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ORIGINAL ARTICLE

Box–Behnken supported development and validation of robust HPTLC method: an application in estimation of punarnavine in leaf, stem, and their callus of *Boerhavia diffusa* Linn

Wasim Ahmad[1] · Ibraheem Husain[2] · Niyaz Ahmad[3] · Mohd Amir[4] · Md. Sarafroz[5] · Mohammad Azam Ansari[6] · Ameeduz Zafar[7] · Abuzer Ali[8] · Rasheeduz Zafar[9] · Kamran Ashraf[10]

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Abstract

Boerhavia diffusa (BD) Linn. (Nyctaginaceae) is one of the most commonly used herbs in the Indian traditional system of medicine for the urinary disorders. The aim of the current investigation was to carry out initiation, development, and maintenance of BD callus cultures and quantitative estimation of punarnavine in plant and callus extracts. Leaves and stem of BD were used as explant for the tissue culture studies using Murashige and Skoog (MS) basal medium. MS Media comprising 2,4-Dichlorophenoxy acetic acid (2,4-D) (1 ppm) and 2,4-D (1 ppm) + Indole-3-acetic acid (IAA) (1.0 ppm) were found to yield friable callus from leaf explant; similarly, 2,4-D (0.3 ppm) + IAA (0.75 ppm) + Kinetin (0.3 ppm) and 2,4-D (0.5 ppm) + Naphthalene acetic acid (NAA) (1.5 ppm) + Kinetin (0.3 ppm) were found to yield friable callus from the stem explant. High-performance thin-layer chromatography method was been developed for the quantitative estimation of punarnavine ($R_f = 0.73$) using mobile phase containing toluene: ethyl acetate: formic acid in the ratio (7.0:2.5:0.7, v/v/v) at 262 nm. The validated method was found linear ($r^2 = 0.9971$) in a wide range (100–1000 ng spot^{-1}), precise, accurate, and robust. The values of limit of detection, LOD = 30.3 ng spot^{-1}, and limit of quantification, LOQ = 100.0 ng spot^{-1}. The robustness of the method was proved by applying the Box–Behnken design (BBD). The developed method found appropriate for the quality control of medicinal plants containing punarnavine as a constituent.

Keywords *Boerhavia diffusa* · Punarnavine · HPTLC · Callus culture

Abbreviations

LOD	Limit of detection
LOQ	Limit of quantification
BBD	Box–Behenken design
ICH	International council for harmonization
HPTLC	High-performance thin-layer chromatography

✉ Wasim Ahmad
wasimahmadansari@yahoo.com

[1] Department of Pharmacy, Mohammad Al-Mana College for Medical Sciences, Al-Safa, Dammam 34222, Kingdom of Saudi Arabia

[2] Department of Pharmacology, Mesco Institute of Pharmacy, Uttar Pradesh, Amroha, India

[3] Department of Pharmaceutics, College of Clinical Pharmacy Imam, Abdulrahman Bin Faisal University, Dammam 31441, Saudi Arabia

[4] Department of Natural Product & Alternative Medicines College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam 31441, Saudi Arabia

[5] Department of Pharmaceutical Chemistry College of Clinical Pharmacy Imam, Abdulrahman Bin Faisal University, Dammam 31441, Saudi Arabia

[6] Department of Epidemic Disease Research, Institutes for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, Dammam 31441, Saudi Arabia

[7] Department of Pharmaceutics, College of Pharmacy, Jouf University, Al-Jouf, Sakaka, Saudi Arabia

[8] Department of Pharmacognosy, College of Pharmacy, Taif University, Taif, Saudi Arabia

[9] Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India

[10] Faculty of Pharmacy, University Teknologi MARA (UITM), Cawangan Selangor, 42300 Kampus Puncak Alam, Seleangor Darul Ehsan, Malaysia

BD	*Boerhavia diffusa*
MS	Murashige and Skoog
TLC	Thin-layer chromatography
Rf	Retention factor
UV	Ultra-violet
2–4, D	2–4, Dichlorophenoxy acetic acid
IAA	Indole acetic acid
NAA	Naphthalene acetic acid
PPM	Parts per million
PNE	Punarnavine
RSD	Relative standard deviation

Introduction

Medicinal plants have been used since long time as remedies to cure the various diseases. Medicinal plants and its constituents are important source of medicine and many other valuable therapeutic substances. Plants and their products have used as medicine by almost all cultures since ancient times. In the present time, medicinal plants are very essential to the international economy, as around 85% of traditional medicinal preparations consist of plants or plant extracts. Last few eras, there has been a resurgence of attentiveness in the study and practice of medicinal plants in health care and in recognition of the importance of medicinal plants to the health system. Due to this, awakening has led to rapidly increase the demand of herbal drugs (Satish and Hsin-sheng 2004).

It is important to immediately develop simple, sensitive, and specific modern principles for the quality, safety, and efficacy of herbal drugs. Medicinal plants used in the phytopharmaceutical formulations are acquired primarily from the naturally growing regions. Due to escalating demands of herbal medicine, plants are being exploited and thus threatening the existence of many endangered plants. On the other hand, plants are also vanishing at an alarming rate due to uncontrolled deforestation and urban development. Hence, biotechnology plays a major role in tracking substitutes to overcome this problem by plant tissue culture method and deliver resources for propagating and preserving valuable endangered medicinal plants (Sheeba et al. 2013).

Boerhavia diffusa L. (BD) commonly known as punarnava is important medicinal plant of the family Nyctaginaceae. *Boerhavia* is known to have two species: one with white flower identified as Shweta punarnava (*B. erecta)* and other with red flower termed as rakta punarnava (BD), which is well known to have medicinal properties (Aradhya et al. 2014; Chaudhary and Dantu 2011; Kapil and Sanjivani 2015).

BD is one of the most popular drugs used in the Indian system of medicine against urinary disorders (Singh and Udupa 1972). The active diuretic principle of BD is an alkaloid punarnavine (PNE) which is mainly acted on renal tubular epithelium (Chowdhary and Sen 1955). PNE molecular formula is $C_{17}H_{22}N_2O$ and about 0.01% in the aerial part, while 0.045% occurs in the roots BD (Sethi and Zafar 2003).

According to Ayurveda, Unani, and Sidha traditional medicinal systems, Punarnava possesses bitter, cooling, and astringent properties. It is useful in various heart diseases, biliousness, anemia, inflammations, blood impurities, leucorrhoea, and also in asthma. The leaves of BD are appetizer, useful in tumours, abdominal pain, spleen enlargement, ophthalmia, and in joints pains, whereas seeds are carminative, expectorant, and useful in scabies (Kirtikar and Basu 1996).

The current research was carried out to investigate the approaches for enhancing the alkaloid production of PNE using plant tissue culture methods and HPTLC method was used for its quantitative estimation. The proposed developed method was validated for the estimation of PNE in BD plant and in in-vitro callus cultures in terms of precision, accuracy, robustness, LOD, and LOQ. This developed validated method may be beneficial for quality control analysis of PNE containing medicinal plants and herbal drug formulations.

Methods

Plant materials

Leaves and stem of BD were collected from the herbal garden, Jamia Hamdard New Delhi. The plant samples were authenticated by Taxonomist Dr. M. P. Sharma, Department of Botany, Jamia Hamdard, New Delhi, and the voucher specimen number (JH/FP/316/BD) was deposited. Fresh leaves and stem sections were used as explants for the in-vitro study.

Explant sterilization

To minimize the contamination produced by endogenous, exogenous bacteria, and fungus, the explants leaves and stem of BD were exposed to varying chemical sterilants for the surface sterilization. This investigation was carried out to choose an appropriate chemical sterilants and also to find the appropriate contact time in between the explant and sterilants. The immature young leaves and stem of BD were washed with very dilute detergent solution in a conical flask and washed with running tape water, and again washed with sterile double-distilled water. Furthermore, the explants, leaf (3 min) and stem (8 min) were surface sterilized with ethanol 70% (v/v) followed by mercuric chloride 0.1% (w/v) solution. Under the aseptic condition, the explants were washed again three-to-four times using sterile double-distilled water to eliminate the traces of chemical sterilant and transferred to a sterile flask.

Media

Murashige and Skoog (MS) solid basal medium (Murashige and Skoog 1962) supplemented 3% sucrose, 1% (w/v) agar, and plant growth hormones.

Initiation and development of callus

For the induction, development, and maintenance of callus, sterilized immature young leaf and stem were cultured on MS solid basal medium comprising several auxins, namely, 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), and cytokinin, viz., kinetin in different concentration either alone or in combination were used for callus initiation and development. The cultures were incubated at 25 ± 2 ºC under light 16 h photoperiod (Nhut et al. 2006; Te-chato et al. 2006; Dodds and Roberts 1995). The callus culture were subcultured in every 4 weeks onto MS medium containing the same concentration of auxins.

Growth measurement

Growth kinetics of established callus of BD measured in terms of increase in fresh weight (FW). The growth rate and growth pattern of established callus of BD; 30 days old leaf and stem calli grown on MS media supplemented with different hormones were cut into pieces of approximately equal size and then under strictly aseptic condition transferred to previously weighed culture tubes having MS media supplemented with the similar hormones on which they were initiated. After transferring the pieces of callus into the respective tubes, each tube was weighed again. The difference in weight (final weight—initial weight) was recorded as fresh weight at 0 weeks. Similarly, the weight was taken before and after transference at the end of 2nd, 4th, 6th, and 8th week (i.e., the interval of 2 weeks).

Phytochemical screening

Various general chemical tests were performed on crude drugs powdered samples, i.e., Leaf, Stem, Leaf callus 1 & 2, and Stem callus 1 & 2 for the detection of different metabolites (Ahmad et al. 2010, 2013, 2014a, b; Wasim et al. 2016).

Isolation of PNE by preparative thin-layer chromatography

Isolation of PNE from BD was done according to the procedure given by Agarwal and Dutt 1935 with a few modifications. Preparative thin-layer chromatography is a reliable method by which PNE can be isolated from plant extracts. Powdered aerial parts of BD (200gm) were exhaustively extracted with ethyl alcohol (1000 mL) and concentrated to 50 mL under reduced pressure at 45 ºC. This solution was used as sample for the preparative thin-layer chromatography using a solvent system diethylamine-cyclohexane (30:70 v/v).

Thin-layer chromatography (TLC) developing chamber was washed, dried, and filled with 100 mL of solvent system. The filter paper was introduced into the developing chamber to saturate it. The sample was applied as band on activated silica gel preparative TLC plate and placed in air-tight chromatographic chamber. After allowing the solvent to run up to the 75% of the length of plate, the chromatogram was taken out from the chamber, air dried, and examined in day light as well as under UV light. The band of PNE (R_f 0.87) (Manu and Kuttan 2009a, b) was marked and silica gel layer was scrapped out in a glass beaker. Sufficient quantity of methanol was added to silica gel powder to dissolve PNE. Furthermore, the solution was filtered, and filtrate was concentrated under reduced pressure to yield off white crystals of PNE. These crystals were tested for various chemical tests and melting point was recorded using Parfitt melting point apparatus (Thermo-scientific, India) without correction (Manu and Kuttan 2009a, b).

Quantitative estimation of PNE in leaves and stem of bd and their callus by high-performance thin-layer chromatography

Preparation of standard solution

Weighed accurately 10 mg of PNE and dissolved in 10 mL of HPLC grade methanol, it was dilute to ten times to give 0.1 mg/mL solution, and was filtered through 0.22-µm syringe filter, prior to use. The stock solutions were used for the development of calibration curve of PNE.

Preparation of Test Solution

The dried powder (1 g) of each leaf and stem of BD, and their callus were extracted by cold extraction method separately in conical flask with methanol (50 mL) and sonicated for 25 min at room temperature. The extracts were filtrated in volumetric flask and volume adjusted up to 50 mL (20 mg/mL) with HPLC grade methanol, again filtered through syringe filter (0.22 µm) prior to application, and used for the analysis of PNE by HPTLC (Ahmad et al. 2014a, b).

HPTLC instrumentation

Chromatography experiment was conducted on CAMAG Linomat V sample applicator (Switzerland) controlled by winCats software using Pre-coated silica gel 20 × 10 cm aluminum TLC plates 60F254 with 0.2 mm thickness (E. Merck, Germany). A constant application rate of 120 nL/s was used. Linear ascending development was conducted in twin trough glass chamber. Eight different volume of standard PNE were applied on TLC plate for the calibration curve of PNE. The slit dimensions were kept at 4.0 × 0.30 mm and the scanning speed was 10 mm/s. Mobile-phase containing toluene:ethyl acetate:formic acid (7.0:2.5:0.7, v/v/v) was employed for the development of TLC plate. The mobile phase optimized at room temperature at relative humidity of 60 ± 5% for 15 min. Tungsten lamp was the source of radiation. The developed TLC plate was dried in air using an air dryer. Densitometric scanning at 262 nm was conducted on CAMAG TLC scanner III (Ahmad et al. 2019).

HPTLC method validation

The proposed HPTLC method was validated according to the ICH guidelines (ICH 1997), for linearity, accuracy, robustness, precision, limit of detection, and quantification. The linearity of the method was assessed by varying concentrations of standard PNE, and LOD and LOQ were also carried out (Wasim et al. 2015; 2019)

The precision of the method was performed by repeatability and intermediate precision. Robustness of the proposed method was carried out by small changes in the analytical methodology at a single concentration level by deliberately changing the composition of mobile phase, analysis temperature (K), and changing the detecting wave length (nm). In the current investigation, robustness of the method was evaluated with the help of Box–Behnken design (Ahmad et al. 2014a, b). Design Expert 8.1 (Stat-Ease, Minneapolis, MN, USA) was used to assess the study.

The accuracy of the HPTLC method was analyzed as recovery by standard addition method. For this, pre-analyzed samples were spiked with 50, 100, and 150% concentrations, and the mixtures were further analyzed in triplicate and mean ± SD were determined.

Results and discussion

Callus culture of leaves and stem of BD showed healthy tissue growth without contamination and no change in color of explants which were observed. This study was aimed to determine specific and suitable hormonal combination and optimize the concentration of auxins for the callus induction. The best combinations for the initiation of the callus from the leaf explant were MS media-containing 2,4-D (1 ppm) alone and 2,4-D + IAA (1 ppm each) in combination. Whereas for the stem explant, MS media-containing 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm) and 2,4-D + NAA + Kinetin (0.5 + 1.5 + 0.3 ppm) showed prominent results for the induction of callus. The above results indicate a role of exogenous plant growth regulators on callus formation in BD using leaf and stem explants. This findings corroborates the previous research reports on callus formation (Ragi and Shibu 2014; Pinki and Khalkho 2012). The above-mentioned combinations of phyto-hormone were further used for development and maintenance of the callus (Table 1, Figs. 1, 2, 3, 4). Callus initiated on leaf was soft friable and cream-colored, whereas stem callus profused and emergence of few roots from the callus.

The growth pattern of cultured tissue was studied and the growth kinetics (in terms of mean fresh weight) were analyzed (Fig. 5). The results indicated that the time period in between 4 and 6th weeks of kinetics study was of exponential growth phase and then stationary phase was started. The maximum growth of callus was obtained in 5th and 6th weeks. After 6th week, the calli grew in same media, but the increase in mean fresh weight was lesser than what it was observed on 4th and 6th week of studies.

The qualitative chemical tests were performed for the presence/absence of various phytoconstituents in leaves and stem and their callus. Results represented in Table 2 exhibited that the leaf, stem, and callus contain alkaloids, glycosides, flavonoids, carbohydrates, phenolic compounds, steroids, tannins, saponins, and amino acids.

Isolation and crystallization of PNE

Preparative TLC using diethylamine cyclohexane (30:70 v/v) as solvent system showed a light blue colour band under UV light (366 nm) represented isolated PNE (Rf 0.87) and the result was consistent with the earlier findings (Manu and Kuttan 2009a, b). PNE off white crystals obtained from preparative TLC process were purified with methanol using recrystallization method, recorded yield was 0.01% w/w. Melting point of crystallized PNE was 236 ± 2 ºC. PNE showed maximum absorption at 262 nm in UV spectroscopy. PNE showed brown precipitate with Dragendorff's reagent and green color with ferric chloride solution, whereas with concentrated sulphuric acid, it gave greenish yellow color. PNE crystals with nitric acid exhibited red color, whereas it displayed no color with hydrochloric acid. These results were consisted with the earlier reports (Manu and Kuttan 2009a, b) that confirmed the isolated crystals were of PNE.

Table 1 Plant growth regulator effect on induction, development, and maintenance of callus

MS media with Growth regulators	No. of days	Observation	Result
MS + 2,4-D (1 ppm)	12–15	Cream-colored friable mass of callus initiated from the periphery of the leaf	+ +
MS + 2,4-D + IAA (1 ppm each)	12–15	White cream-colored soft and friable mass of callus	+ + +
MS + IAA (1 ppm)	20–22	Curling of leaf	No callus
MS + 2,4-D + IAA + Kinetin (0.5 ppm)	15–20	Curling of stem	No callus
MS + 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm)	12–15	Cream-coloured friable mass of Callus initiated from the cut end of the stem	+ +
MS + 2,4-D + NAA + Kinetin (0.5 + 0.5 + 0.3 ppm)	20–25	Curling of stem	No callus
MS + 2,4-D + NAA + Kinetin (0.5 + 1.5 + 0.3 ppm)	12–15	The mass of callus was profused and only few roots emerged from the callus	+ + +
MS + 2,4-D (1 ppm)	45–55	Cream-colored friable mass of callus initiated from the periphery of the leaf	+ + +
MS + 2,4-D + IAA (1 ppm each)	45–55	White Cream colored soft and friable mass of callus	+ + +
MS + 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm)	45–55	Cream-coloured friable mass of Callus initiated from the cut end of the stem	+ + +
MS + 2,4-D + NAA + Kinetin (0.5 + 1.5 + 0.3 ppm)	45–55	The mass of callus was profused and only few roots emerged from the callus	+ + +
MS + 2,4-D (1 ppm)	60–75	Cream colored friable mass of callus initiated from the periphery of the leaf	+ + +
MS + 2,4-D + IAA (1 ppm each)	60–75	White cream-colored soft and friable mass of callus	+ + + +
MS + 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm)	60–75	Cream-coloured friable mass of Callus initiated from the cut end of the stem	+ + + +
MS + 2,4-D + NAA + Kinetin (0.5 + 1.5 + 0.3 ppm)	60–75	The mass of callus was profused and only few roots emerged from the callus	+ + + +

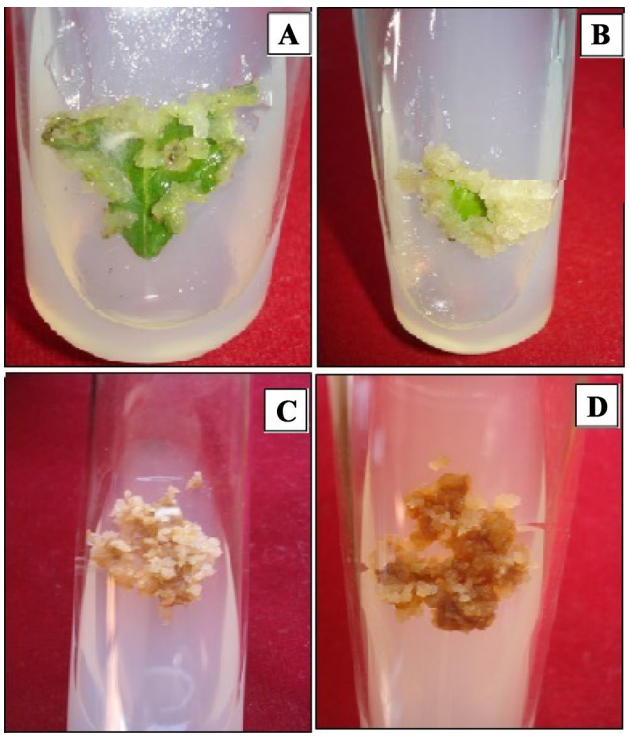

Fig. 1 Leaf callus 1: Developed leaf callus on MS media consisting 2,4-D (1 ppm), **a** initiation of leaf callus; **b** development of callus from leaf explant; **c** initiation of shoot from developed leaf callus culture; and **d** development of shoot from developed leaf callus culture

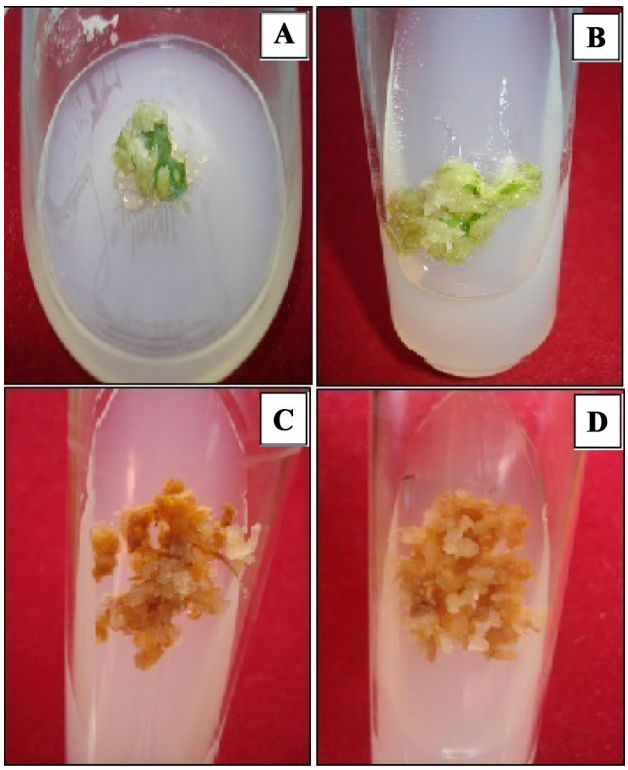

Fig. 2 Leaf callus 2: Developed leaf callus on MS media consisting 2,4-D + IAA (1 ppm each), **a** initiation of leaf callus; **b** development of leaf callus; **c** initiation of shoot from developed leaf callus culture; and **d** development of shoot from developed leaf callus culture

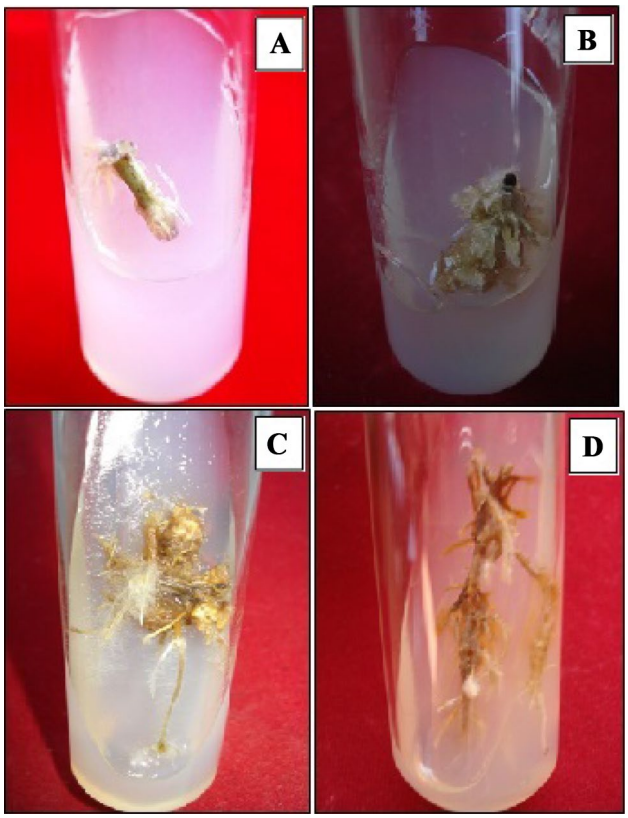

Fig. 3 Stem callus 1: Developed stem callus on MS media consisting 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm), **a** initiation of stem callus; **b** development of stem callus; **c** initiation of root formation from stem callus culture; and **d** development of root formation from stem callus culture

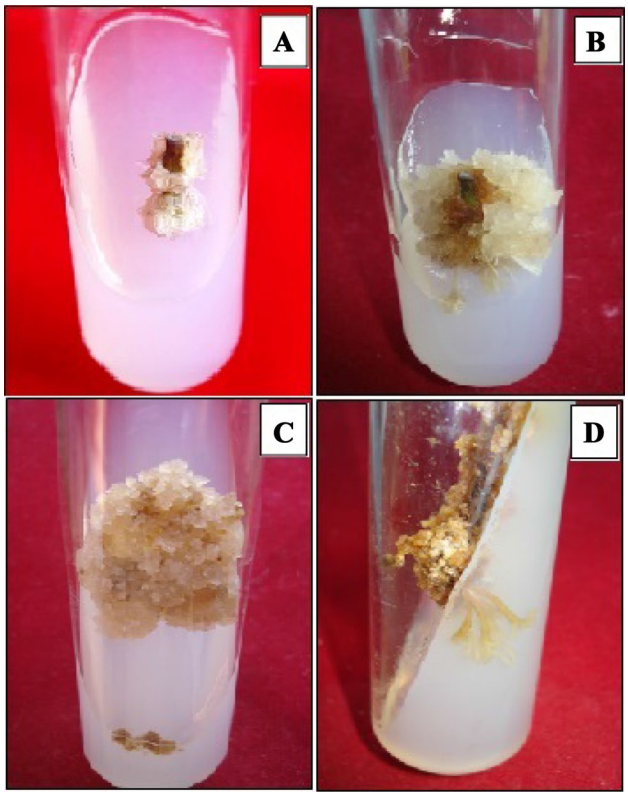

Fig. 4 Stem callus 2: Developed stem callus on MS consisting 2,4-D + IAA + Kinetin (0.5 + 1.5 + 0.3 ppm), **a** initiation of stem callus; **b** development of stem callus; **c** initiation of root formation from stem callus culture; and **d** development of root formation from stem callus culture

Optimization of HPTLC densitometric method

The selection of mobile phase was done after different hit-and-trial methods using various solvents in changed proportions. At the end, when mobile phase containing toluene and ethyl acetate in varying proportions spot was observed with tailing, then addition of formic acid in the composition to improve the resolution of spot and the tailing. Finally, toluene:ethyl acetate:formic acid was used in the proportion of (7.0:2.5:0.7, v/v/v) as mobile phase. This developed and optimized new mobile phase facilitated in attaining very compact spots and well-defined peaks of PNE at R_f 0.73 (Fig. 6).

Validation of parameters

Linearity

Linearity was evaluated by different concentration of PNE in triplicate. A good linearity relationship was found within a range of 100–1000 ng spot^{-1} with a correlation coefficient $r = 0.9971$. The LOD and LOQ of the methods were 30.3 and 100.0 ng spot^{-1}.

Precision

Table 3 shows the precision results of the HPTLC method, which was found in acceptable range.

Accuracy as recovery

Recovery study for the developed HPTLC method was analyzed by spiking with pre-analyzed sample of the standard PNE. The recovery study results showed 96–103% using HPTLC (Table 4).

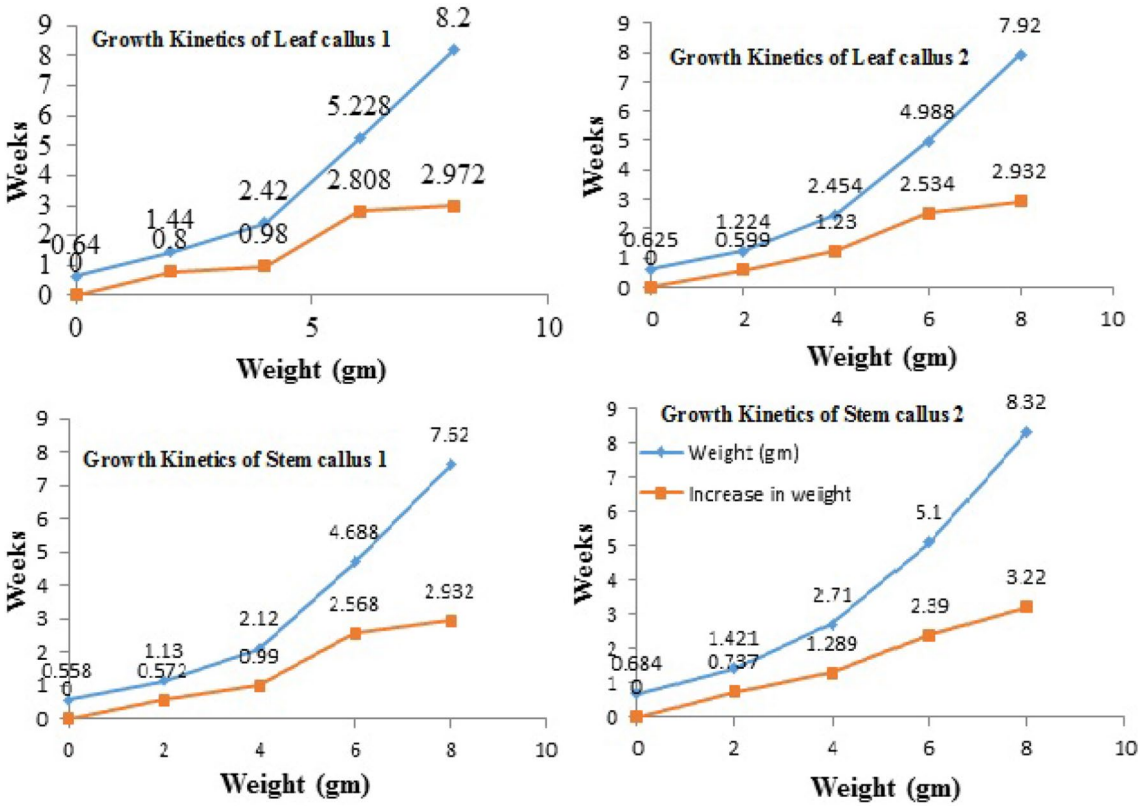

Fig. 5 Graph between increase in fresh weight (g) of callus and number of weeks plot to represent the growth kinetics of calluses obtained from the leaf and stem explants

Table 2 Qualitative chemical tests for presence/absence of various phytoconstituents

S. no	Class of compounds	Leaf extract	Stem extract	Leaf callus 1	Leaf callus 2	Stem callus 1	Stem callus 2
1	Alkaloids	+	+	+	+	+	+
2	Glycosides	+	+	+	+	+	+
3	Flavonoids	+	+	+	+	+	+
4	Carbohydrates	+	+	+	+	+	+
5	Phenolics	+	+	+	+	+	+
6	Steroids	+	+	+	+	+	+
7	Tannins	+	+	+	+	+	+
8	Saponins	+	+	+	+	+	+
9	Amino acids	+	+	+	+	+	+

Note: (+): Present; and (−): Absent

Leaf callus 1: Developed leaf callus on MS media consisting 2,4-D (1 ppm)

Leaf callus 2: Developed leaf callus on MS media consisting 2,4-D + IAA (1 ppm each)

Stem callus 1: Developed stem callus on MS media consisting 2,4-D + IAA + Kinetin (0.3 + 0.75 + 0.3 ppm)

Stem callus 2: Developed stem callus on MS media consisting 2,4-D + NAA + Kinetin (0.5 + 1.5 + 0.3 ppm)

Robustness

For the HPTLC method, robustness was assessed by deliberately little changes in the mobile phase composition, detection wavelength and analysis temperature and the results were evaluated. The design expert software proposed the following polynomial equation for the peak area of PNE:

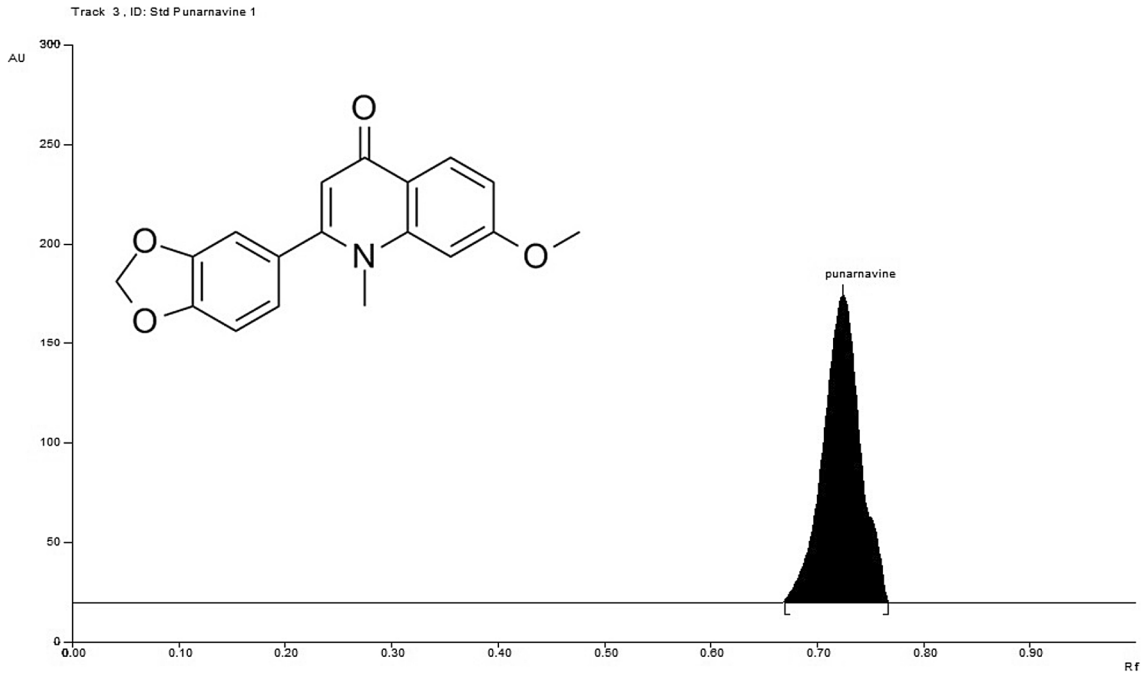

Fig. 6 Typical HPTLC chromatogram of PNE (Rf = 0.73)

Table 3 Precision % RSD of PNE

Precision	PNE % RSD
Intraday ($n=5$)	1.26
	1.16
	0.76
Interday ($n=5$)	0.83
	0.67
	1.03

Table 4 Accuracy of PNE

Excess spike concentration added	% Recovery of PNE
50	96.32 ± 0.58
100	101.01 ± 0.78
150	103.51 ± 0.71

$$\text{Peak area (Y)} = +1986 : 60 + 0.0625\text{A} + 0.3000\text{B} + 0.0875\text{C} + 0.0250\text{AB} + 0.0500\text{AC} + 0.4750\text{BC} - 1.50\text{A}^2 - 1.28\text{B}^2 - 10000\text{C}^2,$$

where *A* is the temperature of analysis (K), *B* is composition of the mobile phase, and *C* is detecting wave length (nm).

The given polynomial equation of PNE demonstrated the positive value in the equation denotes the effect which help the optimization process. On the other hand, negative value shows the reverse connection between the factors and responses (Myers and Montgomery 2002). It is clear from the equation that the factors, such as analysis temperature (*A*), mobile phase composition (C), as well as detection wavelength, displayed positive result on the response (Y) (Fig. 7). The quadratic model was suitable model to the data of the experimental design with three variable factors.

Analysis of PNE

The analysis of PNE in BD extracts of leaf, stem, and callus of leaf and stem was determined by HPTLC method. The concentration of PNE was found to raised by 1.07-, 1.35-, 1.97-folds (Table 5) in leaf callus2 and Stem callus 1 & 2, respectively, while in case of leaf callus 1, there was no significant increase in concentration of the PNE when compared with the plant extracts.

In the present investigations, an attempt was made to develop the tissue culture profile of BD. The results showed increasing the production of PNE on MS medium using the different growth hormonal combinations by tissue culture technique. A validated quantitative estimation method of PNE in BD and its callus was developed by HPTLC with LOD (30.3 ng spot^{-1}) and LOQ (100.0 ng spot^{-1}).

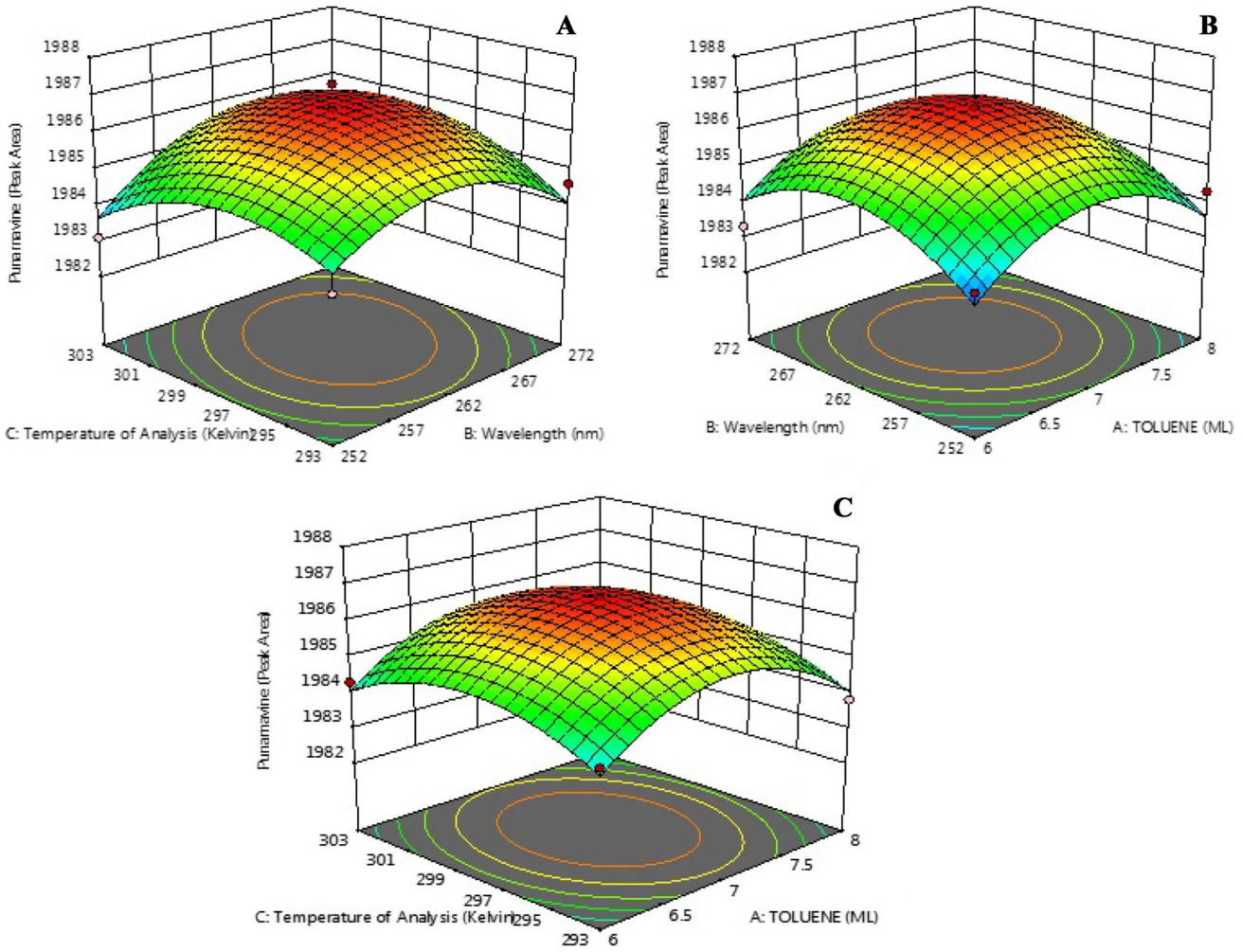

Fig. 7 Three-dimensional graphs of PNE using Box–Behnken experimental design

Table 5 Quantitative estimation of PNE in different combination of callus and crude drugs

S. no	Sample name	Conc. of PNE (µg/spot)	% w/w of PNE	PNE raised by no of folds
1	Leaf	0.150	0.0075	–
2	Stem	0.140	0.0070	–
3	Leaf callus 1	0.121	0.0060	0.80
4	Leaf callus 2	0.161	0.0080	1.07
5	Stem callus 1	0.190	0.0095	1.35
6	Stem callus 2	0.277	0.0138	1.97

Promising results were obtained from the tissue culture studies of BD. Further studies are suggested to assess and improve the growth of cultures as well as the secondary metabolites, especially PNE. This could be achieved using cell suspension culture or supplementing the MS medium with different concentration and combination of plant growth hormones, vitamins, and precursors. The culture may also be tried in immobilized cultures or other advance culture techniques facilitating the ability of callus and may improve the production of useful secondary metabolites.

Conclusion

A simple and efficient protocol for the successful callus induction and regeneration, and with multiple shoot and root formation of important medicinal plant BD has been developed, and can be applied for the conservation of BD which possess valuable medicinal properties. The present investigation confirmed the presence of PNE in developed callus of BD and supported its production through biotechnology-based tissue culture techniques. Quantitative estimation method of PNE has been developed for the first

time both in callus and plant extract by validated HPTLC method. Results obtained from the validation process were encouraging and suitable for the routine analysis of PNE. The proposed HPTLC method for the estimation of PNE is less expensive, simpler, easier, rapid, and more flexible than high-performance liquid chromatography (HPLC). The method can be beneficial for the standardization and quality control of medicinal plants/herbal drugs containing PNE as one of the ingredients.

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Author contribution WA and RZ conceived and designed research. IB, NA, MS, and AZ analyzed data. WA, AA, MAA, and MA conducted the experiment and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they do not have competing interest.

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