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Effect of sample extraction, preparation methods on HPLC quantification of plumbagin in *in vivo* and *in vitro* plant parts of *Plumbago zeylanica* L.

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Plumbagin is an important therapeutic compound of *Plumbago zeylanica* L., the commercial demand of this compound warrants optimizing a suitable method to isolate plumbagin in large scale. The present study was undertaken to obtain the maximum recovery of plumbagin content by employing different extraction methods viz., ultra-assisted extraction (UAE), maceration extraction (ME), soxhlet extraction (SE), serial soxhlet extraction (SSE), and serial maceration extraction (SME) from plant parts of *P. zeylanica*. Plumbagin content of two different sources such as field grown and hardened *in vitro* regenerated plants were quantified using reversed-phase high-performance liquid chromatography (RP-HPLC). For *in vitro* cultures, 6-benzylaminopurine (BAP) and Kinetin (KIN) were used for multiple shoot induction from nodal segments of *P. zeylanica*. Maximum percentage of shoot induction was obtained on MS medium fortified with BAP (6.66 μ M) from nodal segments exposed for 6 weeks. Further multiple shoot proliferation and elongation was achieved in MS medium with a combination of BAP (6.66 μ M) and KIN (4.44 μ M), with the maximum number of shoots (47.3 ± 0.06) and shoot length (2.0 ± 0.06 cm) per explant after 6 weeks of culture. The optimum root induction was observed on MS medium supplemented with 1.23- μ M indole-3 butyric acid (IBA) which produced 10.02 ± 0.2 mean roots with 6.2 ± 0.8 cm root length. Among the extraction methods, the SME method yielded maximum recovery (99.5%) of plumbagin as compared to others. *In vitro* leaf extract yielded high content of plumbagin (152.02 mg/g⁻¹ DW) as compared to other plant parts (root 115.41 mg/g⁻¹ dry weight; stem 98.02 mg/g⁻¹ dry weight) whereas *in vivo* leaf, stem and root samples yielded 96.7, 38.59, and 86.35 mg/g DW of plumbagin, respectively. The present observation suggests that the SME was more efficient for obtaining the maximum recovery of plumbagin and it was confirmed with HPLC quantification. Among the field grown and *in vitro* regenerated plants, the *in vitro* culture shows more accumulation of plumbagin and is found suitable for commercial extraction.

Key words: Micropropagation, cytokinin, serial maceration extraction (SME), optimization, methanol, quantification.

INTRODUCTION

Plumbago zeylanica L. is an important medicinal shrub commonly known as 'Chitramoolam' in Tamil, and

'Chitrak' in Sanskrit belonging to the family Plumbaginaceae (Nisha and Purshotam 2014). It is a

native of South Asia; the species are distributed throughout most of the tropics and subtropics; growing in deciduous woodland, savannas and scrublands from sea level up to 2000 m altitude (Jain et al., 2014). Plumbagin is a natural naphthoquinone compound (5-hydroxy-2-methyl-1, 4-naphthoquinone) found in three genera (*Plumbago*, *Plumbagella*, *Ceratostigma*) of the tribe Plumbagineae (Van der Vijver, 1972) and also from the insectivorous genera such as *Drosera*, *Dionaea*, and *Nepenthes* (Widhalm and Rhodes, 2016). This compound is reported for pharmacological activities like antifertility, antimalarial, antiviral, antimicrobial, anticancer and leishmanicidal (De Paiva et al., 2003; Premakumari et al., 1977; Thaweesak et al., 2011; Aziz et al., 2008). Most importantly, the radio sensitizing effect of plumbagin was demonstrated in *in vitro* cell cultures and in *in vivo* mouse tumour models (Matamoros et al., 2012). Plumbagin being a potential anticancer compound (Cao et al., 2018), the commercial demand for this compound is increasing due to its pharmacological activity. As plumbagin is being isolated from the wild plant population, continuous exploitation may lead to extinction of this plant in future. Therefore, there is a need for developing alternative sources towards plumbagin production. *In vitro* culture (Hu and Wang, 1983) system offers an alternative source of secondary metabolite production in variety of medicinal plants. Various extraction and quantification methods of plumbagin from different plant species have been reported earlier which includes maceration, dynamic maceration, assistance of ultrasonic waves, Soxhlet apparatus, cold maceration and homogenization (De Paiva et al., 2004; Hajnos et al., 2007; Gangopadhyay et al., 2008; Putalun et al., 2010; Chellampillai et al., 2011; Thaweesak et al., 2011) with limited percent of plumbagin recovery. Solvents based on polarity were employed for the extraction of this valuable compound. Hsieh et al. (2005) developed a maceration method using ethanol and water for extraction of plumbagin from the roots of *P. zeylanica*. Chellampillai et al. (2012) developed a novel solvent-free extraction method for plumbagin using hydrophilic liquid Gelucire 44/14. Qualitative and quantitative analyses of plumbagin were carried out from roots of *P. scandens* (De Paiva et al., 2004), leaf and roots of *P. europea* (Muhammad et al., 2009). Gas chromatography-mass spectrometry (GC-MS) based analysis of 1, 4-naphthoquinones in different species of Droseraceae (Bonnet et al., 1984) and normal phase liquid chromatography coupled with UV-detector method (Marston and Hostettmann, 1984) has been studied. Reversed-phase high-performance liquid chromatography (RP-HPLC) method was also used for the analysis of plumbagin (Stensen and Jensen, 1994; Unnikrishnan et al., 2008). Sakamoto et al. (2008) developed enzyme-

linked immunosorbent assay (ELISA) method for detection of plumbagin using monoclonal antibody. Nevertheless, a significant sample preparation step is required for the aforementioned quantification methods. Recovery of the compound is augmented by the methods and selection of solvents used. Although plumbagin was extracted from *P. zeylanica* using various methods, to the best of our knowledge there is no report on the standardized extraction method to maximize the plumbagin content. Hence, in the present study, to find out a suitable extraction method, five different extraction methods were evaluated for maximum recovery of plumbagin from plant parts of *P. zeylanica*. Plumbagin content of *in vivo* and hardened *in vitro* regenerated plant parts of *P. zeylanica* using the extraction method with maximum recovery of plumbagin was standardised and the same has been validated using RP-HPLC.

MATERIALS AND METHODS

Plant

The stem cuttings of the medicinal plant *P. zeylanica* L., was procured from the Centre for Indian Medicinal Heritage, Kanjikode, Kerala, and grown in the Garden of Department of Biotechnology, Bharathiar University, Coimbatore. The Botanical Survey of India, Coimbatore, authenticated the identification; voucher specimen was deposited at the Herbarium, Department of Botany, Bharathiar University, Coimbatore.

In vitro culture

The sterilised nodal explants of *P. zeylanica* were placed on MS media (Murashige and Skoog) 1962 fortified with various concentrations (2.21 to 11.09 μM) of 6-benzylaminopurine (BAP) individually and in combination of BAP (6.66 μM) with Kinetin (2.32 to 11.61 μM). The cultures were maintained at a photoperiod of 16 h light/day at $25 \pm 2^\circ\text{C}$ for multiple shoot regeneration. The regenerated 40-days-old shoots were transferred to MS medium with different concentrations (1.2 to 4.9 μM) of indole-3-butyric acid (IBA) for root induction. The *in vitro* regenerated plantlets were transferred into mixture of sand: soil: vermiculite (1:1:1). The six-month-old *in vitro* hardened plantlets were used for the analysis of plumbagin content.

Chemicals and reagents

Plumbagin (>99% purity) purchased from Sigma Aldrich, USA (Cat. No. P7262) was used as standard. Solvents (HPLC grade) like methanol, hexane, chloroform, ethyl acetate, water and trifluoroacetic acid were obtained from Merck (Mumbai). All other chemicals with the highest purity used for plant tissue culture were purchased from Himedia (Mumbai).

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Sample preparation and extraction

Leaf, stem and root parts of *P. zeylanica* from six-month-old field grown and hardened *in vitro* regenerated plants of the same age were selected for sample preparation after several trails. The plant materials were shade-dried, powdered and sieved (20 to 40 µ mesh). One gram of each plant material was used for extraction of plumbagin.

Extraction methods

For the optimization of extraction and efficient recovery of plumbagin, following extraction techniques viz., ultrasonication, maceration, Soxhlet, serial Soxhlet, and serial maceration, with five different solvents with varying polarity (hexane, chloroform, methanol, ethyl acetate and water) were chosen.

Ultrasonication assisted extraction (UAE) method

UAE was performed according to the method described by Ying et al. (2011). UAE method was performed using ultrasonic apparatus (First source Laboratory solution, LLP). Plant material was soaked in 100 ml of different solvents separately in an Erlenmeyer flask. The flask was placed in an ultrasonic bath for 60 min at 30°C. The samples were centrifuged at 5000 rpm for 10 min and the supernatant was taken for the analysis.

Maceration extraction (ME) method

Extraction of plumbagin was carried out using a method described by Jin et al. (2011) with minor modifications. The dried plant material was extracted with 100 ml of different solvents on a shaker with 150 rpm at 30°C for overnight. The extract was filtered using Whatman No. 1 filter paper.

Soxhlet extraction (SE) method

Okoduwa et al. (2016) method was performed using exhaustive Soxhlet extraction of plumbagin with classical extraction apparatus (Soxhlet apparatus, Borosil) with minor modifications. The plant materials were continuously extracted with cooled, condensed solvents individually for 5 h. After extraction, the methanol solvent was evaporated by concentrating under vacuum with rotary evaporator (Cyber Lab) at 40°C under reduced pressure. The solvent free extract was thereafter evaluated.

Serial Soxhlet extraction (SSE) method

For the SSE method, the plant material was packed in thimbles and was continuously extracted with cooled, condensed solvents from non-polar to polar for 5 h using Soxhlet apparatus (Borosil). After extraction, the methanol solvent was evaporated by concentrating under vacuum with rotary evaporator (Cyber Lab.) at 40°C under reduced pressure. The solvent free methanol extract was thereafter evaluated.

Serial maceration extraction (SME) method

For the extraction of plumbagin, the SME was carried out following the protocol of Balasubramanian et al. (2018). The plant material was extracted with 100 ml of different solvent serially based on the polarity. The samples were then macerated on a shaker with 150

rpm at 30°C overnight. The extract was then filtered through Whatman No. 1 filter paper. For quantification, the extracts obtained using the above different methods were dried using rotary vacuum evaporator (Cyber Lab). 1.0 mg of dried solvent extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%) and filtered using 0.45-µm polyvinylidene difluoride (PVDF) syringe filter. The concentrated filtrate was further used for the quantification of plumbagin using HPLC. All the samples were preserved at -20°C until analysis.

Quantification of plumbagin

Quantification of plumbagin was performed using Waters2998Liquid Chromatography equipped with the Photodiode Array Detector. The data was processed with Empower2 software. The separation was achieved on Symmetry® C18column (4.6 mm × 250 mm, 5 µm). The mobile phase used was methanol: water with 0.1% Trifluoroacetic acid at a ratio of 20:80 v/v. With the flow rate of 1.0 ml/min at 30°C, temperature at 254 nm, injection volume was set at 20 µl. Standard stock solution of plumbagin was prepared with HPLC grade methanol (concentration of 1 mg/ml). The presence of plumbagin in the sample was analysed by comparing it with retention time of the standard plumbagin. The standard and sample solutions were injected in triplicate. The amount of plumbagin present in each sample was calculated by comparing the standard area with sample area as:

$$\text{Plumbagin (mg/g)} = \frac{\text{Peak area of the sample}}{\text{Peak area of the standard}} \times 1000$$

Validation of analytical method

Analytical methods used for plumbagin quantification was validated for specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The specificity of the HPLC method was validated by injecting 20 µl of standard plumbagin (1.0 mg/ml) and methanol (100%) as control individually. Five different concentrations (10 to 50 µg/ml) of the standards were analysed in triplicate and the respective calibration curve was generated.

The linearity between peak areas and the concentration of the plumbagin was calculated using linear regression analysis. Accuracy of the analytical condition was determined by spiking known concentration of the standard to the various extracts obtained using different extraction methods and solvents and calculating the percentage of recovery. The recovery of plumbagin was calculated by subtracting the mass concentration of non-spiked extract using external standard linear regression. The experiment was repeated three times and the replica were evaluated, the values were represented as relative standard deviation (RSD%) and standard error (SE). Recovery (%) and RSD (%) were calculated using the following formula:

$$\text{Recovery (\%)} = \text{RC/IC} \times 100$$

where RC is the recovered concentration and IC is the injected concentration.

$$\text{RSD (\%)} = (\text{SD/M}) \times 100$$

where SD is the standard deviation and M is the mean.

The LOD and LOQ were calculated based on the standard deviation of the y-intercept (σ) and slope of the calibration curve (S) obtained from linear regression. LOD was calculated using the expression $3.3 \sigma/S$ and LOQ was calculated using $10 \sigma/S$.

Table 1. Effect of different concentration of 6-Benzylaminopurine (BAP) and Kinetin (KIN) combination with BAP (6.66 μ M) exposed cultures on multiple shoot induction using nodal explants of *P. zeylanica* L.

BAP (μ M)	6 Weeks		
	Response (%)	Mean shoot number	Mean shoot length (cm)
0.0	0.0 $\pm$ 0.00 ^e	0.0 $\pm$ 0.00 ^e	0.0 $\pm$ 0.00 ^e
2.21	30.0 $\pm$ 1.3 ^d	12.2 $\pm$ 0.5 ^c	0.6 $\pm$ 0.02 ^c
4.44	50.0 $\pm$ 1.1 ^a	15.6 $\pm$ 0.4 ^b	1.2 $\pm$ 0.06 ^a
6.66	52.3 $\pm$ 1.7 ^a	18.4 $\pm$ 0.4 ^a	1.8 $\pm$ 0.01 ^a
8.88	44.4 $\pm$ 0.2 ^b	16.5 $\pm$ 0.2 ^b	0.8 $\pm$ 0.03 ^b
11.10	39.2 $\pm$ 0.4 ^c	8.6 $\pm$ 0.8 ^d	0.3 $\pm$ 0.01 ^d
BAP+KIN (μM)			
0.0	0.0 $\pm$ 0.00 ^f	0.0 $\pm$ 0.00 ^f	0.0 $\pm$ 0.00 ^e
6.66+2.21	60.12 $\pm$ 1.6 ^d	25.1 $\pm$ 0.32 ^b	0.6 $\pm$ 0.08 ^d
6.66+4.44	92.06 $\pm$ 1.8 ^a	47.3 $\pm$ 0.06 ^a	2.0 $\pm$ 0.06 ^a
6.66+6.66	79.06 $\pm$ 0.8 ^b	20.4 $\pm$ 0.08 ^c	1.0 $\pm$ 0.01 ^b
6.66+8.88	72.04 $\pm$ 1.0 ^c	14.8 $\pm$ 0.2 ^d	0.8 $\pm$ 0.01 ^c
6.66+11.10	58.06 $\pm$ 1.0 ^e	8.2 $\pm$ 0.02 ^e	0.2 $\pm$ 0.04 ^e

Values represent mean $\pm$ standard error of 20 replicates per treatment in three repeated experiments. Values within column followed by the same letter are not significantly different. Mean values of three independent experiments ($\pm$) with standard errors. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5 % level.

Statistical analysis

The analysis was put up according to a thoroughly randomized design. All the experiments were repeated three times. The data (percent of regeneration, shoot number and shoot length, root number and root length) were statistically analysed using one-way analysis of variance and pair wise means compared using Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISCUSSION

Plant parts of six-month-old field grown and six-month-old *in vitro* hardened plant parts of *P. zeylanica* was found suitable for extraction of plumbagin. For *in vitro* propagation, the shoot tip and nodal explants are valuable culture technique for the large-scale production of secondary metabolites (Sen and Sharma, 1999). In the present study, after 10 days of inoculation, shoot initiation in nodal explants was observed. The regenerated shoots produced abundant adventitious shoots with subsequent sub-culture. Efficient regeneration response was observed in medium supplemented with 6.66 μ M of BAP with the highest mean number (18.4 $\pm$ 0.4) of shoots per explants at the end of 6 weeks (Table 1). The shoot multiplication rate was decreased at high concentration of BAP, which corroborate with Ashok et al. (2011) where BAP above 6.66 μ M was not effective for shoot regeneration in *Plumbago* species. The primary effect of BAP in inducing multiple shoot has been previously reported (Chen et al., 2001; Huang et al., 2000). In the present study, among the five different concentration (2.2 to 11.10 μ M) of KIN along with BAP (6.66 μ M) tested,

combination of 6.66 μ M BAP along with 4.44 μ M KIN was found to be effective in enhancing shoot induction. The highest mean number of shoots obtained was 47.3 $\pm$ 0.06 (Figure 1a, b and c) with an average shoot length of 2.0 $\pm$ 0.06 cm per explants at the end of 6 weeks. Root initiation was observed within 5 to 7 days after inoculation on MS medium enriched with different concentrations (1.23 to 4.92 μ M) of IBA. Maximum number of roots (10.02 $\pm$ 0.2) was produced when media was supplemented with 1.23 μ M of IBA with an average of 6.2 $\pm$ 0.8 cm root length per explants at the end of 4 weeks (Table 2 and Figure 1d). Similar results were also reported in *Plumbago* spp. (Ashok et al., 2011).

The first prime step in secondary metabolite extraction is to determine the solvent efficiency. For the present study, five different solvents were used based on their increasing polarity viz. hexane, chloroform, methanol, ethyl acetate and water for plumbagin extraction. Previously, solvents like chloroform, toluene, water, ethanol, methanol and n-hexane were used for the extraction of plumbagin from *Plumbago* spp. (De Paiva et al., 2003; Hseih et al., 2005; Gupta et al., 1993; Pawar et al., 2010; Komaraiah et al., 2004). In the present study, a significant variation in the area of the chromatogram was obtained with respect to the solvent used for the isolation. The maximum recovery of plumbagin from the plant parts of *P. zeylanica* was achieved using methanol as solvent in the extraction method, which might be due to the lower boiling point of methanol. Water, a highly polar than all solvents used was least effective in the extraction of plumbagin, while chloroform was found to be suitable solvent for maximum recovery of plumbagin from stem



Figure 1. (a) Bud breaking on BAP combination Kinetin ($6.66+4.44 \mu\text{M}$) from nodal segments after 30 days. (b) Shoot multiplication in BAP combination Kinetin ($6.66+4.44 \mu\text{M}$) exposed cultures growing on MS basal medium after 6 weeks of transfer. (c) Shoot multiplication in BAP combination Kinetin ($6.66+4.44 \mu\text{M}$) exposed cultures growing on MS medium after 6 weeks of incubation. (d) Rooting in the regenerated microshoots on MS media containing $1.23 \mu\text{M}$ IBA after 4 weeks of incubation.

Table 2. Effect of IBA augmented with MS media on root induction from *in vitro* raised *P. zeylanica* L. after 6 weeks.

IBA (μM)	4 Weeks	
	Mean of roots number	Mean of roots number (cm)
0.0	0.00 ± 0.00^d	0.00 ± 0.00^d
1.23	10.02 ± 0.2^a	6.2 ± 0.8^a
2.46	8.6 ± 0.1^b	3.0 ± 0.0^b
3.69	2.4 ± 0.0^c	1.8 ± 0.6^c
4.92	2.3 ± 0.1^c	1.6 ± 0.0^d

Values represent mean $\pm$ standard error of 20 replicates per treatment in three repeated experiments. Values within column followed by the same letter are not significantly different. Mean values of three independent experiments ($\pm$) with standard errors. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5 % level.

Table 3. Accuracy of recovery with standard plumbagin spiked to the leaf, stem and root different extraction methods in field grown *P. zeylanica* L.

Extraction method	Solvent	Recovery (%)			RSD (%)		
		Leaf	Stem	Root	Leaf	Stem	Root
UAE	Hexane	73.0±2.6	43.8±1.3	74.9±0.6	3.62	2.99	0.86
	Chloroform	68.3±2.8	76.0±1.0	64.3±1.1	4.22	1.32	1.71
	Methanol	93.6±1.5	51.4±1.2	95.3±0.9	1.63	2.47	1.01
	Ethyl acetate	69.6±0.6	24.4±0.4	84.3±1.2	0.88	2.01	1.42
	Water	10.6±0.5	9.6±0.5	12.1±0.3	4.94	5.97	2.51
ME	Hexane	58.3±2.8	75.1±0.9	87.9±0.7	4.94	1.27	0.79
	Chloroform	83.3±2.8	23.8±1.3	54.0±1.6	3.46	5.81	3.07
	Methanol	92.3±0.5	63.1±2.7	63.9±0.6	0.62	4.29	1.02
	Ethyl acetate	83.0±2.6	9.6±0.5	74.2±1.0	3.18	5.51	1.34
	Water	51.6±2.8	5.4±0.4	26.6±0.6	5.58	7.40	2.49
SE	Hexane	91.0±1.0	73.6±1.5	45.6±1.0	1.09	2.16	2.24
	Chloroform	72.3±2.5	23.7±1.5	65.8±0.8	3.47	6.63	1.32
	Methanol	83.0±2.6	76.0±1.6	89.4±0.9	3.18	2.11	1.02
	Ethyl acetate	61.6±2.8	15.1±0.3	75.5±1.2	4.68	2.01	1.65
	Water	11.6±0.5	8.8±0.6	16.0±0.8	4.94	7.87	5.0
SSE	Hexane	14.3±0.5	87.1±1.4	54.9±0.5	4.02	1.63	1.06
	Chloroform	9.6±0.5	64.2±1.1	33.7±1.6	5.97	1.72	4.97
	Methanol	24.1±0.9	23.7±1.5	74.3±1.2	3.73	6.63	1.62
	Ethyl acetate	9.6±0.5	14.9±0.7	26.0±0.8	5.97	4.70	3.07
	Water	5.3±0.5	7.6±0.5	14.0±0.2	10.82	0.06	1.42
SME	Hexane	64.1±0.9	72.5±0.6	76.0±0.4	1.48	0.84	0.52
	Chloroform	76.6±1.4	54.7±0.7	70.7±0.6	1.82	1.42	0.94
	Methanol	98.9±0.8	90.8±0.3	99.5±0.2	0.81	0.38	0.20
	Ethyl acetate	9.6±0.5	14.6±0.4	24.8±0.2	5.97	3.37	0.00
	Water	4.6±0.5	5.06±0.6	10.3±0.4	0.12	2.99	4.02

The data were tabulated recovery with standard plumbagin content spiked to the six month old *in vivo* of *P. zeylanica* L plant. Mean values of three independent experiments (±) with standard errors.

part of *P. zeylanica*. These results corroborate with the previous studies, where methanol was found to be pertinent for the extraction of quercetin (Balasubramanian et al., 2018). Methanol was found to be ideal for the separation of phenolic substance from field-grown plants and *in vitro* cultures of *Hypericum* species (*Hypericum perforatum* and *Hypericum androsaemum*) (Dias et al., 1999).

Among the extraction methods such as UAE, ME, SME, SE and SSE evaluated for their efficiency to extract plumbagin from different parts of *P. zeylanica*, the recovery of plumbagin using SME method was efficient as compared to ME, SE, UAE and SSE (Table 3). The extraction conditions such as time duration and temperature for each method were resolved based on the previous report (De Paiva et al., 2004). The relative recovery of plumbagin varied among the extraction

method tested. The highest percent of plumbagin recovery from leaf extract was obtained using SME (98.9%) method, followed by UAE (93.6%), ME (92.3%), SE (83.0%), and SSE (24%). The recovery in stem part was the highest in SME (90.8%) followed by SSE (87%), UAE and SE (76%), and ME (75.1%). Similarly, in root extract, the highest recovery was obtained in SME (90.8%), followed by SSE (87.1%), UAE and SE (76%), and ME (75.1%) (Table 3). Plumbagin extraction efficiency was found to be in the order of SME > ME > SE > UAE > SSE. The poor recovery of plumbagin using SSE method could be due to long solvent-reflex (5h). Likewise, the setback of SE method include time consumption, high quantity solvent requirement and economically non-feasible. Further, plumbagin could be degraded due to long extraction process (De Paiva et al., 2004; Matamoros et al., 2012). UAE method was

Table 4. Serial maceration method (SME) using extraction of different solvent system on Plumbagin content in *in vivo* and *in vitro* of *P. zeylanica* L. plant.

Extraction Method	Solvent	<i>In vivo</i> of <i>P. zeylanica</i> L. (mg g ⁻¹) ^a			<i>In vitro</i> of <i>P. zeylanica</i> L. (mg g ⁻¹) ^b		
		Leaf	Stem	Root	Leaf	Stem	Root
SME	Hexane	0.69	0.99	33.45	2.42	41.0	80.49
	Chloroform	2.4	0.5	26.14	8.26	18.9	45.32
	Methanol	96.7	38.59	86.35	152.02	98.02	115.41
	Ethyl acetate	0.0592	0.005	0.002	5.78	6.33	75.42
	Water	0.0512	0.04	0.04	0.02	0.01	0.13

All the extracts were dried and about 1 mg extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%). About 20 µl of extracts (1 mg ml⁻¹) and standard (1 mg ml⁻¹ plumbagin) were taken as injection volume. mg^a plumbagin equivalent/g⁻¹ DW of *in vivo* of *P. zeylanica* L. extract. mg^b plumbagin equivalent /g⁻¹ DW of *in vitro* of *P. zeylanica* L. extract.

effective for the extraction of plumbagin from stem part; this might be due to the penetration efficacy of the ultrasound. UAE reportedly improved the extraction rate and yielded isoflavone from the stem part of *Pueraria lobata* (Willd.) Ohwi (Huaneng et al., 2007). The SME method was found suitable for maximum plumbagin recovery for *in vitro* culture plants and same was validated using RP-HPLC (Table 4). The conditions for HPLC such as mobile phase composition, temperature and flow rate were optimized to accomplish a good resolution and symmetrically shaped peak for plumbagin in less run-time. Similar separation of plumbagin was reported by Pereira et al. (2015). Although, flow rates of 1.0 ml/min and 0.75 ml/min were reported earlier for the isolation of plumbagin from the plant extract (Stensen and Jensen, 1994; Gangopadhyay et al., 2008; Gupta et al., 1993; Muhammad et al., 2009), in the present study, the resolution of the chromatogram was satisfactory at the flow rate of 1.0 ml/min and can be claimed as significant condition. Similarly, in the present study, methanol: water with 0.1% TFA was efficient with good peak resolution (Figure 2a) of plumbagin when compared with the mobile phase composition, like acetonitrile: water, n-hexane: chloroform-2-propanol, methanol: water reported earlier (Stensen and Jensen, 1994; Gangopadhyay et al., 2008; Gupta et al., 1993; Muhammad et al., 2009). The chromatography peak obtained under the optimized condition applied was efficient and reproducible similar to the earlier findings of Hsieh et al. (2005).

The validation parameters including specificity, linearity, accuracy, precision, limit of Detection (LOD) and limit of quantification (LOQ) were examined according to the ICH Guidelines (2005). Different methods have been reported earlier for the analysis of plumbagin in *Plumbago* spp. (Unnikrishnan et al., 2008; Hsieh et al., 2005; Gupta et al., 1993). However, a full validation report of RP-HPLC method for the comparative analysis of plumbagin from the field-grown and hardened *in vitro* regenerated plant material is reported here for the first time. The least square linear regression data of

plumbagin were used to determine the calibration parameter. Excellent linearity of the standard plumbagin (linear regression of R² = 0.9994) was obtained. The LOD and LOQ values were found to be 2.2 and 2.4 µg/L, respectively, which indicate that the developed method is suitable and sensitive for the determination of plumbagin. The percentage of recovery was good with relative standard deviation of ≤ 2%, which signifies the method is accurate.

The samples prepared from leaf, stem and root parts of *in vivo* and *in vitro* samples of *P. zeylanica* by serial maceration method (SME) using different solvents (hexane, chloroform, methanol, ethyl acetate and water) were used to quantify plumbagin content by HPLC. Methanolic extract of field-grown root samples of *P. zeylanica* showed plumbagin content of 86.35 mg/g dry weight, whereas the content was higher (115.41 mg/g dry weight) *in vitro* roots. This is the first report on the quantification of plumbagin from *in vitro* hardened plant parts of *P. zeylanica*. Dorni et al. (2007) reported the total plumbagin content in the methanolic extract of roots of *Piriformospora indica* L. and *P. zeylanica* L., were 0.569 and 0.247% w/w, respectively. There are reports in other plants (Bhardwaj et al., 2018) which suggest growth hormones applied during *in vitro* propagation may be one of the factor and the other factor hypothesized as formation of chemicals like phenyl amides and accumulation of polyamines during stress conditions during the hardening process of *in vitro* plants (Ramakrishna, 2011). Plumbagin also being a secondary metabolite, the results of the present study could be related to earlier reports. Bonnet et al. (1984) reported an increase in naphthoquinone level up to five times in *in vitro* root and shoot samples of *Drosera intermedia* and *Drosera rotundifolia*. The increase in naphthoquinone level of the plant parts was attributed to *in vitro* culture conditions. HPLC quantification of plumbagin content in methanolic extract of *in vivo* leaf samples of *P. zeylanica* was 96.7 mg/g dry weight, which is lower than the *in vitro* leaf sample (152.02 mg/g dry weight) (Table 4 and Figure 2b, c, d). The results corroborate with the earlier reports

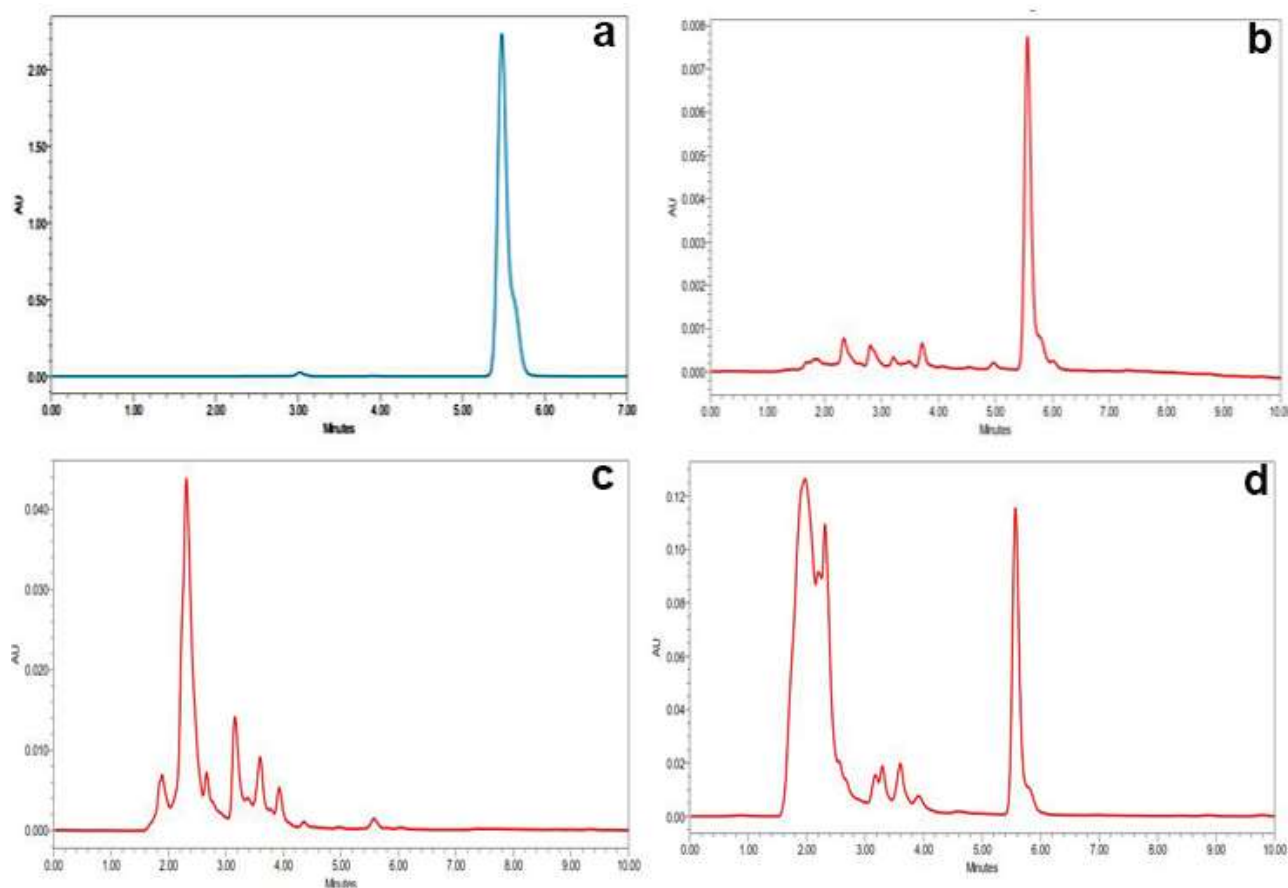


Figure 2. HPLC chromatograms of plumbagin in the *in vitro* *P. zeylanica* L. plant of leaf stem and root methanolic extract of Serial Maceration Method (SME): (a) Standard of plumbagin, (b) methanolic extract of leaf, (c) methanolic extract of stem, (d) methanolic extract of root.

in other plants (Bhardwaj et al., 2018) and Karuppusamy (2009) that the hormones supplied exogenously during tissue culture not only influence shoot proliferation but also *in vitro* bioactive secondary metabolites. Abiotic stress signals creating stress on plants during hardening process was another factor attributed for increased secondary metabolite secretion. Bryant et al. (1983) hypothesized that when plants are stressed, an exchange occurs between carbon to biomass production or formation of defensive secondary compounds. A stress response is induced when plants recognize stress at the cellular level. Secondary metabolites are involved in protective functions in response to both biotic and abiotic stress conditions. The *in vitro* leaf, stem and root of *P. zeylanica* showed 63, 40 and 74%-fold increase in plumbagin content as compared to the field grown parts. The yield of plumbagin from *in vitro* roots of *P. zeylanica* was higher (115.41 mg/g dry weight) than *in vivo* root extract. The *in vitro* leaf samples of *P. zeylanica* showed the highest plumbagin content (152.02 mg/g dry weight) compared to *in vitro* roots (115.41 mg/g dry weight). This finding has implications in plant biodiversity conservation

as *P. zeylanica* L. is also reported to be a threatened taxon and the *in vitro* leaves can be better source of plumbagin rather than *in vivo* and *in vitro* roots, thereby conserving the wild population. *In vitro* propagation and hardening of propagated plantlets of *P. zeylanica* (L.) in greenhouse, is commercially feasible, considering the cost of plumbagin and its pharmacological significance.

Conclusions

The extraction methods optimized in the present study to quantify plumbagin were simple, less time-consuming and reproducible for industrial production. The HPLC method validated for linearity, repeatability and reproducibility was satisfactory. The results from the present study clearly indicate that the SME method-using methanol as solvent is efficient in maximum recovery of plumbagin from *P. zeylanica*. The leaf samples of *in vitro* hardened plants were found to be suitable source for plumbagin isolation than the field grown plants of *P. zeylanica*. Hence, this can be recommended for

production of plumbagin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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