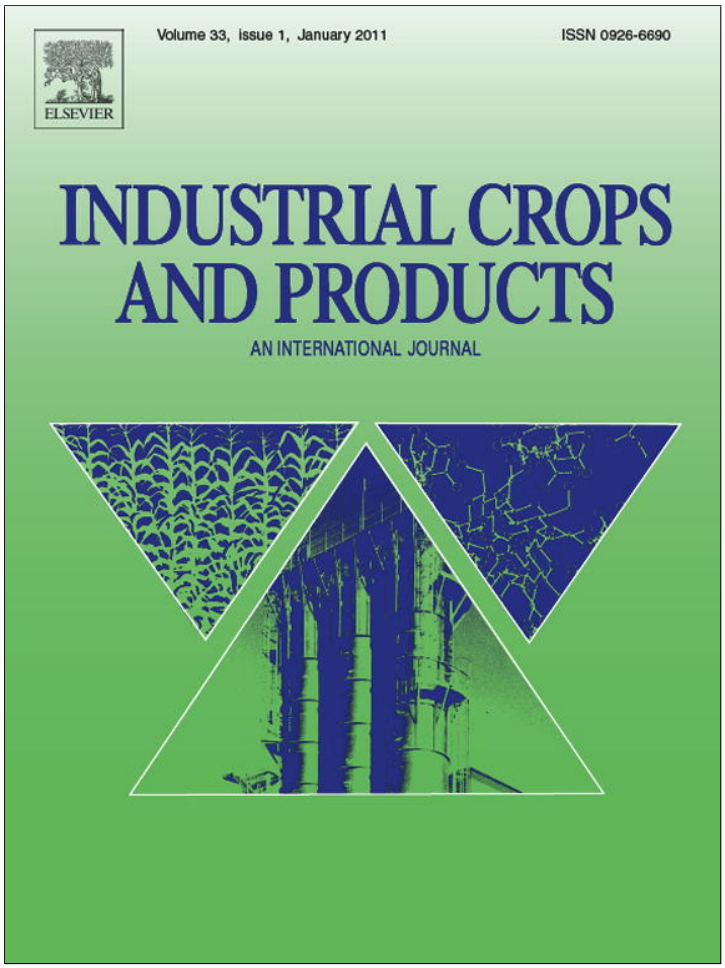

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Screening and quantification of an antiseptic alkylamide, spilanthol from *in vitro* cell and tissue cultures of *Spilanthes acmella* Murr.

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ABSTRACT

The study revealed, for the first time, accumulation of spilanthol, an antiseptic alkylamide, in *in vitro* cultures of *Spilanthes acmella* Murr., a medicinal plant of immense commercial value. To achieve this, *in vitro* shoots were regenerated via direct organogenesis from leaf-disc explants of *Spilanthes*. Shoots were induced in the presence of N^6-benzylaminopurine (BAP) alone or in combination with either α-naphthalene acetic acid (NAA) or Indole-3-acetic acid (IAA) in Murashige and Skoog medium. The best treatment for shoot regeneration was MS + BAP (5.0 μM) + IAA (5.0 μM), which promoted adventitious shoot proliferation in >82% cultures with an average of 5.3 shoots per explant. Regenerated shoots rooted spontaneously with a frequency of 100% on half strength MS medium (major salts reduced to half strength) containing 50 g l^{-1} sucrose. The plantlets were acclimatized successfully with 90% survival rate. Additionally, ploidy stability of the regenerated plants was assessed by flow cytometry which showed that all investigated plants had the similar ploidy as that of the mother plant. For spilanthol identification, peaks eluted from HPLC were analyzed by mass spectrometry with its characteristic fragmentation pattern. For quantification studies, calibration curve was generated, which revealed a higher amount of spilanthol content (3294.36 ± 12.4 μg/g DW) in the leaves of *in vitro* plants compare to those of *in vivo* plants (2703.66 ± 9.6 μg/g DW of spilanthol). An efficient multiplication frequency, ploidy stability and enhanced spilanthol accumulation ensure the efficacy of the protocol developed for this industrially important medicinal plant.

1. Introduction

Spilanthes acmella Murr., commonly known as "Akarkara", of the family Asteraceae, holds an important place in Indian and global scenario owing to its medicinal properties. It is a perennial herb and widely distributed in tropics and subtropics. The recent surge of interest in this plant is mainly due to the spectacular properties of ingredients found in the plant, which has been extensively used to treat mouth ailments, stammering, stomatitis, and throat complaints from antiquity. It also possesses anti-inflammatory, antiseptic, analgesic, antioxidant and cytotoxic properties (Jondiko, 1986; Saritha et al., 2002; Rai et al., 2004; Wu et al., 2008; Prachayasittikul et al., 2009). It is an active constituent of beauty care cosmetics such as fast acting muscle relaxant to accelerate repair of functional wrinkles (Belfer, 2007). Besides, the plant is well known for larvicidal and insecticidal properties (Ramsewak et al., 1999; Saraf and Dixit, 2002; Pandey et al., 2007). These properties of the plant are attributed to a range of diverse chemicals and one which remains the most sought after by scientists is an antiseptic alkylamide (2E, 6Z, 8E)-deca-2,6,8-trienoic acid N-isobutyl amide, commonly known as spilanthol (Khadir et al., 1989). Spilanthol has immense application in pharmaceuticals, food, health and body care products.

Despite the multifaceted uses of *Spilanthes*, no perceptible biotechnological advances have been made for this genus to exploit or enhance its utility. No conclusive tissue culture reports exist, till date, and all the biochemical studies carried out, so far, have been done on plants growing in wild. As we are aware that major limitation to the commercial use of potential metabolites is their very scarce supply from field grown plants due to their seasonal growth, genetic, geographical and climatic variations, and insect and pathogen attack. Establishment of *in vitro* cultures helps to nullify the effect of seasonal variation and favours the facilitated yield and consistent production of active compounds, irrespective of seasons and regions. In this context, micropropagation is an important and beneficial tissue culture technique that can be effectively used for mass propagation of genetically uniform plants as well as for its conservation. The cell culture technique is

Abbreviations: BAP, N^6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, Indole-3-acetic acid; Kinetin, N^6-furfuryladenine; NAA, α-naphthalene acetic acid; pCPA, para-chlorophenoxyacetic acid; TDZ, Thidiazuron.

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complimentary and may provide competitive metabolite production systems when compared to whole plant extraction. Although a few reports are available on tissue cultures (Saritha et al., 2002; Haw and Keng, 2003; Saritha and Naidu, 2008; Pandey and Agrawal, 2009; Singh et al., 2009a, 2009b), conditions for micropropagation were not optimized in either of these reports. Moreover, none of the reports have verified the ploidy stability of *in vitro* derived plantlets, which is the most important aspect for commercial application of any micropropagation protocol.

Therefore, this work is aiming to optimize the conditions to raise high yielding true-to-type plantations of *S. acmella* by *in vitro* culture, to determine the ploidy stability of *in vitro* regenerated plantlets by chromosomal squash preparation and flow-cytometry, and to develop a simple procedure for identification, quantification and enhanced production of spilanthol, a pharmaceutically important alkylamide, by employing cell and tissue culture technology.

2. Materials and methods

2.1. Establishment of leaf-disc culture

Healthy leaves of *Spilanthes* were collected from the campus of Indian Institute of Technology, Guwahati. Leaves were washed with 1% (v/v) savlon (Johnson and Johnson, India) for 20 min, followed by three rinses in sterile distilled water (SDW). Thereafter, the remaining steps were carried out inside the laminar-air-flow cabinet (Saveer Biotech, India). Leaves were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min and rinsed thrice with SDW. Leaf-disc explants were prepared by punching the sterilized leaves with 5 mm sized cork-borer before being cultured with the abaxial side in contact with the media. Murashige and Skoog (MS, 1962) basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, including α-naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), parachlorophenoxyacetic acid (pCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), N^6-benzylaminopurine (BAP), Thidiazuron (TDZ) and N^6-furfuryladenine (Kinetin) were used to induce regeneration of shoots directly from the explants. Unless mentioned otherwise, all media contained 30 g l^{-1} sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). After adjusting the pH to 5.8 with 0.1 N NaOH or 0.1 N HCL, 20 ml medium was dispensed into each 150 mm × 25 mm Borosil rimless glass tubes. Leaf-disc experiments were subcultured in 250 ml Erlenmeyer flasks where each flask contained 50 ml of medium. The culture tubes and flasks were plugged with nonabsorbent cotton wrapped in cheesecloth before autoclaving at 15 psi and 121 °C for 15 min. The thermolabile substances such as IAA and TDZ were filter sterilized using Millipore filters (0.45 μm pore size) and added to autoclaved medium cooled to 50 °C. The medium was then dispensed into each culture vessel inside the laminar-air-flow cabinet. The cultures were maintained at 25 ± 2 °C temperature with 50–60% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (1000–2000 lx). At least twenty four cultures were raised for each treatment and each experiment was repeated at least three times. Observations were recorded at weekly intervals and subcultured regularly into fresh medium at 5-week intervals.

2.2. Scanning electron microscopy

Leaf-discs of *Spilanthes* showing shoot-bud differentiation were fixed in 2.5% glutaraldehyde and dehydrated through a graded alcohol series. After drying, the samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

2.3. Rooting of regenerated shoots and acclimatization

Rooting of *in vitro* developed shoots and acclimatization were carried out as described previously (Singh and Chaturvedi, 2010).

2.4. Flow cytometric analyses

The ploidy stability of the regenerants, derived from *in vitro* leaf-disc cultures, was determined by flow cytometry. For flow cytometry, the nuclear suspension from fresh plant material was prepared by chopping the tissues with scalpel in cold woody plant buffer (WPB; 0.2 M Tris–HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1% (v/v) Triton X-100, pH 7.5 (Loureiro et al., 2007)). The suspension was then filtered through a 30 μm nylon filter membrane (Millipore, USA) to remove fragments and large tissue debris. For staining nuclei, 50 μg ml^{-1} propidium iodide (PI; Fluka) was added to the filtered suspension. To prevent staining of double stranded RNA, 50 μg ml^{-1} of RNAase (Fluka) was also added. Samples were stirred for 20 min and analyzed thereafter. *In vivo* plants (from the open field) were used as the controls for ploidy comparison.

2.5. Preparation of standard solution

Stock solutions (1000 μg ml^{-1}) of dodeca-2(E), 4(E)-dienoic acid isobutylamide (a reference standard for spilanthol) was prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at −20 °C. Quantification was carried out using 5 levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 250–15 μg ml^{-1}. Each concentration of standard was filtered through a 0.22 μm nylon membrane filter (Millipore, USA) before HPLC analysis.

2.6. Preparation of sample solution

To prepare samples, dried powdered cell mass and leaf samples from *in vivo* and *in vitro* grown plants were separately soaked in methanol (analytical grade, Merck, India) for 12 h. The methanolic samples were then centrifuged in a high speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5000 rpm for 10 min. The supernatant was transferred into new tube and the residue was re-extracted thrice with 10 ml methanol. Thereafter, the residue was discarded and the supernatant was pooled, filtered and evaporated to dryness in a rotary evaporator (Buchi Rotavapor R-200, Japan) at 40 °C. The methanolic fraction, thus, obtained was redissolved in HPLC grade methanol, filtered through a 0.22 μm nylon membrane filter prior to analysis and aliquots of 20 μl of clean solution were injected into HPLC system.

2.7. High performance liquid chromatography

Detection and quantification of spilanthol were carried out using Varian Prostar HPLC system (Varian, USA) consisted of Ultraviolet (UV) detector, a prostar binary pump, a 20 μl injection loop, and Hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6 mm × 250 mm.

The mobile phase used for spilanthol was 93% A (acetonitrile) and 7% B (Milli Q water) with a flow rate of 0.5 ml min^{-1}. The eluted samples were detected by UV detector at 237 nm. Spilanthol peaks obtained in HPLC were identified by electrospray ionization mass spectra and by comparing with published data. As there was no commercially available standard for spilanthol, it was tentatively quantified on the basis of another compound; dodeca-2(E), 4(E)-dienoic acid isobutylamide which is structurally similar to spilanthol. Both the compounds have isobutylamide group and long carbon chain.

Linearity of developed method was checked by running the standard compound at five different concentration range of 250–15 μg ml^{-1}. Calibration curve was constructed by plotting the peak area (y) against concentration in μg ml^{-1} of standard solutions (x). The standard equation obtained from the curve, was used for quantification of the spilanthol in the unknown samples. Spilanthol content was reported as, μg/g DW of sample.

Precision of developed assay was evaluated by running the same concentration of standard compounds at least thrice in same day (intraday) and twice at one day intervals (interday). HPLC grade acetonitrile, methanol and analytical grade methanol used for analysis were purchased from Merck, India. Purified water used for HPLC analysis was obtained from Milli Q system.

2.8. Mass spectroscopy

MS detection of spilanthol was carried out on Water quadrupole TOF Premier mass spectrometry with micro channel plate detector (Waters, USA). The analysis was done with an ESI probe source in positive mode and with collision energy of 3 V. The cell entrance and exit voltage was set at 2 V and −10 V, respectively. For spilanthol identification, all peaks that appeared in HPLC were collected, concentrated and than redissolved in methanol prior to analysis. Confirmation of the spilanthol peak was done by comparing the mass spectra of samples and that reported in published literature.

2.9. Statistical analysis

For each experiment, 24 replicates were raised and each experiment was repeated thrice. All the investigated parameters were analyzed using analysis of variance (ANOVA) and significance was determined at $p < 0.05$. The data was analyzed statistically using SPSS (version 16) software and significant differences among the mean values were assessed on the basis of the Duncan's multiple range test. Percentages have been transformed using arcsine transformation before statistical analysis. For spilanthol estimation, observations are an average of three separate analyses.

3. Results

3.1. Establishment of leaf-disc culture

In the present study, leaf-disc explants of 5 mm size were cultured on a range of media. The treatments involved MS basal medium and varying concentrations and combinations of cytokinins and auxins, like BAP, TDZ, Kinetin, NAA, IAA, 2,4-D and pCPA. Leaf-disc cultures showed no response in the absence of growth regulators. The morphogenic response of leaf-discs was greatly influenced by the type of growth regulator used in the medium. On most of the media combinations, leaf-discs either callused or differentiated into shoots.

Among individual cytokinin/auxin treatments, incorporation of BAP at 5.0 μM to MS basal medium had induced direct adventitious shoot organogenesis. In contrast to this, no shoot proliferation observed from leaf-disc explants cultured on MS medium containing either other cytokinins, TDZ, Kinetin or any of the auxins, 2,4-D, NAA or pCPA, alone at tested concentration of 5.0 μM.

Since the shoot proliferation was obtained with the addition of BAP, it was further evaluated at a concentration range of 1.0–7.0 μM. The frequency of shoot proliferation declined at lower concentrations of BAP; BAP at 3.0 μM concentration induced shoot regeneration in 80.3% cultures with only one shoot per explant while no response was seen at BAP 1.0 μM. The higher concentration of BAP (7.0 μM) induced callusing in 100% explants and the callus remained non-regenerative. At its optimal concentration of 5.0 μM, BAP showed highest percentage (100%) of shoot organogenesis with an average of 3.5 adventitious shoots, directly from the explants, without an intervening callus phase.

Further, a combined effect of cytokinin and auxin was evaluated on multiple shoot induction. Incorporation of NAA (1.0 and 5.0 μM) in the BAP containing medium did not show significant effect ($p < 0.05$) on number of shoot-buds differentiation per explants. Although 100% cultures showed regeneration in NAA containing medium, only one shoot-bud proliferated per explants (Table 1). In comparison to BAP alone and BAP + NAA, addition of IAA to MS + BAP medium enhanced the number of shoot-buds per explants significantly ($p < 0.05$) (Table 1). The best response was obtained on MS + BAP (5.0 μM) + IAA (5.0 μM) medium, which promoted differentiation of loads of shoot-buds per explants, directly, in 82.9% cultures. On this medium, the first visible change in the cultured leaf-discs was observed within the first week of culture. The leaf-disc explants enlarged, and adventitious shoot-buds appeared to arise directly from the cut ends (Fig. 1A–E). The developmental ontogeny of shoot organogenesis from leaf-disc explants was recorded by scanning electron microscopy which confirmed the direct differentiation of shoot-buds from the explants (Fig. 1F and G).

Though, numerous shoot-buds were differentiated from leaf-disc explants on MS + BAP (5.0 μM) + IAA (5.0 μM), however, only a few shoot-buds (5–6) developed into distinct shoots of 1.0–3.5 cm length over a period of 5 weeks and growth of rest of the adventitious shoot-buds remained arrested. To overcome this problem, after 5 weeks, distinct shoots were separated from leaf explants and utilized for rooting and remaining part, which was crowded with stunted shoots, was cut into 3–4 small segments, each with a bunch of 4–5 shoots. Thereafter, these segments were subcultured to 250 ml capacity flasks containing the fresh medium of MS + BAP (5.0 μM) + IAA (5.0 μM). With this arrangement, an average of 51 shoots was differentiated from each segment, in 5 weeks (Fig. 2A). In flask, shoots grew well and attained height of 4.5 cm with each shoot having an average of 4–5 nodes after 5 weeks.

Apart from the shoot proliferation, on few combinations, like MS + NAA (5.0 μM), MS + pCPA (5.0 μM), MS + BAP (7.0 μM) and MS + 2,4-D (5.0 μM), cultures responded initially for callusing but the calli did not survive after the first subculture. The best treatment in terms of sustained growth of calli, percent explants callused and the degree of callusing, was the combination of MS + BAP (5.0 μM) + NAA (1.0 μM) + 2,4-D (1.0 μM).

3.2. Rooting of regenerated shoots and ex vitro acclimatization

Following the protocol described by Singh and Chaturvedi (2010), the 3 cm long shoots, from 5-week-old cultures on MS + BAP (5.0 μM) and MS + BAP (5.0 μM) + IAA (5.0 μM) were used for rooting. On 1/2 MS + sucrose (50 g l^{-1}), 100% shoots formed numerous roots directly from the basal cut end of the shoots (Fig. 2B). The plantlets were acclimatized successfully with 90% survival rate (Fig. 2C).

3.3. Flow cytometric analyses

The ploidy stability of *in vitro* regenerants was determined by flow cytometry. Fig. 3A and B shows representative histograms of field grown parent plant (control) and micropropagated plant derived from leaf-disc explants. The presence of single peak at around 200 channel position in histograms of micropropagated plants corresponded to those of control plant suggested that *in vitro* regenerants maintained ploidy stability and no changes in ploidy levels occurred during regeneration process.

Table 1
Effects of growth regulators on callusing and shoot regeneration response from leaf-disc cultures of *Spilanthes*. Growth period: 5 weeks. Control: MS basal medium.

Media	Percentage of explants forming callus	Percentage of explants forming shoot	Number of shoots per culture
MS basal medium	0.0g	0.0d	0.0e
MS + NAA (5.0 μM)	55.1c	0.0d	0.0e
MS + 2,4-D (5.0 μM)	77.7b	0.0d	0.0e
MS + pCPA (5.0 μM)	10.0f	0.0d	0.0e
MS + Kinetin (5.0 μM)	0.0g	0.0d	0.0e
MS + TDZ (5.0 μM)	0.0g	0.0d	0.0e
MS + BAP (1.0 μM)	0.0g	0.0d	0.0e
MS + BAP (3.0 μM)	0.0g	80.3b	1.0d
MS + BAP (5.0 μM)	0.0g	100a	3.5c
MS + BAP (7.0 μM)	100a	0.0d	0.0c
MS + BAP (5.0 μM) + IAA (1.0 μM)	33.0d	67.0c	4.0b
MS + BAP (5.0 μM) + IAA (5.0 μM)	17.1e	82.9b	5.3a
MS + BAP (5.0 μM) + NAA (1.0 μM)	0.0g	100a	1.0d
MS + BAP (5.0 μM) + NAA (5.0 μM)	0.0g	100a	1.0d
MS + BAP (5.0 μM) + NAA (1.0 μM) + 2,4-D (1.0 μM)	100a	0.0d	0.0e

Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

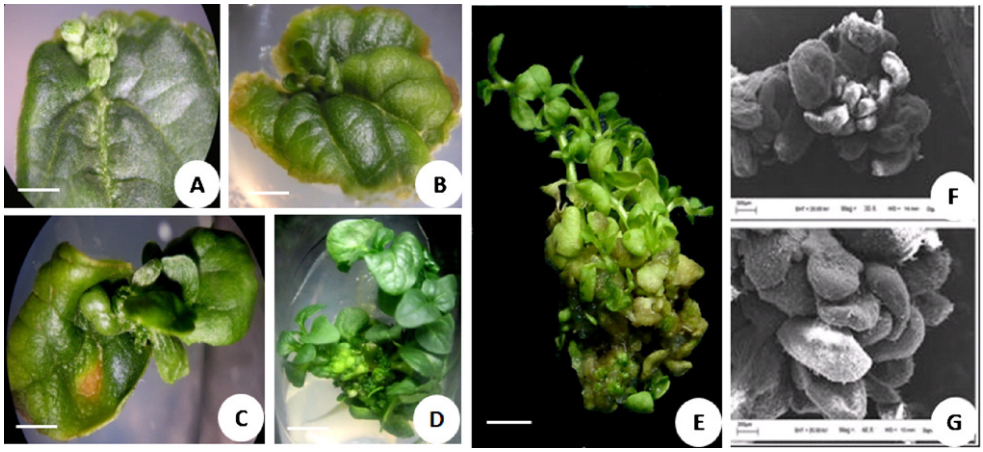

Fig. 1. Shoot proliferation from leaf-disc cultures. (A and B) Leaf-disc cultures on MS + BAP (5.0 μM) + IAA (5.0 μM), showing shoot bud initiation; (A) after one week (bar = 0.12 cm); (B) after two week (bar = 0.18 cm). (C) Same, after 3 weeks, showing development of foliar structures (bar = 0.23 cm). (D) Four-week-old leaf-disc culture showing well developed shoots (bar = 0.9 cm). (E) Five-week-old leaf-disc culture, showing multiple number of shoots some of them have elongated (bar = 0.9 cm). (F) Scanning electron micrograph of 2-week-old leaf-disc cultures showing cluster of leaf primordial (bar = 300 μm). (G) Enlarged view of same (F) with distinct leaf primordial (bar = 200 μm).

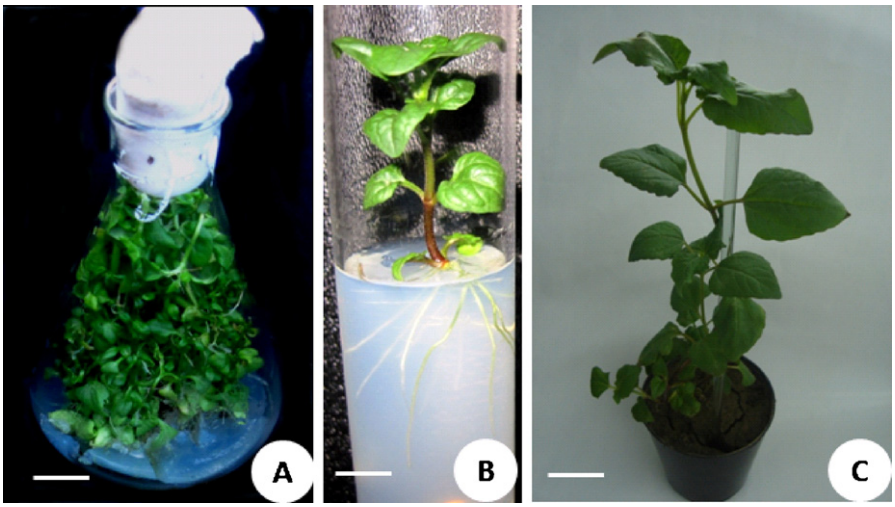

Fig. 2. Shoot proliferation from leaf-disc cultures. (A) Same as Fig. 1E, 5 weeks after transfer to the same medium in 250 ml conical flask, showing proliferation of many well developed shoots (bar = 1.49 cm). (B) A shoot rooted on 1/2 MS + 50 g l^{-1} sucrose. Roots have developed directly from the basal cut end (bar = 1.21 cm). (C) A hardened micropropagated plant, one month after transfer to soil (bar = 2.17 cm).

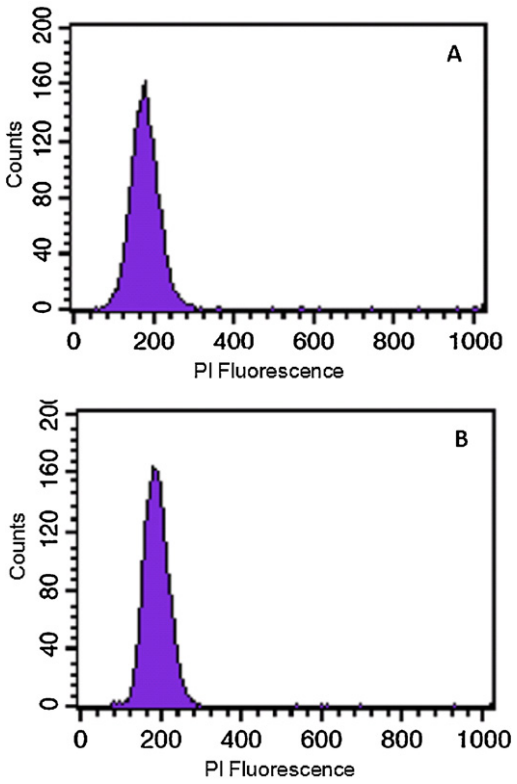

Fig. 3. Flow cytometric analysis. (A) Representative flow-cytometric histogram of nuclei stained with propidium iodide and isolated from leaf tissues of field grown parent plant of *Spilanthes* (control). (B) Same, from micropropagated shoots derived from leaf-disc cultures of *Spilanthes*.

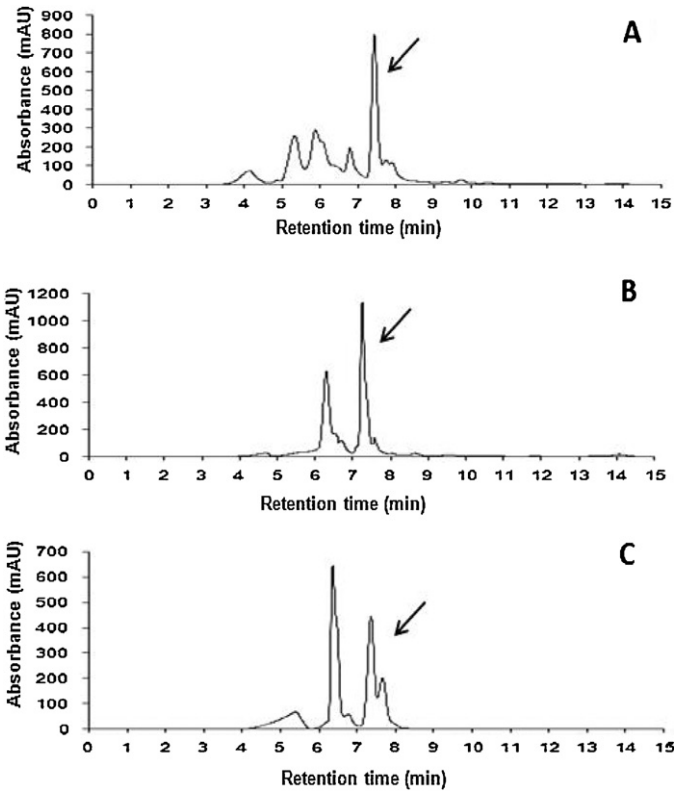

Fig. 4. High Performance Liquid Chromatography for spilanthol analysis. (A) A chromatogram showing the presence of spilanthol (arrow marked) in leaf extract of field grown *Spilanthes* plants (control). (B) A chromatogram showing the presence of spilanthol (arrow marked) in leaf extract of *in vitro* plants proliferated on leaf disc explants. (C) A chromatogram showing the presence of spilanthol (arrow marked) in calli established from leaves of field grown plants.

3.4. Identification of spilanthol

Since spilanthol standard was commercially not available, HPLC and then mass spectroscopic analysis were performed for the identification of spilanthol in samples. Acetonitrile and water at the ratio of 93:7 as the mobile phase was found to be appropriate for satisfactory separation of compounds at a flow rate of 0.5 ml min^{-1}. Typical HPLC profiles of *Spilanthes in vivo* (leaves from field grown plants) and *in vitro* (leaves and calli) extracts are shown in Fig. 4A–C. For spilanthol characterization, all peaks eluted from HPLC, were collected, concentrated and analyzed by mass spectrometry. Samples were analyzed in both positive and negative electrospray ionization mode but, the sensitivity and reproducibility of the dominant ions in the positive electrospray ionization mode was better than in negative ionization mode. Therefore, all HPLC peaks were analyzed in positive mode. Spilanthol was identified by its fragmentation profile which was further confirmed with literature data. It has been observed that the peak eluted at 7.34 ± 0.12 min in HPLC, has characteristic fragmentation pattern of spilanthol. Fig. 5 shows the mass spectrum of spilanthol compound. The spectrum has a base peak at m/z 222 corresponding to the protonated $[M+H]^+$ molecular ion. The ion at m/z 244 was generated due to sodium ion adduct formation $[M+Na]^+$. The characteristic fragment at m/z 149 was formed due to the dissociation of the C–N bond. It is an acyllium ion fragment, which indicates the amount of carbon atoms in the alkyl chain. This fragment is formed by the loss of isobutyl amine group $[MH-C_4H_{11}N]^+$. Mechanism involved in the acyllium ion formation is charge-remote homolytic cleavage that yields a resonant distonic radical cation, which subsequently undergoes hydrogen rearrangement. Another fragment seen at the m/z 123 can be attributed to the $[MH-C_5H_9NO]^+$. The loss of a fragment with specific m/z 99 from the protonated $[M+H]^+$ molecular ion (m/z 222) confirmed that spilanthol contains isobutylamide group. Analysis of samples, each having a peak at approximately 7.34 ± 0.12 min, was all found to contain the fragments diagnostic for spilanthol. In samples, spilanthol was tentatively quantified on the basis of another alkylamide, dodeca-2(E), 4(E)-dienoic acid isobutylamide. Like spilanthol, this compound also has isobutyl amide group and long carbon chain. In mass spectra, this amide form acyllium ion at the m/z 179 (Table 2).

3.5. Calibration curve analysis

In order to quantify the amount of spilanthol in the methanol extracts of *Spilanthes*, calibration curve was prepared with the available standard dodeca-2(E), 4(E)-dienoic acid isobutylamide. Standard showed high linearity at tested concentrations (250–15 μg ml^{-1}) with correlation coefficient (R^2) of 0.987. The linear regression equation for standard was: $y = 1.375x + 36.43$, where x is the concentration of standard (μg ml^{-1}) and y is the total peak area. The equation, thus, generated from the curve by external standard method was used to calculate the amount of compound present in crude samples.

The precision of the developed method was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard samples, at same concentration, were analyzed at least 3 times within the same day and the RSD value obtained was 1.8%. Similarly, for inter-day variability, same concentration of the standard was run at least twice at one day interval and the values for the same figured out to be 2.4%.

Table 2
Fragments generated in MS analysis of spilanthol and dodeca-2(E), 4(E)-dienoic acid isobutylamide.

S. No.	Compound	MH^+	Fragments	References
1	Spilanthol	222	166, 149[a], 123, 121, 81	Boonen et al. (2010)
2	Dodeca-2(E), 4(E)-dienoic acid isobutylamide	252	196, 179[a], 161, 119, 95	Cech et al. (2006)

[a] Acyllium fragment formed by dissociation of the C–N bond to lose the entire amine functional group ($MH^+ - 73$).

3.6. Quantification of spilanthol

By following the protocol as described in Section 2, the methanol extracts of leaves and calli were analyzed by HPLC, for the quantification of spilanthol. HPLC analysis of regenerated plants and *in vitro* callus revealed that the peak for spilanthol was identical to those of field-grown mother plants (Fig. 4A–C). From the external standard equation, the amount of spilanthol in different samples was calculated. Spilanthol content in the methanol extracts of callus was 998.03 ± 15.6 μg/g DW and in the leaves of *in vitro* plants derived from leaf-discs was 3294.36 ± 12.4 μg/g DW. Leaves of field grown mother plants contained 2703.66 ± 9.6 μg/g DW of spilanthol. Statistical analysis showed that leaves of *in vitro* plants derived from leaf-discs contained significantly ($p < 0.05$) higher amount of spilanthol than the leaves of field grown mother plant. In comparison to *in vitro* plants and field grown parent plants, dedifferentiated callus accumulated significantly ($p < 0.05$) lower amount of spilanthol.

4. Discussion

In contrast to the use of other explants like nodal segments, axillary buds and shoot tips, the use of leaf explants to raise *in vitro* cultures would be very convenient for growers and herbal product companies to clone elite individuals of *Spilanthes* for enhancing its cultivation and metabolite production. This is due to the fact that foliar explants are easy to obtain and do not require to sacrifice the mother plant. Till date, only two reports are available on leaf-disc culture of *Spilanthes* (Saritha and Naidu, 2008; Pandey and Agrawal, 2009). However, in these studies, *in vitro* cultures were not analyzed for ploidy stability and metabolite production.

The type and concentration of auxin and cytokinin used in the medium and a possible interaction between exogenous and endogenous concentrations of plant growth regulators have a marked effect on the *in vitro* culture responses (Skoog and Miller, 1957). In the present study, shoot regeneration from leaf-disc cultures was obtained on MS + BAP, MS + BAP + IAA, and MS + BAP + NAA. The best treatment for shoot regeneration was MS + BAP (5.0 μM) + IAA (5.0 μM) which promoted adventitious shoot proliferation in >82% cultures with an average of 5.3 shoots per explant. The requirement of auxin along with cytokinin for obtaining optimal response of shoot-bud differentiation is well documented in a number of plants including *Aegle marmelos* (Hossain et al., 1995), *Pistacia vera* (Tilkat et al., 2009), *Emblica officinalis* (Nayak et al., 2010); *Musa acuminate* (Jafari et al., 2011) and *Arnebia hispidissima* (Shekhawat and Shekhawat, 2011). In this study, shoot proliferation from leaf-disc cultures occurred without an intervening callus formation. This point is of major importance because plants produced by direct organogenesis may exhibit greater genetic stability than those produced via callusing (Karam and Al-Majathoub, 2000).

Adventitious shoot proliferation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, and, thus, it may cause a change in ploidy due to chimera formation which in turn can influence the metabolite content in *in vitro* cultures. For the reason, the ploidy stability of the *in vitro* cultures was checked by flow cytometry which showed that all *in vitro* regenerated shoots were at similar ploidy level as that of the parent plant. Similarly, Libiakova et al. (1995) found that shoots regenerated *in vitro* from mature embryos and cotyledons of seedlings, as well as long term callus cultures of the hybrid *Abies concolor* × *Abies grandis* were

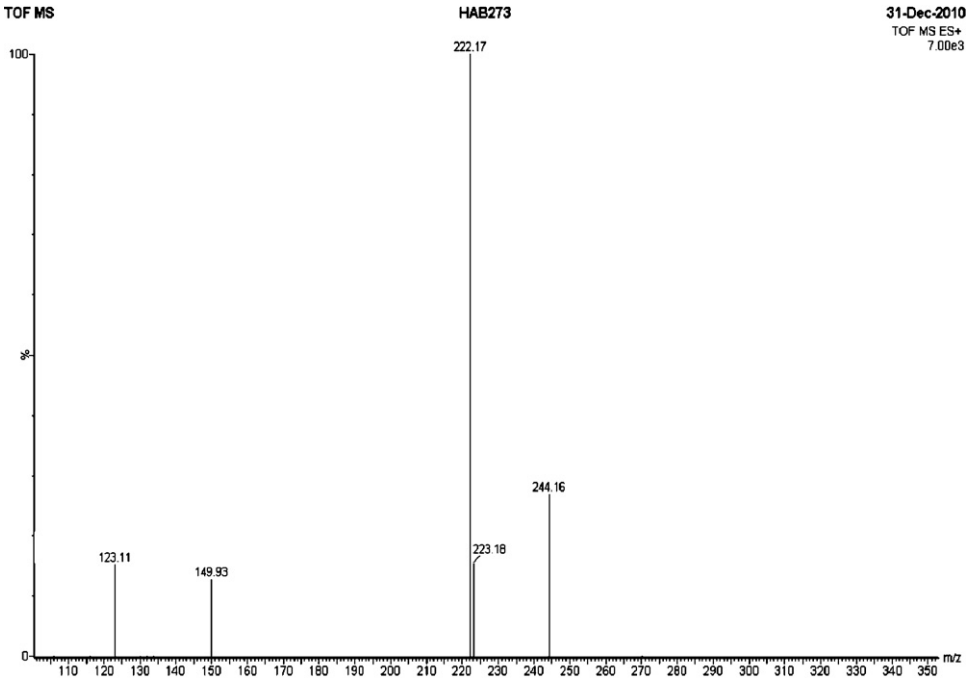

Fig. 5. Mass Spectroscopy. Positive electrospray ionization mass spectra of spilanthol, purified from HPLC fraction of crude extract obtained from cell biomass.

stable, remaining at the diploid level. Thiem and Śliwińska (2003) also showed that there is no variation in the ploidy level among different types of tissue cultures: axillary shoots from the 3rd and 20th passage, shoots from cold storage, and shoots developed from alginate-encapsulated buds. Micropropagated cultures of *Juniperus phoenicea* (Loureiro et al., 2007), and, more recently, of *Rubus fruticosus* (Vujović et al., 2010) also showed ploidy stability.

Another interesting finding of this study was the production of spilanthol from *in vitro* cultures of *Spilanthes*. Results of the HPLC analysis have shown that leaf-disc derived *in vitro* plants accumulated higher amount of spilanthol (3294.36 ± 12.4 μg/g DW) than field grown mother plant (2703.66 ± 9.6 μg/g DW of spilanthol). In comparison to field grown plants and *in vitro* plants, dedifferentiated callus accumulated significantly ($p < 0.05$) lower amount (998.03 ± 15.6 μg/g DW) of spilanthol. The present results agree with those of Massot et al. (2000), where micropropagated shoots accumulated higher secondary metabolites compared to the field-grown plants.

This study confirms the earlier reports which suggested that differentiated (organized or redifferentiated) cells and specialized organs generally produce most secondary products compared to dedifferentiated (unorganized) cells in cultures (Rao and Ravishankar, 2002; Tang et al., 2010). Further, it has been frequently observed that specialized plant organs show different expression pattern of secondary metabolites when inducted into the *in vitro* systems (Łuczkiewicz and Głód, 2005; Lucchesini et al., 2009). Stafford et al. (1986) reported that the accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources. Effect of growth regulators on secondary metabolite production has also been very well documented by other workers (Di Cosmo and Towers, 1984; Moreno et al., 1995; Amit et al., 2005; Jaleel et al., 2009; Nagella and Murthy, 2010; Anuradha et al., 2010; Lee et al., 2011).

Over the past decade, a number of biologically active compounds have been reported in *Spilanthes* (Ramsewak et al., 1999; Prachayasittikul et al., 2009). However, the studies on quantification of spilanthol, an alkylamide, have never been conducted in this plant. Moreover, this is the first study, reporting the spilanthol content in the tissue-culture derived plantlets and cells of *Spilanthes* whereby *in vitro* leaves accumulated higher amount of spilanthol compared to the field grown elite mother plants. These findings have opened up the possibility of producing *Spilanthes* plants with desired metabolite content, throughout the year, consistently, irrespective of the season, which will help the pharmaceutical industry to achieve better yield by using superior quality raw materials.

5. Conclusion

An optimized protocol for regeneration of high yielding true-to-type plantations of *S. acmella* is developed by employing leaf-disc explants of *Spilanthes*. By following this method, more than 155 plants can be produced per explants, in every 10 weeks. This rate of shoot multiplication has not been achieved till date in *Spilanthes*. Moreover, this is the first report, in which ploidy stability of *in vitro* regenerants has also been evaluated by flow cytometry. An interesting finding of this study is the identification and quantification of spilanthol, an antiseptic alkylamide, from *in vivo* and *in vitro* sources whereby *in vitro* leaves produced significantly higher amount of compounds (3294.36 ± 12.4 μg/g DW) than that of field grown mother plant (2703.66 ± 9.6 μg/g DW).

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