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Isolation and quantification of antimalarial *N*-alkylamides from flower-head derived in vitro callus cultures of *Spilanthes paniculata*

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This is the pioneer work reporting on simple procedure for synchronized determination and quantification of two biologically active *N*-alkylamides, (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) and (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA), using in vitro callus cultures from flower-heads of *Spilanthes paniculata*. The extracts were purified using preparative thin layer chromatography (TLC) and finest separation of compounds was optimized using high performance liquid chromatography (HPLC). Eventually, *N*-alkylamides were validated by mass spectrometry. Linearity curve with its regression coefficients (R^2) obtained for both these alkylamides was 0.99. While spilanthol was quantified using tentative standard dodeca-2(*E*),4(*E*)-dienoic acid due to the non-availability of commercial standard and the precision of a developed method was evaluated in terms of relative standard deviation by measuring inter- and intra-days variation 3.52% and 1.74%, respectively. Similarly, calibration curve was obtained for the compound UDA isolated from flower-head explants from field grown parental plant with its inter- and intra-day RSD values as 4.33% and 3.61%, respectively. With this protocol, a very high yield of 2.23 mg/g of spilanthol and 4.30 mg/g dry weight (DW) of UDA, was obtained, simultaneously, from callus cultures. Flower-heads from parent plants, used as control, showed negligible amount of spilanthol and quantity of UDA was marginally higher than that in callus cultures. The highly stable biotherapeutic spilanthol and UDA with *m/z* 222 and *m/z* 230, respectively, showed retardation of malaria parasite development through blockage at ring stage of erythrocytic schizogony and ultimately lead to parasite death. The effect on parasite was additive. This study signifies the utility of in vitro cell cultures for therapeutic compound production, throughout the year, at higher yield for down-stream applications.

[**Key words:** *Spilanthes paniculata*; Asteraceae; In vitro; Callus cultures; *N*-Alkylamides; Spilanthol; High performance liquid chromatography; Electrospray ionization mass spectrometry; Malaria drug; Preparative thin layer chromatography]

The *N*-alkylamides are organoleptic compounds, classified to be one of the most promising groups of secondary metabolites, comprised of condensed saturated or polyunsaturated fatty acid and an amine. These alkylamides are found predominantly in the genus *Spilanthes* of the family Asteraceae (formerly Compositae), which includes more than 300 species, generally distributed in the tropics (1). The entire plant is utilized as a leafy vegetable and spice (2). It has extensively been used in traditional medicine around the world with varied biological activities. Its ethnopharmacological prominence is extensively reported in folklore remedies against toothache, stomatitis, throat complaints and local anesthetic (3). Generally, the extracts and decoctions of various species of *Spilanthes* have shown diverse pharmacological activities and contain strong biologically active compounds though most of them have not been identified and quantified in the extracts (3). More than 0.5% alkylamides, present in the genus, are responsible for local anesthetic, analgesic, antiseptic, sialogogue and insecticidal properties and are probably exploited in traditional medicine (3). The plant is known to contain potent larvicidal activity against Anopheles mosquitoes and helps in the prevention and control of malaria (4,5). Because of these multifold uses, the commercial interest in *Spilanthes* has increased tremendously. The principal pungent and bioactive *N*-alkylamide, reported to be responsible for most of its biological activity, is spilanthol, (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide, which acts as a representative of alkylamides (3). Later other alkylamides, like (2*E*)-*N*-(-2-methylbutyl)-2-undecene-8,10-diynamide, (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, (7*Z*)-*N*-isobutyl-7-tridecene-10,12-diynamide, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide had also been identified from flower-heads of *Spilanthes* (2,6,7).

Compared to its real market potential in the medicinal, food and body care products, attempts on scientific research to explore the potential alternative sources for production of pharmacological compounds from this endemic and traditional medicinal plant are negligible. Nevertheless, despite a few reports demonstrating the biological properties of *Spilanthes* (4,8–10) the quantitative analysis of responsible key components in the extracts are completely ignored. There are no defined approaches sophisticated in characterization of structural similarities and quantitative analysis of these compounds in complex mixtures (4). The phytochemical analysis is very important due to biological activities of these

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prominent compounds to ensure the safety and efficacy of functional food and dietary supplements. These studies can firmly establish the vital relationship between the pharmacology and chemistry of the plants. Therefore, the objective of the present study was to establish the in vitro callus cultures of *Spilanthes paniculata* as an alternative to whole plant extraction and to isolate the key bioactive *N*-alkylamides *via* chemical analysis from these in vitro cell lines for a bioactivity-based approach.

As the flower-heads are reported to be a rich source of active principle in *S. paniculata* (4), the in vitro callus cultures were established from flower-heads as alternative for biomass utilization and consistent and homogeneous production of principal bioactive *N*-alkylamides, such as spilanthol (7,11) and (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) (11). Furthermore, isolation and quantification of spilanthol and UDA, simultaneously, from callus lines have been accomplished for the first time in this report. This is one and only report where UDA has also been reported from any in vitro source. The isolation, purification and high performance liquid chromatography (HPLC) methods adopted for determining the compounds are efficient and reproducible. Needless to mention that such quantifications studies have not been performed earlier on *Spilanthes*. Additionally, suitability of the above claims was further checked in a bioassay against *Plasmodium falciparum* 3D7 where schizonticidal and parasiticidal properties of these two alkylamides was established with noticeable activity.

MATERIALS AND METHODS

Plant material and establishment of flower-head callus cultures Fresh, healthy and young flower heads were collected from *S. paniculata* plants growing in the campus at Indian Institute of technology Guwahati, Assam, India. These explants were washed with Savlon (Johnson and Johnson, India) (1%, v/v) for 15 min followed by rinsing, three times, with sterile distilled water (SDW). Subsequently, under aseptic conditions, the explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min. After three more rinses with SDW, these surface-sterilized flower-heads were carefully inoculated onto the medium. Murashige and Skoog (MS) basal medium (12) augmented with different combinations and concentrations of plant growth regulators, such as α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzylaminopurine (BAP), was used for callus induction and multiplication. Sucrose with 30 g/L acted as a carbon source and the media were solidified with 0.8% (w/v) agar (Hi Media Laboratories, India). The media pH was adjusted using 1 N NaOH/HCl to 5.8. Later, 20 ml of the media was distributed into each 150 × 25 mm Borosil rimless glass tube and the test-tubes were plugged with cotton (non-absorbent) wrapped with muslin cloth. The media were autoclaved for 20 min at (1.06 kg cm^{-2}) and 121°C. All cultures were maintained under cool white fluorescent light (1000–1600 lx) with a photoperiod of 16 h at 25 ± 2°C with relative humidity of 50–60%. After induction of callus, the biomass of cells are repeatedly subcultured and maintained into fresh media of the parental media composition at every 4 weeks of interval.

Determination of dry cell weight The biomass of flower-head callus cells, grown successfully on responding semi-solid media, were harvested at the end of growth period of 4 weeks, washed with distilled water and filtered under vacuum pressure. Consequently, the fresh weight of harvested calli (193.44 g) and also flower-head samples (362.8 g) from field grown parent plant were weighed using high precision analytical balance (Sartorius, India). Further, the undifferentiated masses of cells and flower-head samples were dried in oven at 30 ± 2°C until a constant weight was achieved and also to prevent decomposition of thermolabile bioactive phytochemical compounds. The dried cell biomass (20 g) and flower-head samples (50 g) were utilized for further biochemical studies.

Preparation of dodeca 2(*E*),4(*E*) dienoic acid isobutylamide standard Since there is no commercial standards of spilanthol available, the stock solutions of dodeca 2(*E*),4(*E*) dienoic acid isobutylamide, a structurally similar spilanthol like *N*-alkylamide, was used as reference standard (13). Its stock was prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol (i.e., 1 mg/ml). Quantification of standard was carried over at five different concentrations range from (250 μg/ml to 15 μg/ml) and each concentration of standard was filtered through a 0.22 μm nylon membrane filter (Millipore, USA) before HPLC analysis and was run at least thrice to check the repeatability and precision of results.

Preparation of UDA standard Similarly, due to non-availability of commercial compound of UDA, it was isolated from flower-heads of field grown parental plant using thin layer chromatography (TLC) and HPLC. Quantification of purified UDA standard was carried over at five different concentrations range from (1 mg/ml–0.06 mg/ml) and each concentration of standard was filtered through a 0.22 μm nylon membrane filter (Millipore, USA) before HPLC analysis and was run at least thrice to check the repeatability and precision of results.

Preparation of sample solutions The dried mass of cells (20 g) and flower-heads (50 g) were powdered using porcelain pestle and mortar. They were soaked in methanol analytical grade (Merck, India) for 12 h. The methanolic extract was then centrifuged at 5000 rpm for 10 min in a refrigerated centrifuge (Sigma 4K 15C, Osterode Am Harz, Germany). The centrifuged aliquot was collected and the residue was re-extracted thrice with 10 ml methanol. Thereafter, the residue was discarded and the whole methanolic solution was collected, filtered and then evaporated to dryness in a rotary evaporator (Buchi Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland) at 40°C. The samples were re-dissolved in HPLC grade methanol and filtered through a 0.22 μm nylon membrane filter before analysis. The obtained samples were stored at −20°C until further use. The percent yield of the crude extract was calculated relative to the weight of the dried cells and subjected to initial test against malaria parasite.

Preparative thin layer chromatography of extracts For qualitative analysis, the elution of samples were performed from the extracts of field grown flower-heads and in vitro grown callus samples through preparative thin layer chromatography (PTLC) using hexane:ethylacetate mixture (2:1). Later, the yellowish brown single band was observed in the UV chamber, and the R_f value of the band was calculated. The obtained band was collected and re-dissolved in ethylacetate. The filtered ethylacetate extract was evaporated to dryness using rotary evaporator (Büchi Rotavapor R-200) and re-eluted in PTLC. The procedure was repeated thrice to remove silica and to obtain semi-purified samples. This semi-purified sample was subjected to antimalarial assay in a preliminary assay and excess sample was stored at −20°C until further use.

Semi-purified sample obtained from PTLC was first analyzed by Water quadrupole-T of Premier mass spectrometry (MS) before being screened and purified by HPLC equipped with UV detector (HPLC-UV), to understand the presence of *N*-alkylamides. Immediately after that, this PTLC semi-purified sample from callus and flower-heads were quantitatively estimated using Varian Prostar HPLC system (Varian, USA) consisted of UV detector, a prostar binary pump, a 20 μl injection loop and 4.6 mm × 250 mm dimension Hypersil BDS RP-18 column (Thermo, USA). Aliquot of 20 μl of the samples were injected into the loop. The mobile phase was A (MilliQ water) and B (acetonitrile) with a flow rate of 0.5 ml min^{-1}. The various methodologies attempted for mobile phase for an appropriate separation of phytochemical compounds are listed in Table 1. The eluted samples were detected by UV detector at 237 nm and the two prominent major peaks eluted are collected in separate vials.

Thereafter, both the collected peaks were concentrated at −50°C using lyophilizer. The presence of alkylamides, spilanthol and UDA, were confirmed in the two purified peaks by electrospray ionization (ESI) massspectrometry (MS) and by comparing these spectra with the previously published reports. Besides, due to the non-availability of commercial standards of spilanthol and UDA, spilanthol was tentatively quantified in the samples on the basis of structurally similar alkylamide, dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide, which consists of isobutylamide group and long carbon chain. However, the UDA in the samples was identified and quantified from UDA purified from flower-heads of field grown parent plants and used as a standard.

Linearity and precision of developed method The prepared five various concentrations of reference standards, dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide (structurally similar spilanthol) and UDA were allowed to run by HPLC to check the linearity of developed method. The calibration curve was generated by plotting the peak area (y) against concentration in μg/ml of standard solutions to generate the standard equations for quantification of the two important alkylamide, spilanthol and UDA in unknown samples. The amounts of spilanthol and UDA were reported as mg/g DW of the samples. The correlation coefficient (R^2) were also generated using Microsoft Office (Excel) edition 2003 by fitting the linear trend line to the standard curves obtained for each of the two compounds.

Precision of developed assay was evaluated by running the same concentration of standard compounds at least three times on the same day (intraday) and twice at one day intervals (interday). The values are calculated in terms of relative standard deviation (RSD).

$$\%\ \text{RSD} = (\text{standard deviation}/\text{mean}) \times 100 \quad (1)$$

TABLE 1. Various isocratic methodologies using HPLC.

Methodology	
MilliQ water (%)	Acetonitrile (%)
7	93
10	90
20	80
30	70
40	60

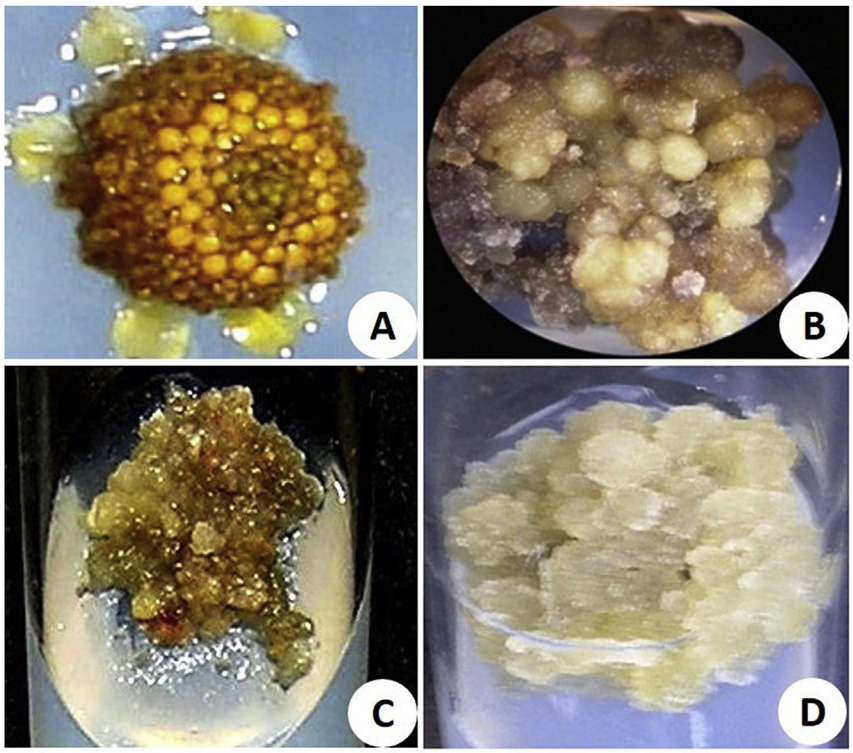

FIG. 1. Establishment of flower-head callus cultures from *S. paniculata*. (A) A-day-old flower-head explant on Murashige and Skoog medium supplemented with auxin and/or cytokinin (×0.18 cm). (B) Same as panel A, after 4 weeks of inoculation showing callus initiation from individual flowers in the flower-head (×0.11 cm). (C) Same as panel B, after 8 weeks of inoculation showing brown and wet undifferentiated calli (×0.17). (D) Same as panel C, after 12 weeks showing profusely growing, healthy, fresh, cream and friable calli (×0.72 cm).

Identification and confirmation of compounds by mass spectrometry Detection of spilanthol and UDA by MS was carried out on Water quadrapole-T of Premier mass spectrometry with micro channel plate detector (Waters, USA). The analysis was done with an ESI probe source in positive mode with collision energy of 3 V. The cell entrance and exit voltage were set at 2 V and −10 V, respectively. All peaks that appeared through HPLC were collected, concentrated through lyophilizer and then re-dissolved in methanol prior to analysis. The confirmation of spilanthol and UDA peaks were done by comparing the mass spectra of samples from previously published literature.

Malaria parasite culture *P. falciparum* 3D7 strain was maintained in human red blood corpuscles (RBCs) (blood group O+) suspended in RPMI 1640 media containing 25 mM HEPES, 0.4% glucose, 0.2% sodium bicarbonate, 0.5% albumax, 50 mg/L hypoxanthine, 40 μg/ml gentamycin and 25 μg/ml amphotericin B, at 37°C in 5% CO_2 (14). The study was first carried out directly on crude and semi-purified TLC extracts obtained from in vitro callus and flower-heads from parent plant to understand the possible biological activity of the obtained extracts. Later on receiving the positive results, biological assays were conducted in detailed on HPLC purified spilanthol and UDA.

Determination of schizonticidal activity of HPLC purified extracts To determine schizonticidal activity, parasites were synchronized with D-sorbitol and schizonticidal activity was performed as described by Parveen et al. (15). In brief, ring synchronized parasite culture (1% parasitemia and 3% haematocrit) was incubated with different concentrations (0–50 μg/ml) of plant extracts as well as active compounds from HPLC column. The parasite culture was allowed to grow along with plant extract or active compound for another 36 h and a thin blood smear was prepared. The smear was stained with Giemsa and the number of schizont containing RBCs were counted under 100× objective (Nikon YS100) with oil immersion. A total 5 different fields were counted and the results were analyzed to determine schizonticidal activity as described (16).

Determination of parasiticidal activity of HPLC purified samples The parasite culture was incubated with active purified compounds for 36 h, washed twice with complete RPMI 1640 media to remove the compounds. Thereafter, the parasite was allowed to propagate in complete media without compound for another 72 h. A thin smear of parasite culture was stained with Giemsa and level of parasitemia was determined under 100× oil immersion to calculate parasiticidal activity of crude and HPLC purified active compounds as described previously by Parveen et al. (15).

RESULTS AND DISCUSSION

Establishment of callus cultures from flower-head explants and extraction yield The sterilized flower head explants were inoculated (Fig. 1A) on MS basal medium comprised of auxins and/or cytokinin. Callus induction was observed on all the media combinations like, MS + BAP (9 μM), MS + 2,4-D (5 μM) and MS + NAA (1 μM), MS + BAP (9 μM) + NAA (1 μM), MS + BAP (9 μM) + 2,4-D (1 μM) and MS + BAP (4 μM) + 2,4-D (2 μM). However, the calli showed browning and did not sustained growth beyond first subculture of two weeks duration on the parent medium. Therefore, these calli were transferred to a different set of growth regulator combinations consisting of two auxins and one cytokinin. When MS medium was supplemented with 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM), it supported profusely growing, brown, friable growth of callus within 12 weeks (Fig. 1B–D). Therefore, these calli were further maintained and multiplied on this medium by sub-culturing at every 4 weeks interval. This process of repeated sub-culturing at regular intervals was adopted in *Lantana camara* leaf-disc cultures to generate the massively growing cell biomass and to reduce the browning of calli due to phenolic compound accumulation (17–19). Recently, the callus cultures have been selectively used for the production of various groups of secondary metabolites (17–20). The reason for their selection is that the plant cells are biosynthetically totipotent and retain the same genetic information as that of the parent plant (20) with an added advantage of increased cell biomass with high productivity cell lines.

With this strategy of selective use of cell lines, in the current study, the cell biomass differentiated from flower-head explants yielded 6.4% (w/w) of the crude extracts from 20 g DW of the cells. The bioactivity of crude extract was determined against malaria

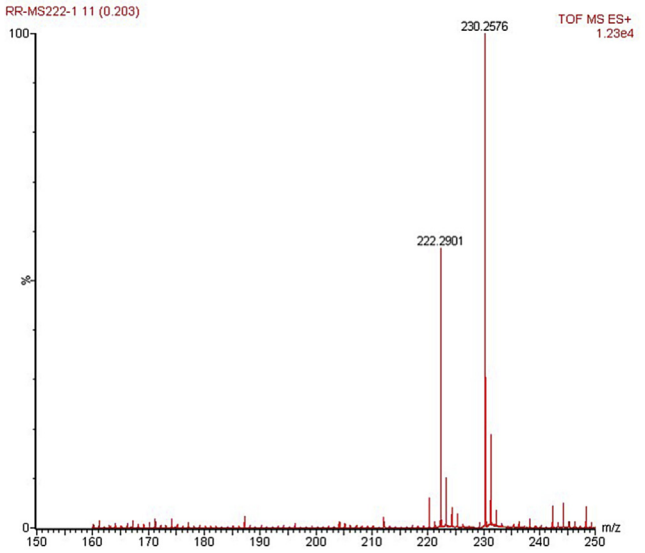

FIG. 2. Positive electrospray ionization mass spectrum of (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8 decatrienamide (spilanthol) at 222.2901 *m*/*z* and (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) at 230.2576 *m*/*z* in semi-purified PTLC extracts.

parasite. Following this, the extract was further processed to isolate the purified *N*-alkylamides, spilanthol and UDA, their quantification and bioassays, like schizonticidal and parasiticidal activities against malaria parasite was performed.

Identification, isolation and quantification of *N*-alkylamides from cell cultures of *S. paniculata* The acquired cream, fresh, friable and healthy callus from Fig. 1D was utilized for extraction and analysis of *N*-isobutylamides. The purification, isolation, characterization and quantification of *N*-alkylamides were performed by PTLC, HPLC, MS and FT-IR spectroscopy.

Purification of extracts by preparative TLC By following the protocol as described in material and methods, the crude extracts from in vitro cell lines and flower-head samples were semi-purified by preparative TLC to yield yellowish dark brown band from in vitro cell lines (221 mg, $R_f = 0.5$) and field grown flower-heads (583 mg, $R_f = 0.5$) and then, it was visualized under UV chamber. The band was further purified by re-dissolving in ethylacetate and eluting in solvent mixture, thrice, as mentioned in materials and methods section. In a preliminary test, the band showed schizonticidal and parasiticidal activity. The confirmation on the presence of two compounds, spilanthol [*m*/*z* 222] and UDA [*m*/*z* 230] in the band was performed using mass spectrometry (Fig. 2). Knowing the presence of two compounds, posteriorly, the acquired semi-purified sample was taken to HPLC for further analysis.

Isolation and identification of spilanthol and UDA The semi-purified sample from TLC exhibited mixtures of alkylamides and, hence, was subjected to HPLC-UV method isocratically (Table 1) by adjusting the ratio of MilliQ water and acetonitrile in the mobile phase for subsequent isolation and quantification of alkylamides (Fig. 3A–E). The mobile phase of MilliQ water at 7% and acetonitrile at 93% provided two prominent peaks, where the first peak approached at retention time 5.9 ± 0.09 provided area 47.50%, whereas, the second prominent peak at retention time 6.5 ± 0.08 yielded area of 20.93% with improper separation. Consequently, the highest polarity of the solvent, MilliQ water was transiently increased to 10% and acetonitrile of 90% provided the similar retention time at 5.9 ± 0.09 and 6.5 ± 0.09 as that of

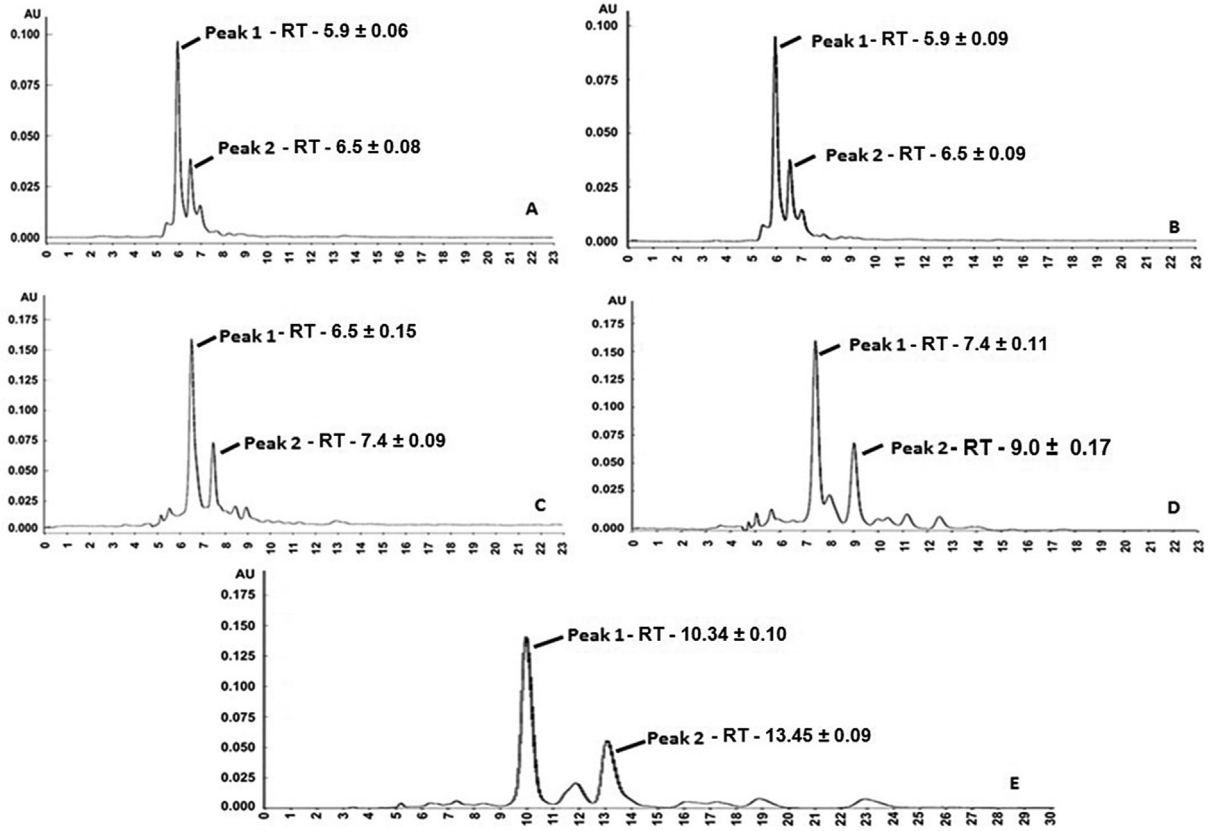

FIG. 3. Isolation and purification of alkylamides through HPLC isocratic methodology. (A) Mobile phase ACN:MilliQ (93:7), (B) mobile phase ACN:MilliQ (90:10), (C) mobile phase ACN:MilliQ (80:20), (D) mobile phase ACN:MilliQ (70:30), (E) mobile phase ACN:MilliQ (60:40).

TABLE 2. Calibration curve for the standard compounds.

Standard	Calibration curve	R^2	RSD (interday, %)	RSD (intraday, %)
Dodeca-2(*E*), 4(*E*)-dienoic acid isobutylamide (structurally similar to spilanthol)	$y = 0.8559x + 2.82$	0.99	3.52	1.74
UDA	$y = 159.66x + 7.19$	0.99	4.33	3.61

TABLE 3. Quantification of spilanthol and UDA in callus cultures and flower-head explants.

N-Alkylamide	Retention time	MS spectra [*m*/*z*]	Total amount (mg/g DW)	
			In vitro callus cultures from flower-head	Flower-head
Spilanthol	13.45 ± 0.09	221	2.23 ± 0.04	0.83 ± 0.12
UDA	10.34 ± 0.10	230	4.30 ± 0.22	5.29 ± 0.05

isocratic method attempted using 7%:93% ratio of MilliQ water and acetonitrile with area percentage 43.53% and 19.36% corresponding to retention time, respectively. Later, MilliQ water was adjusted to 20% and acetonitrile of 80% offered shifting of two prominent peaks at different retention time at 6.5 ± 0.15 with area percentage and 7.4 ± 0.09 with 17.16% area percentage. Eventually, MilliQ water was increased to 30% and acetonitrile was auto-adjusted to 70% to attain further peak shifting from 6.5 ± 0.15 to 7.4 ± 0.11 with 34.53% area percentage whereas, 7.4 ± 0.09 to 9 ± 0.17 with 18.93% area percentage over previous methodology. Further, polarity of mobile phase, MilliQ water was increased to 40% and acetonitrile to 60%, generated the best chromatogram with distinct separation and further shifting of two major peaks at retention time 10.34 ± 0.10 (peak 1) with area percentage of 38.94% and another peak at 13.45 ± 0.09 (peak 2) with area percentage of 20.19% (Fig. 3E). However, equivalent ratio (50%) of MilliQ water and acetonitrile yielded mixture of peaks with improper separation of phytochemical constituents. Therefore, of various isocratic methodologies tried, MilliQ water 40% and HPLC grade acetonitrile 60% gave the best and satisfactory separation of two distinct *N*-alkylamides, spilanthol and UDA, from field grown flower-head and callus extracts and hence, was used throughout the experiment.

Previously, the structural elucidation, isolation and identification of organic biomolecules moieties, alkylamides were determined from the *Spilanthes* species by employing the combinatorial

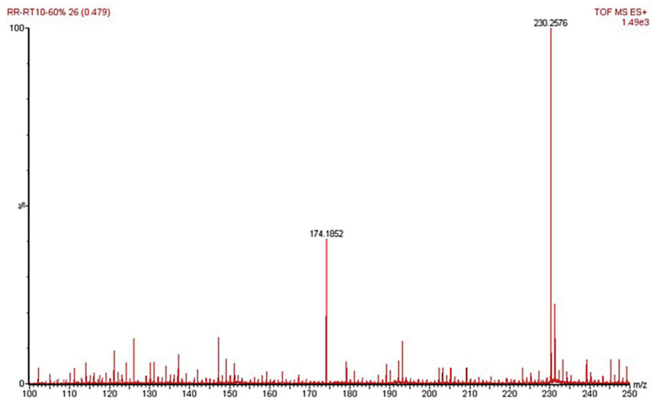

FIG. 5. Mass spectrometry of purified compound (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) eluted at retention time of 10.34 ± 0.10.

analytical techniques, like NMR, MS and GC–MS (2,21–25). However, these approaches are associated with a few disadvantages, such as lengthy purification process, lack of identification and quantification of organic compounds in mixture of sample. In a few others reports (8,9), LC–MS with atmospheric pressure chemical ionization and electron impact ionization was employed for analysis of alkylamides from mixtures but again these techniques do not facilitate quantification of compounds in mixtures. Compare to these, the present study reported a new simplest strategy of two step methods by employing PTLC and HPLC, which has strengthened the efficacy on isolation of the significant alkylamides from even the crude methanolic extracts prepared from field grown flower-head and unorganized callus samples of *S. paniculata*.

Linearity and precision of developed method After appropriate confirmation on the presence of spilanthol and UDA, the total content estimation was performed. The calibration curves for the two standards were generated at tested concentrations as mentioned in material and methods. They showed high accuracy, efficient linearity and reproducibility with correlation coefficients (R^2) of 0.99 for both the standards. The linearity curve for spilanthol was obtained using structurally similar alkylamide standard dodeca-2(*E*),4(*E*)-dienoic acid due to the non-availability of commercial standard. The precision of the developed method in terms of relative standard deviation (RSD) mentioned in Table 2 was evaluated by measuring inter- and intra-day variations by following the procedure mentioned in Materials and methods. The interday RSD values were 3.52% and 4.33% for spilanthol and

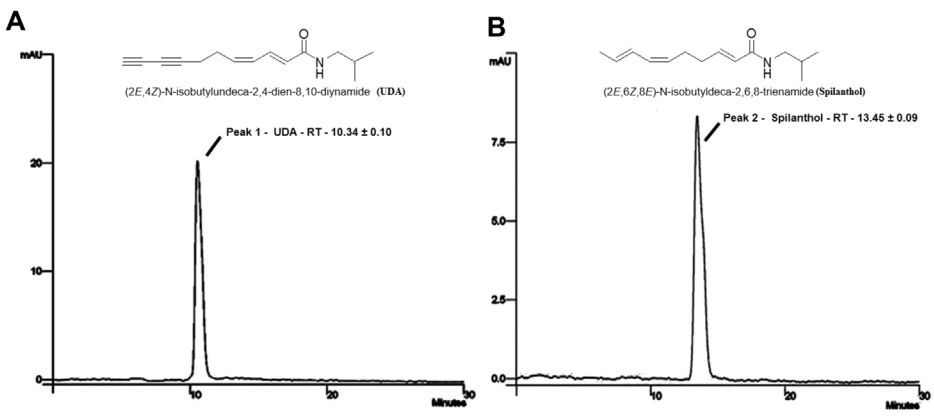

FIG. 4. (A) Isolation of (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) at retention time of 10.34 ± 0.10 min with *m*/*z* 230.2576. (B) Isolation of (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) at the retention time of 13.45 ± 0.09 min with *m*/*z* 222.2901.

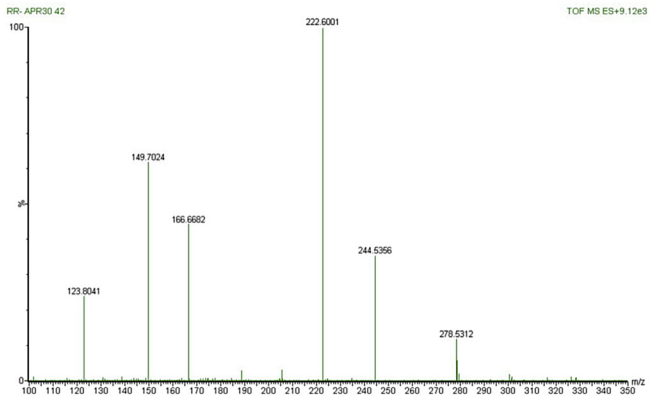

FIG. 6. Mass spectrometry of compound (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) eluted at retention time of 13.45 ± 0.09.

TABLE 4. Parasiticidal and schizonticidal activities of methanolic crude and HPLC purified compounds from in vitro cultures of *S. paniculata*.

Plant extracts/ compound	Schizonticidal activity IC_{50} (μg/ml)	Parasiticidal activity IC_{50} (μg/ml)
UDA	14.64 ± 0.35	16.42 ± 0.39
Spilanthol	23.22 ± 0.59	17.72 ± 0.16

UDA, respectively. Similarly, within the same day, the RSD values figured out to be 1.74% and 3.61% for spilanthol and UDA, respectively.

Quantification of spilanthol and UDA The amount of spilanthol and UDA in flower-head and callus extracts was quantified from the generated calibration curve. The in vitro callus accumulated distinctly higher spilanthol content as 2.23 ± 0.04 mg/g DW compare to that from the field grown flower-head samples, which contained only 0.83 ± 0.12 mg/g DW (Table 3). The quantity of UDA in in vitro callus cultures was observed as 4.30 ± 0.22 mg/g DW, which was marginally lesser than that in flower-heads as 5.29 ± 0.05. The biosynthesis of

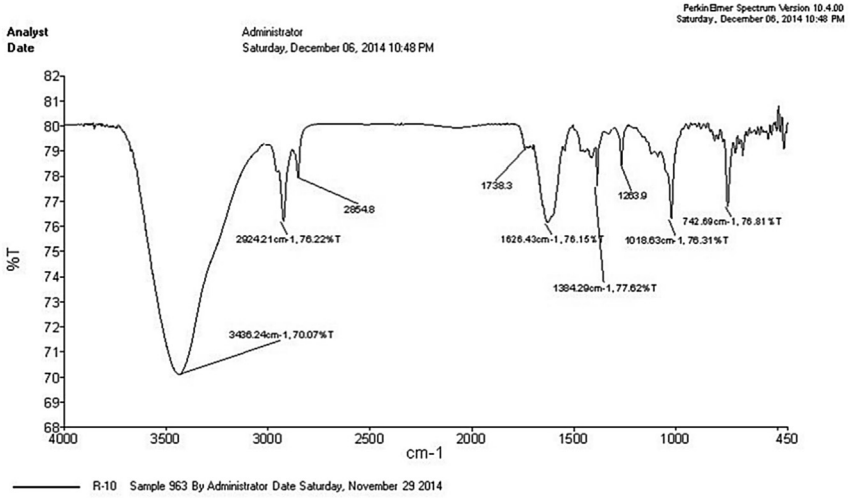

FIG. 7. FT-IR spectra of purified (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) eluted at retention time of 10.34 ± 0.10.

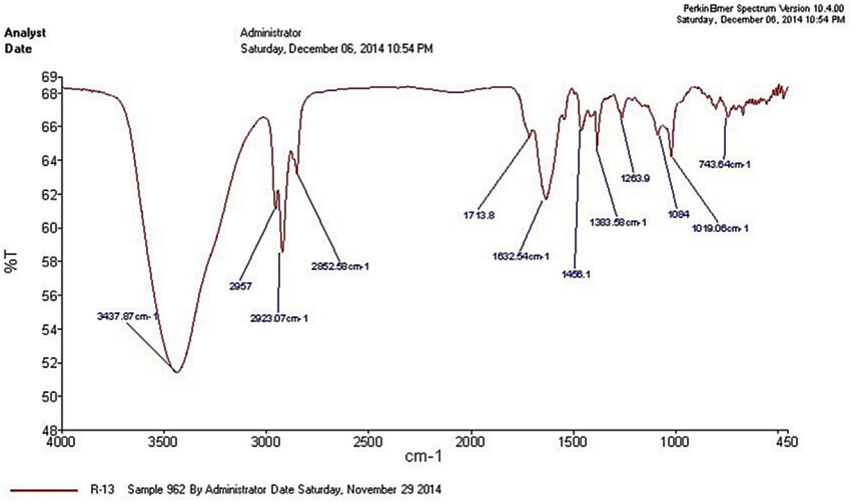

FIG. 8. FT-IR spectra of purified (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) eluted at retention time of 13.45 ± 0.09.

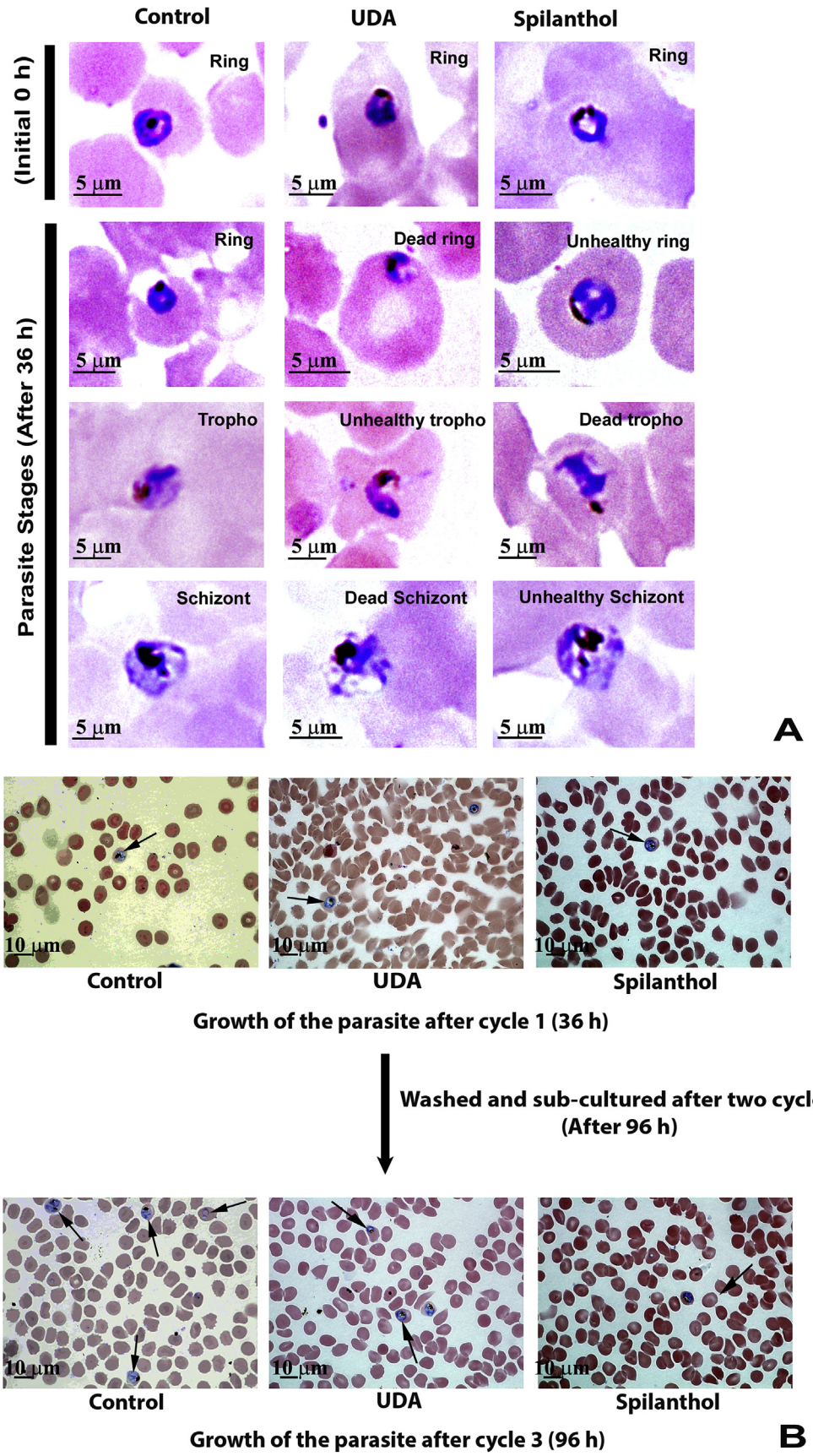

FIG. 9. Microscopic evidence of erythrocytic antimalarial activity of *Spilanthes paniculata*. (A) Stages of parasites in RBC of untreated (control) and treated samples after 36 h. (B) Growth of the parasites after cycle 1 and 3.

plant metabolites in in vitro callus cultures may not give the characteristics yield of intact plants (18,21,26–30). The cells in culture grow under heterotrophic conditions and the nutrient medium in general affect the biosynthetic potential of in vitro grown cells.

As indicated in the introduction section that there are a few reports on *Spilanthes* indicating the pharmacological activities of crude extracts prepared from field frown samples or in vitro tissue but none of them have quantified the bioactive compounds. A few others indicated spilanthol as the principle compound responsible for most of the bioactivity of *Spilanthes* (10). The present study provides an excellent reproducible protocol for simultaneous determination, purification and quantification of the two prominent biologically active *N*-alkylamides, spilanthol and UDA with noticeably high accumulation as 2.23 ± 0.04 mg/g DW and 4.30 ± 0.22 mg/g DW, respectively, in the selected in vitro cell lines of *S. paniculata*.

Analysis of alkylamides by mass spectrometry The two distinct peaks eluted in HPLC chromatogram at retention time of 10.34 ± 0.10 min (peak 1) (Fig. 4A) and 13.45 ± 0.09 min (peak 2) (Fig. 4B) were collected separately in two vials to identify the compounds based on characteristic collision-induced dissociation (CID) fragmentation patterns by mass spectrometry in positive mode due to its reproducibility. Peak 1 was identified by MS–MS spectra as UDA with its fragmented molecular ion spectra at 230 *m*/*z* (Fig. 5). In earlier reports, this compound was identified from ethanolic extracts of wild grown entire plants and flower-heads of *Spilanthes acmella* with loss of entire alkyl group [-56 *m*/*z*] attached directly to the amine by the dissociation of C–N bond of an isobutylamide and produced the product ions of 174 *m*/*z* (31–33).

The [MH^+–222] in purified peak-2 was a fragmented spilanthol with two stable fragment ions [MH^+–149] and [MH^+–166] where, [MH^+–149] fragment was formed by the dissociation C–N bond from the isobutylamide to lose the entire amine functional group and [MH^+–166] was formed by the dissociation of C–N bond from the isobutylamide to lose the alkyl group directly attached with the amine group [MH^+- 73] [MH^+–56] (Fig. 6). This is consistent with the previously published reports (11,34).

FT-IR spectroscopy analysis of isolated UDA and spilanthol The two pure compounds, UDA (peak 1) and spilanthol (peak 2) eluted by HPLC (Fig. 4A and B) were subjected to IR spectra to confirm the presence of functional groups. Peak 1 compound, UDA eluted at RT 10.34 ± 0.10 provided IR ν_{max} film (cm^{-1}): 3436, 2924, 2845, 1626, 1263, 1018, 742 (Fig. 7). The conjugation of double bonds to an amide bond was contributed by (1738 cm^{-1}) whereas, diene group (*E*,*Z* or *Z*,*E*) conjugation was afforded by (1018 cm^{-1} and 742 cm^{-1}). Similarly, peak 2 compound, spilanthol eluted at RT 13.45 ± 0.09 provided IR ν_{max} film (cm^{-1}): 3437, 2923, 2852, 1632, 1383, 1019, 743 (Fig. 8). The conjugation of double bond to an amide bond was contributed by (1713 cm^{-1}) whereas, diene group (*E*,*Z* or *Z*,*E*) conjugation was afforded by (1019 cm^{-1} and 743 cm^{-1}). The interesting findings of the two phytochemical constituents clearly indicated that the compound consists of carbonyl group and long alkyl chain conjugated with amide and devoid of any aromatic functional groups.

Antimalarial activity of purified bioactive compounds from *S. paniculata* The crude extract, TLC semi-purified extract and HPLC purified UDA and spilanthol extracts were tested for erythrocyte antimalarial activity, particularly schizonticidal and parasiticidal. The crude extracts and semi-purified extracts, obtained from callus cultures and flower-head explants, showed noticeable biological activity against the parasite (data not shown). Therefore, the semi-purified extracts were further processed to isolate the pure compounds of spilanthol and UDA to treat the malaria parasite. The HPLC purified compounds, UDA and spilanthol exhibited schizonticidal activity with an IC_{50} value of 14.64 ± 0.35 μg/ml and 23.22 ± 0.59 μg/ml, respectively (Table 4). Both, UDA and spilanthol, also demonstrated retardation of parasite growth with prominent parasiticidal activity with IC_{50} value of 16.42 ± 0.39 μg/ml and 17.72 ± 0.16 μg/ml, respectively. The compound treated parasite culture displayed dead or sick parasites with dead rings (Fig. 9A) In comparison, control culture treated with solvent is giving healthy parasite with all stages (Fig. 9B, marked with black arrow). Overall, UDA exhibit higher antimalarial activity as compared to spilanthol. In another experiment, the parasite was treated with a mixture containing 3:1, 1:1 or 1:3 proportions of UDA and spilanthol to explore synergistic interactions of these active compounds for antimalarial activity (Table 5). A very strong synergistic effect of UDA and spilanthol was observed. However, UDA alone showed distinctly superior schizonticial and parasiticidal activities against *Pf* 3D7 strains.

TABLE 5. Combinatorial effect of purified active compounds from *S. paniculata* callus cultures towards antimalarial activity.

Compound mixture	Schizonticidal activity IC_{50} (μg/ml)	Minimum killing concentration IC_{50} (μg/ml)
UDA:spilanthol = 3:1 ratio	17.01 ± 0.61	17.8 ± 0.42
UDA:spilanthol = 1:1 ratio	18.35 ± 0.59	19.55 ± 0.3
UDA:spilanthol = 1:3 ratio	21.58 ± 0.64	21.63 ± 0.79

In the current study, this simplified complementary method provides an excellent procedure for isolation and quantification of *N*-alkylamides, spilanthol and UDA. Both these compounds were simultaneously present in the same callus cultures and demonstrated the prominent parasiticidal activity against malaria parasite. This identifies the merit of cell cultures as a constant source of medicinally potential compounds at higher amount all through the year, irrespective of seasonal variations.

References

1. **Robinson, H., Powell, A. M., King, R. M., and Weedin, J. F.:** Chromosome numbers in Compositae: XII Heliantheae, vol. 52, p. 28. Smithsonian Institution Press, Washington, DC (1981).
2. **Nakatani, N. and Nagashima, M.:** Pungent alkamides from *Spilanthes acmella* L. var. oleracea Clarke, Biosci. Biotechnol. Biochem., **56**, 759–762 (1992).
3. **Paulraj, J., Govindarajan, R., and Palpu, P.:** The genus Spilanthes ethnopharmacology, phytochemistry, and pharmacological properties: a review, Adv. Pharmacol. Sci., **2013**, 1–22 (2013).
4. **Ramsewak, R. S., Erickson, A. J., and Nai, M. G.:** Bioactive *N*-isobutylamides from the flower buds of *Spilanthes acmella*, Phytochemistry, **51**, 729–732 (1999).
5. **Pandey, V., Agrawal, V., Raghavendra, K., and Dash, A. P.:** Strong larvicidal activity of three species of *Spilanthes* (Akarakara) against malaria (*Anopheles stephensi Liston*, *Anopheles culicifacies*, species C) and filarial vector (*Culex quinquefaciatus* Say), Parasitol. Res., **102**, 171–174 (2007).
6. **Gerber, E.:** About the chemical constituents of paracress (*Spilanthes oleracea*, Jacquin), Arch. Pharm., **241**, 270–289 (1903).
7. **Ghokale, V. G. and Bhide, B. V.:** Chemical investigation of *Spilanthes acmella* (Murr.), J. Chem. Soc., **1**, 250–252 (1945).
8. **Nagashima, M. and Nakatani, N.:** LC-MS analysis and structure determination of pungent alkamides from *Spilanthes acmella* L. flowers, Lebensm. Wiss. Technol., **25**, 417–421 (1992).
9. **Nagashima, M. and Nakatani, N.:** A new alkamide from *Spilanthes acmella* L. Chem. Express, **7**, 153–156 (1992).
10. **Pandey, V. and Agrawal, V.:** Efficient micropropagation protocol of *Spilanthes acmella* L. possessing strong antimalarial activity, In Vitro Cell. Dev. Biol. Plant, **45**, 491–499 (2009).
11. **Bae, S. S., Ehrmann, B. M., Ettefagh, K. A., and Cech, N. S.:** A validated liquid chromatography-electrospray ionization-mass spectrometry method for quantification of spilanthol in *Spilanthes acmella* (L.) Murr. Phytochem. Anal., **21**, 438–443 (2010).
12. **Murashige, T. and Skoog, F.:** A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant., **15**, 473–497 (1962).

13. **Singh, M. and Chaturvedi, R.:** Evaluation of nutrient uptake and physical parameters on cell biomass growth and production of spilanthol in suspension cultures of *Spilanthes acmella* Murr. Bioprocess Biosyst. Eng., **35**, 943–951 (2012).
14. **Trivedi, V., Chand, P., Srivastava, K., Puri, S. K., Maulik, P. R., and Bandyopadhyay, U.:** Clotrimazole inhibits hemoperoxidase of *Plasmodium falciparum* and induces oxidative stress proposed antimalarial mechanism of clotrimazole, J. Biol. Chem., **280**, 41129–41136 (2005).
15. **Parveen, A., Chakraborty, A., Konreddy, A. K., Chakravarty, H., Sharon, A., Trivedi, V., and Bal, C.:** Skeletal hybridization and PfRIO-2 kinase modeling for synthesis of α-pyrone analogs as anti-malarial agent, Eur. J. Med. Chem., **70**, 607–612 (2013).
16. **Balaji, S. N., Ahsan, M. J., Jadav, S. S., and Trivedi, V.:** Molecular modelling, synthesis, and antimalarial potentials of curcumin analogues containing heterocyclic ring, Arab. J. Chem., http://dx.doi.org/10.1016/j.arabjc.2015.04.011 (2015).
17. **Srivastava, P., Kasoju, N., Bora, U., and Chaturvedi, R.:** Dedifferentiation of leaf explants and cytotoxic activity of an aqueous extract of cell cultures of *Lantana camara* L, Plant Cell Tissue Org. Cult., **99**, 1–7 (2009).
18. **Srivastava, P., Kasoju, N., Bora, U., and Chaturvedi, R.:** Simultaneous determination and quantification of three pentacyclic triterpenoids—betulinic acid, oleanolic acid, and ursolic acid—in cell cultures of *Lantana camara* L. Biotechnol. Bioprocess Eng., **15**, 1038–1046 (2010).
19. **Srivastava, P. and Chaturvedi, R.:** Accumulation of betulinic, oleanolic, and ursolic acids in in vitro cell cultures of *Lantana camara* L. and their significant cytotoxic effects on HeLa cell lines, In Vitro Cell. Dev. Biol. Plant, **46**, 549–557 (2010).
20. **Alfermann, A. W. and Petersen, M.:** Natural product formation by plant cell biotechnology, Plant Cell Tissue Org. Cult., **43**, 199–205 (1995).
21. **Yasuda, I., Takeya, K., and Itokawa, H.:** The geometric structure of spilanthol, Chem. Pharm. Bull., **28**, 2251–2253 (1980).
22. **Borges-Del-Castillo, J., Vazquez-Bueno, P., Secundino-Lucas, M., Martinez-Martir, A. I., and Joseph-Nathan, P.:** The *N*-2-phemylethyl cinnamanide from *Spilanthes ocymifolia*, Phytochemistry, **23**, 2671–2672 (1984).
23. **Jondiko, I. J. O.:** A mosquito larvicide in *Spilanthes mauritiana*, Phytochemistry, **25**, 2289–2290 (1986).
24. **Molinatorres, J., Salgado-Garciglia, R., Ramirez-Chavez, E., and Delrio, R. E.:** Purely olefinic alkamides in *Heliopsis longipes* and *Acmella* (*Spilanthes*) *oppositifolia*, Biochem. Syst. Ecol., **24**, 43–47 (1996).
25. **Stashenko, E. E., Puertas, M. A., and Combariza, M. Y.:** Volatile secondary metabolites from *Spilanthes americana* obtained by simultaneous steam distillation-solvent extraction and supercritical fluid extraction, J. Chromatogr. A, **752**, 223–232 (1996).
26. **Rao, S. R. and Ravishankar, G. A.:** Plant cell cultures: chemical factories of secondary metabolites, Biotechnol. Adv., **20**, 101–153 (2002).
27. **Butenko, R. G.:** Experimental morphogenesis and differentiation in plant cell culture, 35th Timiryazev Lecture, pp. 45–48. Nauka, Moscow (1975).
28. **Nosov, A. M.:** Functions of plant secondary metabolites in-vivo and in-vitro, Russ. J. Plant Physiol., **41**, 767–771 (1994).
29. **Dörnenburg, H. and Knorr, D.:** Strategies for the improvement of secondary metabolite production in plant cell cultures, Enzyme Microb. Technol., **17**, 674–684 (1995).
30. **Kol'chugina, I. B. and Markarova, E. N.:** Photoheterotrophic callus culture *Ficus elastica*. The formation of polyisoprene synthesis, Mosc. Univ. Biol. Sci. Bull., **64**, 28–31 (2009).
31. **Boonen, J., Baert, B., Roche, N., Burvenich, C., and De Spiegeleer, B.:** Transdermal behavior of the *N*-alkylamide spilanthol (affinin) from *Spilanthes acmella* (Compositae) extracts, J. Ethanopharmacol., **127**, 77–84 (2010).
32. **Cech, N. B., Tutor, K., Doty, B. A., Spelman, K., Sasagawa, M., Raner, G. M., and Wenner, C. A.:** Liver enzyme-mediated oxidation of *Echinacea purpurea* alkylamides: production of novel metabolites and changes in immunomodulatory activity, Planta Med., **72**, 1372–1377 (2006).
33. **Sharma, V., Boonen, J., Chauhan, N. S., Thakur, M., De Spiegeleer, B., and Dixit, V. K.:** *Spilanthes acmella* ethanolic flower extract: LC–MS alkylamide profiling and its effects on sexual behavior in male rats, Phytomedicine, **18**, 1161–1169 (2011).
34. **Boonen, J., Baert, B., Burvenich, C., Blondeel, P., De Saeger, S., and De Spiegeleer, B.:** LC–MS profiling of *N*-alkylamides in *Spilanthes acmella* extract and the transmucosal behaviour of its main bio-active spilanthol, J. Pharm. Biomed. Anal., **53**, 243–249 (2010).