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Original Article

Comparative HPTLC analysis of bioactive marker barbaloin from *in vitro* and naturally grown *Aloe vera*

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ABSTRACT

Aloe vera (L.) Burm. f., Xanthorrhoeaceae, a succulent, produces barbaloin, a bioactive compounds used in various pharmaceutical products. Extracts prepared from the leaves have been widely used as bittering agents, taste modifiers and also as cathartic agent against severe constipation. Barbaloin is reported for its anti-inflammatory, anticancer, antiviral and anticancer activities and these properties are mostly mediated by its antioxidative capacity. Presently, a study has been conducted on the comparative High Performance Thin Layer Chromatography analysis of barbaloin from the dried leaf skin powder of *in vivo* and *in vitro* grown *A. vera*. Shoot tips of *A. vera* were cultured in Murashige and Skoog media supplemented with different combination of 6-benzylaminopurine and 1-naphthaleneacetic acid. [Best multiplication response was noted in benzylaminopurine (2.0 mg/l) + 1-naphthaleneacetic acid (0.1 mg/l) supplemented Murashige and Skoog media]. The quantitative determination of barbaloin was performed on silica gel 60 F_{254} HPTLC plates as stationary phase. The linear ascending development was carried out in a twin trough glass chamber saturated with a mobile phase consisting of ethyl acetate: methanol: water (100:16.5:13.5) at room temperature ($22 \pm 2\,^{\circ}C$). CAMAG Thin Layer Chromatography scanner-3 equipped with CATS software (version: 1.4.4.6337) was used for spectrodensitometric scanning and analysis in the ultraviolet region at $\lambda = 366$ nm. The method was validated for linearity, precision and accuracy. Correlation coefficient, limit of detection, limit of quantification as well as recovery values were found to be satisfactory. Out of the five populations studied, the leaf skin of *A. vera* collected from Jodhpur (Rajasthan, India) and raised *in vitro* was found to contain higher amount of barbaloin (2.78%) when compared to its naturally growing counterparts (2.46%) and other plant populations.

Introduction

Aloe vera (L.) Burm. f., Xanthorrhoeaceae, is a succulent plant indigenous to Northern Africa and Mediterranean countries and has become naturalized almost in all parts of India (Klein and Penneys, 1988). The original commercial use of the plant was meant for the production of a latex substance called barbaloin (**1**) or aloin (molecular formula: $C_{21}H_{22}O_9$) which is yellow-brown in color with and lingering taste and was used as laxative until world war-II (Saeed et al., 2004). *A. vera* has exhibited anticancer, wounds healing, immunomodulatory, antiviral, anti-inflammatory, dental protective, laxative, antiseptic, gastroprotective, moisturizing and anti-aging properties (Surjushe et al., 2008; Park et al., 2011; Yonehara et al., 2015; Hashemi et al., 2015; Mangaiyarkarasi et al., 2015). Oral administration of *A. vera* for the treatment of diabetes mellitus and dyslipidemia has also been investigated (Ngo et al., 2010). Besides barbaloin, the other main ingredient of *A. vera* is called leaf gel, a clear, colorless and tasteless substance, which covers inner portions of the leaves (Reynolds and Dweck, 1999). The gel is used for commercial use in pharmaceuticals, functionals foods and cosmetics (Hamman, 2008). Sterols present in *A. vera* gel stimulated collagen and hyaluronic acid synthesis by human dermal fibroblasts (Tanaka et al., 2015). *A. vera* gel also promoted cesarean wound healing in women (Molazem et al., 2014). Barbaloin (also named aloin), the *C*-glucoside of aloe emodin anthrone, localizes in the outer rind of the plant has been reported to constitute up to 30% of the plant's dried leaf exudates (Groom and Reynolds, 1987) and proposed as a part of the defense mechanisms against herbivores (Gutterman and Chauser-Volfson, 2000; Chang et al., 2006). Barbaloin was reported for its histamine release inhibitory, anti-inflammatory, antiviral, antimicrobial, anticancer, antioxidant and cathartic effects (Patel et al., 2012)

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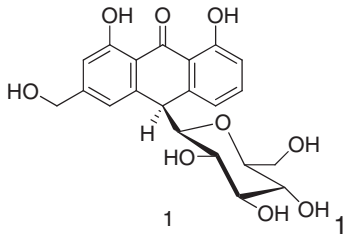
In another *Aloe* species *A. ferox*, aloesin, aloeresin a and anthraquinone (as barbaloin) was estimated by a reversed-phase high-performance liquid chromatographic (HPLC) method (Zahn et al., 2008). In leaf exudate of *A. secundiflora* the major components were analyzed by HPLC-mass spectroscopy (Rebecca et al., 2003). Barbaloin in aloe capsule was also determined by HPLC methods (Chen et al., 2002). Aloin was selectively determined in different matrices by HPTLC densitometry in fluorescence mode (Coran et al., 2011). Earlier, barbaloin was estimated in *A. vera* and in its commercial products (Pandey et al., 2012). An efficient micropropagation protocol was adopted in *A. vera* in Murashige and Skoog (MS) medium supplemented with growth regulators (Aggarwal and Barna, 2004). Rapid propagation by the shoot generation calli was achieved in polyvinylpyrrolidone (PVP) and growth regulator supplemented MS medium (Roy and Sarkar, 1991). However, no attempt has yet been made to quantify barbaloin (**1**) comparatively in natural or *in vitro* populations of *A. vera*. Since barbaloin is considered as an important biologically active compound in *A. vera*, the present investigation depicts a validated HPTLC protocol for determination of barbaloin from *in situ* and *in vitro* grown populations of *A. vera*.



Materials and methods

Plant material and chemicals

Different populations of *Aloe vera* (L.) Burm. f., Xanthorrhoeaceae, were collected from five districts (from four different states of India) *viz.* Jalandhar (Punjab), Jodhpur (Rajasthan), Varanasi and Lucknow (Uttar Pradesh) and Bhubaneswar (Odisha). Plants with 9–11 leaves and of almost same size and age were harvested in their vegetative stage during the month of June 2012 to September 2012 and maintained at the herbal medicinal garden, Lovely Professional University, Punjab. The materials were identified and authenticated on the basis of morphological characters by a botanist in the Department of Botany, Banaras Hindu University, Varanasi. A voucher specimen (Voucher No. 080912) was deposited at the Department of Biotechnology, Lovely Professional University for future reference. All solvents (HPLC grade) were obtained from E. Merck (Mumbai, India). Standard barbaloin (~97% pure) was purchased from Sigma–Aldrich (USA).

Micropropagation of Aloe vera

MS (Murashige and Skoog) medium supplemented with 6-benzylaminopurine (BAP) (0.5–2.0 mg/l) and 1-naphthaleneacetic acid (NAA) (0.1–1.0 mg/l) and agar (0.8%) was used for shoot proliferation. After 4–5 weeks of culture period, the *in vitro* grown plantlets with newly form shoots were taken out aseptically and the shoots were excised from the parent plant with the help of sterile scalpel blade and forceps and inoculated into new bottles containing solid MS medium with different set of growth regulators (PGR) as mentioned earlier. Newly formed shoots measuring 3–4 cm in length were excised individually from the parent plant and were transferred into the two types of rooting media: MS media supplemented with NAA (0–2.0 mg/l) and indole-3-butyric acid (IBA) (0–2.0 mg/l) individually. All cultures were incubated under 16 h photoperiod (cool, white fluorescent light (30 μM/s)) and temperature of 25 ± 1° C with 50–80% relative humidity. After 30 days of culture on rooting media, the plantlets were shifted to plastic pots for hardening prior to final transfer to natural conditions. For hardening, plants with newly formed roots were taken out from the culture bottles with utmost care to prevent any damage to the roots. The plants were then dipped in warm water to remove any traces of agar following which the plants were dipped in 1% (w/v) solution of Bavistin to prevent any fungal infection in the newly developed plants. The plantlets were then planted in plastic pots containing 1:1 mixture of soil and manure.

Preparation of sample and standard solution

The shed dried leaf skin of *A. vera* were powdered in a mixer grinder (Champ Essentials, Morphy Richards, India). Leaf skin (0.1 g each from *in situ* and *in vitro* grown plants) were separately extracted with methanol (2× 20 ml) for 15 min under reflux on a water bath at 70 °C. The extracts were filtered through Whatman no: 1 filter paper (separately for *in situ* and *in vitro* samples) and were evaporated under vacuum using a rotary evaporator (Eyela, N-1100, China) to furnish a solid mass of extract. The extract was kept in freezing temperature free of methanol because barbaloin (**1**) is converted into aloe emodin and a number of unknown compounds as the glucose part is removed from the compound when kept in methanol (Chang et al., 2006). The extract (0.1 g/ml) obtained from each sample were prepared in HPLC-grade methanol for quantitative analysis. Stock solutions of barbaloin were prepared by dissolving 10 mg of the compound in 10 ml of methanol.

Chromatographic conditions

The HPTLC system was composed of a CAMAG (Muttenz, Switzerland) Linomat-5 automatic sample applicator and CAMAG TLC scanner-3 provided with CATS software (version: 1.4.4.6337). The stationary phase was composed of pre-coated silica gel 60 F_{254} HPTLC plates (20 cm x 10 cm; with 0.25 mm thickness). Samples were administered to the plates as 5 mm wide bands *via* Linomat-5 automatic sample applicator (with nitrogen flow) equipped with a 100 μl Hamilton syringe. Delivery rate from the Hamilton syringe was fixed at 100 nl/s. Linear ascending mode of development up to a distance of 80 mm, with ethyl acetate: methanol: water (100:16.5:13.5) as mobile phase (Wagner and Bladt, 1996; Gutterman and Chauser-Volfson, 2000), was implemented at room temperature (22 ± 2 °C) and 50% relative humidity in a CAMAG twin trough glass chamber (20 cm × 10 cm) saturated earlier with mobile phase vapour for 20 min. After development, the plates were dried at 100 °C for 10 min, derivatized with 100 ml of 10% (v/v) alcoholic KOH solution, following which densitometric scanning was executed at 366 nm (Wagner and Bladt, 1996). The slit dimensions were 5 mm × 0.45 mm and the scanning speed was 100 nm/s. For calibration and to estimate the linearity, marker stocks (0.3, 0.6, 0.9, 1.2, 1.5 μl) were administered to the plate to furnish amounts in the range 300–1800 ng per band. Peak areas were plotted against the corresponding concentrations and regression analysis was executed to generate the calibration equation. To analyze plant samples, 1.0 μl extract of each of the samples was administered to the plate. After development, derivatization, scanning and measurement of peak area the amount of barbaloin (**1**) was determined, assuming the purity of the marker to be 100%. Chromatograms are shown in Figs. 2 and 3.

Method validation

The method was validated *via* calculating linearity, peak purity, limit of detection (LOD), limit of quantification (LOQ),

Fig. 1. Different stages of tissue culture in *Aloe vera* plants: a. explant source, b. (1–4). shoot proliferation after 8 weeks of culture on MS medium, c. (1–4): *in vitro* root induction on microshoots and acclimatization of rooted plantlets.

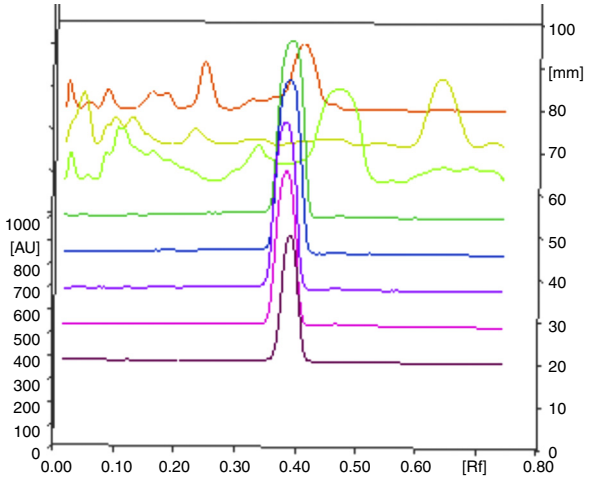


Fig. 2. HPTLC fingerprinting of barbaloin along with the methanolic extract of different parts of *in vitro* grown *A. vera* plants (1–5: standard; 6: light green-gel, 7: yellow-root; 8: red- leaf skin) from Jodhpur.

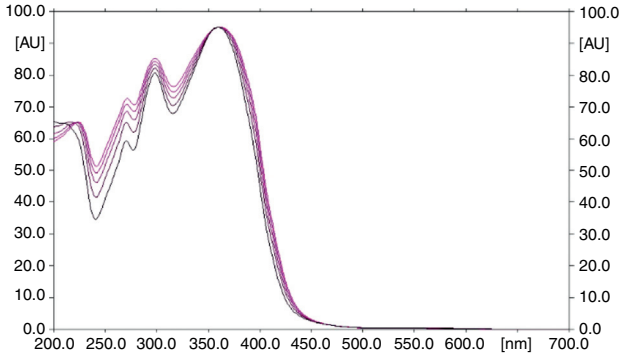


Fig. 3. Overlay spetra of barbaloin.

repeatability, percentage recovery, intra-day and intermediate precision. Each of the standard solutions of barbaloin (0.3, 0.6, 0.9, 1.2, 1.5 μg per band) was administered in triplicate. The calibration plot was developed by plotting peak area against the amount of barbaloin and linearity range was resolved. Instrument precision was examined by scanning the same barbaloin band (1 μg) six times. The mean, standard deviation, and coefficient of variation (CV) (%) were determined for peak area and retention factor (R_f). Repeatability was determined by analyzing the band for barbaloin after application of standard solution to the plate ($n = 10$), and determining % CV ($n = 10$). The accuracy of the method was measured by determination of recovery at four levels, after addition of 0, 50, 100 and 150% barbaloin to the sample. A known amount of standard barbaloin was added to 100 mg powdered plant material (containing approximately 2.5 mg barbaloin), the *A. vera* leaf skin samples were extracted and the amounts of barbaloin were determined as described above. Recovery was calculated for each of the three levels. Precision was studied by analyzing three bands of sample solution per plate on three plates (intra-day precision) and by analyzing three bands of sample solution per plate on second day (inter-day and intermediate precision) and calculating % CV. The specificity of the method was calculated from the absorbance spectrum of barbaloin standard and the corresponding peak in the test samples in the range 200–800 nm. Different dilutions of the standard solutions were administered with methanol as blank and the LOD and LOQ were determined.

Table 1
In vitro multiple shoot formation from lateral shoots of *Aloe vera* as influenced by the growth regulators (4 replicates per treatment).

MS + growth regulators (mg/l)		% of cultures showing shoot proliferation	Average number of shoot per explant (after 4 weeks) (mean ± SE)	Average number of shoot per explant (after 8 weeks) (mean ± SE)
BAP	NAA			
0.5	0.5	50	1.32 ± 0.22	3.3 ± 0.2
0.5	1.0	44.2	1.3 ± 0.31	2.21 ± 0.6
1.0	0.5	70.2	2.00 ± 0.32	5.2 ± 0.3
1.0	0	65.34	2.33 ± 0.36	4.1 ± 0.3
2.0	0.5	83.2	2.8 ± 0.52	5.6 ± 0.2
2.0	0.1	100	4.1 ± 0.25	8.12 ± 0.3

Data represent the mean of 3 replicates for each treatment.

Determination of barbaloin in samples

Sample extracts and standard solution mixtures (0.3, 0.6, 0.9, 1.2, 1.5 μl) were administered to 20 cm × 10 cm silica gel_{60} HPTLC plates and analyzed as described above. Peak areas were noted and a calibration plot was generated by plotting peak area against the amount of standard barbaloin (**1**) administered. The calibration plot was used to determine the amounts of standards present in the samples.

Results and discussion

Effect of PGR on organogenesis

In order to achieve multiple shoot induction, various combinations of cytokinin and auxin were investigated. Appearance of shoots from lateral shoot explants (suckers) was found after two weeks of culture in all the hormone combinations (Table 1). Shoot buds initiated were light green to yellowish in color and were formed either as single or in clusters. The maximum number of shoot bud (4) per explant was noted in MS media supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA within four weeks of culture.

Effect of PGR on shoot multiplication and elongation

Shoot buds proliferated in MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA were multiplied into clusters of small buds and were elongated when subcultured in the same regeneration media. Maximum shoots were obtained within eight weeks of culture in the same medium (Table 1). However, MS media containing 0.5 mg/l BAP and 1.0 mg/l NAA has yielded significantly lower number of shoots.

Rooting in regenerated shoots

Rooting was observed within two weeks in all rooting media (Fig. 3). Maximum (87%) rooting was found in MS media supplemented separately with 0.5 mg/l NAA and 2.0 mg/l IBA within two weeks of culture (Table 2). Significant difference in root number was noted after two weeks of culture in two rooting media. The root length was significantly different and the maximum root elongation was observed in the aforesaid medium (Table 2). Roots regenerated in this medium were relatively thick and strong compared to other medium even during initial stages of development.

Acclimatization of rooted plantlets

Well developed rooted plantlets (3.0–5 cm) were obtained after two months of culture on MS basal medium which were then transferred to pots containing soil and sand of 1:1 ratio and 100% of the explants were survived during and after the acclimatization process. The stages of micropropagation are presented in Fig. 1.

Method validation by HPTLC

Preparatory TLC studies showed that the solvent system ethyl acetate:methanol:water (100:16.5:13.5) was ideal as mobile phase which produced a single spot with an R_f 0.4 for barbaloin (**1**) and well resolved spots were found for the test samples. The spots were observed at 366 nm after spraying with 10% (v/v) ethanolic KOH. The three dimensional densitogram patterns of the test samples and barbaloin demonstrated that the peaks corresponding to R_f 0.4 were superimposable in all the samples. The spectrum characteristics corresponding to this peak were also noted to match exactly denoting the compounds corresponding to R_f of the standards and the test samples to be identical. The peak purity test was performed by comparing the absorption spectra of the standard barbaloin and the test samples; clear superimpossibility specified the purity of the peak (Fig. 2). Linearity of the calibration curve was found between 0.3 and 1.5 μg. The correlation coefficient for a calibration curve between 0.3 and 1.5 μg was found to be 0.998 for barbaloin. The regression equation for the calibration plot for barbaloin is summarized in Table 3. Percentage of barbaloin was determined by using the peak area parameter. This HPTLC method was validated for precision, accuracy and repeatability. The method is specific for barbaloin because it resolved the compound (R_f = 0.4) well in the presence of other components in *A. vera* (Fig. 2). A linear relationship was achieved between response (peak area) and amount of barbaloin in the range 0.3–1.5 μg per band (Fig. 4).

Table 2
Effect of different concentrations of IBA and NAA on the number of root and root length in *Aloe vera*.

MS + growth regulators (mg/l)		% of shoots producing roots (mean ± SE)	Number of roots per shoot (mean ± SE)	Average root length in (cm) (mean ± SE)
IBA	NAA			
0.5	0.5	43.21 ± 1.5	1.17 ± 0.18	1.76 ± 0.3
0.0	1.0	16.7 ± 2.1	1.98 ± 0.37	1.13 ± 0.2
1.5	2.0	64.22 ± 1.3	4.19 ± 0.15	2.11 ± 0.2
2.0	0.5	87.56 ± 2.0	7.14 ± 0.16	3.47 ± 0.4
1.0	0.0	42.19 ± 1.3	4.01 ± 0.21	1.96 ± 0.2

Data represent the mean of 3 replicates for each treatment.

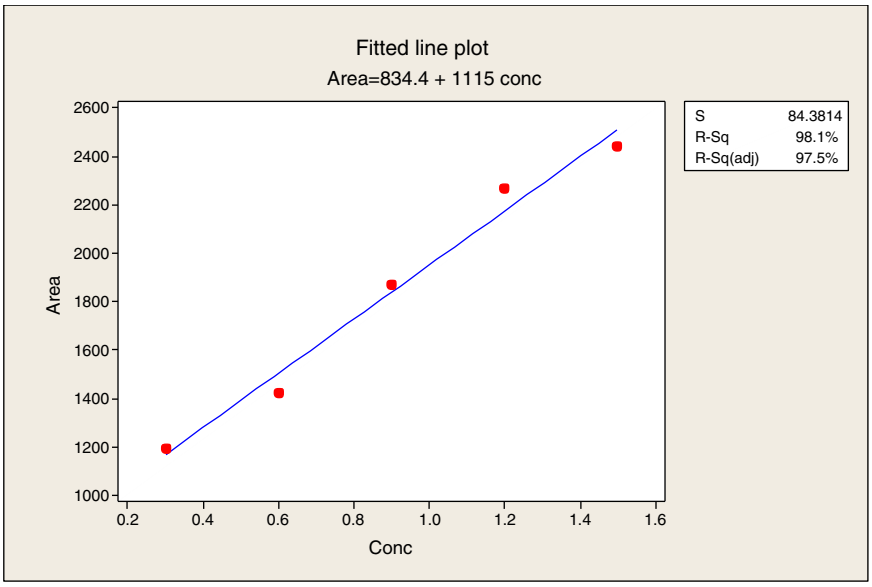


Fig. 4. Standard curve of barbaloin.

Table 3
Method validation parameters for the quantification of barbaloin.

Parameters	Results
R_f	0.4
Linearity range (ng/spot)	300–1500 ng
Regression equation (area)	$Y = 834 + 1115 \times X$
LOD	50 ng
LOQ	150 ng
Standard deviation (SD)	5.36
Correlation coefficient (R^2)	0.99

Table 4
Intra- and inter-day precision study (intermediate precision) the quantification of barbaloin.

Marker compound	Concentration (μg)	Intra-day	Inter-day
Barbaloin	0.5	1.34	1.45
	1.0	1.65	1.23
	1.5	1.39	1.56

Instrument precision was observed by scanning the same spot of barbaloin ten times (% CV = 0.52; $n = 10$). The repeatability of the method was investigated by analyzing ten applications of the same standard solution (% CV = 0.62; $n = 10$). The accuracy of the method was demonstrated at four levels (0, 50, 100, and 150%) *via* addition of known amounts of barbaloin to leaf skin extract of *A. vera*. Recovery at the four levels was noted to be 100, 100.5, 100.25 and 99.25% respectively (Table 5). Repeatability and precision were estimated by measuring intra-day and inter-day variation. Little intra-day and inter-day variation was noted (Table 4). Specificity of the method was calculated by acquiring the spectrum of barbaloin standard and the corresponding peak in the test samples in the range 200–800 nm. The spectra obtained from the barbaloin and the barbaloin present in leaf powder matched exactly, indicating no interference from other plant constituents (Fig. 3). LOD and LOQ were determined (Table 3) by using the equations LOD = $3 \times N/B$ and LOQ = $10 \times N/B$, where N is the standard deviation of the peak area of the standard ($n = 3$), taken as a measure of the noise and B is the slope of the corresponding calibration plot.

Table 6
Barbaloin content in various populations (*in situ* and *in vitro*) of *Aloe vera*.

Populations	Barbaloin content (*in situ* populations) (%)	Barbaloin content (*in vitro* populations) (%)
Jalandhar (Punjab)	2.23	2.32
Jodhpur (Rajasthan)	2.46	2.78
Varanasi (Uttar Pradesh)	2.34	2.38
Lucknow (Uttar Pradesh)	2.15	2.27
Bhubaneswar (Odisha)	2.20	2.29

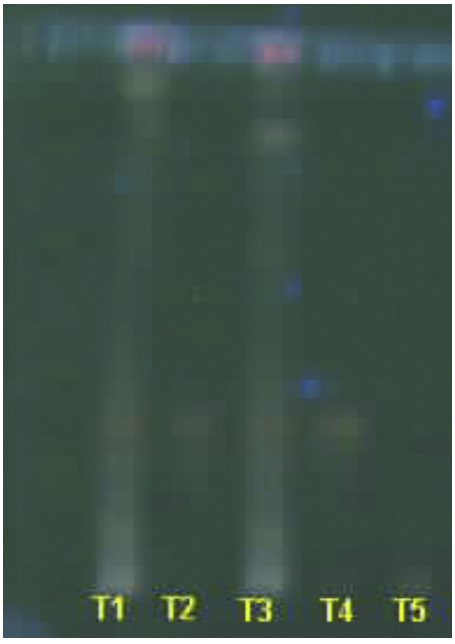
Barbaloin content in samples

The present method was used to determine the amount of barbaloin (**1**) in leaf skin extracts of *A. vera*. The identification of barbaloin was done on the basis of R_f values (Fig. 3) and comparison of spectra (λ_{max} 362 nm) (Fig. 3). A good separation of barbaloin was achieved (Fig. 2). Calibration curve for barbaloin (Fig. 4) was found to be linear as shown in Table 3. The chromatograms of various parts of *in vitro* raised *A. vera* from Jodhpur district are shown in Fig. 2. Leaf skin of *in vitro* cultured *A. vera* from Jodhpur district was found to contain the highest amount of barbaloin (2.78%) followed by the *in situ* grown leaf skin samples from the same district (2.46%). The *in situ* grown leaf skin sample collected from Lucknow district was found to contain the minimum amount of barbaloin (2.15%). Intrapopulational variations in barbaloin content among

Table 5
Recovery study of barbaloin by proposed HPTLC method.

Marker compound	Amount present in the sample (μg/mg)	Amount added (μg)	Amount found[a] (μg)	Recovery (%)	Average recovery (%)
Barbaloin	27.8	15	42.8 ± 0.34	98.64	98.9
	27.8	30	60.86 ± 0.45	98.36	
	27.8	45	72.9 ± 0.32	99.72	

[a] Relative standard deviation (% RSD, $n = 3$).



T1=methanolic leaf extract (100 μg) (tissue cultured)
T2=standard (barbolin) (4%)
T3=methanolic leaf extract (100 μg) (naturally grown)
T4=standard (barbolin) (8%)
T5=methanolic gel extract (200 μg)

Fig. 5. A chromatogram.

in situ and *in vitro* grown plant samples are tabulated in Table 6. A chromatogram (photograph of TLC plate is provided in Fig. 5).

A number of factors such as genotype, culture medium (including PGR and their combinations), physical environment and developmental stage of the explant affect the adventitious shoot regeneration from tissue cultured explants (Zhang et al., 1998; Bai and Qu, 2001). Moreover, the *in vitro* regeneration of direct adventitious shoots is an essential component to produce plants from elite materials as to avoid formation of somaclones. In this present study, BAP and NAA were selected for shoot regeneration and multiplication as these are reportedly among the PGRs used most often for the shoot organogenesis (Kantia and Kothari, 2002). Shoot amplification occurred in almost all the hormone combinations but a combination of BAP and NAA was noted crucial for direct shoot regeneration. According to Chaudhuri and Mukundan (2001), the best medium for shoot induction from shoot tips of *A. vera* was MS medium supplemented with 10 mg/l BAP, 160 mg/l adenine sulphate and 0.1 mg/l IBA. However, this is in contrast to the results obtained in the present investigation as when BAP at a higher concentration and in combination with NAA was added, it reduced the shoot production. Other studies indicate that BAP is more efficient than NAA for shoot proliferation in *A. vera* (Velcheva et al., 2005; Debiasi et al., 2007). According to the literature, BAP is better than other cytokinins for shoot initiation and proliferation. The shoots showed rooting in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA. Some researchers reported rooting in hormone-free medium (Natali et al., 1990; Aggarwal and Barna, 2004) while some others showed the presence of PGR is necessary for rooting (Meyer and Staden, 1991; Abrie and Staden, 2001; Velcheva et al., 2005).

Micropropagation is being considered as an advanced tool for the production of high value phytochemicals with medicinal potential (Debnath et al., 2006). Growth regulators used *in vitro* was found to enhance some biological activity and modulate phytochemical content in tissue culture plantlets which are otherwise different in their acclimatized counterparts. In one study, various cytokinines were found to influence phenolic acids and antioxidant activity of tissue cultured and acclimatized *Merwilla plumbea* (Aremu et al., 2013). In our present investigation, we have found enhanced barbaloin content from the leaves of micropropagated plants growing under the influence of two growth regulators. Similarly plant cell cultures are also reported as a source of micro-propagation of virus-free plants useful for novel secondary metabolite production (Sato, 2013). *In vitro* propagation also serves as a valuable tool for propagation avoiding the overexploitation of natural populations and to apply biotechnology-based approaches (Gonçalves and Romano, 2013).

In the present study, a simple and validated HPTLC method for the separation and quantification of barbaloin in *A. vera* leaves from *in vitro* and naturally grown plants was presented. It also quantifies barbaloin content from *in vitro* and *in situ* grown plant samples. *In vitro* derived leaves were found to contain more barbaloin than *in situ* grown samples. Besides being useful as a reproducible method for quantification of barbaloin from plant samples, the results also indicate the possible enhancement of secondary metabolite production from tissue cultured plant. Biotic and abiotic stresses developed during tissue cultural condition may be exploited in order to modulate some plant metabolite with possible therapeutic value.

Author contributions

DKP monitored the experiments and prepared the manuscript draft. SP performed the experiments. AD designed the experimentation and finalized the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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