mL/min and a sample size of 15µL (Crouch et al. 1990). The extracts obtained through soxhlet extraction were evaporated to dryness, taken together and dissolved in chloroform for quantitation of total plumbagin. Quantitation was done by comparison with the standard curve prepared using different concentrations of authentic plumbagin, each taken in triplicate.

The extracts were also analysed using XP 3001Xplorer spectrophotometer (PG instruments, Germany), in comparison with pure plumbagin showing absorbance at 254 and 415nm (Crouch et al. 1990). The red colored eluent was further analysed by drop wise addition of 1N HCl to check for any colour change that may be due to reversal of the chemical reactions occurred during curing. Pure plumbagin dissolved in lime-water and in 1N NaOH were also analysed. Both these solutions were then acidified by adding 1N HCl drop wise and their absorbance measured.

To identify the changes occurring during curing of the roots as well as to find out if cured hairy roots can be used as an alternative to cured tuberous roots, LC-MS analysis of these samples was conducted. Liquid chromatography of the samples was performed with Alliance 2795 (Waters) separation module bearing a X-Terra C18 column (dimensions of 100 x 2.1 mm, particle size of 5 µm). Flow rate was 0.15 mL/min with isocratic mobile phase of methanol: water in the ratio 80:20, the pH adjusted to 3.5 and injection volume was 15-20µL. The Micromass Q-ToF micro mass spectrometer consisted of an Electrospray Ionisation (ESI) source and was operated in the positive mode. Nebulizaton was with nitrogen gas at 6-7 bar, with a source temperature of 120°C. Desolvation gas at 250°C was delivered at 550Lts/Hr. The Capillary Voltage was 2725V. The cone gas was delivered at 18 Lts/Hr and the cone voltage was 26V. The instrument operated at a resolution of 5000 with a scan time of 0.5 sec.

Up-scaling of Hairy roots in Air-sparged Bioreactor

The hairy roots grown in shake flasks were scaled-up in a bench-top, stirred tank bioreactor of 2L working capacity (Infors, Switzerland). The central shaft of the top-driven system had a mechanical agitator consisting of 6-blade impeller for proper mixing of medium. Influent air was passed through a hydrophobic membrane filter (Millipore; 0.2µm) into the single air-sparger for providing aeration. The thick glass double walled culture vessel has water circulated around, through a condenser with the help of a water bath circulator. To minimize the medium evaporation during culture, an inbuilt exhaust gas condenser is attached to the unit. The growth chamber has a detachable autoclavable inert metal-mesh that prevents submerging of inoculated roots in the

medium and provides anchorage surface to the growing roots. This also protected the cultures from shear stress created by agitator. The culture vessel encompasses a removable baffle assembly with different probes for assessing and controlling various culture conditions of dissolved oxygen (DO), pH and temperature. The values maintained in the reactor were 200 rpm agitation speed, 160 mg/L DO, pH 5.8 and 25 ºC.

Bioreactor containing MS basal medium (2L) within the stirred tank was sterilized at 121°C for 30 min. Hairy root inoculum for scaling-up was prepared by sub culturing about 1% w/v of hairy roots grown for three weeks in shake-flasks. The actively growing hairy root inoculum was chopped and transferred aseptically through inoculation port into the growth chamber. The culture room conditions for the bioreactor were maintained as described for shake-flask cultures.

Time-course biomass measurement and plumbagin level (Growth analysis) of hairy roots from bioreactor and shake-flasks was made (Kittipongpatana et al. 1998; Rajasekaran et al. 2004) with 1% w/v inoculum at 1 week interval over 3 weeks of culture and each experiment consisted of three replicates.

Statistical test

The data was subjected to ANOVA and the mean values of different treatments compared using DMRT. $p < 0.05$ was considered to be significant.

Results

Bacterial survival test

A. rhizogenes colonies were not observed in the Yeast-Mannitol Agar plates, which showed the absence of bacteria in the hairy roots.

Phytochemical analysis

Air drying showed a weight reduction of 83% in tuberous roots, 89% in hairy roots and curing increased the reduction in weight by 0.29% and 3.3% in tuberous and hairy roots respectively.

Screening of the root samples for different classes of secondary metabolites showed positive color reactions for alkaloids, steroids and flavanoids. Uniform distribution of compounds was observed as the result of TLC fingerprinting of the extracts (Fig. 1c, d, e).

The HPLC chromatogram of each extract of tuberous and hairy roots showed remarkable similarity with respect to the number of peaks and corresponding retention times. The cured roots displayed a chemical

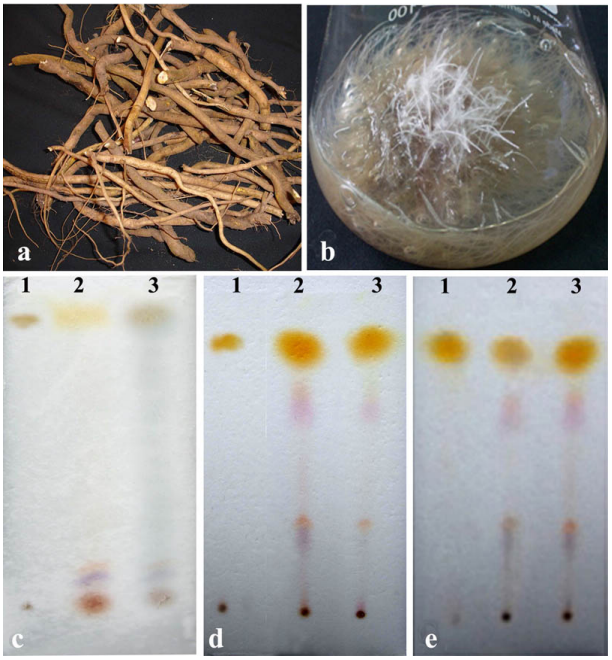

Fig. 1. a Tuberous roots of one year old *P. rosea* plants. **b** Three weeks grown hairy root culture of *P. rosea* in shake flasks (1% w/v inoculum). **c** TLC profile of Hexane extract (**1** standard plumbagin. **2** tuberous roots. **3** hairy roots). **d** TLC profile of chlorophom extract(**1** standard plumbagin. **2** tuberous roots. **3** hairy roots). **e** TLC profile of methanolic extract (**1** standard plumbagin. **2** tuberous roots. **3** hairy roots).

profile similar to the uncured roots, with diminished level of compounds. For each root sample, Hexane extracts gave 3 peaks (at average rt. times 4.57, 6.93, 8.04) (Fig. 2 b, c, d, e), chloroform extracts 4 peaks (3.22, 3.55, 4.43, 8.34) (Fig. 2f, g, h, i) and methanol extracts 4 peaks (2.89, 4.66, 5.45, 8.39) (Fig. 2j, k, l, m). The peaks for plumbagin were identified by running of the standard plumbagin (Fig. 2a)

The study also demonstrated that plumbagin is the major component in all extracts and curing reduced its level. The HPLC analysis of 12 samples from soxhlet extraction indicates that elution of plumbagin to each solvent is significantly varied; tuberous roots eluting more to methanol and hairy roots to chloroform. Plumbagin was the principal component in all the root samples, the total amounts were: 1.15 ± 0.08% DW in tuberous roots, 1.32 ± 0.09% DW in hairy roots**,** 0.372 ± 0.0263% DW in cured tuberous roots and 0.061± 0.0043% DW in cured hairy roots (Fig. 3). Curing reduced the amount of plumbagin by 68% (to 0.372 ± 0.0263% DW) in tuberous roots and the reduction was greater in hairy roots, 95.4% (to 0.061± 0.0043% DW). Similar gradation of plumbagin concentration was observed through the analysis of extracts using UV spectrophotometry (Fig. 4). This implies, 100 g DW of cured tuberous root is equivalent to 16 g DW of tuberous roots or 28.2 g DW of hairy roots with

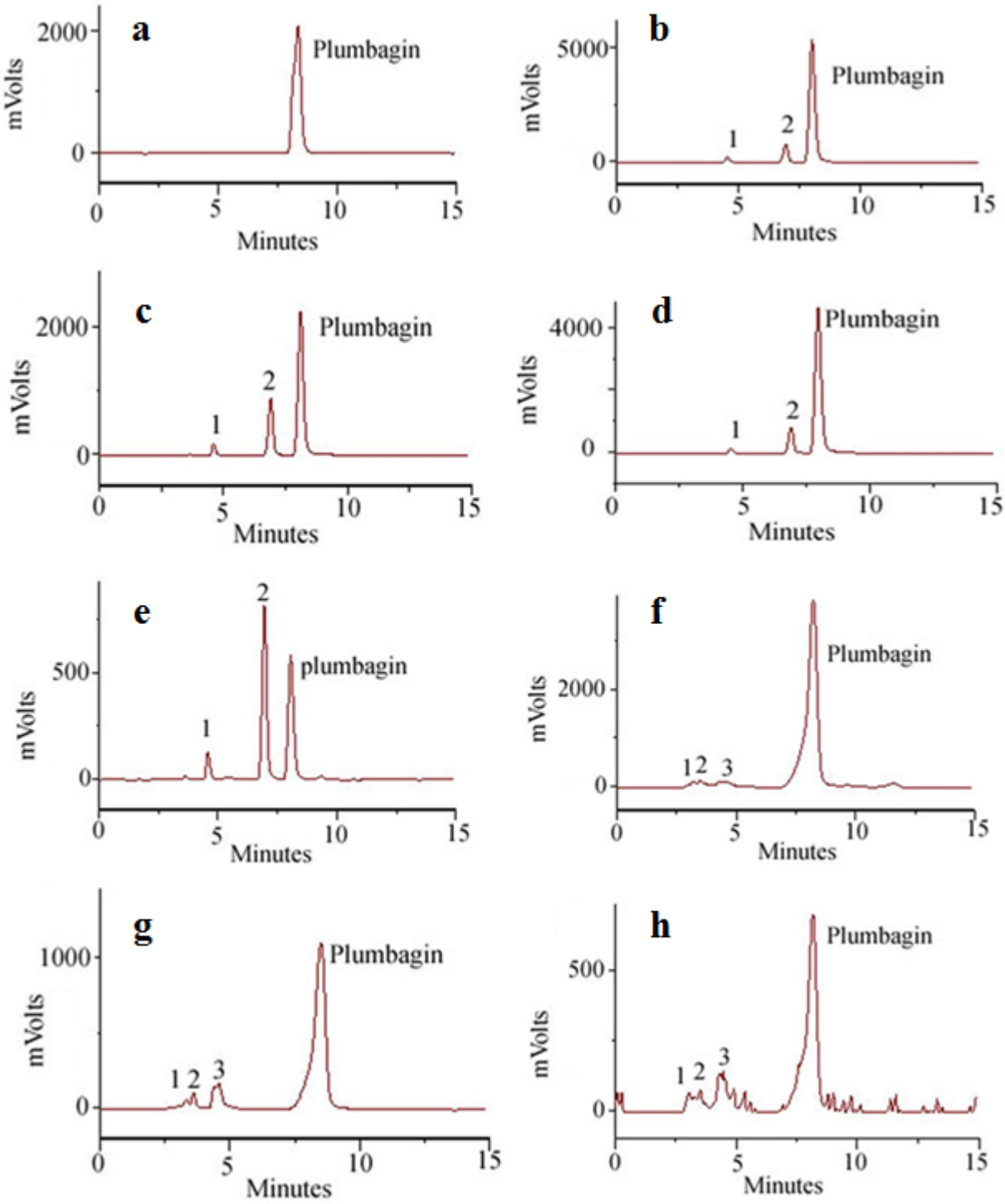

Fig. 2. HPLC profile of different extracts of the four root samples of *P. rosea* showing uniform distribution of compounds. **a** Standard plumbagin. **b** Hexane extract of tuberous roots. **c** Hexane extract of hairy roots. **d** Hexane extract of cured tuberous roots. **e** Hexane extract of cured hairy roots. **f** Chloroform extract of tuberous roots. **g** Chloroform extract of hairy roots. **h** Chloroform extract of cured tuberous roots. **i** Chloroform extract of cured hairy roots. **j** Methanol extract of tuberous roots. **k** Methanol extract of hairy roots. **l** Methanol extract of cured tuberous roots. **m** Methanol extract of cured hairy roots. (Please see next page for **i-m**.)

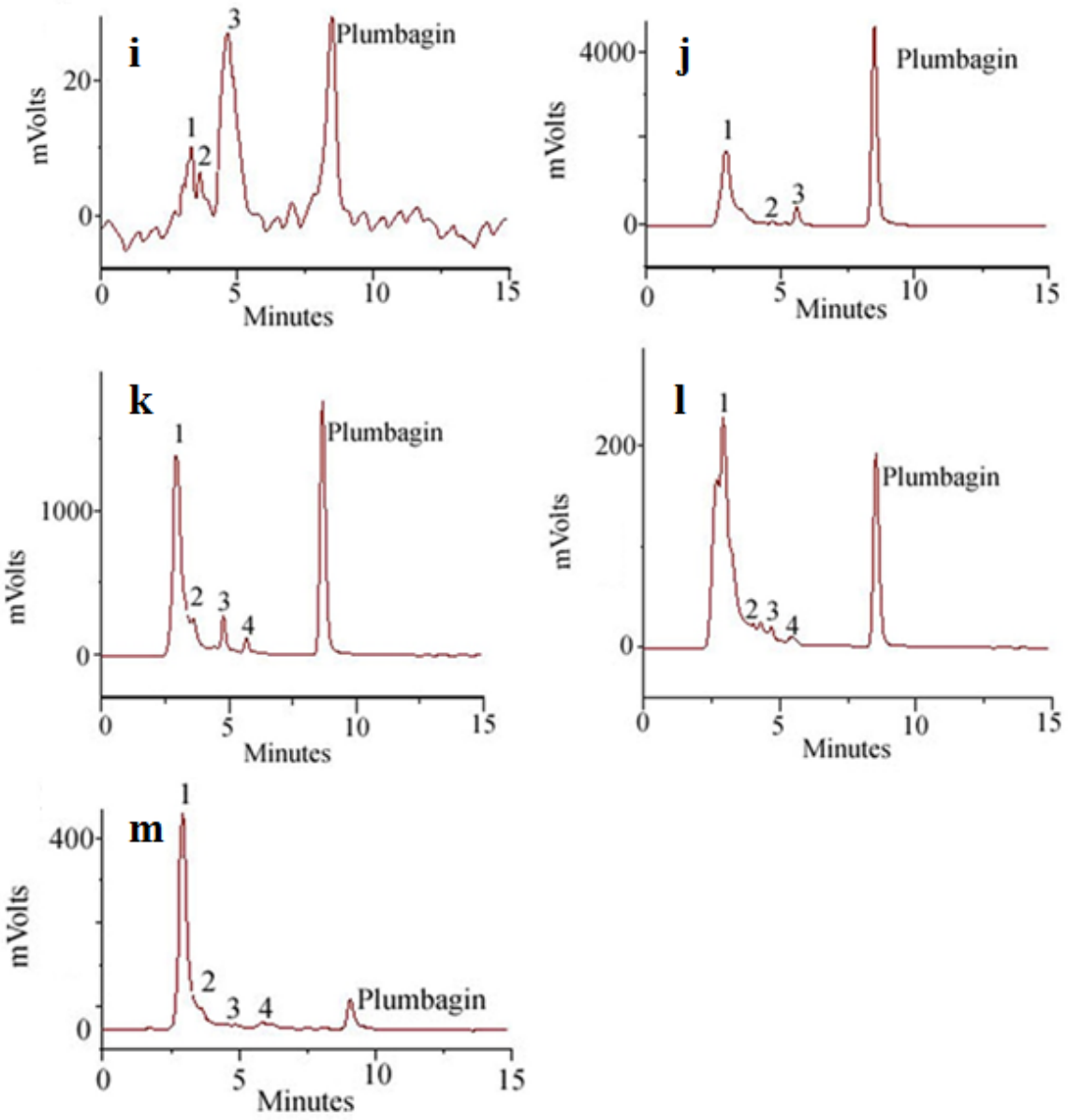

Fig. 2. (Continued from previous page.) HPLC profile of different extracts of the four root samples of *P. rosea* showing uniform distribution of compounds. **i** Chloroform extract of cured hairy roots. **j** Methanol extract of tuberous roots. **k** Methanol extract of hairy roots. **l** Methanol extract of cured tuberous roots. **m** Methanol extract of cured hairy roots. (Please see previous page for **a**-**h**.).

respect to percentage of plumbagin.

The spectrum of the red colored eluent showed absorbance at 525 nm instead of 415 nm (λ maxima of pure plumbagin). The red colored eluent turned into yellow on addition of 1 N HCl and its absorbance reverted to 415 nm. Pure plumbagin dissolved in 1N NaOH and in lime-water, developed red color which was measured at 525 nm. Adding 1N HCl reversed their color into yellow (the color of plumbagin). The red eluent and the eluent that turned yellow by addition of 1 N HCl showed some additional peaks when compared to the spectra of pure plumbagin in alkaline and acidic pH (data not shown). This indicated the presence of other compounds in the eluent in addition to plumbagin.

The cured and uncured roots were studied by LC-MS method showed the characteristic decrease of plumbagin in the cured root sample (Fig. 5 a-d). The mass spectrum showed the $[M+H]^+$ peak at 190 along with characteristic peaks at 161,147, 133, 121 and 105

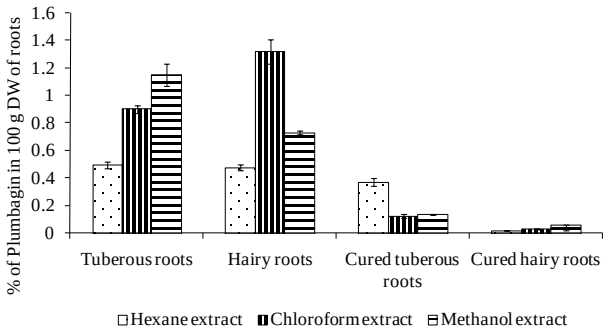

Fig. 3. The elution properties of plumbagin among different solvent extracts of each root samples of *P. rosea* compared and quantified based on HPLC analysis.

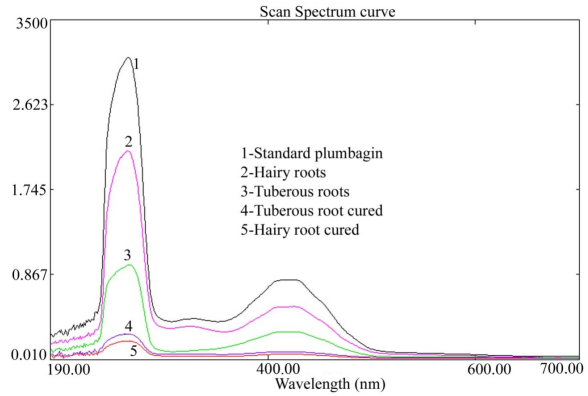

Fig. 4. UV spectrum of authentic plumbagin (absorbance at 415 nm and 254 nm) and chloroform extracts of different root samples of *P. rosea*.

for plumbagin (Fig. 5e). In agreement to the observations through HPLC, LC-MS data of the cured tuberous roots also showed characteristic decrease of plumbagin in the cured roots. Reduction of plumbagin was greater in cured hairy roots compared to the cured tuberous roots.

Up-scaling in bioreactor

Hairy roots grown in the bioreactor (Fig. 6a) showed 11.3 fold increase (Growth index GI= 10.3) in root biomass after 3 weeks whereas it was only 5.39 fold increase (GI= 4.39) in the shake-flask cultures (Fig. 6b). Plumbagin level of the hairy roots cultured in bioreactor and shake flasks was 1.56% DW and 1.32% DW respectively (Fig. 6b).

Discussion

A. rhizogenes mediated transformation is a natural genetic transformation, the bacteria being a soil microflora closely associated with dicotyledonous plants. Such transformants have been established for crops like *Arachis hypogaea*, have received authorization and are free from legal controls of GMOs in countries like Japan (Mishiba et al. 2006; Venkatachalam et al. 2000). Inspite of all these, hairy roots, tissue culture plants or hairy root derived transformants have not yet been analysed with the objective of using either in the traditional preparations or otherwise, as a substitute to their natural counterparts. Additionally, though hairy root culture of *P. rosea* has been established as an alternative to its tuberous roots with respect to plumbagin production, its applicability as an alternative to the tuberous roots as a whole has not been analysed.

Since the doubt of presence of transforming bacteria prevents the direct application of hairy roots in medicinal preparations, hairy roots taken for the present study were subjected to bacterial survival test though these were established through a series of decontamination and repeated subculture for a long period (Satheeshkuamr et al. 2009). Further, this is a test followed by several workers before the *ex-vitro* application of hairy roots or transformed plants (Mishiba et al. 2006) and here we confirm the absence of bacteria in *P. rosea* hairy roots.

While air drying the root samples for extraction, the greater reduction in weight of hairy roots when compared to tuberous roots might be due to the difference in tissue types of the two roots. As a result of curing, greater amount of compounds are lost by elution from hairy roots than tuberous roots and this justifies the higher reduction of weight in cured hairy roots. This can be taken into account while considering utilization of hairy roots in traditional preparations which incorporate the whole root biomass and not decoctions.

The phytochemical screening, TLC, HPLC and UV spectrophotometric analysis of extracts from each root sample (hairy, tuberous, cured tuberous and cured hairy roots) showed uniform distribution of similar compounds. There is no evident influence of *in vitro* cultivation on the phytochemical profile of hairy roots.

The difference in the elution properties of plumbagin between the two types of roots during the process of curing might be due to the difference in the tissue types of the two roots. The curing process does not significantly alter the chemical composition and the outcome was merely a reduction in the percentage (quantity) of each constituent, including plumbagin. This is evident from the smaller size of the corresponding peaks in the HPLC profiles of the cured samples and the presence of additional peaks in the spectrum of the red colored eluent. Spectrophotometric analysis also showed that the gradation of plumbagin quantity in the root samples was in accordance with the observations from HPLC based

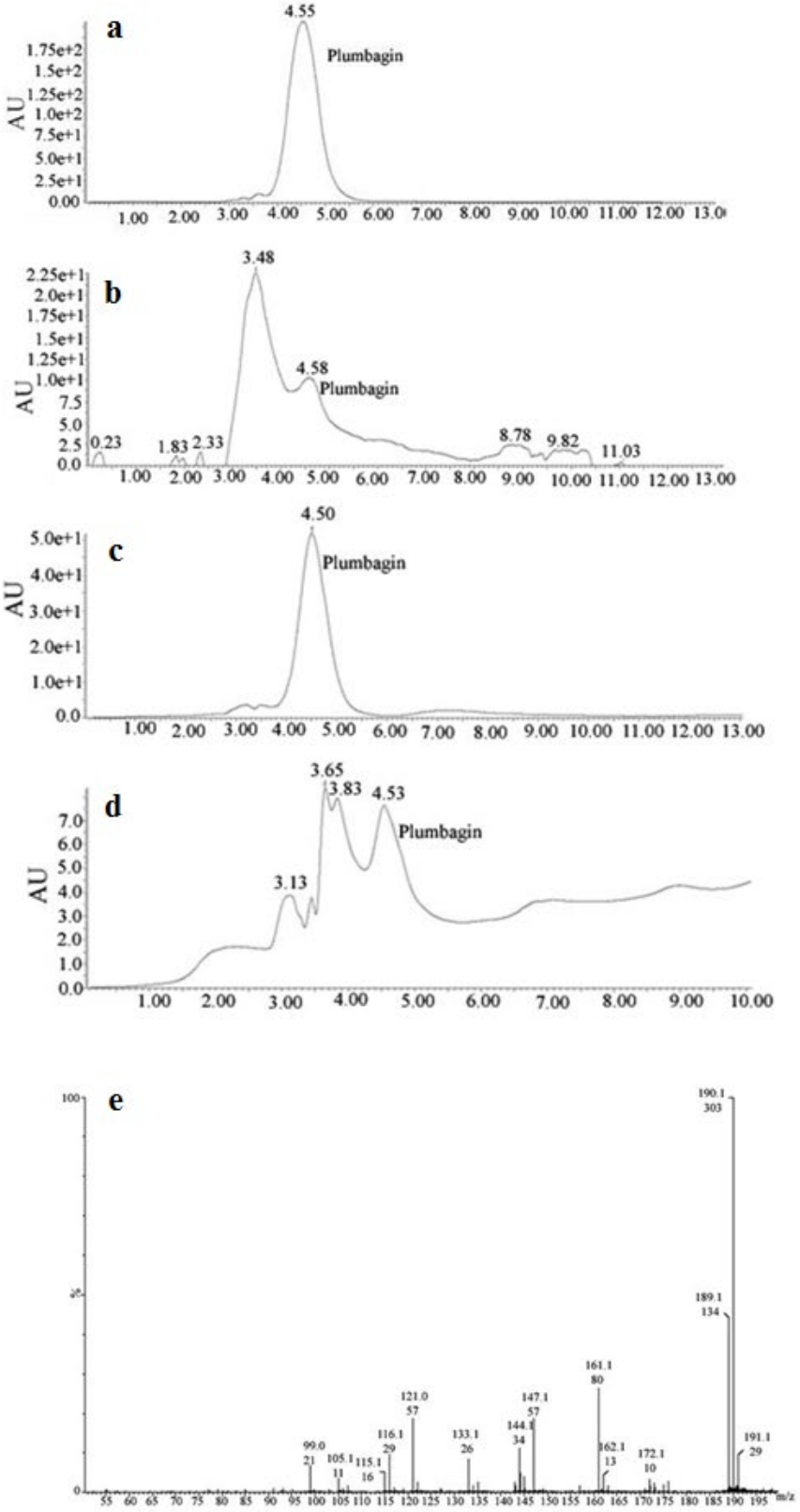

Fig. 5. LC-MS spectrum. **a** tuberous root extract **b** cured tuberous root extract. **c** Hairy root extract. **d** cured hairy root extract. **e** Mass spectrum of plumbagin at 4.5 minutes retention time.

quantitation, i.e., the decreasing order being hairy roots, tuberous roots, cured tuberous roots and cured hairy roots. It also indicates that curing must be standardised with respect to the time of treatment and/or concentration of the solution used for curing, according to the amount of plumbagin that must be left in the roots, either tuberuos or hairy, for incorporation into traditional preparation. The LC-MS study also corroborates with the HPLC analysis confirmed that plumbagin is the major compound in both the roots and curing reduced the level significantly. The mass spectrum showed the $[M+H]^+$ peak at 190 along with the characteristic peaks at 161,147, 133, 121 and 105 for plumbagin is in agreement with the published reports (Hsieh et al. 2005)

It is already known from the published reports that plumbagin has a different absorbance with red color in alkaline pH (above 7.6) (Harborne and Turner 1973) and remains yellow in acidic pH. This color difference might be due to the formation of salt or the presence of quinonoid structure of plumbagin which largely contributes to the color developing mechanisms and was confirmed here through spectrophotometric analysis. These imply that plumbagin is the component accounting for the red color of the eluent and the curing process was traditionally meant for the reduction of plumbagin, however, other compounds are also eluted. Though the complete fingerprinting has not been done in this experiment, we place the preliminary evidence that the process of curing does not bring a significant difference in the overall phytochemical profile of the roots. Apparently, a mere reduction of toxicity can be done by using lesser amount of the uncured tuberous roots or hairy roots than using larger amount of cured roots.

The results on scaling up of *P. rosea* hairy roots clearly indicate their large scale culture feasibility. The results also substantiate the published reports on hairy roots of *P. rosea* that both biomass and plumbagin level was increased in biorector cultures and the plumbagin production was asoociated with growth of the roots (Gangopadhyay et al. 2011). Mass production systems have been established for secondary metabolite production through hairy root cultures of several medicinal plants (Sim and Chang 1993; Palazon et al. 2003), but the direct application of these hairy roots in traditional preparation is yet to be analysed.

All these collectively substantiate the possible use of hairy roots in place of tuberous roots and also suggest that curing procedure can be excluded in the traditional preparations. Additionally, the successful up-scaling of *P. rosea* hairy roots in bioreactor indicates the possibility of utilization of this large-scale root biomass production system at the level of traditional industries. The novelty of the study

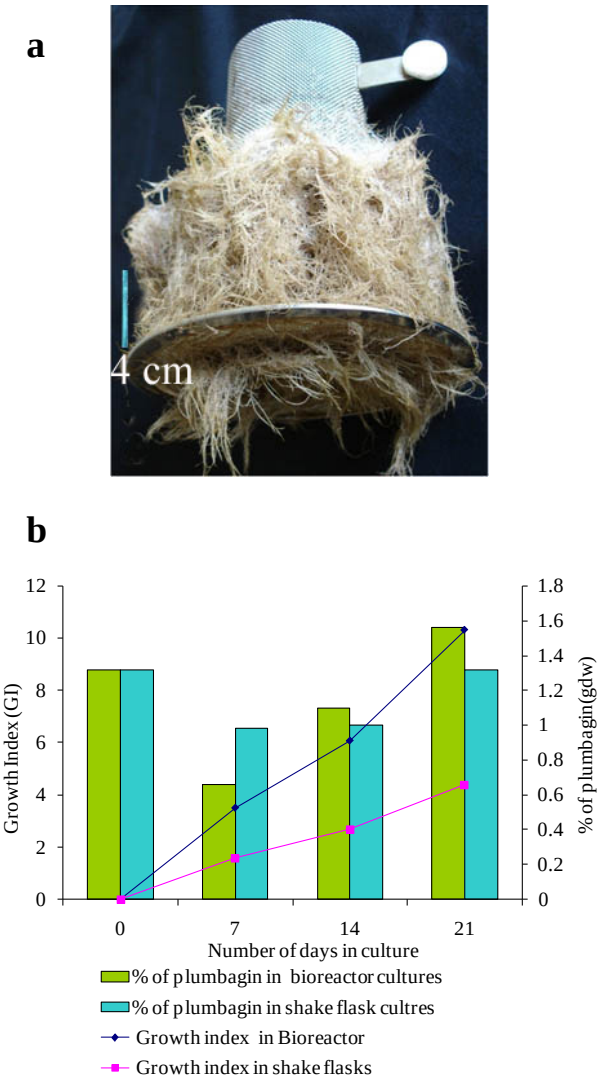

Fig. 6. a *P. rosea* hairy root biomass produced in bioreactor with 1% w/v inoculum over three weeks. **b** Growth indices of *P. rosea* hairy root cultures in shake flasks and bioreactor.

lies not only in proposing an additional application to the well-established hairy root culture system in traditional medicine, but also in suggesting an approach to reduce the quantity of usage of tuberous roots, and thereby prevent destruction of the rare plant from its natural habitat. Further comprehensive phytochemical/pharmacological studies are imperative, not only in *P. rosea* but also in other medicinal plants, to affirm whether hairy roots are an exact replica of the field-grown roots and whether these can be incorporated in traditional medicine.

Acknowledgments

This study was supported by research funds from Council of Scientific & Industrial Research, New Delhi and facilities from Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Thiruvanan-

thapuram, Kerala. The authors also acknowledge the analytical facilities provided by SAIF, Panjab University, Chandigarh.

References

Ahmad A, Banerjee S, Wang Z, Kong D, Sarkar HF 2008 Plumbagin-induced apoptosis of human breast cancer cells is mediated by inactivation of NF-kB and Bcl-2. J. Cell Biochem. 105: 1461-1471.

Ahmad I, Mehmood Z, Mohammad F 1998 Screening of some Indian medicinal plants for their antimicrobial properties. J. Ethnopharmacol. 62: 183-193.

Chetia S, Handique PJ 2000 A high frequency *in vitro* shoots multiplication of *Plumbago indica* – a rare medicinal plant. Curr. Sci. 78: 1187-1189.

Crouch IJ, Finnie JF, Staden J 1990 Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. Plant Cell Tiss. Organ Cult. 21: 79-82.

CSIR 1989 A Dictionary of India Raw Materials and Industrial Products 3rd edition. Publication and information Directorate CSIR, New Delhi.

Dorni CAI, Vidyalakshmi KS, Vasanthi HR, Rajamanickam GV, Dubey GP 2006 Anti-inflammatory activity of *Plumbago capensis*. Pharmacogn. Mag. 2: 239-243.

Dubey NK, Kumar R, Tripathi P 2004 Global promotion of herbal medicine: India's opportunity. Curr. Sci. 86: 37-41.

Evans WC 1996 Trease and Evans's Pharmacognosy 14th edition.W.B Saunder Company, London.

Gangopadhyay M, Dewanjee S, Bhattacharya, S 2011 Enhanced plumbagin production in elicited *Plumbago indica* hairy root cultures. J. Biosc. Bioeng. 3: 706-710.

Gangopadhyaya M, Sircar D, Mitra A, Bhattacharya S 2008 Hairy root culture of *Plumbago indica* as a potential source for plumbagin. Biol. Plantarum 52: 533-537.

Harborne JB 1973 Phytochemical methods. Chapman and Hall, London.

Harborne JB 1998 Phytochemical Methods - A Guide to Modern Techniques of Plant Analysis. Chapman and Hall, London.

Harborne JB, Turner BL 1973 Phytochemical methods. Chapman and Hall, London

Hsieh Y, Lin L, Tsai T 2005 Determination and identification of plumbagin from the roots of *Plumbago zeylanica* L. by liquid chromatography with tandem mass spectrometry. J. Chromatogr. 1083: 141-145.

Hu ZB, Du M 2006 Hairy Root and Its Application in Plant Genetic Engineering. J. Integr. Plant Biol. 48: 2121-2127.

Itoigawa M, Takeya K, Furukawa H 1991 Cardiotonic action of plumbagin on guinea-pig papillary muscle. Planta Med. 57:317-319.

Kirtikar KR, Basu BD 1975 Indian medicinal plants 2nd edition. M/S Bishen Singh Pal Singh, Delhi: 1465-1472.

Kittipongpatana N, Hock RS, Porter JR 1998 Production of solasodine by hairy root, callus, and cell suspension cultures of *Solanum aviculare* Forst. Plant Cell Tiss. Organ Cult. 52: 133-143.

Likhitwitayawuid K, Kaewamatawong R, Ruangrungsi N, Krungkrai J 1998 Antimalarial naphthoquinones from *Nepenthes thorelii*. Planta Med. 64: 237-241.

Mallavadhani UV, Sahu G, Muralidhar J 2002 Screening *Plumbago* species for bioactive marker plumbagin. Pharm. Biol. 40: 508-511.

Mishiba K, Nishihara M, Abe Y, Nakatsuka T, Kawamura H, Kodama K, Takesawa T, Abe J, Yamamura S 2006 Production of dwarf potted gentian using wild-type *Agrobacterium rhizogenes*. Plant Biotechnol. 23: 33-38.

Moos NS 1976 Single Drug Remedies. Vaidyasarathy Press (P) (Ltd), Kottayam, Kerala.

Nair S, Nair RRK, Srinivas P, Srinivas G, Pillai MR 2007 Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. Mol. Carcinog. 4: 722-733.

Palazón J, Mallol A, Eibl R, Lettenbauer C, Cusidó RM,. Piñol MT 2003 Growth and ginsenoside production in hairy root cultures of *Panax ginseng* using a novel bioreactor. Planta Med. 69: 344-349.

Parimala R, Sachdanandam P 1993 Effect of Plumbagin on some glucose metabolizing enzymes studied in rats in experimental hepatoma. Mol. Cell Biochem. 12: 59-63.

Rajasekaran T, Ravishankar GA, Reddy BO 2004 *In vitro* growth of *Tagetes patula* L. hairy roots, production of thiophenes and its mosquito larvicidal activity. Indian J. Biotech. 3: 92-96.

Satheeshkumar K, Jose B, Soniya EV, Seeni S 2009 Isolation of morphovariants through plant regeneration in *Agrobacterium rhizogenes* induced hairy root cultures of *Plumbago rosea* L. Indian J. Biotech. 8: 435-441.

Savitha BC, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA 2006 Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor. Process Biochem. 41: 50-60.

Shanavaskhan AE, Binu S, Muraleedharan CU, Santhoshkumar ES, Pushpangadan P 1997 Detoxification techniques of traditional physicians of Kerala, India on some toxic herbal drugs. Fitoterapia 68: 69-74.

Shastri PK 1994 2nd Adhyaya Rasatharangini 11th edition. Sri Jainendra Press, New Delhi

Sim SJ, Chang HN 1993 Increased shikonin production by hairy roots of *Lithospermum erythrorhizon* in two phase bubble column reactor. Biotech. Lett. 15: 145-150.

Singh VU, Bisht SK, Rao S, Uma Devi P, Udupa N 1997 Reduced toxicity and enhanced antitumour efficacy of plumbagin using poly (lactic-co-glycolic) biodegradable injectable implant. Indian J. Pharmacol. 29: 168-172.

Srivastava S, Srivastava AK 2007 Hairy root culture for mass-production of high-value secondary metabolites. Crit. Rev. Biotechnol. 27: 29-43.

Tilak JC, Adhikari S, Devasagayam TPA 2004 Antioxidant properties of *Plumbago zeylanica*, an Indian medicinal plant and its active ingredient, plumbagin. Redox Rep. 9: 219-227.

Trease GE, Evans WC 1989 Textbook of Pharmacognosy. Balliere, London.

Van der Vijver LM 1972 Distribution of plumbagin in the Plumbaginaceae. Phytochemistry 11: 3247-3248.

Venkatachalam P, Geetha N, Khandelwal A, Shaila MS, Lakshmi Sita G 2000 *Agrobacterium*-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. Curr. Sci. 78: 1130-1136.

Wermuth CG 2004 Multitargeted drugs: the end of the "one-target-one-disease" philosophy? Drug Discov. Today 9: 826-827.

Dr. K Satheeshkumar, doing research for the last 30 years in plant tissue culture, secondary metabolite production using cell, root and hairy-root cultures of medicinal plants from shake flasks to bioreactor level.

Mr. Binoy Jose, a researcher in plant tissue culture, bio-production of plant specific compounds and hairy root culture technology. Mr. Binoy also interested in inducing genetic variability in *Plumbago rosea* L.

Ms Dhanaya B Pillai focuses on optimization of conditions for production of plumbagin using transformed hairy root cultures of *Plumbago rosea* L. in bioreactor.

Dr. PN Krishnan, a researcher in Plant Biotechnology and Bioinformatics.