MEDIA OPTIMIZATION, BATCH KINETICS AND PRODUCTION OF BIOACTIVE ALKYLAMIDES IN *IN VITRO* CELL LINES OF *SPILANTHES PANICULATA* WALL. EX. DC

THESIS SUBMITTED TO IIT GUWAHATI FOR THE PARTIAL FULFILLMENT OF DEGREE

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JUNE, 2016

Dedicated to My Parents

and

Teachers

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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STATEMENT

I, hereby, declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Professor Rakhi Chaturvedi.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: 6th June, 2016 **Radhika R.**

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Media optimization, batch kinetics and production of bioactive alkylamides in *in vitro* cell lines of *Spilanthes paniculata Wall. EX. DC.*"** by Radhika, R. for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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Abstract

The higher plants are widely distributed all over the world has enormous varieties of secondary metabolites was hunted by living being particularly for food and pharmaceutical needs, to utilize unconditionally for health benefits. This implies that phytochemicals play a significant role in the entire biological entity. In a worldwide, despite synthetic drugs, population is approaching towards natural remedies, due to its no side effect efficacy. Recently, most of the prescribed drugs were industrialized using several plant materials. Owing to the strength of bioactive metabolites in different fields, investigations on improving the production of same phytochemicals are increasing in recent decades through various techniques and technologies. Mostly, synthesis of bioactive metabolites is highly unique to the plant kingdom, rather than any other micro-organisms and animals. In the next decennia, the investigations on secondary metabolites from different plant species are increasing due to the renewed interest in the use of medicinal plants and its products. These products serves as an alternative to synthetically produced pharmaceuticals for the prevention and treatment of ailments and diseases. Therefore, to overcome annihilation of the entire plant family, there is an absolute necessity to approach a trustful biotechnological tool known as plant tissue culture technology is an alternative methodology especially to retrieve consistent and continuous production of secondary plant product rather sacrificing whole plant system. Moreover, change in the chemical profile occurs due to various climatical factors and environmental fluxes could be avoided through cell culture technology.

Spilanthes paniculata is a flowering herbaceous genus belonging to the family Asteraceae, a highly valuable plant species. Apart from the genus *Spilanthes*, other plant species falls in the family Asteraceae has taken the credit of ethanopharmacological importance in the field of biomedicine owing to its medicinal properties. This perennial medicinal plant *S. paniculata* is highly recorded for its folklore remedies. These attributes are due to the presence of organoleptic *N*-alkylamides are polyunsaturated fatty acids strewn in the entire biological kingdom considered to be the most superior novel drug as it possesses vast medicinal properties. But, majority of research works are carried out from the plant growing in the field in terms of phytochemical analysis and evaluation of its biological activity. Besides, only few works are reported scientifically through plant cell cultures on identification of alkylamides. Hence, the development of *in vitro* cultures plays an important role in the conservation of vital plant material *S. paniculata* by optimizing appropriate

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culture conditions for the constant production of alkylamide, spilanthol. The present thesis work examines the production of secondary metabolites and improvement of bioactive chemicals for its quality and quantity through a plant biotechnological tool, plant cell and organ culture. The phytochemical investigation on isolation was preceded through *in vitro* cell cultures and further, improvement of spilanthol production from elite cell lines was determined through multivariate statistical tool. Extensively, the identified alkylamides paved a new path to develop a potential anti-plasmodial drug against malarial parasites.

The current thesis is partitioned into five chapters, where,

Chapter 1: Reveals the introduction and review of literature reported till date with respect to plant tissue cultures, analysis of bioactive phytochemicals from field grown plants as well as *invitro* cell lines and also regarding the studies on biological assays.

Chapter 2: Describes about materials procured from renowned companies and all the methodologies adopted for the present work.

Chapter 3: The results obtained by implementing various protocols are investigated and presented. The tables and graphs are included within the text whereas relevant figures are compiled in the form of plates positioned at the end of thesis.

Chapter 4: Discussed regarding the insight of results described in the previous chapter 3 by referring and comparing with previous reported works.

Chapter 5: Finally, the entire conclusion was withdrawn from the detailed work studied in this thesis work and its future prospects. Further, it was preceded by list of bibliography and appendix comprised of taxonomical classifications of the plant *Spilanthes paniculata*. Lastly, thesis was also enclosed in terms of research output with lists of conference proceedings.

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List of abbreviations

ACN	-	Acetonitrile
2,4-D	-	2,4-Dichlorophenoxyacetic acid
BAP	-	N^6- benzylaminopurine
CCD	-	Central Composite Design
DCW		Dry cell weight
ESI	-	Electron spray ionization
FDA	-	Fluorescein diacetate
FW	-	Fresh weight
GABA	-	Gamma-aminobutyric acid
Hg_2Cl_2	-	Mercuric chloride
HPLC	-	High performance liquid chromatography
IL	-	Interleukin
iNOs	-	Inducible NO synthase
MeOH	-	Methanol
MS	-	Murashige and Skoog's medium
MS	-	Mass Spectrometry
NAA	-	α-naphthaleneacetic acid
NO	-	Nitric oxide
NSAID's	-	Non-steroidal anti-inflammatory drugs
PB	-	Plackett-Burman
PTLC	-	Preparative thin layer chromatography
RSM	-	Response Surface Methodology
Rt	-	Retention Time
SDW	-	Sterile Distilled Water
tTCL	-	Transverse Thin Cell Layer
TNF-α	-	Tumor Necrosis Factor-α
UV	-	Ultraviolet

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List of units

amu	-	atomic mass unit
cm	-	centimeter
DF	-	degree of freedom
ºC	-	degree of Celsius
g	-	gram
h	-	hour
LSD	-	least significant difference
nm	-	nanometer
MS	-	mean squares
µl	-	microliter
µm	-	micrometer
min	-	minute
mM	-	millimolar
rpm	-	revolution per minute
%RSD	-	percent relative standard deviation
SD	-	standard deviation
SE	-	standard error
SS	-	sum of squares
µ	-	specific growth rate

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Chapter 1

Introduction and Literature Review

1.1. General

Spilanthes paniculata Wall. EX. DC. is a flowering annual herbaceous plant that belongs to the family Asteraceae, widely distributed in tropical and subtropical regions. It is commonly known as Pellitary in English and Akarkara in Hindi. Generally, *Spilanthes* species are grown at an altitude of 1800 m and in moist, damp soils with high humidity (Jayaweera*,* 1981).

1. *Spilanthes paniculata*, a hairy herbal plant, grows around 40-60 cm height with numerous erect stem and with tapering roots along the nodes. The axillary buds are arranged on opposite, triangular or lanceolate leaves with wavy margins. *S. paniculata* bears solitary inflorescence with yellow, cone-shaped or ovoid flowers with long pedicel (Tiwari *et al*. 2011). Even though 60 species are speckled all over the world, in India, there are five different species, such as *S. paniculata* Wall. EX. DC, *S. calva* L. and *S. mauritiana* (A.Rich. ex Pers.) DC.*,* *S. acmella* (L.) Murr. and *S. acmella* L. *var oleraceae* Clarke are available (Jansen, 1981; Anonymous 1989).

Since 1920, *Spilanthes* species are highly valuable raw material, well documented for its folklore remedies and as flavors in food. Usually, *Spilanthes* spp. are preferred for culinary purposes, as leafy vegetables, spices, flavoring agents in salads, stews and soups (Christophe, 2006). Moreover, all over the world the extracts and decoctions of *Spilanthes* spp. are used to treat diseases, such as cough, flu, rabies, stomatitis, stammering tuberculosis and snake bite by administering topically or orally (Santesson, 1996; Nakatani and Nagashima 1992; Ramsewak *et al*. 1999). Due to the prominence of highly infectious disease, the extraction of alkylamides from *Spilanthes* spp. was recorded for potent anti-plasmodial activity (Mbeunkui *et al*. 2011; Spelman *et al*. 2011). It also attribute larvicidal and bioinsecticidal activity (Ramsewak *et al*. 1999; Saraf and Dixit 2002; Pandey *et al*. 2007). Recently, it was also reported to have anthelminthic activity (Singh *et al*. 2013). Apart from medicinal properties, these phytochemical metabolites are key ingredients in as they decrease facial wrinkles by defending from oxidative stress (Rios 2012; Molina-Torres *et al.*1996; Artaria *et al*. 2011; Demarne and Passaro 2005; Yotsawimonwat *et al*. 2010). All these properties are due to the

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presence of bioactive *N*-alkylamides and other phytochemical constituents, such as coumarin, phenolics and terpenoids (Prachayasittikul *et al*. 2009), present in different species of the genus *Spilanthes.*

In general, the genus *Spilanthes* grows throughout the year and conventionally it was propagated through seeds, which are grey or black in colour. However, the seeds lose their viability in a short span of time. Moreover, the nature of androecium in *Spilanthes* species is strictly protandry, which generates heterozygosity by preventing self-pollination. Furthermore, inflorescence colour of the plant attracts large number of insects and facilitates entomophily. Besides, such natural progressions may not be suitable for the continuous production of secondary metabolites due to genetic variation (Singh and Chaturvedi, 2010). Additionally, it may also lead to the change in chemical profile and can directly affect the demand of the pharmaceutical industry. The majority of the people in the developing countries rely mostly in the plant-based therapy molecules for their health benefits.

1.2. Studies on *in vitro* cell and tissue cultures

Plant cell culture technology has opened up an alternate route for the production of *in vitro* cultures with respect to the conservation of plant biodiversity. This technology acts as a bridge between resource and the final products without any deterioration of materials grown naturally in the field. To prevent over exploitation and extinction of this species Saritha *et al.* (2002) developed a protocol of shoot proliferation from hypocotyl explants obtained from 1-week-old seedlings on MS medium augmented with BAP (2.2 µM) and NAA (0.54 µM). Further proliferation was achieved by repeatedly sub-culturing the shoot on multiplication medium. About 95% of the *in vitro* shoots developed roots after transfer to half strength MS medium supplemented with IBA (4.9 µM) (Saritha et al. 2002). Subsequently, Haw and Keng (2003) obtained multiple shoots from axillary buds on MS medium supplemented with BAP (8.8 µM). The shoots developed into complete plantlets on a lower concentration of BAP at 2.2 µM. However, higher dose of BAP resulted in vitrification of *in vitro* plantlets (Leng *et al*. 2004). Later, Saritha and Naidu (2008) showed direct shoot regeneration on MS medium fortified with BAP (13.2 µM) and IAA (5.7 µM) using *in vitro* grown mature leaves. Later, indirect shoot regeneration through green and compact callus was obtained using sliced nodal segments to get transverse thin cell layer (tTCL). Singh *et al*. (2009b) were able to regenerate *S. acmella* with high efficiency. *In vitro* developed shoots from shoot-tip explants were encapsulated in calcium alginate beads on hormones free full strength MS medium. This

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encapsulated alginate beads were stored at 4º C up to 60 days to support germplasm storage, distribution and a short term conservation of this species (Singh *et al*. 2009a). A high efficiency regeneration of plants from nodal segments each with two axillary buds produced 20.3-fold recurrent multiple of shoots. An increase in concentration beyond 5 µM of BAP resulted in stunted growth of shoots (Singh and Chaturvedi, 2010). The half strength MS medium with an auxin, IBA at 4.9 µM gave maximum frequency of root formation. Yadav and Singh (2010) observed development of greenish, yellow and fragile callus, in addition to micropropagation of shoots, on MS medium with BAP (8.8 µM) and 2,4-D (4.5 µM). Shoot-tips from aseptic seedlings on MS medium with TDZ (0.25 µM) responded well with a mean of 30 shoots per explant on semi-solid medium. However, the response was much greater in liquid medium (Sharma and Shahzad, 2013). Stability of ploidy status among in vitro regenerated plants of *S. acmella* was assessed by using flow cytometry. The results revealed true-to-type regenerants (Singh and Chaturvedi, 2012). An overview of plant tissue culture studies on *Spilanthes* species has been described in **(Table 1)**.

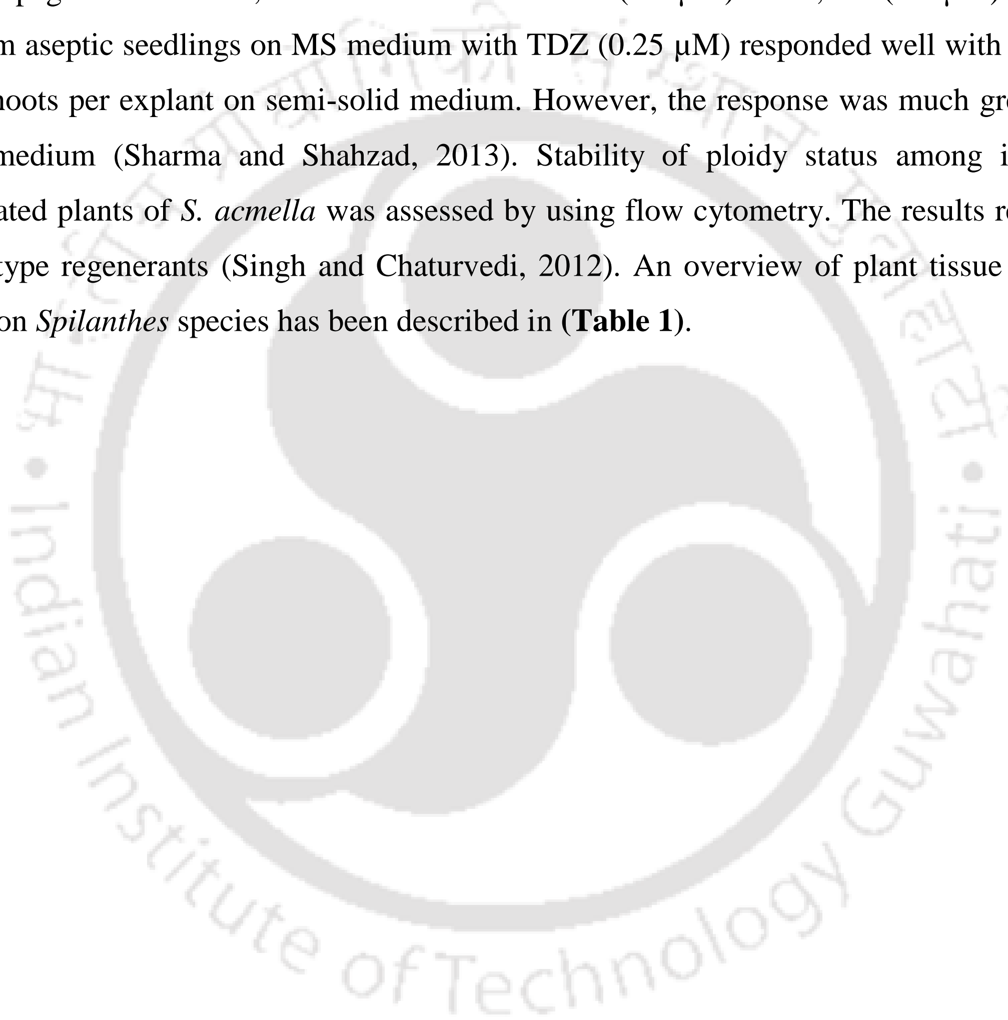

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Table 1: An overview of plant tissue culture studies on *Spilanthes* species

S.no	Explant	Media supplemented with PGRs			References
		Direct/Indirect *via* callus	Shoot proliferation	Rooting	
1.	Hypocotyls	Direct	MS + BAP (2.2 µM) + NAA (0.54 µM)	½ MS + IBA (4.9 µM)	Saritha *et al*. 2002
2.	Axillary buds	Direct	MS + BAP (8.8 µM)	MS + BAP (2.2 µM)	Haw and Keng, 2003
3.	Axillary buds	Direct	MS + BAP (2.2 µM)	MS + BAP (2.2 µM)	Leng *et al*. 2004
4.	*In vitro* leaves	Direct	MS + BAP (13.3 µM) + IAA (5.7 µM)	½ MS + IBA (4.9 µM)	Saritha and Naidu, 2008
5.	*In vitro* leaves	MS + BAP (10 µM) + NAA (1 µM)	MS + BAP (10 µM) + NAA (1 µM)	½ MS + IBA (0.1 µM)	Pandey and Agrawal, 2009
6.	Shoot tips	MS full strength liquid medium without growth harmones			Singh *et al*. 2009a
7.	Nodal segment	Direct	MS + BAP (22.2 µM)	MS basal medium	Singh *et al*. 2009b
8.	Nodal segment	Direct	MS + BAP (5 µM)	½ MS + Sucrose (5 %)	Singh and Chaturvedi, 2010
9.	Nodal segment	MS + BAP (8.8 µM) + 2,4-D (4.5 µM)	MS+BAP(13.3 µM) + IAA (5.7 µM)	½ MS + IBA (4.9 µM)	Yadav and Singh, 2010
10.	Field grown leaves	MS+BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM)	MS + BAP (5 µM)	½ MS + Sucrose (5 %)	Singh and Chaturvedi, 2012a
11.	Shoot tips	Direct	MS + TDZ (0.25 µM)	MS + NAA (2.5 µM)	Sharma and Shahzad, 2013
12.	Field grown leaves	MS + BAP (2.2 µM) + NAA (26.8 µM); 2,4,D (22.6 µM)	MS + BAP (8.8 µM) + zeatin (4.5 µM)	½ MS + NAA (5.4 µM)	Niratker *et al*. 2014

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Most of the tissue culture studies are reported only on *S. acmella*. However, a few other *Spilanthes* species, such as *S. paniculata, S. calva, S. mauritiana* and *S. olereacea* have higher medicinal properties. This has greatly driven the need for tissue culture protocols for the establishment of *in vitro* raised plantlets, which could serve as raw material for phytochemical production. It will also help in the conservation of wild populations (Tiwari *et al*. 2010). Thus, the established *in vitro* raised plant materials can serve as source for analysis of secondary metabolites using analytical methods for future consumption.

1.3. Studies on secondary metabolite production and its analysis

Secondary metabolites are organic compounds synthesized naturally by central metabolism of primary metabolites. Secondary metabolite is defined as an organic chemical diversified in the entire biological entity (Kossel, 1891). Generally, plant moieties are often considered to be low in occurrence when compared to major molecules. However, it plays a major role in adaptation of plant system to environment by interacting with ecosystem for plants' fitness. Moreover, varieties of secondary metabolites produced by higher plants are recurrently consumed for food, health benefits and pharmaceutical needs (Harborne 2001). Globally, despite synthetic drugs, growing population is approaching towards natural remedies, due to its no minimal side effects. Moreover, most of the prescribed drugs are industrialized using various plant materials (Payne *et al*. 1991; Pezzuto 1995). Additionally, biosynthesis of bioactive metabolites are highly unique to the plant kingdom. It is also fourfold greater than micro-organisms and animals. Although plants synthesize enormous beneficial chemicals, analyses and characterization of compounds are still evolving (Cox and Balick, 1994). This can also help in identification and classification of plant species to perform controlled experimentation. Owing to the strength of bioactive metabolites in different fields, investigations on improved production of metabolites are increasing in recent decades through various technologies (Kim *et al*. 2005). Thus, there is an urgent need for the adoption of an attractive alternative technology for the constant recovery of secondary metabolites.

Plant cell, tissue and organ culture extensively emerged as an alternative biotechnological approach especially for improving the production of secondary plant compounds using dedifferentiated cell cultures. Zenk (1975) reported the production of anthroquinones from dedifferentiated cells of *Morinda citrifolia.* Cell culture technology is a highly promising tool, where the cells have a higher rate of metabolism rather than differentiated plants. This can offer valuable chemicals throughout the year compared to traditional plant extraction

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techniques. Additionally, faster proliferation of cells and a condensed biosynthetic cycle can be observed. The formation of secondary metabolites can take place within a short span of life cycle of about (2-4 weeks) (Dörnenburg and Knorr, 1995). Further, consistent and economically viable plant products such as ginsenosides, rosmarinic acid, shikonin, diosgenin and ubiquinone are accumulated in various plant cell cultures when compared to that of intact plant system growing wild (Dicosmo and Misawa, 1995).

1.3.1. Analysis of phytochemicals

The genus *Spilanthes* has different organic chemicals, such as flavonoids, glycosides, alkaloids, tannins, saponins, sesquiterpenes, anthraquinones, terpenoids, coumarins and oxygenated compounds. Nevertheless, predominant phytochemicals are estimated to be fatty acid alkylamides. In general, *N*-alkylamides (NAAs) are organoleptic compounds, which are classified to be one of the most promising groups of secondary metabolites. NAAs are present in more than twenty five plant families with high ethanopharmocological prominence. Plant derived *N*-alkylamides are comprised of condensed saturated or polyunsaturated fatty acid and an amine. Among diversified plant species, *Spilanthes* species are widely used in traditional medicine due to its biological activities. (Boonen *et al*. 2012). However, to ensure the safety and efficacy of functional food and dietary supplements phytochemical analyses are very important. This can firmly establish the vital relationship between the pharmacology and chemistry of plants.

In 1903, the most prominent and active alkylamide, spilanthol was obtained from flower head crude extracts of *S. oleracea*. Later, it was isolated from flower head ethanolic extracts of *S. acmella* (Gerber, 1903; Ghokale and Bhide, 1945). Later, aerial parts of *S. oleracea* were extracted using methanol followed by fractionation with *n*-hexane. Further, sample was subjected through silica gel column to elucidate the geometric structure of pungent taste alkylamide (2*E*,6*Z*,8*E*) *N*-isobutyl-2,6,8 decatrienamide through proton (H^1) and carbon-13 (^{13}C) nuclear magnetic resonance spectra (Yasuga *et al*. 1980). In the same way, mosquito larvicidal compound *N*-isobutyl-2*E*,4*E*,8*E*,10*Z*-dodeca-2,4,8,10-tetraenamide was isolated from aerial parts of *S. mauritiana* (Jondiko, 1986). Similarly, Borges-Del-Castillo *et al*. (1984) reported the presence of simple aromatic amide *N*-2-phenylethylcinnamamide and other three compounds such as stigmasterol, taraxasteryl and lupeyl acetates from leaves of *S. ocymifollia* species. Further, hexane, chloroform, ethylacetate, and methanolic extracts obtained from aerial parts of *S. acmella* afforded other bioactive chemicals, such as vanillic

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acid, trans-ferulic acid, trans-isoferulic acid, scopoletin and triterpenoids, such as *β*-sitostenone, stigmasterol and stigmasteryl-3-*O*-β-D-glucopyranosides, 3-acetylaleuritolic acid, as well as stigmasteryl-and *β*-sitosteryl-3-*O*-β-D-glucopyranosides through gradient elution column chromatography (Prachayasittikul *et al*. 2009).

From field grown flower heads of *S. acmella*, four different alkylamides such as spilanthol, 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, 2*E*,7*Z*-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, and 7*Z*-*N*-isobutyl-7-tridecene-10,12-diynamide were extracted. The compounds were identified and confirmed by thin layer chromatography, high performance liquid chromatography, mass spectroscopy and infra- red spectroscopy (Nakatani and Nagashima, 1992). Similarly, the flower heads of *S. acmella* from hexane extracts are fractionated using medium pressure liquid chromatography to yield spilanthol along with another compound undeca-2*E*,7Z,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide (Ramsewak *et al*. 1999). Likewise, *N*-isobutyl alkylamide from *S. alba* species are also reported (Bohlman *et al*. 1980).

A series of promising alkylamide was isolated and purified from flower head ethanolic extracts of *S. acmella* and analyzed through HPLC/ESI-MS using an embedded column. The gradient mobile phase experiment This provided (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide with m/z 230, (2*Z*)-*N*-phenethyl-2-nonene-6,8-diynamide with m/z 252, (2*E*,6*Z*,8*E*)-N-isobutyl-2,6,8-decatrienamide m/z 222, (2*E*,7*Z*)-N-isobutyl-2,7-tridecadiene-10,12-diynamide with m/z 258, (2E,7Z)-N-isobutyl-2,7-decadienamide m/z 224, (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide m/z 236, (2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide with m/z 248, where mass spectra of amides was examined by ESI using a capillary voltage of 3 kV (Sharma *et al*. 2011). Spilanthol content from flower heads and stems was effectively extracted through solvent fluid extraction (SFE) and evaluated the total amount of compounds using GC-MS, respectively. Further, lower level production of few other nitrogenated organic chemicals are also identified as *N*-(isobutyl)-6Z,8E-decadienamide, *N*-(2-methylbutyl)-2E,6Z,8E-decatrienamide and *N*-(2-phenylethyl)-2E,6Z,8E-decatrienamide in *S. acmella* (Dias *et al*. 2012).

Steam distillation-solvent and supercritical fluid extraction methods are also used to produce sesquiterpenes (α and β-bisabolenes, caryophyllene and cadinenes) from stems of *S. americana*. Moreover, nitrogenated compounds and oxygenated compounds are also reported from leaves and flowers, respectively (Stashenko *et al*. 1996). However, most of the

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previously reported data described about the qualitative analysis of organic compounds. Later, simplified identification and quantification was performed from whole plants of *S. acmella*. Spilanthol was specifically quantified as 1.4 mg/g DW of plant material through the effectiveness of HPLC-esiMS, whereas other amides are confirmed by spectroscopical analysis as (2*Z*)-*N*-isobutyl-2-nonene-6,8-diynamide, (2*E*,4*Z*)-*N*-isobutly-2,4-undecadiene-8,10-diynamide, (2*E*)-*N*-isobutyl-2-undecene-8,10-diynamide, (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol), (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide, (2*E*,4*E*,8*Z*,10*E*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide (Bae *et al*. 2010). Later, purified phytochemical constituents such as (*z*)-Non-2-en-6,8-diynoic acid isobutylamide, spilanthol, (2*E*)-*N*-isobutylundeca-2-ene-8,10-diynamide, spilanthic acid-2-methylbutylamide are obtained from flower-head methanolic crude extracts of *S. acmella* using centrifugal partition chromatography and the chemical product was confirmed through other spectroscopical methods (Mbeunkui *et al*. 2011).

Apart from major constituents of phytochemicals, *Spilanthes* species are also rich in essential oils. The essential oils, such as β-caryophyllene, (*Z*)-β-ocimene, germacrene D, limonene, and myrcene are produced from inflorescences of *S. acmella* (Baruah and Leclercq, 1993). Similarly, caryophyllene, caryophyllene oxide, limonene and myrcene are isolated from inflorescences of *S. calva* (Begum *et al*. 2008) and (*E*)-2-hexenol, 2-tridecanone, germacrene D, hexanol, β-caryophyllene and (*Z*)-3-hexenol from whole plants of *S. acmella* (Jirovetz *et al*. 2005). Subsequently, β-caryophyllene (30.2%), γ-cadinene (13.3%) and thymol (18.3%) oils were also characterized from *S. acmella* (Lemos *et al*. 1991). Further, β-caryophyllene, γ-cadinene and thymol are also characterized from *S. acmella* (Lemos *et al*. 1991). Later, (Yadav, 2012) revealed the presence of carbohydrates, tannins, alkaloids, steroids, carotenoids, fixed oils and fats, sesquiterpenes and amino acids from aqueous and ethanolic extracts of leaf and roots of *S. acmella.* Similarly, amino acids such as amino-n-butyric acid, aspartic acid, glycine, histidine, proline and tyrosine from the root extracts of *S. acmella* are analyzed through thin layer chromatography (Mondal *et al*. 1998).

Most of the phytochemical extractions from *Spilanthes* have been done using wild plants. However, few reports have suggested the utilization of *in vitro* plant cultures for isolation of valuable organic components. This alternative strategy rapidly helps in the conservation of biodiversity (George and Sherrington, 1984). Moreover, the amount of spilanthol production

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was reported to be higher in *in vitro* cultures when compared to that of field grown plants. Additionally, an isomer *N*-isobutyl-2*E*,4*E*,8*E*,10*E* dodecatetraenamide was identified only from *in vitro* raised plantlets of *S. acmella* through mass spectroscopy (Leng *et al*. 2011). Similarly, *n*-hexadecanoic acid and tetradecanoic acid are also reported from *in vitro* plant cell cultures (Leng *et al*. 2011). Later, Singh and Chaturvedi (2012a) revealed that spilanthol production was reported to be higher in *in vitro* leaves rather than wild grown leaves of *S. acmella*.).

1.3.2. Dynamics of various physical parameters in in vitro cell cultures on metabolite production

Generally, cell suspension cultures offer a condensed biosynthetic cycle to study kinetics of growth and production of metabolites within a short span. This methodology can provide highly valuable medicinal compounds in good quantities (Dörnenburg and Knorr, 1995). In *Panax notoginseng,* the dynamic metabolism of monosaccharides and disaccharide in cell suspension cultures favoured the maximum production of ginseng saponin metabolite with initial sucrose level 40 g l^{-1} on 26th day. They also reported that uptake of nitrate was relatively slower when compared to that of phosphate consumption (Zhang *et al*. 1996). Similarly, kinetic profile of carbohydrates, inorganics salts intake and taxol yield is reported from stem derived callus cultures of *Taxus cuspidata*. They have also reported the kinetic trend of pH towards alkanization, nitrate assimilation, faster consumption of phosphate and total carbohydrates. This greatly supported the biomass production and yield of taxol from *T. cuspidata* (Fett-Neto, 1994). Later, batch kinetic study from cell suspension cultures of *Lantana camara* is reported to produce maximum total triterpenoids. Further, it was reported that biomass production of plant cells varied with the carbohydrate source, such as maltose and glucose (Srivastava *et al*. 2011). Of the various carbohydrates, sucrose is the most energy source for most plant cell lines and bears profound effect on quantity of metabolites produced from various plant species.

In *S. oleracea,* Singh and Chaturvedi (2012b) reported that dynamics of various factors such as fresh and dry weight, pH, carbohydrates and other nutrients affected the production of biomass and spilanthol production. Plant growth regulators, especially auxin and cytokinin also affected the production of biomass and withanolide-A in *Withania somnifera* (Nagella *et al*. 2010). The other parameters such as pH, type and concentrations of nutrients and sucrose also have an influence on biomass yield and withanolide-A production.

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1.3.3. Statistical optimization of media constituents

Secondary metabolites can be increased by examining the important nutrients affecting the plant cell biomass. However, by varying single factor keeping other variables constant, variation in production of metabolite can be observed. This method is also termed as classical or conventional routes, which is found to be chaotic, laborious, tedious, and expensive and also time consuming with increasing number of experiments. Even though, increase and decrease of secondary metabolites are observed through conventional methods, devoid of information on interactions between optimized factors and optimum conditions of respective factors is reported to be one of the major disadvantage (Ratnam *et al*. 2005; Haider and Pakshirajan. 2007; Hanchinal *et al*. 2008). Therefore, such limitations of a classic method can be eliminated by the mathematical matrices model. This plays a significant role in selecting variables, influencing response. Further, this model helps to find out the optimal concentrations of screened variable collectively by statistical experimental design using regression analysis. Statistical techniques contributes to the increased product yield, reduced process variability by regression analysis and closer validation of output response when compared to insignificant target requirements, reduced development, overall cost of production with limiting number of experiments. Therefore, statistical strategies specifically includes the execution of Placket-Burman (PB) design to identify the significant factors followed by Response surface methodology (RSM) which can be used to ensure the possible interactions effects between variables by employing central composite design (CCD) (Karemore *et al.* 2013; Bezerra *et al* 2008).

1.3.3.1. Plackett-Burman design

The Plackett-Burman (PB) is a two level statistical design for screening the crucial factors affecting the response (Plackett and Burman, 1946). Generally, PB design allows twelve runs of experimental matrix, up to eleven factors for analysis of response. When compared to two-level full factorial design, it provides sixty four numbers of experiments for only six factors. Thus, it allows the analysis of dependent variables in a lesser period of time with lesser number of statistical experiments. The variables necessary for effective response are tested all the time in two level ranges either at (-) and (+) which represents the lower and higher limits, respectively. The effect of response can be evaluated by the difference between higher and lower limit to the total number of trials (N) described in the following equation,

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$$\textbf{Effect} = \frac{2(\Sigma S_i^{+} - \Sigma S_i^{-})}{N}$$

where, S_i^+, S_i^- refers to the dependent variable in the experimental design has the highest and the lowest ranges of a given constituents, whereas, i, represents the variation from initial number of experiments to the final composition. Despite the economy of experimental runs, PB majorly examines the effects of main factors affecting the response. The larger effects of variables are identified by ratio of factor mean square to the error mean square values represented through *F*-test. Further, optimization of highly significant or most effective factors are chosen to find out the exact concentrations by omitting insignificant or smaller effects on response value. (Sanjeeviroyar *et al*. 2009; Wu *et al*. 2007).

1.3.3.2. Central Composite Design

Central composite design is a chemometric and most commonly used popular tool for three dimensional (3D) response surfaces, initially presented by Box and Wilson (1951). It is used for optimization of any effective parameter concentrations with the aim of scaling up good quantities of desired products. This multivariate mathematical model comprised of two parts, cubical and star points that strongly develops the relationship between dependent and independent variables. The part of cube directly related to a factorial screening design, whereas, portion of a star or axial points describes the additional set of points displaced from the center of cube on radii passing center values on each face of cube (Ebrahimzadeh *et al.* 2012). The implications of CCD extended for designing any varieties of total number of experiments which can be calculated using,

$$\mathbf{N = 2^k + 2k + m}$$

where, N is the total number of experiments, 2^k is the factorial points from the center of cubic points, 2k is referred to as star points fixed axially from the center and m is the replication of center points in the mathematical matrices plane. For four independent variables with seven center points, the total number experiments can be calculated as $N = 2^4 + (2 \times 4) + 7$. It produces thirty one experiments for the improvement and evaluation of response. Thus, by careful selection of number of independent variables, comparable dependent variable results can be achieved through RSM which provides both linear and quadratic models by avoiding smaller number of experiments through full-factorial three level design criterions. Moreover, the factorial design (2^k) in the experimental region contribute the estimation of interaction terms and the distance from center points specified to star or alpha (2k or α) regions. This has

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a greater attention towards the understanding of quadratic terms. In addition, estimation of internal pure error can be attained through center points (n_c). The distribution of variance and region of operability in the region of interest often influenced to a greater extent by the center points and axial points, respectively (Natarajan *et al*. 2011).

Suitable conditions for the taken parameters such as extraction time, temperature, solvent–solid ratio, mean particle size and solvent compositions are provided by statistical methodology to improve the production of oleanolic acid from field grown plants of *L. camara* (Banik and pandey, 2008). Using statistical methods, the variables, like extraction time, temperature, and liquid/solid ratio were optimized in *Portulaca oleracea* species. The optimized variables resulted in higher production of polyunsaturated fatty acids (Stroescu *et al*. 2013). Further, Puri *et al*. (2012) reported enzymatic extraction of highest non-caloric sweetener stevioside from leaves of *Stevia rebaudiana* through CCD. However, all these optimization studies are implemented in the field grown plants shifted transiently towards *in vitro* cultures for the production of important secondary metabolites. Maximum biomass and azadirachtin production is reported from optimum concentration of carbon source and major nutrients, which is optimized using statistical method for cell suspension cultures of *Azadirachta indica* (Prakash and Srivastava, 2005). Similarly, higher production of azadirachtin is reported from redifferentiated calli obtained from zygotic embryos by optimizing media constituents using statistical techniques (Singh and Chaturvedi, 2012c). The enhanced production of valtrate from adventitious roots of *Valeriana amurensis* is observed by use of elicitors and precursors at optimized concentrations (Cui *et al*. 2012). Later, RSM is implemented to increase the yield of secondary metabolites from *in vitro* cell cultures, such as lycopene using various extraction parameters from *Solanum lycopersicum* (Lu *et al*. 2008) and capsaicinoid from immobilized cultures of *Capsicum frustescens* (Suvarnalatha *et al*. 1993).

Apart from the improvisation of secondary metabolite production using statistical tools, optimization of best media combination on efficient micropropagation of *Decalepis hamiltonii* was also examined by three quality factors, such as multiple shoots, number of leaves and shoot length (George *et al*. 2000). Gutiérrez-Miceli *et al*. (2010) also optimized plant growth regulators for multiplication of shoots and roots from *Dianthus caryophyllus*. Moreover, best media combinations for the growth of undifferentiated mass of cells from *Mucuna pruriens* are also reported using RSM (Sundaram *et al*. 2013). Therefore, statistical model can be used for improvement of media combinations for efficient propagation and also

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for secondary metabolite production. Furthermore, it also resulted in confirmation of output response over insignificant target requirements, reduced development and also overall cost of production (Rao *et al*. 2000).

1.4. Therapeutic applications of genus *Spilanthes*

The diversified human diseases, such as immune deficiencies, neurological disorders, heart diseases, stroke, diabetes mellitus, cancers and certain other diseases may occur due to excessive generation of reactive oxygen species (ROS) and other radicals produced normally by the physiological events. This may be relatively proportional to the damage of carbohydrates, deoxyribonucleic acid (DNA) and comprehensively tends to oxidative stress which can leave indications to natural degenerative processes (Urankar *et al*. 2013). Since 19th century, modern medicines are widely used rather than traditional medicine. Moreover, over use of synthetic drugs resulted in adverse reactions, which have driven back the attention towards therapeutic use of pharmaceutical herbal plants over past fifty years. Traditional medicines are an alternative and readily available source and widely aid in prevention and treatment of ailments since Neanderthal period (Tyagi and Delanty, 2003). The presence of phytoconstituents is used as pharmaceuticals, drinks and also as dietary supplements. Few pharmaceutical metabolites, like digitalis, codeine, morphine, reserpine, taxol, vincristine and vinblastine are used in treatment of cancers (Asada and Shuller, 1989; Dicosmo *et al*. 1989; Endo *et al*. 1988; Nagella and Murthy, 2010). This attracted researchers and industrialists and promoted the growth of industries to produce nutraceuticals and pharmaceuticals. The traditional preparation of plant products or drug preparation from wild population for plant products and drug development negatively affect the vegetation and genetic diversity. Moreover, there will be a change in chemical profiling due to various climate factors and environmental fluxes. The use of plant species for metabolite production has increasing in the past few years due to renewed interest in the use of medicinal plants and its products (Sivanandhan *et al.* 2014; Wink, 1988). In 1974, World Health Organization encouraged the developing countries to utilize natural products from herbal plants to meet the demand (Winslow and Kroll, 1998). Moreover, the global market also turning towards herbal plant, as it is easily accessible and affordable to treat various life threatening diseases.

1.4.1. Traditional medicinal uses

Spilanthes species is known for its traditional medicinal properties because of the presence of rich bioactive metabolites. Generally, extracts and decoctions of *Spilanthes* species have

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shown various pharmacological activities. This is due to the presence of strong biologically active compounds. Each and every part of *Spilanthes* species, such as roots, stems, leaves, inflorescences and the whole herbal plant material are utilized among traditional tribal community (Dubey *et al*. 2013).

The decoction prepared from roots of *Spilanthes* species are reported for its remedial effects on health problems, such as cold, flu, asthma, dental caries, cholera, rheumatism, odontalgia, purgative, laxative, typhus fever, scabies and psoriasis. Additionally, leaves are reported for curative material of sialagogue, sore throat, stimulant and local anesthetic. It can also acts as an antiscorbutic, lithotriptic, immunomodulatory and adaptogenic in nature. Furthermore, it extends as an inexpensive raw bioactive material for the treatment of skin diseases caused by microorganisms, such as bacteria and fungi. The juice from leaves taken after crushing can be used as vulnerary whereas pounded leaves are used in the form of poultice. Therefore, the *Spilanthes* species are also consumed in terms of salad or vegetables (Santesson, 1926; Storey and Salem, 1997; Frahm and Gudat, 1980). Further, flower-head or inflorescence are efficiently used to treat stammering in children, mouth washes, toothache, stomatitis, throat complaints, infections in gums, curing of paralysis in tongue; it includes solving of periodontosis issues. Furthermore, it is reported to act as a substituent for tincture of pyrethrum to heal inflammation of jaw-bone. Likewise, whole plants are used for treatment of articular rheumatism, dysentery, tuberculosis, soreness or bruising. It is also reported to serve as remedy against poisonous snake bites (Shimada and Gomi 1995, Pandey *et al*. 2007; Adler, WO Pat. 2006059196; Rastogi, 1993; Yoganarsimhan, 1996; Tiwari *et al*. 2010). These herbal ingredients are a key benefit of beauty care cosmetics as they decrease facial wrinkles and also help to defend oxidative stress. Further, herbal extract can be used as an alternative drug for synthetically produced drug, botox. This can help in preventing the skin from adverse effects (Demarne and Passaro, 2005). However, more than 0.5% alkylamides are responsible for anesthetic, analgesic, antiseptic, sialogogue and insecticidal properties. Moreover, these pharmacological activities are explored in traditional medicine. Because of these multifold uses, the commercial interest in *Spilanthes* has increased tremendously. However, most of the secondary metabolites are not identified and quantified from the extracts. Therefore, *Spilanthes* species is cultivated in different parts of the world for horticultural, insecticidal, medicinal, and culinary purposes (Jansen, 1981).

1.4.2. Pharmacological applications

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Due to immense potentiality of *Spilanthes* species, most of the extracts from different plant parts are analyzed for its pharmacological applications to authenticate the traditional uses. This can help to commercialize the product obtained from plant species to exhibit *in vivo* and *in vitro*. The ethylacetate extract obtained from aerial parts of *S. acmella*, exhibit immediate vasorelaxation activity in a dose dependent manner. Moreover, it is also reported for antioxidant activity using diphenylpicryl hydrazine (DPPH) and superoxide dimutase assay in phenylephrine induced rat (Wongsawatkul *et al*. 2008). Similarly, methanolic extracts obtained from leaves and stems of *S. acmella* are also reported for its highest antioxidant activity (Tanwer *et al*. 2010) as well as from leaves of *S. paniculata* (Hossain *et al*. 2012). This describes the medicinal impact of plant species which can prevent and treat diseases developed by free radicals and antioxidants (Hossain *et al*. 2012). Moreover, ethanolic extracts of *S. acmella* showed the effect of copulatory behavior and improved penile erection in male rats by the release of reduced Mount latency (ML), Intromission latency (IL) and Penile erection index (PEjI). Further, increased release of nitric oxide *in vitro* describes potent sexual behavior which can be due to the presence of alkylamides and its action of androgen in the treated model (Sharma *et al*. 2011). Later, other pharmacological activities, such as anti-inflammatory property in rats and anti-hyperalgesic activity are also reported from flower heads of *S. acmella* (Ratnasooriya and Pierris, 2005).

Similarly, anti-nociceptive activity is reported from ethanolic extract of *S. paniculata.* Moreover, Sana *et al*. (2014) reported that increase in concentration from root extract of *S. acmella* attribute antiradical potential. Further, another pharmacological application is reported from aqueous extract of aerial part from *S. acmella* against animal models, such as guinea pigs, frogs and albino rats. This resulted in local anesthetic activity, which is examined through skin in pigs and also in frogs. Further, it is also significantly reported for antipyretic activity (Chakraborthy *et al*. 2010). Later, cold water extract from flower heads of *S. acmella* is reported to have diuretic activity (Ratnasooriya *et al*. 2004) while the leaf extract of *S. acmella* possess hypertension as diuretics (Yadav *et al*. 2011). Later, DMSO sample obtained from dried flower heads of *S. acmella* revealed anti-fungal activity against

Fusarium oxysporium, *F. moniliformis, Aspergillus niger* and *A. parasiticus* However, best and maximum fungal inhibitory action is reported in *Candida albicans* from root extracts of *Spilanthes calva* (Rani and Murthy, 2006; Vyas *et al*. 2009). Later, Fabry *et al*. (1996) also reported to exhibit antifungal activity against *Aspergillus* sp. from *Spilanthes mauritiana*. Later, roots and flowers of *S. mauritiana* are reported to have anti-bacterial activity against

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Staphylococcus, *Enterococcus*, *Pseudomonas*, *Escherichia*, *Klebsialla* and *Salmonella* species (Fabry *et al*. 1998). Furthermore, root extracts from *S. calva* are used against *Streptococcus mutans* and *Lactobacillus acidophilus* showed inhibitory action against bacteria (Vyas *et al*. 2009). The leaves and flower heads of *S. acmella* can act as growth inhibitors and helps in protecting the crops from lepidopteran *Chilo partellus*. Moreover, aqueous extracts from both the plant parts showed larvicidal activity against stem borer. It is also reported that leaves and flower head aqueous extract resulted in reduced larval survival rate (Raghuvanshi *et al*. 2010).

Later, hexane extract of flowers from *S. acmella* also reported to have lethality against *Anopheles stephensii*. Further, it is also reported to act against malarial vector *A. culicifacies* and filarial vector *Culex quinquefasciatus,* respectively (Pandey *et al*. 2007). Moreover, the isolated compound spilanthol from *S. acmella* species are reported to have greater effect against larvae's of *Culex, Anopheles* and *Aedes* species (Sarf and Dixit, 2002).

This overcomes the effect of chemical insecticides banned by WHO (1981) after the failure of National Malaria Eradication Programme. Further, promising results are reported to have anti-insecticidal activity from leaves and flower extracts of *S. calva*. This can overcome biodegradable nature of various insecticides or pesticides, which can threaten environmental safety (Dolui and Debnath, 2010). Consequently, acetylenic amide undeca-2*E*-en-8,10-diynoic acid isobutylamide and olefinic amides spilanthol and undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide from dried flower-head hexane extract of *S. acmella* are reported to be very active against *Aedes aegyptii* through mosquitocidal assays. Later, acetylenic amide showed improved activity against *Helicoverpa zea* neonates whereas, olefinic amides showed comparatively lesser activity (Ramsewak *et al*. 1999). Furthermore, (*z*) Non 2 en 6,8 diynoic acid isobutylamide, spilanthol, (2*E*) *N*-isobutyl undeca 2 ene 8,10ıdiynamide, spilanthic acid methylbutylamide compounds isolated from flower head methanolic extract of *S. acmella* are reported to have antiplasmodial biological activity against asexual erythrocyte stages of *P. falciparum* against chloroquine sensitive strain (D10). However, semi-purified compounds significantly reported to have anti-plasmodial activity rather than purified compounds (Mbeunkui *et al*. 2011). The polyunsaturated fatty acid (PUFA) alkylamide from ethanol extracts of dried flowers of *S. acmella* are treated in lipopolysaccharide-activated murine macrophage model, RAW 264.7. This exhibited the reduction of proinflammatory mediators at the transcription and translational levels, such as LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) mRNA and protein expression. The dose

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dependent declination of LPS-stimulated inflammatory cytokines IL-1β, IL-6, and TNF-α production is also observed along with the restrained action of spilanthol towards the LPS-induced nuclear NF-κB DNA binding activity and phosphorylation of cytoplasmic inhibitor-κB. Thus, *N*-isobutylamide involves in the inhibition of NF-κB attenuates the LPS-induced inflammatory responses (Wu *et al*. 2008). Savadi *et al*. (2010) used ethanolic extract of *S. acmella* from leaves and reported immune-stimulant activity. Usually, the complicate biological system does not allow penetrating blood circulation, when *N*-alkylamides (NAAs) are controlled orally or dermally. However, one of the multifunction amides, spilanthol is highly associated with central nervous system. Therefore, it is capable of penetrating the blood-brain barrier and oral mucosa (Veryser *et al*. 2014). Boonen and colleagues also evidently described about transdermal behavior of spilanthol by topical application on the split thickness human skin in a static Franz diffusion cell system using *S. acmella* extract. They have also reported permeability co-efficient, diffusion coefficient and partition coefficient to understand the transdermal efficiency of spilanthol (Boonen *et al*. 2010). But, the earlier data clearly reported about *Spilanthes* species either traditionally or pharmacologically only from field grown plants. However, only meager reports exist on this plant species from *in vitro* cultures to assess its bioactivity prospective. The superior antioxidant activity and anthelminthic activity was reported from dedifferentiated cells of *S. acmella*.

Motivation to define objectives

The evidential data reported that *Spilanthes* species are considered to be an elite medicinal plant in retaining wide array of "*N*-alkylamides". Of various species in India, *Spilanthes paniculata* is holding a very high significance of medicinal importance both, traditionally and pharmacologically. Henceforth, there is a greater necessity to conserve the plant system before it reaches extinction state and also to contribute and satisfy the demands of the society. Moreover, seasonal inconsistencies, geographical location and pathogen attack of the plants lead to decreased production of valuable secondary metabolites, which are of uncountable worth in the field of medicine. The above mentioned aspects motivated to establish an alternative tool for utilization of secondary metabolites.

1.5. Objectives of the present study

From the literature review, the reported data has adequately revealed that the *Spilanthes* species is a potent medicinal herbal plant. The demands for the biologically active compounds from herbal plants are increasing, day-by-day, at industrial level. However,

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production of natural chemical from higher plants through biosynthetic pathway is very meager in amount. Moreover, most of the biological studies conducted so far are reported to be from field grown plants. Nevertheless, change in the environmental conditions can cause variation in chemical profile, quantity and also can have an impact on efficiency of biological activity. Hence, there is an urgent need to improve the production of bioactive metabolites without sacrificing filed grown natural plantation of this species. This can offer commercialization of quality products to the society for the benefits of good health and conditions.

Specific Objectives

In the present study, the following objectives are aimed towards higher production of *N*-alkylamides, spilanthol and UDA and to validate the pharmacological applications of *in vitro* cell cultures of *S. paniculata*,

1. Establishment and maintenance of callus cultures using young leaves and flower heads of *S. paniculata*

2. Screening and quantification of bioactive *N*-alkylamides from *in vitro* callus cultures of *S. paniculata*

3. Establishment of cell suspension culture and batch kinetics of *S. paniculata*

4. Media optimization for callus cultures by using statistical tool to find out the best producing cell lines

5. Bioassay activity of alkylamides derived from *S. paniculata*.

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Chapter 2

Materials and Methods

This chapter describes about the materials acquired from different sources. It is divided into three sections. The first section comprised of tissue culture experiments on *Spilanthes paniculata*, the second section contains details regarding phytochemical analysis and the third section on biological assays.

2.1. Materials

The leaves and flower-head explants from *S. paniculata*, growing in the campus of Indian Institute of Technology Guwahati (IITG), were collected to establish *in vitro* cell and tissue cultures. The medium constituents of Murashige and Skoog (1962) were purchased from Merck, India. The plant growth regulators, fluorescein diacetate (FDA) were procured from Sigma, USA, whereas, glassware and plasticware were purchased from Borosil, India and Tarsons, India, respectively.

The phytochemical investigation was carried out using solvents, such as HPLC grade acetonitrile, analytical grade methanol, ethylacetate and hexane, purchased from Merck, India. The High Pressure Liquid Chromatography (HPLC) analysis of bioactive metabolite, spilanthol was performed by using purified Milli-Q system. To quantify spilanthol, structurally similar dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide, obtained from Chromadex, India was used as standard compound. Estimation of nitrate and phosphate was done using standard potassium nitrate (KNO_3) and potassium dihydrogen phosphate (KH_2PO_4), respectively. To estimate phosphate content, solutions were purchased from Merck, India, and the reagent was prepared by mixing concentrated sulphuric acid, potassium antimonyl tartarate solution, ammonium molybdate $(NH_4)_2MoO_4.4H_2O$) solution and ascorbic acid. The calibration curve was generated after analyzing standards and samples using UV spectrophotometer (Cary 50, Netherlands). The carbohydrate estimation was performed using High Pressure Anion Exchange Chromatography (HPAEC) procured from Dionex Corporation, USA. The biological assay against malarial parasites was performed by

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procuring materials from sigma, USA. All the chemicals and instruments procured are listed in **Table 2.1**.

Table 2.1: List of chemicals and instruments used for the experimental studies

Chemicals	Instruments
Agar, Hi Media, India	Elix water, Millipore, USA
Albumax, Sigma, USA	Glassware, Borosil, India
Ammonium molybdate solution, Merck, India	High Pressure Anion Exchange Chromatography (HPAEC), Dionex Corporation, USA
Analytical grade methanol, Merck, India	High Pressure Liquid Chromatography (HPLC), Varian prostar, USA
Amphotericin B, Sigma, USA	High speed refrigerated centrifuge, Sigma, Osterode Am Harz, Germany
Ascorbic acid, Merck, India	Laminar-air-flow cabinet, Saveer Biotech, India
Concentrated sulphuric acid, Merck, India	Mass spectrometer, Waters, USA
Dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide, obtained from Chromadex, India	Nylon membrane filter (0.22 µm), Millipore, USA
Ethylacetate, Merck, India	Plasticware, Tarson, India
Fluorescein diacetate (FDA), Sigma	Rotary evaporator, Buchi Rotavapor R-200, Japan
Gentamycin, Sigma, USA	UV spectrophotometer (Cary 50, Netherlands
Glucose, Sigma, USA	-
Hexane, Merck, India	-
HPLC grade acetonitrile, Merck, India	-
Hypoxanthine, Sigma, USA	-
Mercuric chloride, Merck, India	-
Potassium antimonyl tartarate, Merck, India	-
Potassium nitrate, Merck, India	-
Potassium dihydrogen phosphate, Merck, India	-
Savlon, Johnson and Johnson, India	-

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2.2. Methods

2.2.1 Establishment of in vitro plant cell cultures of S. paniculata

i. Leaf-disc culture

Firstly, the collected plant materials are washed with distilled water and kept on a blotting paper to remove excess water. Then, the leaves were washed with 1% (v/v) Savlon (Johnson and Johnson, India) for 15 min and rinsed thrice with sterile distilled water (SDW). Further, these explants were surface sterilized using mercuric chloride ($HgCl_2$) (0.1%) for 5 min, inside the laminar-air-flow cabinet (Saveer Biotech, India). The excess $HgCl_2$ was removed by rinsing three times using SDW. By punching the sterilized leaves using 10 mm sized cork-borer, the circular disc was obtained. The abaxial side of leaf–disc explants was inoculated on Murashige and Skoog (1962) medium containing various concentrations and combinations of plant growth regulators. The auxins, such as α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), para-chlorophenoxy acetic acid (pCPA) and cytokinins, such as N6-benzylaminopurine (BAP) and 6-furfurylamino purine (Kinetin), were used to induce dedifferentiated mass of cells. At least eighteen cultures were initiated for each treatment and each experiment was repeated at least three times. The observations were documented regularly at weekly intervals. Further, the established cultures were subcultured and maintained recurrently into fresh medium at four week intervals.

ii. Flower head culture

The flower-head explants were washed initially with distilled water and then washed with 1% (v/v) Savlon for 15 min, followed by three rinses with sterile distilled water (SDW). Further, under aseptic conditions in the laminar-air-flow cabinet, those washed explants were treated with 0.1% mercuric chloride ($HgCl_2$) for 5 min. Finally, SDW wash was given for at least three times. The surface sterilized flower-heads were carefully inoculated on media. MS basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, which includes naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), N6-benzylaminopurine (BAP), kinetin, *para*-chloro phenoxy acetic acid (pCPA) was used for callus induction and multiplication. Each and every experiment was conducted with eighteen replicates. The observations were taken at every four weeks intervals. The proliferation of callus development was recorded from total aseptic flower-head cultures.

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After induction of callus, the biomass of cells were repeatedly subcultured onto the fresh media of the original composition at 4 week interval and maintained.

iii. Establishment of suspension cultures

After 4-5 passages of repeated subcultures, healthy, green and friable callus, obtained on semi-solid medium, was used to establish cell suspension cultures. The liquid medium containing MS basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, which includes NAA, 2,4-D and BAP without an addition of agar was prepared to inoculate 0.1g of fresh calli in 50 ml of media contained in 250 ml capacity of Erlenmeyer flask. The suspended cells were incubated, in a dark, under shaking conditions using orbital shaker at 120 rotations per minute (RPM) at 25 ± 2ºC. Further, to prevent aggregation, it was subcultured in a fresh liquid medium at every 18th day of cell growth.

iv. Culture media

The cultures, obtained from two different explants, were cultured on MS basal medium (**Table 2.2**). The medium was augmented with various growth regulators and other adjuvants as described in **Table 2.3**. Until and unless mentioned otherwise, all media contained 3% sucrose and was solidified with 0.8% agar (Hi Media, India). The chemicals were of analytical grade, and Milli-Q water was used to prepare stock solutions and culture media. All the stock solutions were prepared separately with macronutrients of concentrations (20 x), micronutrients (200 x), iron (200 x) and vitamins (200 x) and they was stored at 4ºC until further use. The stock solutions of (1x 10^{-3} M) growth regulators was prepared and stored at 4ºC. Further, myoinositol and sucrose were measured and added into the culture medium during preparation. Later, medium was made to final volume with Elix water (Millipore, USA) and then it was adjusted to pH 5.8 with 0.1N NaOH/HCL. Successively, 20ml medium was dispensed into 150mm×25mm glass tubes without rim. Then, culture tubes were plugged with non-absorbent cotton wrapped in cheese cloth before autoclaving at 15 psi and 121ºC for 15min. After autoclaving, the dispensed media in glass tubes were allowed to cool down and kept in a slant position to generate surface area for the initiation of undifferentiated mass of cells from leaf-disc and flower-head explants.

v. Inoculation

The sterilization of two different explants, including inoculations were carried out inside the laminar-air-flow chamber. Initially, before starting the work, the platform and the inner

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surface of the laminar air flow cabinet was cleaned thoroughly with 70% alcohol and exposed to Ultra-Violet (UV) for about 15 min. The instruments used for inoculation (forceps, scalpels etc.) and Petri plates were wrapped in aluminum foil and autoclaved. At the time of inoculation, at regular intervals, the autoclaved instruments were dipped in 90% ethanol, flamed and cooled before use. During inoculation or subcultures, the glass tubes and flasks were unplugged and plugged near the flame.

vi. Culture conditions

The cultures were generally maintained at 25±2ºC temperature with relative humidity of about 50–60% and photoperiod of 16h provided with diffused light (1000–2000 lx). Unless mentioned otherwise, at least eighteen cultures were used for each treatment. Each experiment was repeated at least three times.

vii. Observations of cultures

The transitional morphological changes of cultures were observed and recorded at weekly intervals or whenever necessary. The final periodical observation was recorded at the end of fourth week. The explants showing callus formation was recorded and percent callusing was calculated.

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Table 2.2: Constituents of MS (Murashige and Skoog, 1962) basal medium.

Media components	Concentrations (mg/l)
Major elements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor elements	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.60
$Na_2MoSO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic components	
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine.HCl	0.50
Thiamine.HCl	0.10
Glycine	2.00
Iron constituents	
$FeSO_4.7H_2O$	27.80
$Na_2EDTA.4H_2O$	37.30
Carbohydrate	
Sucrose	30,000.00
Gelling agent	
Agar	8,000.00

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Table 2.3: Growth regulators, amino acids and other compounds used as additives to MS basal medium

Additives	Manufacturer
Auxins	
2,4-D	Sigma, USA
NAA	Sigma, USA
IAA	Sigma, USA
pCPA	Sigma, USA
Cytokinins	
BAP	Sigma, USA
Kinetin	Sigma, USA
Aminoacids	
L-Glutamine	Sigma, USA
L-serine	Sigma, USA
Other organic compounds	
Glucose	Merck, India
Fructose	Merck, India

2.2.2. Phytochemical analysis of in vitro cultures

i. Determination of dry cell weight

The cell biomass from leaf-disc and flower-head explants was successfully grown on semi-solid medium till 4 weeks. At the end of four week growth period it was harvested, washed with distilled water and vacuum filtered. Then fresh weight of harvested calli was weighed using high precision analytical balance (Sartorius, India). Further, undifferentiated mass of cells 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 obtained dried cells were utilized for further biochemical studies. In case of batch kinetic studies, suspended cells were harvested at a regular interval of three days from liquid medium and dried in the same way as performed for the cells grown on semi-solid media.

ii. Preparation of dodeca 2(*E*),4(*E*) dienoic acid isobutylamide standard

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The stock solutions of dodeca 2(*E*),4(*E*) dienoic acid isobutylamide (a reference standard non-commercial compound structurally similar to spilanthol) was prepared by dissolving 5mg of compound in 5ml of HPLC grade methanol (i.e., 1mg/ml). Quantification of standard was carried out at five different concentration range from 250 μg/ml to 15 μg/ml and each concentration of standard was filtered through 0.22 μm nylon membrane filter (Millipore, USA) before HPLC analysis and run at least thrice to check the repeatability and precision of results.

iii. Preparation of undeca-2E-en-8,10-dienoic acid isobutylamide *(UDA) standard*

Since commercial UDA is not available, it was isolated from flower-heads of field grown parental plant through thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Quantification of purified UDA standard was carried over at five different concentrations range from (1 mg/ml to 0.06 mg/ml) and each concentration of standard was filtered through a 0.22 μm nylon membrane filter (Millipore, USA) before HPLC analysis. Standard was run at least thrice to check the repeatability and precision of results.

iv. Preparation of sample solution

The dried cell mass from two different explants, leaf-disc and flower-head explants, were powdered using porcelain pestle and mortar and they was soaked in analytical grade methanol (Merck, India) for 12h. The methanolic extracts were then centrifuged in a high speed refrigerated centrifuge (Sigma 4K 15C, Osterode Am Harz, Germany) at 5000 rpm for 10 min. After centrifuging the samples, supernatant was collected. Then, the obtained residue was re-extracted thrice by using (10 mL) of methanol. Thereafter, the residue was discarded and whole methanolic solution was pooled separately. Further, pooled supernatant was filtered using Whatman paper and evaporated to dryness in a rotary evaporator (Buchi Rotavapor R-200, Japan) at 40 °C. Later, evaporated samples were re-dissolved in HPLC grade methanol and filtered through 0.22 μm nylon membrane filter before analysis. The obtained samples were stored at -20 ºC until further use.

v. Preparative thin layer chromatography (PTLC) of extracts

For qualitative analysis, the elution of samples were performed from the extracts of field grown flower-heads and leaf explants as well as from *in vitro* grown calli, through PTLC using hexane:ethyl acetate mixture (2:1). A yellowish brown band was observed in the UV

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chamber, and the R_f value of the band was calculated. The obtained band was cut out and re-dissolved in ethylacetate. The ethylacetate extract was evaporated to dryness using rotary evaporator 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 test and excess sample was stored at -20ºC until further use.

vi. High performance liquid chromatography

a. Leaf-disc explant

An *N*-alkylamide, (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) was identified and quantified using Varian prostar HPLC system (Varian, USA) containing 4.6 mm x 250 mm hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6mm×250mm. The mobile phase was 93% A (acetonitrile) and 7% B (MilliQ water) with a flow rate of 0.5 $mLmin^{-1}$. The eluted samples were detected by UV detector at 237 nm (Singh and Chaturvedi, 2012).

b. Flower- head field grown and callus extracts

The identification of two different *N*-alkylamides, spilanthol and (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide (UDA) was achieved by isolating them from 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 X 250 mm. The mobile phase was A (acetonitrile) and B (MilliQ water) with a flow rate of 0.5 mL min^{-1}. The eluted samples were detected by UV detector at 237 nm. The various methodologies attempted for an appropriate separation of phytochemical compounds are listed in (**Table 2.4**).

Table 2.4: Various isocratic methodologies using HPLC

S.No	Methodology	
	Acetronitrile%	MilliQ water %
1.	93	7
2.	90	10
3.	80	20
4.	70	30
5.	60	40

The two prominent major peaks were collected separately in a vial and were concentrated at -50ºC using lyophilizer. The presence of alkylamides, spilanthol and UDA, were confirmed in

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the two purified peaks by electron spray ionization (ESI) mass spectra (MS) and 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 was used as a standard.

vii. Linearity and precision

Five different concentrations of standards of dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide and (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide (UDA isolated from flower-head) were run in HPLC to develop standard curve, which was used to quantify the amount of phytochemical compounds present in the samples. The calibration curve was generated by plotting the peak area (y) against concentration in µg/ml of standard solutions. The standard equation obtained from the curve was used for quantification of an important alkylamide spilanthol. Spilanthol and UDA content from *in vitro* cell lines and explants of field grown plant were reported as mg/g DW of sample. The correlation coefficient (R^2) was generated using by fitting the linear trend line to obtain the standard curve. Precision of developed analysis was evaluated by loading the same sample at same concentration of standard compound, at least three times, on same day (intraday) and twice at one day intervals (interday). The values were calculated in terms of relative standard deviation (RSD).

% RSD = (Standard deviation/Mean) x 100

viii. Mass spectrometry (MS)

MS detection of spilanthol was carried out on Water quadrapole-Tof Premier mass spectrometer 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. For spilanthol identification, all peaks that appeared through HPLC were collected, concentrated through lyophiliser and then re-dissolved in methanol prior to analysis. The confirmation of spilanthol and UDA peak was done by comparing the mass spectra of samples with previously published reports (Singh and Chaturvedi, 2012; Bae *et al*. 2010).

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ix. Batch kinetic studies of cell suspension cultures

a. Batch kinetic studies

Growth curve and kinetics of nutrient uptake was determined by establishing cell suspension cultures from callus. The fresh cells were harvested at three day interval as mentioned in section 2.2.2.i. The pH and residual nutrients, such as nitrate, phosphate and carbohydrate contents, were measured at three days interval up to 30 days. The specific growth rate of calli was calculated by:

$$\mu = \ln (MT_2 - MT_1)/T_2 - T_1; T_2 > T_1$$

where, MT_1 and MT_2 were biomasses at the different times points (T_1 and T_2). Nitrate was determined by using potassium nitrate (KNO_3) was considered as a standard to make the linearity curve. Firstly, the samples were acidified using salicylic acid followed by addition of sodium hydroxide (NaOH). Then, absorbance was documented in a UV–visible spectrophotometer at 410nm (Cary 50, Netherlands).

Similarly, the residual nutrient phosphate was measured by ascorbic acid assay. Initially, combined reagent was prepared by mixing concentrated sulphuric acid, potassium antimonyl tartarate solution, ammonium molybdate $(NH_4)_2MoO_4.4H_2O$) solution and ascorbic acid. The calibration curve was generated by the standard potassium dihydrogen phosphate (KH_2PO_4). Later, the combined reagent are added to the samples and incubated for 10 min. Then, the absorbance was recorded at 880nm using spectrophotometer (Cary 50, Netherlands).

Carbohydrates (monosaccharide and disaccharide) analysis was carried out using High Performance Anion Exchange Chromatography (HPAEC). Cell suspension cultures from callus collected at every 3days interval was filtered using 0.22-µm nylon membrane filter before HPAEC analysis. Later, the filtrate sample was analyzed on HPAEC ICS-3000 system (Dionex Corporation, USA) using CarboPac PA-20 analytical column (3×150 mm) and CarboPac PA-20 guard column (3×30 mm). The temperature of the column was adjusted to 30 °C and the injection volume was 25 µl. The eluent used was 0.5 M NaOH at a flow rate of 0.5 ml/min. The linear calibration curve was made by three standards sucrose, glucose and fructose for quantification of respective carbohydrates from the samples.

b. Carbon source

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Two monosaccharides (glucose and fructose) and a disaccharide (sucrose) at 3% concentration were fed to the flower head callus suspension cultures to understand the biomass and metabolite production. Later, 250 ml Erlenmeyer flasks containing 100 ml of medium was inoculated with 0.2 g of cells with different carbon sources. Further, cell biomass was harvested at the end of 21 days to analyze dry cell weight and secondary metabolite production from suspension cells.

c. Agitation speed

The callus cultures (0.2 g) were inoculated in 250ml Erlenmeyer flask consisting of 100ml of liquid medium containing MS basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, which includes NAA, 2,4-D and BAP without an addition of agar to evaluate the agitation speed on the basis of fresh and dry cell weight and also their viability at the end of growth cycle. Cells were incubated under darkness in an orbital shaking incubator 60, 90, 120, 150 and 180 RPM to identify the effect of agitation speeds. The cells were allowed to grow and harvested at the end of 21 days to re-inoculate in the same medium composition. Then, it was incubated at various RPM as above for a period of 21 days and then, fresh and dry cell weight was recorded. The cell viability test was performed using 1% FDA solution at every passage of cells.

x. <u>Statistical analysis</u>

<u>a. Plackett-Burman (PB) design</u>

The Plackett–Burman design was used to evaluate the relative significance of five independent variables affecting the production of *N*-alkylamide, spilanthol. The variables were MS major salts, carbon source as sucrose and three different plant growth regulators, like BAP, 2, 4-D and NAA. Based on the first-order polynomial model the Plackett–Burman experimental design is expressed as:

$$\mathbf{Y = \beta_0 + \Sigma \beta_i X_i}$$

Where, Y is the probability of the target response, β_i is the linear coefficient, β_0 is the scaling constant and, X_i is the level of an individual factor influencing the response. All these factors chosen were tested at two different levels, a high (+1) and a low (−1) **(Table 2.5)** with 12 treatment combinations. The preliminary experiments (data not shown) conducted in our laboratory for optimization of media constituents to improve the response at both higher and lower levels. This PB model is used to screen and evaluate only the important factors, which

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had larger impact on response, but it is devoid of interaction amongst the selected factors. The higher and lower level of each variable was set in such a way to categorize the media constituents with appropriate significant influence on spilanthol production. All experiments are conducted in three replicates and the obtained average value of response was considered for statistical analysis.

The variables, which were significant at 95% level ($p<0.05$), considered to have a higher effect on spilanthol production were obtained from the regression analysis. These significant variables were further optimized using central composite design (CCD) by neglecting trivial factors obtained through PB. The experimental design and statistical analysis of the data were developed by using Minitab 16.1 statistical software package.

Table 2.5: Plackett-Burman design

Media Constituents	High level (+1)	Low level (-1)
MS	1.25	0.75
Sucrose	4%	2%
BAP	7 µM	3 µM
2,4-D	1.40 µM	0.60 µM
NAA	1.40 µM	0.60 µM

xi. Response surface methodology (RSM)

Screened significant factors from Plackett–Burman design criterion are fed to 2^n factorial CCD which is a response surface method was implemented to determine optimum level of significant factors for maximum response. Therefore, five different coded values $-\alpha$, -1, 0, +1, $+\alpha$ were calculated for significant factors such as MS major salts, sucrose, BAP and 2,4-D by the following equation (Paul *et al*. 1992):

$$\text{Coded value} = \frac{\text{actual level} - (\text{high level} + \text{low level}) / 2}{(\text{High level - low level}) / 2}$$

where, $\alpha = 2^{(n/4)}$, here n is the number of factors lead to an α value ±2 and 0 is considered to be central point along with ±1 of higher and lower levels. This resulted in 31 experiments with 16 quadrant points, 8 axial points and 7 central cubical points. Numerical optimization process was used to optimize the level of significant factors responsible for greater response and also to correlate the interaction between response (spilanthol content) and variables. The

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second-order polynomial model and the data fitted into the multiple regression procedure were developed for the respective response and were expressed for analysis as:

$$\mathbf{Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i + \Sigma\Sigma\beta_{ij} X_i X_j}$$

where, Y is the predicted response, β_i is the linear, β_{ii} is the quadratic, β_{ij} is the interaction coefficient, β_0 is the scaling constant, and X_i, X_j were coded independent variables influencing the response. The design of experiment of RSM includes the analysis of variance (ANOVA), where the effect and regression coefficients of individual linear, quadratic and interaction terms were developed using Minitab 16.1.1. statistical software package. The analysis of variance can be calculated by the following standard equation to validate the effect and to assess the statistical significance of the model.

$$\mathbf{Effect = \frac{2\{\Sigma R(H) - \Sigma R(L)\}}{N}}$$

where, R(H) refers all components response at high levels, R(L) refers all components response at low levels and N is the number of factors. The significance of all the terms in the polynomial was judged statistically by computing F value at a probability ($p<0.05$). From the regression models, the regression coefficients were used to make statistical calculations to generate response surface curves. To test the model accuracy, R^2, adjusted R^2 (R_{adj}^2) and predicted R^2 (R_{pred}^2) were estimated.

xii. Response optimizer

The extent of second-order polynomial equation for the response of significant variables from CCD was determined using the Minitab response optimizer under a global solution of desirability equal to 1 to obtain the optimal levels of individual variables. This model helps in generating further more experimental number with a maximal or an optimal limit in attaining the largest response. The accuracy of the value was corroborated by correlating the predicted values from the mathematical model and the measured values from the given experiment under the same conditions.

2.2.3 Biological assay against malaria parasite

xiii. Malaria parasite culture

Plasmodium falciparum 3D7 strain was maintained in human RBCs (Blood group O+) suspended in RPMI 1640 media containing 25 mM HEPES, 0.4% glucose, 0.2% sodium

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bicarbonate, 0.5% albumax, 50 mg/l hypoxanthine, 40 µg/mL gentamycin and 25 µg/ml amphotericin B, at 37ºC in 5% CO_2 (Trivedi *et al*. 2005). The study was first carried out directly on crude extracts obtained from *in vitro* callus and flower-heads from parent plant to understand the biological activity of the obtained extracts. Later, biological assays were conducted in detail using HPLC purified spilanthol and UDA.

xiv. Determination of schizonticidal activity of crude and HPLC purified extracts

To determine schizonticidal activity, parasites were synchronized with D-sorbitol and schizonticidal activity was performed as described earlier (Parveen *et al*. 2013). In brief, ring synchronized parasite culture (1% parasitemia and 3% haematocrit) was incubated with different concentrations (0-50µg/ml) of plant extract as well as active compounds from HPLC column. Schizonticidal activity was determined after examining Giemsa stained smears under oil immersion microscope (Nikon 80 Ti) (Balaji *et al*. 2015).

xv. Determination of parasiticidal activity of crude and HPLC purified samples

The parasite culture was treated with sample for 48h and then washed twice with complete RPMI 1640 media. Thereafter, the parasite was allowed to propagate in complete media for another 72 hours. The parasiticidal activity of crude and HPLC purified samples was determined after examining Giemsa stained smears under oil immersion microscope (Nikon 80 Ti) (Balaji *et al*. 2015).

2.2.4. Statistical analysis

The data was proceeded for analysis of variance (ANOVA) and means were compared by using the software SPSS 20 package. Standard deviation (SD) for all parameters taken for study was calculated and represented as bars in the graph. The differences in the values of mean were evaluated by Least square differences (LSD) and Duncan multiple range tests. The percentage conversion using arcsine was performed before ANOVA. The significance of the model towards the response was determined by probability value less than 5% ($P<0.05$).

The analyses on secondary metabolite, spilanthol and UDA production from two different *in vitro* cell lines were an average of three separate replicates. In case of batch kinetic studies and biological assay, all the experiments were conducted in triplicates to check the repeatability of obtained data. Results were presented as mean ± SD.

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Chapter 3

Results

The current chapter illustrates the results obtained from experiments on *in vitro* cell lines of *Spilanthes paniculata*. The investigations on tissue culture studies and analysis of metabolite production were subdivided into three sections as follows,

Section 3.1: Establishment of *in vitro* cell lines from two different explants, leaves and flower-heads

Section 3.2: Biochemical analysis of phytochemical compounds, spilanthol and UDA, in *in vitro* cell biomasses obtained from two explant sources

Section 3.3: Analyses on biological activities of two *N*-alkylamides, spilanthol and UDA, against malarial parasite *Plasmodium falciparum* 3D7.

3.1. Establishment of *in vitro* cultures

The *in vitro* cultures of *S. paniculata* were established from two different explants, leaves and flower-heads. The results of these experiments are represented below:

3.1.1. Leaf-disc culture

Leaf-disc explants of 10 mm size were prepared from sterilized leaves using cork-borer. The explants were inoculated on MS basal medium or MS medium supplemented with various combinations of plant growth regulators, such as BAP, 2,4-D, NAA, pCPA, Kinetin. The presence of one auxin or one cytokinin in media combinations or combinatorial effects of growth regulators resulted in the development of callus. The basal media lacking growth regulators showed no response whereas media composition with auxin or cytokinin, such as MS + 2,4-D (5.0 µM), MS + NAA (5.0 µM), MS + Kinetin (1 µM) initiated callus. Moreover, medium with cytokinin MS + BAP (9.0 µM) induced profuse and massive callusing. Although initiation of callus was observed in all combinations of plant growth regulators, the growth of cultures was not sustained at the first subculture of two weeks. Therefore, these calli were transferred to a different set of growth regulator combination

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consisting of at least one cytokinin and two auxins on MS + BAP (5 µM) + 2, 4-D (1 µM) + NAA (1 µM), (**Table 3.1**). Hence, repeated subcultures at 4 week intervals, up to two passages, were performed to avoid brown colour development in the callus. After 8-week of subculture, the calli became green, friable with light-brown tinge. Later, after 15 weeks of cultures, the calli completely turned to healthy, friable and green mass and also remained in an unorganized state (**Figure 1**). The calli were multiplied and subsequently maintained on the parental media composition, at every 4- week interval.

3.1.2. Flower-head culture

The sterilized flower head cultures were excised at the base and inoculated on MS basal medium devoid of growth regulators or supplemented with auxin/cytokinin. The media combinations used were MS + BAP (9 µM), MS + 2, 4-D (5 µM), MS + NAA (5 µM) and MS + pCPA (5 µM). The initiation of callus was observed on all combinations of plant growth regulators. However, the calli showed browning and did not sustain growth beyond first subculture of two weeks on the parent medium. Therefore, these calli were transferred to a different set of growth regulator combinations consisting of two auxins and one cytokinin **(Table 3.2).** When cultured in MS medium supplemented with 2, 4-D (1 µM) + NAA (1 µM) + BAP (5 µM), nodulated brown calli was produced at 4-week. Later, by 8-week the cultures formed green, friable and slightly brown undifferentiated mass of cells., Then, on successive subcultures it remained brown, friable callus till 12 weeks (**Figure 2**). Therefore, these calli were further maintained and multiplied on this medium by sub-culturing at every 4 weeks interval.

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Table 3.1. Effect of various growth regulators on callus development from leaf-disc cultures of *S. paniculata*. Growth period: 4 weeks. Control: MS basal medium.

S. no	Media compositions	Percentage of leaf-disc culture developing callus*
1.	MS basal medium	0g
2.	MS+ 2,4-D (5µM)	60.13c
3.	MS+NAA(5µM)	0g
4.	MS+BAP(9µM)	80.12b
5.	MS+ Kn(5µM)	0g
6.	MS+BAP (9µM)+NAA (1µM)	22.17f
7.	MS+BAP (4µM)+2,4-D (3µM)	40.14e
8.	MS+BAP (1µM)+2,4-D (2µM)	52.54d
9.	MS+BAP (5µM)+2,4-D (5µM)	0g
10.	MS+BAP (5µM)+ NAA (5µM)	0g
11.	MS+BAP (5µM) + 2,4-D (1µM) + NAA (1µM)	100a

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

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Table 3.2. Effect of various growth regulators on callus development from flower-head cultures of *S. paniculata*. Growth period: 4 weeks. Control: MS basal medium.

S. no	Media compositions	Percentage of flower-head culture developing callus
1.	MS basal medium	0g
2.	MS+ 2,4-D (1µM)	0g
3.	MS+ 2,4-D (5µM)	72.83b
4.	MS+NAA(1µM)	35.77e
5.	MS+NAA(5µM)	0g
6.	MS+BAP(1µM)	0g
7.	MS+BAP(3µM)	0g
8.	MS+BAP(5µM)	0g
9.	MS+BAP(9µM)	100a
10.	MS+BAP (1µM)+2,4-D (4µM)	10.12f
11.	MS+BAP (2µM)+2,4-D (3µM)	15.99e
12.	MS+BAP (4µM)+2,4-D (2µM)	69.57c
13.	MS+BAP (9µM)+2,4-D (4µM)	41.31d
14.	MS+BAP (5µM)+2,4-D (5µM)	0g
15.	MS+BAP (5µM)+ NAA (5µM)	0g
16.	MS+BAP (5µM) + 2,4-D (1µM) + NAA (1µM)	100a

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

3.2. Analysis of bioactive phytochemicals from *in vitro* cell lines of *S. paniculata*

The acquired fresh, green, friable and healthy calli were utilized for extraction and analysis of *N*-isobutylamides, which have wide biological perspective towards upcoming field of medicine. The isolation, purification and characteristics of an *N*-alkylamides were performed by Preparative Thin Layer Chromatography (PTLC), High Performance Liquid Chromatography (HPLC), Mass Spectroscopy (MS) and Infra-Red Spectroscopy (IR).

3.2.1. Identification and estimation N-alkylamide production

i. Analysis of calibration curve

Using Dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide as standard, a calibration curve was generated at different concentrations (0.25 mg/ml to 0.015 mg/ml) of standards with high

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accuracy. This was efficient and reproducible. The obtained linear curve y = 0.85 x + 2.82 with its regression coefficients R^2 (0.99), where, **x** is the concentration of standard and **y** is the total peak area. It was used to quantify the amount of spilanthol, in callus cultures obtained from two different explants, leaves and flower-heads, and from parent plants as well. The precision of a developed method could be evaluated in terms of relative standard deviation (RSD) by measuring inter-and intra-days variations. The same concentrations of the standard were repeated thrice, within the same day and on alternate days with its RSD value 3.52 % and 1.74 %, respectively.

Similarly, the compound UDA was first isolated from field grown flower-head explants through TLC and then, the calibration curve was generated at different concentrations range of 1 mg/ml to 0.06 mg/ml with high accuracy and reproducibility. The obtained linear curve, y = 159.66x + 7.19, with its regression coefficients R^2 (0.99), where, x is the concentration of a standard and y is the total peak area. It was used to quantify the amount of UDA from dedifferentiated cells obtained from flower-heads as well as from flower-heads from parent plants. The precision of a developed method could be evaluated in terms of relative standard deviation (RSD) by measuring inter-and intra-days variations. The same concentrations of the standard were repeated thrice, within the same and on alternate days with its RSD value 4.33 % and 3.61 %, respectively.

ii. Analysis of *N*-alkylamides by HPLC

Spilanthol was quantified, using HPLC, from methanolic extracts prepared from undifferentiated *in vitro* calli and leaves from field grown parent plant. The sample (20 μL) was injected to flow isocratically at 0.5 $mlmin^{-1}$ with acetonitrile (93%) water (7%). A characteristic chromatogram of spilanthol was obtained at retention time 7.32 ± 0.36 (**Figure 3**). The amount of spilanthol was quantified by calibration curve generated from standard.

The semi-purified PTLC sample from flower-head explants and calli exhibited mixtures of alkylamides, which were confirmed using mass spectrometry (**Figure 4**). It was subjected to HPLC-UV method isocratically (**Table 2.4**) by adjusting the ratio of water and acetonitrile in the mobile phase for subsequent isolation and quantification of alkylamides. When the polarity of mobile phase, MilliQ water was increased to 40% (from 7%) and acetonitrile to 60%, (as mentioned in materials and method section) a chromatogram with distinct separation 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% % was

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generated (**Figure 5**). Further, the two peaks (peak 1 and peak 2) were eluted in a separate vial and utilized for the confirmation of compounds. Of various isocratic methodologies tried, MilliQ water 40% and HPLC grade acetonitrile 60% gave the best separation of two distinct *N*-alkylamides, spilanthol and UDA, from field grown flower-head and callus extracts and, hence, was used throughout the experiment. The two distinct peaks eluted in HPLC chromatogram at retention time 10.34 ± 0.10 min (peak 1) (**Figure 6**) and 13.45 ± 0.09 min (peak 2) (**Figure 7**) were collected separately in two vials to identify the compounds based on characteristic collision-induced dissociation (CID) fragmentation patterns by mass spectroscopy in positive mode due to its reproducibility.

iii. Analysis of alkylamides by mass spectrometry

The eluted peaks were collected and the presence of spilanthol was evidently confirmed based on characteristic collision-induced dissociation fragmentation patterns by mass spectroscopy in positive mode due to its reproducibility. The [MH^+ - 222] is a fragmented spilanthol with two stable fragment ions [MH^+-149] and [MH^+ - 166] where, [MH^+-149] fragment is formed by the dissociation C-N bond from the isobutylamide to lose the entire amine functional group and [MH^+-166] is 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] (**Figure 8**) (Boonen *et al* 2010; Cech *et al*. 2010).

The MS-MS spectra of purified lyophilized compounds obtained through HPLC was analyzed by fragmentation pattern of collision induced dissociation (CID) of mass spectrometer. The fraction collected at RT 10.34 ± 0.10 with its molecular ion peak at m/z 230.0 $[M+H]^+$ is UDA. The product ion $[M-56]^+$ = 174] was generated by positive ion mode of electron spray ionization by losing an entire alkyl group attached directly to the amine by the dissociation of C-N bond of an isobutylamide (**Figure 9**) (Cech *et al*. 2010)

iv. Infra-Red Spectroscopy (IR)

The two pure compounds eluted by HPLC were subjected to FTIR to understand and confirm the presence of functional groups identified *via* mass spectrometry. The compound (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide eluted at RT 10.34 ± 0.10 provided IR ν_{max} film (cm^{-1}) : 3436, 2924, 2845, 1626, 1263, 1018, 742, whereas, Spilanthol eluted at RT 13.45 ± 0.09 provided IR ν_{max} film (cm^{-1}): 3437, 2923, 2852, 1632, 1383, 1019, 743 (**Figure 10, 11**).

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v. Quantification of spilanthol and UDA from cell biomass

From the developed calibration curve (as mentioned in the materials and methods), both spilanthol and UDA were quantified. Spilanthol in undifferentiated mass of cells from leaf-disc and flower-head cultures was 1.75 ± 0.03 mg/g DW and 2.23 ± 0.04 mg/g DW, respectively. Compared to *in vitro* callus cultures, leaf and flower-head samples from field grown parent plant accumulated very low amount of spilanthol, 0.26± 0.11 mg/g DW and 0.83 ± 0.12 mg/g DW in leaves and flower-heads, respectively (**Table 3.3**). Moreover, UDA accumulation was found only in flower-heads callus cultures, which contained 4.30 ± 0.22 mg/g DW while the quantity of the same in flower heads from parent plant was higher as 5.29 ± 0.05 mg/g DW.

Table 3.3: Evaluation of *N*-alkylamides

S. No	Explants	*N*-Alkylamide	Total amount (mg/g DW)
1.	Leaf-disc callus culture	Spilanthol	1.75 ± 0.03
2.	Flower-head callus culture	Spilanthol	2.23 ± 0.04
3.	Flower-head callus culture	UDA	4.30 ± 0.22
4.	Leaves (Field grown)	Spilanthol	0.26± 0.11
5.	Flower-head (Field grown)	Spilanthol	0.83 ± 0.12
6.	Flower-head (Field grown)	UDA	5.29 ± 0.05

3.2.2. Batch kinetic studies

i. Establishment of cell suspension cultures from flower-head callus culture

The calli from flower-head explants, obtained on semi-solid medium, were utilized to raise suspension cultures in liquid medium on MS + BAP (5µM) + 2, 4-D (1µM) + NAA (1µM). The profuse and massive growth of callus (288.67 g/l) was obtained in dark within three weeks compared to that of callus (4g/l) grown on semi-solid medium.

ii. Growth curve analysis and production of *N*-isobutylamide

Fresh weight and dry weight of the cells were evaluated to demonstrate kinetics, biomass and spilanthol production from suspended cells of flower-head callus cultures. The inoculated cells were at lag phase up to 6th day. Then the culture entered into the exponential phase till 21st day. Immediately after 21st day, there was a change in colour of the cells and the

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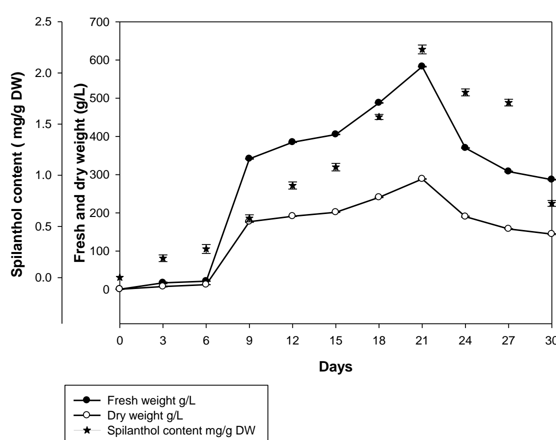
medium. It confirmed that dedifferentiated cells started dying till 30th day and devoid of stationary phase. The specific growth rate (μ) of cell biomass was calculated to be 0.296 day^{-1} during the exponential phase (**Graph 3.1**). The cells harvested at every three day interval were used to identify and quantify the amide, spilanthol. Hence, the methanolic extracts from *in vitro* cell lines were subjected to TLC for purifying particular amide at R_f 0.5 and then, yellowish dark brown band was visualized in UV chamber and, thereafter, this semi-purified sample was taken to HPLC for further analysis.

The sample was isocratically controlled to flow at 0.5 ml min^{-1} with a mobile phase acetonitrile HPLC grade 60% and MilliQ water 40% for the isolation of spilanthol. The desired compound was eluted at 13.45 ± 0.09 and the obtained characteristic dominant peak was identified as spilanthol as shown in **Figure 5e**. Then, spilanthol was quantified by calibration curve (y = 0.85x + 2.82) generated from the standard. The eluted peaks were collected and the presence of spilanthol was evidently confirmed based on characteristic collision-induced dissociation fragmentation patterns by mass spectroscopy in positive mode due to its reproducibility.



Graph 3.1. Growth kinetic curve and production of spilanthol from cell suspension cultures of *S. paniculata*

iii. Kinetics of spilanthol production

The production of spilanthol in cell suspension cultures raised from flower-head was found to be growth associated. The viable cells were highly responsible for the production of spilanthol. But, once the cells entered death phase the amount of spilanthol was lesser than even log phase. Based on Luedeking-Piret model, kinetics of spilanthol could be explained

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more clearly with the equation depends on biomass and growth rate in a linear fashion are as follows,

$r_p = dP/dt = \alpha\, dx/dt + \beta x$

where α and β are the constants of product formation. It is observed that spilanthol production was directly proportional to the growth rate. However, due to absence of stationary phase, βx was considered to be zero **(Graph 3.1)**. The total amount of Spilanthol was highest at 21st day with 2.23 mg/g dry cell weight (DCW). The extracellular production of amide was not observed in a batch culture.

iv. Effect of pH and kinetics of intake of nutrients on cell growth

The nitrate and phosphate were two major nutrients affecting the growth of cultures in suspension cultures. The nitrate from media was consumed by the cells at a slower rate than phosphate. The nitrate uptake by undifferentiated mass of cells was carried out till the end of exponential phase of 21 days, whereas, phosphate uptake was greatly increased and completely consumed before 18 days. Thus, intake of nitrate was faster from 0 to 6th day, followed by slower rate upto 18th days. Eventhough, the complete consumption of phosphate and nitrate was recorded, these two nutrients was found to be leaked at the end phase of the growth cycle.

The medium pH was altered during the growth cycle of suspension cultures. Initially, the media was adjusted to 5.8 before autoclaving for the inoculation of cells. Later, the hydrogen ion activity decreased gradually (5.8 to 4.93, 4.20, 3.43, 3.23, 3.07, 3.08, 5.45, 5.68, 5.75 and 5.85) (**Graph 3.2**). Moreover, intensity of pigmentation of media and cells were completely changed, once the suspended cells shifted to death phase.

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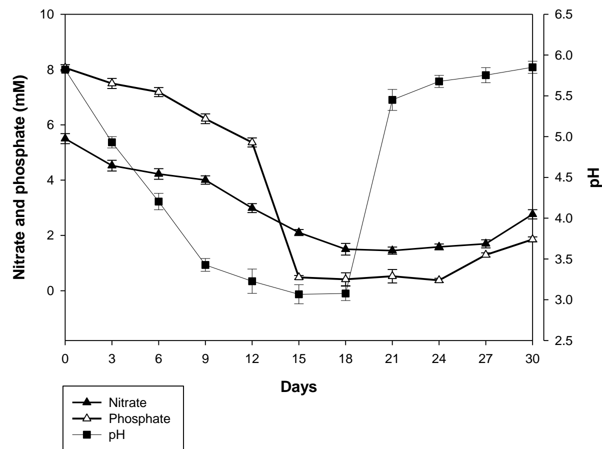
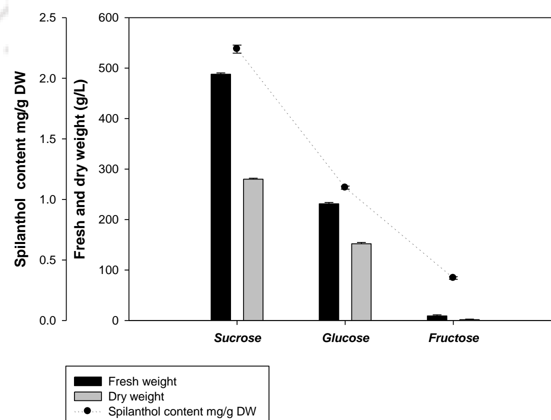


Graph 3.2. pH profile, kinetics of nitrate and phosphate consumption in cell suspension cultures of *S. paniculata*

v. Effect of carbon sources on biomass accumulation and production of spilanthol

The effect of carbon source, like disaccharide and monosaccharides, on metabolite production from cell biomass was determined. The data depicts that carbohydrates plays a major role in the metabolite production **Graph 3.3**. The maximum production of spilanthol as 2.23 ± 0.05 mg/g DW with higher fresh biomass yield of 280.10 ± 1.76 g/l was obtained when sucrose was present in the medium. The biomass production of the cultures grown on glucose supplemented medium was 153.13 ± 2.30 g/l with its metabolite yield of about 2.10 ± 0.81mg/g DW, followed by biomass production of 1.15 ± 1.09 g/l on fructose supplemented medium but with by no metabolite production.



Graph 3.3. Effect of carbohydrates on biomass and metabolite production from cell suspension cultures of *S. paniculata*

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vi. Estimation of carbohydrates using HPAEC

The carbohydrate consumption by suspended cells was analyzed using high performance anion exchange chromatography. The pulse amperometric system detected sucrose, glucose and fructose at the retention time 3.5, 4.3 and 3.1 minute, respectively. The linearity of the standard curve was used to evaluate the total consumption of carbohydrates by cell suspension cultures (**Table 3.4**). Results show that disaccharide molecule sucrose was consumed relatively at a faster rate from 0 to 3rd day, and then, sucrose was hydrolyzed to monosaccharides, glucose and fructose. The kinetics of glucose intake was found to be faster from 6th day followed by fructose at a very slower rate. Moreover, the sucrose was completely hydrolyzed before 18 days of culture. Hence, the biomass production was purely supported by the complete intake of glucose up to 21 days. But, the fructose intake by the cells continued till 30 days at a lesser rate. Thus, the kinetics of carbohydrate estimation illustrates that the plant cells consumed total carbohydrates without any storage of carbon source.

Table 3.4: Linearity curve for carbohydrate standards

S.no	Carbohydrates	Linear curve	R^2 value
1.	Sucrose	y=0.0061x+0.156	97%
2.	Glucose	y=0.0011x+0.016	98%
3.	Fructose	y=0.0903+0.3465	96%

vii. Effect of RPM on cell survival and its viability

The speed of agitation was optimized by varying RPM, as they directly affect the growth and cell viability of cultures. Of various RPM tried the highest fresh and dry cell weight, 553.69 ± 7.84 g/l and 275.10 ± 0.76 g/l, respectively, was recorded at 120 RPM (**Figure 12C; (Graph 3.4**)). On the other hand, at 60 RPM the cells formed clumps (**Figure 12A**), followed by 90 RPM (**Figure 12B**), where the cells were loosely attached in the clump. The cells agitated at 150 and 180 RPM **(Figure 12D-E)** was devoid of free floating cells due to shearing effect. However, at the lowest (60 and 90 RPM) and highest (150 and 180 RPM) agitation speed, the aggregation and shearing of cells increased which lead to the death of cells.

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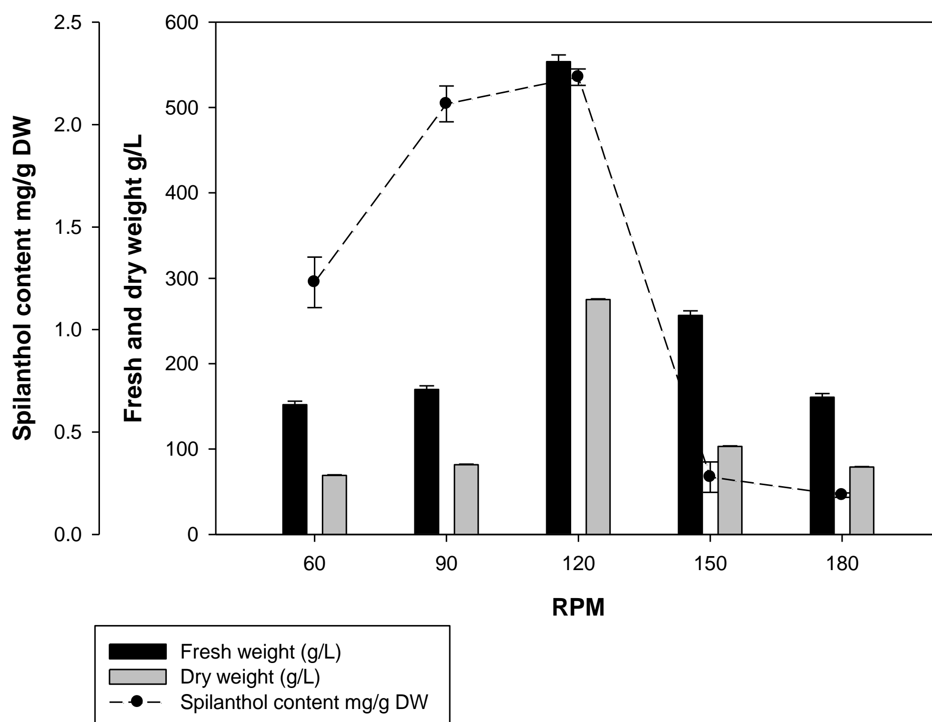

Graph 3.4. Effect of speed of RPM and production of spilanthol from cell suspension cultures of *S. paniculata*

3.2.3. Development of multivariate model using statistical tool for spilanthol production

i. Statistical optimization of media constituents from leaf-disc callus cultures

a. Screening of media constituents using Plackett-Burman (PB) design criterion

The effect of five individual factors towards spilanthol production were screened by the Plackett Burman (PB) design criterion (**Table 3.5**) and analyzed in terms of variable effects, *p*-value and *t*-value. The significance of the variables and the adequacy of the model were scrutinized by the regression analysis. Amongst five different variables, -MS major salts (X_1), sucrose (X_2), BAP (X_3), 2,4-D (X_4) and NAA (X_5)-studied, four variables -MS major salts,

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sucrose, BAP and 2,4-D were chosen due to its significant effect on spilanthol production as seen with the regression analysis (**Table 3.6**). The pareto chart highlights the degree of effect of factors where the data refer the effects on production of spilanthol in the descending order as shown in (**Figure 13**). The length of the bar is directly proportional to absolute *t*-value. The main effect plot depicts the concentration level (high/low) at which the constituents are effective in increasing the production of response. Hence, the effect of salts and sucrose was positive at high concentration, whereas the various plant growth regulators, such as 2,4-D was positive at higher level of concentration, but BAP has inverse significant effect. NAA, had no significant influence on production of spilanthol (**Figure 14**). Therefore, by neglecting the insignificant variable the first order model equation can be written as:

$$Y=5.25 + 2.78X_1 + 3.25X_2 - 2.60X_3 + 2.93X_4$$

The coefficient of determination (R^2) of the model was 0.903, where the model could be explained up to 90.3% variation of the data. The yield of spilanthol obtained from the PB design ranges from (0.05 to 2.13 mg/g DW). This indicates that further optimization is essential to obtain maximum response.

Table 3.5. Screening the level of media constituents on spilanthol production using Plackett-Burman design criterion from cell biomass of leaf-disc cultures

Run order	Major salts Low-0.75 High-1.25	Sucrose (%) Low-2 High-4	BAP (µM) Low-3 High-7	2,4-D (µM) Low-0.6 High-1.4	NAA (µM) Low-0.6 High-1.4	Spilanthol amount (mg/g DW)
1	1.25	2.00	7.00	0.60	0.60	0.05
2	1.25	4.00	3.00	1.40	0.60	2.13
3	0.75	4.00	7.00	0.60	1.40	0.26
4	1.25	2.00	3.00	1.40	0.60	0.49
5	1.25	4.00	7.00	1.40	1.40	1.54
6	1.25	4.00	7.00	0.60	1.40	0.30
7	0.75	4.00	7.00	1.40	0.60	0.46
8	0.75	2.00	3.00	1.40	1.40	0.30
9	0.75	2.00	3.00	1.40	1.40	0.26
10	1.25	2.00	3.00	0.60	1.40	0.31
11	0.75	4.00	3.00	0.60	0.60	0.41
12	0.75	2.00	3.00	0.60	0.60	0.06

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Table 3.6. Estimated effects and coefficients for spilanthol content from cell biomass of leaf-disc cultures

Term	Effect	Coefficient	T	P
Constant	0.000	5.250	6.710	0.001
MS	5.567	2.783	3.560	0.012
Sucrose	6.500	3.250	4.150	0.006
BAP	-5.200	-2.600	-3.320	0.016
2,4-D	5.867	2.933	3.750	0.010
NAA	-1.500	-0.750	-0.960	0.375

R^2 = 90.31% $R_{(pred)}^2$ = 61.25% $R_{(adj)}^2$ = 82.24%

b. Evaluation of an optimum concentration of factors influencing spilanthol production.

The combined effect was examined by the central composite design (CCD) (**Table 3.8**) for the screened independent factors obtained from PB design affecting the production of spilanthol. A CCD provided a total of thirty one experiments with $2^4 = 16$ cubic points, plus 7 central points and 2x4 = 8 axial points (α value) along with experimental and predicted value. The cubic points, central points and axial points are added to together to obtain a total of 31 experiments. The coded and uncoded values are given in the (**Table 3.7**).

Table 3.7. Media constituents and their coded and actual values used for optimization of media constituents using central composite design from cell biomass of leaf-disc cultures

Treatment of variables	Coded values				
	-2	-1	0	+1	+2
MS	0.50	0.75	1.00	1.25	1.50
Sucrose	1.00	2.00	3.00	4.00	5.00
BAP	1.00	3.00	5.00	7.00	9.00
2,4-D	0.20	0.60	1.00	1.40	1.80

The Minitab developed a design and a mathematical model, which depicts the quadratic relationship between the spilanthol production and the media constituents by applying multiple regression analysis on the experimental data, which gives the following second order polynomial coefficient equation for each term as:

Y = 1.7924 + $0.1880X_1$ (MS major salts) - $0.1555X_2$ (sucrose) - $0.1019X_3$ (BAP) + $0.2542X_4$ (2,4-D) - $0.3005X_1^2$ (MS major salts x MS major salts)-$0.2473X_2^2$ (sucrose x sucrose) - $0.2158X_3^2$ (BAP x BAP) - $0.0160X_4^2$ (2,4-D x 2,4-D) + $0.2684X_1X_2$ (MS major salts x

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sucrose) + $0.0148X_1X_3$ (MS major salts x BAP) + $0.3200X_1X_4$ (MS major salts x 2,4-D) + $0.3101X_2X_3$ (sucrose x BAP) + 0.3101 X_2X_4 (sucrose x 2,4-D)–$0.0008X_3X_4$ (BAP x 2,4-D)

The linear significance of the media constituents ($p<0.05$) MS major salts, sucrose, BAP and 2,4-D on spilanthol production are shown in (**Table 3.6**). The regression analysis determines that *p*-value corresponds to the linear effect of MS major salts and 2,4-D has positive coefficients, whereas sucrose and BAP has negative significance on the response. 2,4-D had greater impact on response with high positive coefficient (+0.25) followed by MS major salts (+0.19), while sucrose and BAP has negative linear effect with (-0.16) and (-0.10), respectively. The quadratic terms of the factors are significant with negative coefficient, nevertheless mutual interaction between 2,4-D is insignificant ($p>0.05$). Therefore, the impact of the linear effect of sucrose and BAP as well as the quadratic terms of MS major salts, sucrose and BAP on response towards spilanthol is increasing as well as decreasing when the level of all these variables are increased at certain levels. Moreover, it is quite evident that *p*-value corresponding the interaction between MS major salts vs. sucrose, MS major salts vs. 2,4-D and sucrose vs. 2,4-D were significant with positive coefficient while MS major salts vs. BAP, sucrose vs. BAP and BAP vs. 2,4-D are insignificant with positive coefficient (**Table 3.9**).

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Table 3.8. Central composite design criteria from cell biomass of leaf-disc cultures

Run order	MS major salts	Sucrose	BAP	2,4-D	Spilanthol amount (mg/g DW)		Studentized residuals
					Experimental	Predicted	
1	0.75	2	3	0.6	1.66	1.67	-0.03
2	1.25	2	3	0.6	0.81	0.86	-0.86
3	0.75	4	3	0.6	0.21	0.20	0.25
4	1.25	4	3	0.6	0.45	0.47	-0.35
5	0.75	2	7	0.6	1.40	1.46	-1.05
6	1.25	2	7	0.6	0.65	0.66	-0.25
7	0.75	4	7	0.6	0.06	-0.01	1.05
8	1.25	4	7	0.6	0.29	0.27	0.35
9	0.75	2	3	1.4	0.87	0.91	-0.67
10	1.25	2	3	1.4	1.31	1.39	-1.34
11	0.75	4	3	1.4	0.69	0.69	0.1
12	1.25	4	3	1.4	2.17	2.24	-1.12
13	0.75	2	7	1.4	0.66	0.71	-0.85
14	1.25	2	7	1.4	1.10	1.19	-1.48
15	0.75	4	7	1.4	0.45	0.48	-0.52
16	1.25	4	7	1.4	2.08	2.04	0.68
17	0.50	3	5	1.0	0.21	0.20	0.12
18	1.50	3	5	1.0	1.04	0.96	1.69
19	1.00	1	5	1.0	1.25	1.10	2.98
20	1.00	5	5	1.0	0.42	0.48	-1.17
21	1.00	3	1	1.0	0.93	0.86	1.48
22	1.00	3	9	1.0	0.47	0.45	0.32
23	1.00	3	5	0.2	1.19	1.27	-1.17
24	1.00	3	5	1.8	2.34	2.28	0.80
25	1.00	3	5	1.0	1.74	1.78	-0.57
26	1.00	3	5	1.0	1.81	1.78	0.43
27	1.00	3	5	1.0	1.94	1.78	2.25
28	1.00	3	5	1.0	1.81	1.78	0.51
29	1.00	3	5	1.0	1.76	1.78	-0.26
30	1.00	3	5	1.0	1.75	1.78	-0.32
31	1.00	3	5	1.0	1.74	1.78	-0.46
Validation model							
32	1.5	5	4.82	1.8	2.81	3.72	-

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Table 3.9. Estimated regression coefficients from cell biomass of leaf-disc cultures producing spilanthol

Term	Coefficient	*T*	*P*
Constant	1.79	59.22	0.000
MS major salts	0.19	11.50	0.000
Sucrose	-0.16	-9.52	0.000
BAP	-0.10	-6.23	0.000
2,4-D	0.25	15.55	0.000
MS major salts x MS major salts	-0.30	-20.07	0.000
Sucrose x sucrose	-0.25	-16.52	0.000
BAP x BAP	-0.28	-18.80	0.000
2,4-D x 2,4-D	-0.02	-1.07	0.302
MS major salts x sucrose	0.27	13.41	0.000
MS major salts x BAP	0.01	0.74	0.470
MS major salts x 2,4-D	0.32	15.99	0.000
Sucrose x BAP	0.01	0.63	0.539
Sucrose x 2,4-D	0.31	15.49	0.000
BAP x 2,4-D	0.00	-0.04	0.966

The adequate precision of response surface statistical model was obtained by the analysis of variance (ANOVA) to validate the regression model as shown in (**Table 3.9).** The value of F-test higher than the critical value determines the adequate variation occurred in the experimental data at the p<0.05 level of significance. The squared regression static $R^2 = 0.99$, whereas $R_{pred}^2$and R_{adj}^2 were 96.54% and 98.54% respectively, expressed the goodness of fit of the model. Thus, the variation of the model is explainable upto 99.22% and also indicates high significance of the model. When the R value is closer to the value 1, the observed and predicted values remains highly significant. The *p*>0.344 corresponding to the lack of fit precisely explains the fit of the model as shown in (**Table 3.10)** and revealed the effects of media constituents. The normal distribution plot is an important tool to diagnose the homogeneous scatter of the errors and the residuals above and below the X-axis. Thus, confirmed the normality assumption and the independence of the residuals. This plot indicates the residuals are independent of the value of spilanthol and therefore, fits the adequacy of the model **(Figure 15)** (Sanjeeviroyar *et al*., 2009).

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Table 3.10. Analysis of variance for the spilanthol production from leaf-disc *in vitro* cell lines

Source	DF	Sequential SS	Adjusted SS	Adjusted MS	*F*	*P*
Model	14	13.11	13.11	0.94	146.06	0.000
Linear	4	3.23	3.23	0.81	125.9	0.000
Square	4	5.55	5.55	1.39	216.29	0.000
Interaction	6	4.34	4.34	0.72	112.68	0.000
Lack of fit	10	0.07	0.07	0.01	1.43	0.344
Pure error	6	0.03	0.03	0.01		
Total	30	13.22				

R^2 = 99.22% $R_{(pred)}^2$ = 96.54% $R_{(adj)}^2$ = 98.54%

DF, degree of freedom; Sequential SS, Sequential sum of square; Adjusted SS, Adjusted sum of square; Adjusted MS, Adjusted mean square; *F*, *F*-statistics test to determine significance and *P*, probability.

The response surface contour plot of the mutual interaction between the factors on spilanthol production from *S. paniculata* were predicted based on the nature of shapes of contour whether elliptical, circular or saddle. The interaction between major salts *vs* sucrose are found to be elliptical, indicates high significance of variables as shown in **Figure 16A**. When the α-level of MS major salts *vs* 2,4-D was increasing the response was found to be increasing upto 2.5 mg/g DW as can be seen in **Figure 16B,** followed by sucrose *vs* 2,4-D as shown in **Figure 16C** with its regression coefficients 0.32 and 0.31, respectively. **Figure 16D-E** shows the circular nature of surface contours depicts the lesser significant of factors on response. This experimental study leads to the maximum production of spilanthol upto 2.34 mg/g DW of plant cells, which is higher than the predicted value of 2.28 mg/g DW of the cells.

c. Model validation

From the experimental data the optimum values of four media constituents obtained by resolving the second order polynomial equation were: $1^1/_2$ MS major salts, sucrose (5%), BAP (4.82 µM) and 2, 4-D (1.8 µM). To validate the model the above mentioned experiment was performed to obtain 2.81 mg/g DW from *S. paniculata* **(Table 3.8)**. The amount of production was slightly far closer to the predicted value of about 3.72 mg/g DW of cells.

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ii. Statistical optimization of media constituents used in flower head callus cultures

a. *Screening of media constituents using PB design criterion*

The PB design criterion (**Table 3.11**) was used to screen the effectiveness of five individual factors are major salts, sucrose, BAP, 2,4-D and NAA which were analyzed for spilanthol production. In order to obtain the significant variables affecting the response for maximum production, twelve different full factorial media combinations were analyzed in terms of variable effects, *p*-value and *t*-value with a maximal and a minimal range. The significance of variables and the adequacy of the model can be screened by regression analysis.

Table 3.11. Plackett-Burman design criterion for screening various factors affecting spilanthol production from flower head cell lines

Run Order	MS	Sucrose	BAP	2,4-D	NAA	Spilanthol amount (mg/g DW)
1	1.25	2	7	0.6	0.6	1.50
2	1.25	4	3	1.4	0.6	1.19
3	0.75	4	7	0.6	1.4	0.45
4	1.25	2	7	1.4	0.6	2.45
5	1.25	4	3	1.4	1.4	1.54
6	1.25	4	7	0.6	1.4	0.81
7	0.75	4	7	1.4	0.6	1.06
8	0.75	2	7	1.4	1.4	1.92
9	0.75	2	3	1.4	1.4	1.70
10	1.25	2	3	0.6	1.4	1.32
11	0.75	4	3	0.6	0.6	0.27
12	0.75	2	3	0.6	0.6	0.98

Of five variables, NAA was not significant ($p>0.05$), whereas, other four factors MS, sucrose, BAP and 2,4-D were significant ($p<0.05$) as indicated in (**Table 3.12**). Therefore, the model is clearly indicating that, amongst five different variables MS major salts (X_1), sucrose (X_2), BAP (X_3), 2, 4-D (X_4) and NAA (X_5) studied, four variables, such as MS major salts, sucrose, BAP and 2, 4-D were selected as a significant model terms for further optimization to study the interaction effects of those variables. Generally, a larger *t*-value and the lesser p-value indicate the high significance of the model term. The four considerable significant factors influencing spilanthol production was evidently proved through regression analysis with ($p<0.05$). The main effect plot shown in **Figure 17** shows that the effect of variables at the chosen maximal and minimal range, where, NAA is not effecting the production of an alkylamide either at higher or lower level of concentrations. However, MS and two growth

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regulators BAP and 2, 4-D is responding at the higher limit rather than lower level of concentration. Further, sucrose was influencing the production of spilanthol at lower level of concentration. Moreover, the degree of influence of an independent variables demonstrating the size of the effects on production of spilanthol in the descending order is described as pareto chart as shown in **Figure 18**. The length of the bar is directly proportional to the absolute *t*-test value. Therefore, by neglecting the insignificant variable the first order model equation can be written as:

$$Y = 4.44 + 0.70X_1 - 1.34X_2 + 0.35X_3 + 1.33X_4$$

The regression analysis describes the coefficient of determination (R^2), where the model was able to explain up to 97.74% variation of the data, $R(pred)^2 = 90.97\%$, $R(adj)^2 = 95.86\%$. The scope of production of spilanthol obtained from PB design varied from 0.65 to 2.23 mg/g DW. This implies that further optimization of concentration of media constituents are highly crucial to improve the maximum response.

Table 3.12. Estimated effects and coefficients for spilanthol content from flower head *in vitro* cell lines

Term	Effect	Coeff	T	P
Constant		4.442	35.050	0.000
MS	1.408	0.704	5.560	0.001
Sucrose	-2.675	-1.337	-10.550	0.000
BAP	0.698	0.349	2.750	0.033
2,4-D	2.652	1.326	10.460	0.000
NAA	0.165	0.083	0.650	0.539

$R^2 = 97.74\%$ $R(pred)^2 = 90.97\%$ $R(adj)^2 = 95.86\%$

b. Central composite design (CCD)

Improvisation of spilanthol production was done using central composite design (**Table 3.14**). Also, the examination of the interactive effect of 5 factors, obtained from PB, on spilanthol production was determined by CCD. This resulted in thirty one experiments using coded and uncoded values (Table 3.7) Statistical software developed a model which illustrates the second order polynomial equation where the relationship between spilanthol production and selected model were analyzed by multiple regression analysis.

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The experimental matrices of linear significance of the media constituents of MS major salts, sucrose, BAP and 2, 4-D on spilanthol production is developed through CCD (**Table 3.12**). The effectiveness of a linear, square and interaction variables was evaluated by the probability value lesser than 5% ($p<0.05$). The smaller p value represents the higher significance of quadrant terms corresponding to coefficients.

Table 3.13. Estimated regression coefficients influencing the spilanthol production from flower head *in vitro* cell lines

Term	Coeff	T	P
Constant	2.49	66.30	0.000
MS	0.02	0.78	0.446
Sucrose	0.08	3.82	0.002
BAP	0.04	1.93	0.071
2,4-D	0.36	17.79	0.000
MSXMS	-0.52	-28.24	0.000
Sucrose X sucrose	-0.58	-31.40	0.000
BAP X BAP	-0.56	-30.26	0.000
2,4-D X 2,4-D	-0.06	-3.22	0.005
MS X sucrose	-0.07	-2.87	0.011
MS X BAP	0.01	0.40	0.693
MS X 2,4-D	-0.05	-2.11	0.051
Sucrose X BAP	-0.01	-0.25	0.805
Sucrose X 2,4-D	0.03	1.06	0.306
BAP X 2, 4-D	0.06	2.32	0.034

Therefore, amongst linear effect of individual variables, p-value corresponds to 2,4-D had greatest effect on Spilanthol production with positive coefficient (0.26) followed by sucrose with its coefficient (0.08) and probability ($p<0.002$). Of four linear effects, MS and BAP had its ($p>0.05$) with its coefficient (0.02 and 0.04), respectively and also recorded to be insignificant. The regression analysis determined that interaction effect of individual factors of MS major salts, sucrose, BAP and 2, 4-D corresponds to p-value lesser than 0.05 with -0.52, -0.58, -0.56 and -0.06 coefficients respectively. In terms of mutual interaction effect of 2,4-D between variables of major salts and sucrose, MS and 2,4-D, BAP and 2,4-D had larger impact on response with ($p<0.011$, $P<0.051$ and $p<0.034$) with its coefficients values (-0.07, -0.05 and 0.06), respectively. Other interaction effects are not statistically significant which has p-value greater than 5% ($p>0.05$) with coefficients of MS with BAP (0.01), sucrose with

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BAP and 2, 4-D (-0.01 and 0.03) respectively (**Table 3.13**). Thus, an experimental data which gives the following quadratic second order polynomial coefficient equation for each term as:

$Y=2.49+0.02X_1+0.08X_2+0.04X_3+0.36X_4-0.52X_1^2-0.58X_2^2-0.56X_3^2-0.06X_4^2-0.07X_1X_2+0.01X1X3-0.05X_1X_4-0.01X_2X_3+0.03X_2X_4+0.06X_3X_4$

The goodness of fit and adequate precision of the mathematical model could be determined by analysis of variance (ANOVA) to validate the quadratic regression model as shown in **Table 3.13**. The coefficient of determination R^2 = 99.39%, predicted R^2 = 97.12% and adjusted R^2 = 98.86% indicates the variation of media constituents towards Spilanthol production is attributed to the individual factors. This clearly implies that fit of the model could explain upto R^2 = 0.99 along with F-statistics test value (186.04) more than the critical value determines the adequate variation occurred in the experimental data with probability (p<0.05). In addition, the second order polynomial well fitted to a model, as the variances of lack of fit value is lesser than the tabulated F value with P-value greater than 5% (0.217) as shown in **Table 3.15**. This exhibited that mathematical matrices model is found to be satisfactory on the effect of media constituents on spilanthol production. The distribution of an errors and residuals above and below X-axis could be determined by a vital tool normal distribution plot. In consequence, confirmed the suitability of multivariate matrices model and also independence of residuals. This normal plot indicated that residuals were independent of the value of spilanthol and, therefore, fits the adequacy of the model (**Figure 19**)

The response surface plot (**Figure 20**) demonstrates the mutual interaction between the factors on spilanthol production from flower head callus cultures of *S. paniculata.* The interaction between variables could be better explained by different shapes of the surface plots like elliptical, circular and saddle points (Haider *et al*. 2007). Firstly, **Figure 20** represents that mutual interaction between the variables, like MS and sucrose, MS and 2, 4-D, BAP and 2, 4-D had higher significance and maximum response within the experimental region with slight appearance of elliptical shape on spilanthol production. Secondly, **Figure 20B & D** illustrated that mutual interaction between MS and BAP and

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Table 3.14. Central composite design criteria from cell biomass of flower head cultures

Run Order	MS	Sucrose	BAP	2,4-D	Spilanthol content mg/g DW Experimental	Spilanthol content mg/g DW Predicted	St Residuals
1	0.75	2	3	0.6	0.26	0.23	0.47
2	1.25	2	3	0.6	0.41	0.49	-1.23
3	0.75	4	3	0.6	0.39	0.49	-1.52
4	1.25	4	3	0.6	0.42	0.46	-0.65
5	0.75	2	7	0.6	0.06	0.19	-1.96
6	1.25	2	7	0.6	0.56	0.49	1.17
7	0.75	4	7	0.6	0.53	0.42	1.74
8	1.25	4	7	0.6	0.41	0.43	-0.35
9	0.75	2	3	1.4	0.84	0.89	-0.77
10	1.25	2	3	1.4	0.91	0.94	-0.44
11	0.75	4	3	1.4	1.26	1.25	0.13
12	1.25	4	3	1.4	1.07	1.02	0.84
13	0.75	2	7	1.4	1.20	1.08	1.95
14	1.25	2	7	1.4	1.19	1.16	0.40
15	0.75	4	7	1.4	1.42	1.41	0.12
16	1.25	4	7	1.4	1.27	1.22	0.83
17	0.5	3	5	1	0.36	0.36	0.01
18	1.5	3	5	1	0.41	0.42	-0.19
19	1	1	5	1	0.02	0.01	0.3
20	1	5	5	1	0.28	0.31	-0.48
21	1	3	1	1	0.27	0.16	1.68
22	1	3	9	1	0.20	0.32	-1.86
23	1	3	5	0.2	1.61	1.53	1.26
24	1	3	5	1.8	2.88	2.97	-1.44
25	1	3	5	1	2.41	2.49	-0.87
26	1	3	5	1	2.53	2.49	0.43
27	1	3	5	1	2.40	2.49	-0.98
28	1	3	5	1	2.61	2.49	1.3
29	1	3	5	1	2.45	2.49	-0.43
30	1	3	5	1	2.56	2.49	0.76
31	1	3	5	1	2.47	2.49	-0.22
Validation of a model							
32	0.97	3.10	5.28	1.8	2.94	2.99	-

sucrose and BAP is highly insignificant with circular shape followed by **Figure 20E**, the interaction effect of variables, sucrose and 2, 4-D had a response outside the experimental region with no effect on production of a response factor Spilanthol. Thus, the experimental

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quadratic model was used to obtain a Spilanthol of about 2.88 mg/g DW from flower head callus cultures of *S. paniculata*.

Table 3.15. Analysis of variance for Spilanthol production from flower head *in vitro* cell lines

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Model	14	25.72	25.72	1.84	186.04	0.000
Linear	4	3.31	3.31	0.83	83.85	0.000
Square	4	22.21	22.21	5.55	562.45	0.000
Interaction	6	0.19	0.19	0.03	3.23	0.028
Lack-of-fit	10	0.12	0.12	0.01	1.93	0.217
Pure error	6	0.04	0.04	0.01		
Total	30	25.88				

R^2 = 99.39% R^2(pred) = 97.12% R^2(adj) = 98.86%

c. *Model validation*

The media combinations of MS major salts (0.97), sucrose (3.10%), BAP (5.28µM) and 2, 4-D (1.8 µM) resulted into the predicted value of 2.99 mg/g DW of spilanthol to validate multivariate regression model. The optimum values of these four media constituents gave 2.94 mg/g DW of spilanthol (**Table 3.14**). The independent residual obtained through normal distribution and lack of fit ($p>0.05$) increased the production of spilanthol from 2.88 mg/g DW to 2.94 mg/g DW.

3.3. Bioassays

3.3.1. Antimalarial activity of an isolated compound against malarial parasites

The crude extract, TLC semi-purified extract and HPLC purified UDA and spilanthol extracts were tested for erythrocyte antimalarial activity, as schizonticidal and parasiticidal compound. The crude extracts and semi-purified extracts, obtained from callus cultures and flower-head explants, showed biological activity against the parasite in an initial experiment. 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 IC_{50} value of 14.64 ± 0.35 µg/ml and 23.22 ± 0.59 µg/ml, respectively (**Table 3.16**). Both, UDA and spilanthol, also demonstrated prominent parasiticidal activity with IC_{50}value of 16.42 ± 0.39 µg/ml and 17.72 ± 0.16 µg/ml, respectively. The treated parasitized cells displayed sick appearance with dead rings (**Figure 20**). Overall, UDA exhibited a higher antimalarial activity over spilanthol. In another

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experiment, the parasite was treated with a mixture containing 3:1, 1:1 or 1:3 proportions of UDA and spilanthol (**Table 3.17**). However, purified UDA showed superior parasiticidal activity against *Pf* 3D7 strain over spilanthol and the combinations of isolated compounds.

Table 3.16. The parasiticidal and schizonticidal activity of methanolic crude and HPLC purified compounds from *in vitro* cultures of *S. paniculata*

S. no	Plant extracts/ Compound name	Schizonticidal activity IC_{50} (µg/ml)	Parasiticidal activity IC_{50} (µg/ml)
1.	UDA	14.64 ± 0.35	16.42 ± 0.39
2.	Spilanthol	23.22 ± 0.59	17.72 ± 0.16

Table 3.17. Combinatorial effect of purified extracts from *S. paniculata* towards antimalarial activity

S. no	Compound Mixture	Schizonticidal activity IC_{50} (µg/ml)	Minimum Killing concentration (MKC) IC_{50} (µg/ml)
1	UDA:Spilanthol = 3:1	17.01 ± 0.61	17.8 ± 0.42
2	UDA:Spilanthol = 1:1	18.35 ± 0.59	19.55 ± 0.3
3	UDA:Spilanthol = 1:3	21.58 ± 0.64	21.63 ± 0.79

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Chapter 4

Discussion

Worldwide, insect-borne diseases are one of the major causes for most of the illness and death. Generally, malaria, lymphatic filariasis, chikungunya, yellow fever, Japanese encephalitis are caused by vectors leading to death every year (Prathibha *et al*. 2014). For the past many decades several people have been affected by malaria in endemic areas (Kumar *et al*. 2014). There are 207 million confirmed cases of malaria and 627 million deaths globally, including young children (WHO, 2012), until the existence of few anti-malarial drugs in tropical and sub-tropical countries. Of various protozoan parasites, predominant *Plasmodium* species, such as *Plasmodium. falciparum* and *P. vivax* transmitted by female *Anopheles* mosquitoes can occur sympatrically in human population and within infected individuals (Krogstad, 1999; Service and Townson, 2002). Lack of immunity may favour the infectious disease malaria greatly in pregnant women and infants. In India, malarial incidences are fluctuating between 2-3 million per year which contribute to nearly 40% of all cases outside African countries. To overcome this disease, plant derived secondary metabolites can be utilized for the development of effective anti-malarial drugs. Malarial drugs obtained from plant sources are limited to quinine, antifolates and artemisinin derivatives, which impart a therapeutic potential on infection. The inconsistency of drug resistance against complex diseases urgently need attention for the development of attentive, affordable, resistant and putative drug combination which can counteract the parasite adaptability (Grellier *et al*. 2008; Saleh *et al*. 2013).

Since 5000 BC, the importance of natural constituents from plant species are renowned from various civilizations includes Hindu, Greek, Egyptian, Chinese, Mesopotamian, Biblical and many other archaic scripts or societies. In recent years, medicinal plants are recognized to be a vital bioactive metabolite resources for the development of lead compounds in the field of medicine for illness and public health care. The World Health Organization also assessed that majority of the population are greatly dependent on resurgence of natural products obtained from plant species due to their enormous survival and benefits (Tiwari *et al*. 2010).

In consequence, vincristine, vinblastine, paclitaxel and artemisinin drugs are prepared solely from natural plant source. However, overuse of the plant may cause depletion of plant biodiversity or germplasm. It is also a time consuming process, which requires development

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of plant system in field for its utilization as herbal medicine (Dhami and Mishra, 2015). Further, geographical regions and environmental fluctuations also affect the type and concentration of phytochemical constituents. These uncertainties during pre- and -post harvesting of plants primarily can unveil spatio-temporal fluctuation in the chemical profile and intensify the risk of usage of metabolites in various fields (Foster *et al*. 2011). This is a big challenge for the development of quality assessed natural phytochemical products. Therefore, to ensure safety use of effective medicinal plants, there is an urgent need for the development of plant source for constant supply of biologically active secondary metabolites. Thus, plant cell culture can serve as an alternative method for the conservation of germplasm as well as for constant production of medicinal metabolites.

4.1. Tissue culture studies

Since 2002, micropropagation in *Spilanthes* was carried out by using varying auxins and cytokinins in MS based medium. The development of roots from shoots has been accomplished mainly by using half strength MS medium supplemented with auxin and/or cytokinin. Sometimes rooting has been achieved in the medium devoid of growth regulators (Saritha *et al*. 2002; Haw and Keng, 2003; Leng *et al*. 2004; Saritha and Naidu, 2008; Pandey and Agrawal, 2009; Singh *et al*. 2009a; Singh *et al*. 2009b; Singh and Chaturvedi, 2010; Singh and Yadav, 2010; Singh and Chaturvedi, 2012; Sharma and Shahzad, 2013; Niratker *et al*. 2014).

Callus is a mass of actively dividing undifferentiated cells obtained from *in vitro* plant tissues when grown on nutrient medium. These callus cultures are biosynthetically totipotent, which means that they retain the complete genetic information to produce the range of chemicals found in the parent plant. It is an attractive alternative to whole plant extraction for the production of high-value secondary metabolites (Rao and Ravishankar 2002; Ravishankar et al. 1999; Dornenburg and Knorr 1997; Scragg, 1997; Alfermann and Petersen 1995).

For the first time, the young leaf and flower-head explants were being used to establish callus cultures from *S. paniculata*. The young explants are good material to initiate in vitro cultures since their cell wall is physiologically and biochemically active in nature (Mishra and Bhatnagar, 1995). The leaf-disc cultures provided good biomass of cells when grown on MS medium supplemented with either BAP or 2,4-D. When plant growth regulators are supplemented in combinations, it helped to develop moderate amount of callus and the biomass produced was at par with that produced in the medium containing the growth

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regulators individually. Therefore, appropriate concentrations of both auxin (2,4-D or NAA) and cytokinin (BAP) were supplemented in media to maximize the production of cell biomass. Similarly, flower-head explants developed good biomass of cells when media was supplemented with at least one auxin (2,4-D/NAA) or one cytokinin (BAP). However, when PGR were supplemented in combinations, it yielded good cell biomass. The best combination which gave 100 % response to yield cell biomass from flower-head callus was observed to be MS+ BAP (5µM) + 2,4-D (1µM) + NAA (1µM).

The calli generated from the two explants, leaf-discs and flower-heads, were subcultured to increase the biomass. However, during subculture, browning of calli or exudation of phenolics in the medium was observed. The phenolic substances exudate from the cut ends leading to the browning of tissues or callus cells. Browning can be avoided by using adsorbing agents, such as activated charcoal (AC), polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP). Use of antioxidants in the medium or soaking the explants in antioxidant solution is other alternatives. However, the use of additives may have adverse effect on metabolite production. Frequent transfer of explants to fresh medium at particular intervals or sealing the cut ends of the explants with paraffin wax can be practiced to prevent harmful effects generated by browning (Naik *et al*. 1999; Rout *et al*. 1999; Wu and Du Doit, 2004). In the present study, the browning of calli was resolved by frequent subculture of calli on fresh composition of the same medium constituents. After three to four passages fresh, friable and cream and greenish callus lines was obtained from leaves and flower-head explants. In the same way, auxin and cytokinin favoured the development of callus from nodal segments of *S. acmella* and *S. paniculata* (Yadav and Singh, 2010; Varsha and Ghulam, 2013; Perez-Parson *et al*. 1994, Čellárová and Kimakova, 1999). The accumulation of secondary products in callus cultures are completely depends on the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources.

4.2. Analysis of secondary metabolites from *in vitro* cultures

Spilanthes species encompasses wide variety of biologically active phytochemicals, which are largely due to the presence of abundant amount of alkylamides in field grown plants (Nakatani and Nagashima, 1992; Prachayasittikul *et al.* 2009; Bae *et al*. 2010). The demand of these medicinal alkylamides is increasing due to varied ethnopharmaceutical uses. The plant cell cultures can serve as an alternative method for constant production of these metabolites throughout the years to meet the demand. Consequently, whole plant extraction

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and change in profile of bioactive compounds due to various climatic and environmental instabilities can be avoided. Till date, no reports are available on metabolite production, specifically alkylamides from *in vitro* cultures of *S. paniculata*. The present study describes the screening of *N*-alkylamides from *in vitro* cultures, established from leaf-disc and flower-head explants, using simple and high-throughput analytical methodologies, evaluation of metabolites through batch culture study and also the assessment of secondary products by varying media constituents for higher production of phytochemical compounds.

An important analytical technique, HPLC-UV method was utilized to isolate secondary product, spilanthol, from the undifferentiated mass of cells obtained from leaves and flower-head explants of *S. paniculata*. The two explants were selected for cell biomass growth based on the previous studies whereby Singh and Chaturvedi (2012a) reported the yield of spilanthol from leaf-disc callus cultures of *S. acmella* and Leng *et al*. (2011) noticed its presence in filed grown inflorescence.

The spilanthol was analyzed through two-step process. Initially, preparative thin layer chromatography (PTLC) is attempted to isolate spilanthol followed by HPLC-UV method. The collected sample from PTLC showed mixtures of alkylamides, which is confirmed through mass spectrometry. Further, the same sample was used for the separation of compounds through HPLC. When, the polarity of solvent increased, two important compounds such as spilanthol and (2*E*,4*Z*)**-***N***-**isobutyl-2,4-undeca-8,10-diynamide (UDA) are separated from the field grown as well from the unorganized cultures of flower-head explants **(Figure 5)**. However, when polarity is increased beyond optimum separation limit, merging of peaks in chromatogram is observed. Previously, structural elucidation, isolation and identification of organic biomolecules moieties, alkylamides are determined from *Spilanthes* species using combinatorial analytical techniques NMR, MS and GC-MS. However, these approaches have few disadvantages, such as lengthy purification, identification of mixtures, which simultaneously leads to lack of quantification of desired organic compounds (Yasuda *et al*. 1980; Borges-Del-Castillo *et al.* 1984; Jondiko, 1986; Nakatani and Nagashima, 1992, Ramsewak *et al.* 1999; Molinatorres *et al*. 1996; Stashenko *et al.* 1996).

The interesting findings of two phytochemical constituents clearly indicated that it consists of carbonyl group and long alkyl chain conjugated with amide. Moreover, it is devoid of any aromatic functional groups. After appropriate confirmation of spilanthol from cell biomass of leaf explant as well as the presences of spilanthol and (2*E*,4*Z*)**-***N***-**isobutyl-2,4-undeca-8,10-diynamide from flower-heads, it is estimated for total content of *N*-alkylamides. From cell

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biomass of leaf, spilanthol was quantified as 1.75 ± 0.03 mg/g DW, which was higher than those present in leaves (0.26 ± 0.11 mg/g DW) from field grown plants. Similarly, the spilanthol was also quantified from the flower-head callus cultures as 2.23 ± 0.04 mg/g DW, which was higher than that obtained from cell biomass of leaves and flower-head of field grown parent plants (0.83 mg/g DW). Additionally, another compound (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide (UDA) was also quantified from cell biomass of flower-head (4.30 ± 0.22 mg/g DW) as well from flower-head from field grown plants (5.29 ± 0.05 mg/g DW). For the first time, these two compounds were screened and quantified from the flower-head *in vitro* cell biomass of *S. paniculata*.

Generally, the yield of secondary product *N*-alkylamides content from various plant species differ based on geographical distributions, mode of growth and cultivation of the plant system. The selection of plants parts, use of fresh and dry materials as well as the extraction procedures also has a greater impact on production of secondary metabolites (Cech *et al*. 2006; Bae, 2007; Li *et al*. 2007; Spelman *et al*. 2009). But, use of alteration in concentrations of media constituents with growth regulators can also play a significant role in the production or concentrations either in the increment or declination of secondary metabolite from intact cells when compared to that of field grown plants (Dicosmo and Towers 1984; Moreno *et al.* 1995; Jaleel *et al*. 2009; Nagella and Murthy, 2010; Anuradha *et al*. 2010). The selection of plant part and type of *in vitro* cell lines also show variations in the production of secondary metabolites (Łuczkiewicz and Głód, 2005; Lucchesini *et al*. 2009).

In the past two decades, interest on meta-group of drug analysis is increasing due to its importance in the biomedical field for the discovery of new lead compounds. Although, significance of *N*-alkylamides has driven the attention to identify the chief compound spilanthol and other plant chemicals (Ramsewak *et al*. 1999; Prachayasittikul *et al*. 2009). There are few other reports on quantification of spilanthol and identification of other derivatives of alkylamides (Boonen *et al*. 2010; Cech *et al*. 2010; Sharma *et al*.2010).

In the current study, cell suspension cultures of *S. paniculata* were also raised which yielded good cell biomass and spilanthol production. Doubling time of cells under dark condition started approximately at the end of 6th day and the cells grew up to 21st day. The rate of cell growth was indicated in terms of dry cell weight (DCW). There was a sharp decrease in the growth of cells immediately after 21st day (after exponential phase) indicated that it devoid of stationary phase. The cell suspension cultures from *S. acmella* were established by Singh and Chaturvedi (2012c) where the exponential phase of cells was observed up to 18 days. When

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compared to growth cycle of herbs, like *Spilanthes*, the growth cycle of woody species are very slow with doubling time of 30 to 40 days (Liu *et al*., 1990; Uden *et al*., 1990. Usually the plant cells take a few time to adapt to new environment, which can be stated as lag phase of suspension cultures. Further, when cells trend towards exponential phase, it proliferates massively to produce the desired secondary products (Ouyang *et al*., 2005; Liu *et al*., 2007). Thus, high dependency of log phase of plant cells on nutrient medium supplemented with plant growth regulators (PGRs), nitrogen and carbon sources, accumulated enormous cell biomass and secondary products. Similar works on various plant species, such as *Cistanche salsa*, *Artemesia annua* are also reported (Dornenburg and Knorr, 1995; Sivanandhan *et al*., 2013; Baldi and Dixit, 2008). Moreover, in the present study, productions of metabolite from viable cells of *S. paniculata* are observed to be growth related with higher spilanthol content (2.23 mg/g DW). Similarly, *Lantana camara* (Srivastava et al., 2011) and *Panax quinquefolium* (Kochan and Chimel, 2011) also produced growth associated phytochemicals. However, Fetto-Netto demonstrated that taxol produced from *T. cuspidata* is non-growth related (Fetto-Netto et al., 1993a). Later, in the present study, throughout the growth curve, the product was endogenously accumulated in intact cells. However, in few other species phytochemicals, like terpenoids and other secondary products, are observed exogenously and leached out into the culture medium (Renaudin and Guern, 1990, Balbeuna *et al*., 2009).

The current study, indicated that the consumptions of sucrose and phosphate in the exponential phase of cell growth cycle was rapid followed by nitrate. Moreover, sucrose served as an ideal energy source for biomass accumulation and secondary metabolite biosynthesis in cell cultures. Depending upon the plant species, supplement of sucrose concentration in *in vitro* cultures varies specifically with respect to the production of bioactive metabolites (Rao and Ravishankar, 2002). Sucrose affects osmotic pressure of cells and also stimulated the mitochondrial activity for synthesis of secondary products (Su, 1995). This was confirmed in cell suspension cultures of *Panax ginseng* augmented with 3% sucrose to produce ginsenoside (Lian *et al.,* 2002). However, in few species higher amount of sucrose favoured the production of secondary metabolite. But, in few other species lesser amount of sucrose or no sucrose favored the metabolite production in cell cultures (Knobloch and Berlin 1980; Naik *et al*., 2010).

Similarly, same concept was attempted in the flower-head suspension cultures of *S. paniculata* to yield spilanthol, where higher amount of metabolite is observed in the medium complemented with sucrose followed by glucose. However, no metabolite is produced in

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medium containing fructose. The suspended cells starved for carbohydrates during the growth phase of cell cycle as hydrolysis of disaccharide has been analyzed by HPAEC at the end of third day which lead to the accumulation of glucose and fructose. The hydrolysis of carbohydrates seems to be catalyzed by a wall-bound or extracellular acid invertase (Masuda et al., 1988). The estimation of sugar from field grown plant parts of olive is determined by anion exchange chromatography. As the cells are starves, carbohydrates get completely depleted resulting in cell death. This has been documented in several species, such as *Taxus cuspidata* (Fetto-Netto *et al*., 1993b), *Lantana camara* (Srivastava et al., 2011) and *Spilanthes acmella* (Singh and Chaturvedi, 2012c). The reason being that sucrose in the medium gets hydrolyzed into glucose and fructose. The cells prefers uptake of glucose as a primary source followed by fructose. Glucose is consumed by the cells faster while the fructose is metabolized at a slower rate. It resulted into starvation like situation in the cell suspension and causes the cells to enter into the death phase of growth cycle (Srivastava et al., 2011).

Phosphate and nitrate are the two most important medium components, which affect cell physiology and metabolism in *in vitro* plant cell cultures (Wen and Zhong, 1997). Uptake of phosphate by suspended cells of *S. paniculata* was completely utilized before 18 days. Intake of phosphate was faster upto six days, but nitrate uptake was slower than phosphate (upto nine days). Compare to this, in *S. acmella* and *T. cuspidata* cell suspension cultures, intake of phosphate was much faster upto three days and nitrate was upto six days (Fetto-Netto et al., 1993b; Singh and Chaturvedi, 2012c). The change in pH of medium may be due to ammonium consumption of cells by trending towards alkanization. This phenomenon was due to the formation of OH^- ion equivalents and release of H^+ ions which lead to the acidification of medium. Additionally, deterioration of cells at the death phase of cell cycle is probably due to depletion of medium nutrients. The trend towards a leakage of phosphate into the medium immediately after the exponential phase could be related to cell lysis of a small population of cells. However, better performances of cultures were obtained due to carbohydrate source- sucrose and nitrogen and phosphate salts concentrations in medium (Bonga and Ardekas, 1992; Marschner, 1986).

The agitation speed for cell suspension culture of *S. paniculata* was optimized as 120 RPM. Similar agitation speed of cell suspension cultures of a few other plant species are reported (Singh and Chaturvedi, 2012c; Srivastava *et al*., 2011). Shaking of cell suspension culture has a greater impact on viability of cells. Therefore, viability of living cells was tested by staining

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the cells with fluorescein diacetate (FDA) which gave coloration due to the release of fluorescein by the esterase activity *via* the entrance of non-polar dye into the cells. Fluorescein diacetate is found to be the optimal dye for staining viable plant cells. In the plant cell, they are hydrolyzed to highly polar fluorescent compounds. Because of their polar nature, these compounds are unable to diffuse across the plasma membrane and are retained within viable cells, producing an intense green fluorescence within the cytoplasm (Huang et al., 1986).

For the first time, the media optimization was done in the current work using Plackett-Burman (PB) design (Plackett and Burman, 1946) and central composite design (CCD). Using PB design, the leaf-disc callus cultures was influenced by the factors sucrose with its highest *t*-value followed by 2,4-D and MS, while BAP obtained negative *t*-value on spilanthol production. Similarly, flower-head callus cultures was affected by 2,4-D with greater positive *t*-value followed by MS and BAP, whereas, sucrose had a negative *t*-value on improvement of spilanthol production. This statistical model using two-level multifactorial design overcame single factor optimization by maintaining other factors unchanged at a time. PB design helped to reduce the number of experiments and time to found the optimal level of the significant factor using RSM based CCD to achieve best conditions (Sanjeeviroyar *et al*. 2009; Karemore *et al*. 2013).

Therefore, all four significant factors obtained from PB design are subjected through CCD to understand the interaction effects on metabolite production of spilanthol from both leaf-disc and flower-head cell cultures. Initially, interaction effect of independent variables through pseudo-second-order kinetics using CCD provided 2.34 mg/g DW from leaf-disc callus cultures. Firstly, the interaction effect of MS with 2,4-D has strong positive effect which is affecting the production of spilanthol with highest *t*-value. Secondly, sucrose with 2,4-D followed by MS with sucrose also has strong positive interaction effect supporting spilanthol production from leaf-disc callus cultures. Further, the interaction effect of MS with BAP, sucrose with BAP has positive effect with low *t*-value and BAP with 2,4-D has negative effect with negative *t*-value. However, these interaction effect of MS with BAP, sucrose with BAP and BAP with 2,4-D are insignificant and did not affect the leaf-disc cultures on production of spilanthol. This was analyzed by regression model. Similarly, the interaction effect of independent variables through pseudo-second-order kinetics using CCD provided 2.88 mg/g DW from flower-head dedifferentiated cell cultures. This is due to the fact that square model of sucrose had strong negative effect with highest *t*-value which has greatest

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influence on production of spilanthol followed by BAP, MS and 2,4D. Further, the interaction effect of major salts with sucrose has strong negative on flower head cultures on spilanthol production with highest *t*-value. In addition, BAP with 2,4-D has strong positive effect on production of spilanthol. Further, the interaction effect of major salts with 2,4-D has strong negative effect with high *t*-value which also have largest impact on increase of spilanthol. Moreover, production of spilanthol from both leaf-disc and flower head cultures are comparatively higher than the production obtained through PB design.

The significance and the effect of variables on improved spilanthol production were evaluated due to *t*-value and *p*-value. The higher *t*-value and lesser *p*-value determine the model significant with the corresponding coefficient value (Mukherjee *et al*. 2008). However, statistically significant variables can be determined by analysis of variance (ANOVA) for testing the null hypotheses on the factors of model (Montgomery, 1991). The higher Fisher's *F*-value is evident enough to indicate about the importance of factors affecting spilanthol production by regression model (Montgomery, 1991). The linear, square and interaction terms are considered to be significant of regression model at the confidence limit greater than 95% ($P< 0.05$) (Montgomery, 1991; Singh *et al*. 2010).

The normal probability plot demonstrates that errors were distributed normally and the variance was also independent of spilanthol production. Thus, the adequacy of model was well supported towards response target by equal scattering of residual errors on its axis. Outliers of significant variables affecting the response fall within the experimental α value or star points. According to the mathematical model, studentized residual varies with respect to dependent and independent variables (Anderson and Whitcomb, 2005). The statistical tool validated a mathematical model through response optimizer to find out optimal concentrations of significant independent variables. From the experimental data, the optimum values of four medium constituents were obtained by resolving the second order polynomial equation. Under optimal concentrations of medium constituents, leaf-disc callus cultures yielded 2.81mg/g DW of spilanthol, which was closer to predicted value of 3.72 mg/g DW of cells. Compare to this, before optimization, leaf-disc callus cultures produced 1.75 mg/g DW of spilanthol which was higher than leaves of field grown parent plant as 0.26 mg/g DW. Similarly, under optimal conditions, flower-head *in vitro* callus cultures yielded 2.94 mg/g DW of spilanthol with its predicted value 2.99 mg/g DW whose desirability value is equal to 1. Haaland (1989) and Kaushik *et al*. (2006) illustrated that mathematical multivariate model, the R^2 value of linear model and second order model was very close to 1, which indicated the

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statistical significant and the strength of the model, as the regression value was ideally between 0 and 1. The adjusted R^2 value refers high significance of model, as the values were observed to be adequate in representing the actual relationship between responses (Box *et al*. 1978; Rahman *et al*. 2010).

4.3. Biological assay

The genus *Spilanthes* is widely documented for its traditional value and very few reports are scientifically distinct to record about the therapeutic activities from the plant growing wildly. Moreover, most of the workers had experienced various potent activities from the extracts of *Spilanthes* species (Tiwari *et al*. 2011). However, the bioactive compounds from *in vitro* cell cultures from *S. paniculata* against parasites causing infectious diseases are not yet revealed. The current study is the first report to investigate the significant schizonticidal and parasiticidal activity of crude extracts and the isolated *N*-alkylamides from *in vitro* unorganized cultures of *S. paniculata*. Therefore, the methanolic crude extracts and two isolated compounds spilanthol and (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide(UDA) were used to examined the anti-malarial activity 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 biological activity against the parasite Iin initial experiment. 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. 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 ± 0.16µg/ml, respectively. Treated parasite culture displayed dead or sick parasites with dead rings. In comparison, control culture treated with solvent is giving healthy parasite with all stages (Fig. 21, marked with black arrow). Overall, UDA exhibits higher antimalarial activity 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. A very strong synergistic effect of UDA and spilanthol was observed. However, UDA alone showed distinctly superior schizonticial and parasiticidal activities against *Pf3D7* strains. Spelman and co-workers also

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attempted anti-malarial activity by using isolated spilanthol and the commercial compound undeca-2E-ene-8,10-diynoic acid isobutylamide against the chloroquine-resistant K1 strain and mildly chloroquine-resistant strain PFB of *Plasmodium falciparum*. However, the activity of isolated compound spilanthol was reported to be better when compared to commercial compound undeca-2E-ene-8,10-diynoic acid isobutylamide against chloroquine-resistant K1 strain of *Plasmodium falciparum*. Later, higher activity of spilanthol was reported against mildly chloroquine-resistant strain PFB of *Plasmodium falciparum* (Spelman *et al*. 2011). Likewise, centrifugal partition chromatography (CPC) fractions of *S. acmella* exhibited superior anti-plasmodial activity against chloroquine sensitive strains of *P. falciparum* than purified alkylamides However, the activity was observed to be lesser when the parasites were treated by the purified compounds (Mbeunkui *et al*. 2011). In the same way, complete lethality against late third/ fourth instar larvae was achieved at the minimal doses from flower head crude hexane extracts of *S. acmella* rather than *S. Calva* or *S. paniculata* against malarial and filarial vectors (Pandey *et al*. 2007). Indeed, the herbal medicinal products (HMPs) from the medicinal plant material can promptly cause notable variation in the chemical profile due to geo-biological sources, harvesting techniques, post-harvest processing, storage conditions, and recurrent use of pesticides, frequent adulteration and microbial contamination. These significant factors incur routine complications in determination of potency, purity, consistency, therapeutic efficiency and safety of HMPs. This causes severe problems in the effective use of plant chemicals, quality of the product of medicinal herbs and consumer's safety (Sidhu *et al*. 2011).

In the present study, *N*-alkylamides isolated from leaf and flower-head callus extracts of *S. paniculata* afforded a great challenge to combat malaria significantly. It is also increasing the scope to treat other communicable infectious diseases. The increment of anti-malarial activity by the interaction of isolated compounds suggests the possibility for development of new combinatorial drug formulations against the life threatening diseases.

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Chapter 5

Conclusion and Future prospects

The present study emphasized the importance of *S. paniculata as* an herbaceous medicinal plant. Using cell culture technology, the cell biomass can be generated throughout the year, irrespective of the seasons and regions, in a shorter duration, for metabolite production. The present report confirms this unique capacity of plant cells while performing plant tissue culture, biochemical and integrated analytical studies on *S. paniculata*. The ethanopharmocological relevance of *in vitro* cell cultures in prevention or treatment of malaria was confirmed. The exclusive findings of the current work is summarized below,

- The current study developed reproducible protocol for the establishment of unorganized callus cultures from two different explants, leaves and flower-heads that are the treasure trove of metabolites in *S. paniculata*.
- The leaf-disc and flower-head explants induced calli on MS medium supplemented with at least one auxin, like 2,4-D (5.0 µM), NAA (5.0 µM) or one cytokin, like Kinetin (1 µM), BAP (9.0 µM). However, further multiplication of calli was achieved on MS medium consisting of a combination of two auxins and one cytokinin; MS + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) supported profusely growing calli within 4 weeks.
- Two *N*-alkylamides, Spilanthol and (2*E*,4*Z*) Undeca 8,10 dienoic acid isobutylamide (UDA) were isolated from field grown and *in vitro* callus cultures of *S. paniculata* by using high-throughput screening technique HPLC. Further the presence of bioactive compounds is confirmed through mass spectrometry followed by functional characterization through infra-red spectroscopy.
- The amount of anesthetic compound spilanthol in leaf-disc callus cultures was quantified as 1.75 mg/g DW, whereas, flower-head callus cultures recorded highest amount of Spilanthol as 2.23 mg/g DW. Compared to this, leaf and flower-head explants from field grown parent plants contained significantly lower amount of Spilanthol as 0.26 mg/ml and 0.83 mg/ml, respectively. Direct

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impact of source explant is seen on metabolite production in callus cultures; leaf explant from parent plant accumulated lowest amount of spilanthol and so does the calli originated from it.

- The amount of compound (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide (UDA) isolated from *in vitro* flower-head callus cultures was quantified as 4.30 mg/ml whereas, the field grown flower-heads provided 5.29 mg/ml.

- Cell suspension cultures from flower-head callus cultures were successfully established to analyze the production *N*-isobutylamide. Plant cell cultivation is a suitable unconventional technique for the production of highly desired secondary products that are greatly affected by the nutrients in the culture medium and the carbohydrate sources.

- Placket-Burman (PB) design was applied to identify the significant nutrient media constituents in leaf-disc callus cultures. Among the five variables tested, Murashige and Skoog (MS) major salts, sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzylaminopurine (BAP), were found to have significant effect on spilanthol production. The optimal concentrations of the four variables were determined using central composite design (CCD), which is widely used response surface methodology (RSM) design. The most suitable concentration of variables for spilanthol production were, 1½ MS, 5% sucrose, 4.82µM BAP and 1.8µM 2,4-D. At these optimal parameters, the maximum yield of Spilanthol was obtained experimentally as 2.81 mg/g DW, which was found to be very close to its predicted value of 3.72 mg/g DW. The developed mathematical model was found to fit well with the experimental data by which the higher production of metabolite was achieved as compared to non-optimized media constituents. Before optimization, the callus cultures and leaves from parental plant (control) yielded 1.75 mg/g DW and 0.26 mg/g DW spilanthol, respectively.

- The attempts on increasing the production of Spilanthol was performed by RSM from flower-head callus cultures as well. Of five different variables chosen, four significant independent factors, MS major salts, sucrose, BAP and 2, 4-D, were screened by Plackett-Burman design. The optimum concentration of screened factors was evaluated using central composite design to achieve the

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maximum production of Spilanthol. It generated a media combinations of MS major salts (0.97), sucrose (3.10%), BAP (5.28µM) and 2, 4-D (1.8 µM) with the predicted value of 2.99 mg/g DW to validate multivariate regression model. At these optimal parameters, the maximum yield of Spilanthol was obtained experimentally as 2.94 mg/g DW and was very close to the predicted value. Before optimization, the callus cultures and flower-heads from parental plant (control) yielded 2.23 mg/g DW and 0.83 mg/g DW Spilanthol, respectively

- The two isolated *N*-alkylamides UDA and Spilanthol were evaluated for anti-malarial activity. The anti-malarial activity of UDA was recorded with its schizonticidal activity as IC_{50}14.64 ± 0.35µg/ml and parasiticidal activity as IC_{50} 16.42 ± 0.39µg/ml, whereas, Spilanthol possessed schizonticidal activity as IC_{50} 23.22 ± 0.59µg/ml and parasiticidal activity as IC_{50}17.72 ± 0.16µg/ml. The compound treated parasite culture displayed dead or sick parasites with dead rings. Overall, UDA exhibit higher antimalarial activity as compared to Spilanthol.
- A very strong synergistic effect of UDA and Spilanthol was observed. However, UDA alone showed distinctly superior schizonticidal and parasiticidal activities against *Pf3D7* strains

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.

Future prospects

- Large scale up of cultures in bioreactors from *S. paniculata*
- The biological assays using biologically active phytochemical constituents

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1. Adler, R.J., 2005. Compositions for the acute and/or long term treatment of periodontal diseases using herb extracts. US 11/720,768. Chem. Abstr.
2. Anderson, M.J., Whitcomb, P.J. 2005. RSM simplified: optimizing processes using response surface methods for design of experiments. Productivity press, New York.
3. Anonymous, 1989. The wealth of India: a dictionary of Indian raw materials and industrial products. Council of Scientific and Industrial Research, New Delhi, 10, 11-12.
4. Anuradha, V.E., Jaleel, C.A., Salem, M.A., Gomathinayagam, M., Panneerselvam, R. 2010. Plant growth regulators induced changes in antioxidant potential and andrographolide content in *Andrographis paniculata* Wall. Ex Nees. Pest. biochem. physiol. 98, 312-316.
5. Artaria, C., Maramaldi, G., Bonfigli, A., Rigano, L., Appendino, G. 2011. Lifting properties of the alkamide fraction from the fruit husks of Zanthoxylumbungeanum. Int j cosmetic sci. 33, 328-333.
6. Asada, M., Shuler, M.L. 1989. Stimulation of ajmalicine production and excretion from *Catharanthus roseus*: effects of adsorption in situ, elicitors and alginate immobilization. Applied microbiol biotech. 30, 475-481.
7. Bae, S.S., Ehrmann, B.M., Ettefagh, K.A., Cech, N.S. 2010. A validated liquid chromatography-electrospray ionization-mass spectrometry method for quantification of spilanthol in *Spilanthes acmella* (L.) Murr. Phytochem anal. 21, 438-443.
8. Bais, H.P., Green, J.B., Walker, T.S., Okemo, P.O., Vivanco, J.M. 2002. *In vitro* propagation of *Spilanthes mauritiana*DC., an endangered medicinal herb, through axillary bud cultures. *In vitro* cell. Dev. Biol-Plant. 38, 598-601.
9. Balaji, S.N., Ahsan, M.J., Jadav, S.S., Trivedi, V., Molecular modelling, synthesis, and antimalarial potentials of curcumin analogues containing heterocyclic ring, Arabian J. Chem. (2015) doi:10.1016/j.arabjc.2015.04.011.
10. Balbuena ,T.S., Santa-Catarina, C., Silveira, V., Kato, M.J., Floh, E.I. 2009. *In vitro* morphogenesis and cell suspension culture establishment in *Piper solmsianum* C. DC.(Piperaceae). Acta Bot. Bras. 23, 274-281.
11. Baldi, A., Dixit, V.K. 2008. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresour. technol. 99, 4609-4614.

TH-1738_10610620

12. Banik, R.M., Pandey, D.K. 2008. Optimizing conditions for oleanolic acid extraction from *Lantana camara* roots using response surface methodology. Ind crops prod. 27, 241-248.
13. Begum, J., Bhuiyan, M.N.I., Chowdhury, J.U. 2008. Essential oil from inflorescence of *Spilanthes calva* DC. Bangladesh J. Bot. 37, 217-218.
14. Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta. 76, 965-977.
15. Bonga, J.M., von Aderkas, P. 1992. *In vitro* culture of trees. Springer science &business media. Boston, pp. 4.
16. Boonen, J., Baert, B., Roche, N., Burvenich, C., De Spiegeleer, B. 2010a. Transdermal behavior of the *N*-alkylamide spilanthol (affinin) from *Spilanthes acmella* (Compositae) extracts. J. Ethanopharmacol. 127, 77-84.
17. Boonen, J., Baert, B., Burvenich, C., Blondeel, P., De Saeger, S., De Spiegeleer, B. 2010b. LC–MS profiling of N-alkylamides in *Spilanthes acmella* extract and the transmucosal behaviour of its main bio-active spilanthol. J. Pharmabiomed. Anal. 53, 243-249.
18. Boonen, J., Bronselaer, A., Nielandt, J., Veryser, L., De Tré, G., De Spiegeleer, B. 2012. Alkamid database: chemistry, occurrence and functionality of plant *N*-alylamides. J Ethanopharmacol. 142, 563-590.
19. Borges-Del-Castillo, J., Vazquez-Bueno, P., Secundino-Lucas, M., Martinez-Martir, A.I., Joseph-Nathan, P. 1984.The *N*-2-phemylethyl cinnamanide from *Spilanthes ocymifolia*. Phytochemistry. 23, 2671-2672.
20. Bowman, L., Geiger, E. 1984. Optimization of fermentation conditions for alcohol production. Biotechnol. Bioeng. 26, 1492-1497.
21. Box, G.E., Wilson, K.B. 1951. On the experimental attainment of optimum conditions. J royal statistical society. Series B (Methodological). 13, 1-45.
22. Box, G.E.P., Hunter, W.G., Hunter, J.S. 1978. Statistics for Experimenters. John Wiley and Sons, New York.
23. Burbulis, N., Kupriene, R., Blinstrubiene, A. 2008. Callus induction and plant regeneration from somatic tissue in spring rapeseed (*Brassica napus* L.). Biologija. 54, 258-63.

TH-1738_10610620

24. Cataldi, T.R., Campa, C., De Benedetto, G.E. 2000. Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. Fresen J. Anal Chem. 368, 739-758.
25. Cech, N.B., Tutor, K., Doty, B.A., Spelman, K., Sasagawa, M., Raner, G.M., Wenner, C.A. 2006. Liver enzyme-mediated oxidation of *Echinacea purpurea* alkylamides: production of novel metabolites and changes in immunomodulatory activity. Planta Med. 72, 1372-1377.
26. Čellárová, E., Kimakova, K. 1999. Morphoregulatory effect of plant growth regulators on *Hypericum perforatum* L. seedlings. Acta Biotechnologica. 19, 163-169.
27. Chakraborty, A., Devi, B.R., Sanjebam, R., Khumbong, S., Thokchom, I.S. 2010. Preliminary studies on local anesthetic and antipyretic activities of Spilanthes acmella Murr. in experimental animal models. Indian J. Pharm. 42, 277-279.
28. Chattopadhyay, S., Farkya, S., Srivastava, A.K., Bisaria, V.S. 2002.Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. Biotechnol. Bioprocess Eng. 7:138-149.
29. Cheynier, V., Feinberg, M., Chararas, C., Ducauze, C. 1983. Application of response surface methodology to evaluation of bioconversion experimental conditions. Applied Environ. Microb. 45, 634-639.
30. Christophe, W. 2006. Ethnopharmacology of Medicinal Plants. Asia and the Pacific, Humana Press, USA.
31. Cochran, W.G., Cox, G.M. 1957. Experimental designs. John wiley and sons, Inc. New York.
32. Cox, P.A., Balick, M.J. 1994. The ethnobotanical approach to drug discovery. Scientific American. pp. 60-65.
33. Cui, L., Wang, Z.Y., Zhou, X.H. 2012. Optimization of elicitors and precursors to enhance valtrate production in adventitious roots of *Valeriana amurensis* Smir. Ex Kom. Plant Cell. Tiss. Org. Cult. 108, 411-420.
34. Dandin, V.S., Naik, P.M., Murthy, H.N., Park, S.Y., Lee, E.J., Paek, Y. 2014. Rapid regeneration and analysis of genetic fidelity and scopoletin contents of micropropagated plants of *Spilanthes oleracea* L. J. Hort. Sci. Biotech. 89, 79-85.
35. Demarne, F., Passaro, G. 2005. Use of an *Acmella oleracea* extract for its botox-like effect in an anti-wrinkle cosmetic composition. FR Pat. 286513, Chem. Abstr. 143, 138654.

TH-1738_10610620

36. Deming, S.N., Morgan, S.L. 1987. Experimental design: A chemometric approach. Elsevier, Oxford.
37. Dhami, N., Mishra, A,D. 2015. Phytochemical variation: How to resolve the quality controversies of herbal medicinal products?. J. Herbal Med. 5, 118-127.
38. Dias, A.M.A., Santos, P., Seabra, I.J., Júnior, R.N.C., Braga, M.E.M., De Sousa, H.C. 2012. Spilanthol from *Spilanthes acmella* flowers leaves and stems obtained by selective supercritical carbon dioxide extraction. J. Supercrit. Fluids. 61, 62-70.
39. DiCosmo, F., Facchini, P.J., Kraml, M.M. 1989. Cultured plant cells-the chemical factory within. Chemistry in Britain. 25, 1001-1004.
40. Dicosmo, F., Misawa, M. 1995. Plant cell and tissue culture: alternatives for metabolite production. Biotech. Adv. 13, 425-453.
41. DiCosmo, F., Towers, G.H. 1984. Stress and secondary metabolism in cultured plant cells. In: Phytochemical adaptations to stress. Springer US. pp. 97-175.
42. Dörnenburg, H., Knorr, D. 1995. Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme. Microb. Technol. 17, 674-84.
43. Dolui, A.K., Debnath, M. 2010. Antifeedant activity of plant extracts to an insect *Helopeltis theivora*. J. Environbiol. 31, 557-559.
44. Dubey, S., Maity, S., Singh, M., Saraf, S.A., Saha, S. 2013. Phytochemistry, pharmacology and toxicology of *Spilanthes acmella*: a review. Adv. Pharmacol. Sci. 2013, 1-9.
45. Ebrahimzadeh, H., Tavassoli, N., Sadeghi, O., Amini, M.M. 2012. Optimization of solid-phase extraction using artificial neural networks and response surface methodology in combination with experimental design for determination of gold by atomic absorption spectrometry in industrial wastewater samples. Talanta. 97, 211-217.
46. Endo, T., Goodbody, A., Vukovic, J., Misawa, M. 1988. Enzymes from *Catharanthus roseus* cell suspension cultures that couple vindoline and catharanthine to form 3′, 4′-anhydrovinblastine. Phytochemistry. 27, 2147-2149.
47. Fabry, W., Okemo, P., Ansorg, R. 1996. Fungistatic and fungicidal activity of East African medicinal plants. Mycoses. 39, 67-70.
48. Fabry, W., Okemo, P.O., Ansorg, R. 1998. Antibacterial activity of East African medicinal plants. J Ethnopharmacol. 60, 79-84.

TH-1738_10610620

49. Fett-Neto, A.G., Melanson, S.J., Sakata, K., Dicosmo, F. 1993. Improved growth and taxol yield in developing calli of *Taxus cuspidata* by medium composition modifications. Biotechnol. 11, 731-734.
50. Fett-Neto, A.G., Zhang, W.Y., Dicosmo, F. 1994. Kinetics of taxol production, growth, and nutrient uptake in cell suspensions of *Taxus cuspidata*. Biotechnol. Bioeng. 44, 205-210.
51. Frahm, E., Gudat, K. 1980. Percutaneous treatment of acute sport injuries with an extract of *Spilanthes paniculata*. Therapy week, German. 30, 8186-8190.
52. Foster, S. 2011. A brief history of adulteration of herbs, spices and botanical drugs. Herbal Gram. 92, 42–57.
53. George, E.F., Sherrington, P.D. 1984. Plant propagation by tissue culture. Exegetics Ltd. Edington.
54. George, J., Bais, H.P., Ravishankar, G.A., Manilal, P. 2000. Optimization of media constituents for shoot regeneration from leaf callus cultures of *Decalepis hamiltonii* Wight. & Arn. Hort science. 35, 296-299.
55. Gerber, E. 1903. About the chemical constituents of paracress (*Spilanthes oleracea*, Jacquin). Arch Pharm. 241, 270-289.
56. Ghokale, V.G., Bhide, B.V. 1945. Chemical investigation of *Spilanthes acmella* (Murr.). Ind. J. Chem Soc. 1, 250-252.
57. Grellier, P., Depoix, D., Schrevel, J., Florent, I. 2008. Discovery of new targets for antimalarial chemotherapy. Parasite. 15, 219-225.
58. Gutiérrez-Miceli, F.A., Arias, L., Juarez-Rodríguez, N., Abud-Archila, M., Amaro-Reyes, A., Dendooven, L. 2010. Optimization of growth regulators and silver nitrate for micropropagation of *Dianthus caryophyllus*L. with the aid of a response surface experimental design. *In vitro C*ell. Dev. Biol – Plant. 46, 57-63.
59. Ha, D.B.D., Norreel, B., Masset, A. 2008. Regeneration of *Asparagus officinalis* L. through callus cultures derived from Protoplasts. J. Exp. Bot. 26, 263-270.
60. Haaland, P.D. 1989. Experimental design in biotechnology. CRC press, New york.
61. Haider, M.A., Pakshirajan, K. 2007. Screening and optimization of media constituents for enhancing lipolytic activity by a soil microorganism using statistically designed experiments. Appl. Biochem. Biotechnol. 141, 377-390.
62. Hanchinal, V.M., Survase, S.A., Sawant, S.K., Annapur, U.S. 2008. Response surface methodology in media optimization for production of ß-carotene from *Daucus carota*, Plant Cell Tiss. Org. Cult. 93, 123-132.

TH-1738_10610620

63. Harborne, J.B. 2001. Twenty-five years of chemical ecology. Nat. Prod. Rep. 18, 361-379.
64. Haw, A.B., Keng, C.L. 2003. Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots. J. Appl. Hort. 5, 65-68.
65. Hossain, H., Shahid-Ud-Daula, A.F., Jahan, I.A., Nimmi, I., Hasan, K., Haq, M.M. 2012. Evaluation of anti-nociceptive and antioxidant potential from the leaves of *Spilanthes paniculata* growing in Bangladesh. Int. J. Pharm. Phytopharmacol. Res. 1, 178-186.
66. Huang, C.N., Cornejo, M.J., Bush, D.S., Jones, R.L. 1986. Estimating viability of plant protoplasts using double and single staining. Protoplasma. 135, 80-87.
67. Jaleel, C.A., Riadh, K., Gopi, R., Manivannan, P., Inès, J., Al-Juburi, H.J., Chang-Xing, Z., Hong-Bo, S., Panneerselvam, R. 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. Acta Physiol. Plant. 31, 427-436.
68. Jansen, R.K. 1981. Systematics of *Spilanthes* (Compositae-Heliantheae). Systematic Bot. 6, 231–232.
69. Jayaweera, D.M.A., 1981. Medicinal plants, Part III. National science council of Srilanka, Columbo.
70. Jirovetz, L., Gerhard, B., Andrea, W., Mohamed, P.S., George, T.A. 2005. Essential oil analysis of *Spilanthes acmella* Murr. Fresh plants from Southern India. J. Essent. Oil Res. 17, 429-431.
71. Jondiko, I.J.O. 1986. A mosquito larvicide in *Spilanthes mauritiana*. Phytochemistry. 25, 2289-2290.
72. Junaid, A., Mujib, A., Sharma, M.P., Tang, W. 2007. Growth regulators affect primary and secondary somatic embryogenesis in Madagaskar periwinkle (*Catharanthus roseus* (L.) G. Don) at morphological and biochemical levels. Plant growth regulation. 51:271-281.
73. Karemore, A., Pal, R., Sen, R. 2013. Strategic enhancement of algal biomass and lipid in *Chlorococcuminfusionum* as bioenergy feedstock. Algal Res. 2, 113-121.
74. Kaushik, R., Saran, S., Isar, J., Saxena, R.K. 2006, Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipaseproduction by *Aspergillus carneus*. J molcatalb: enzym, 40:121–126.

TH-1738_10610620

75. Khadir, H.A., Zakaria, M.B., Ketchil, A.A., Azirum, M.S. 1989. Toxicity and electrophysiological effects of *Spilanthes acmella* Murr. extracts on *Periplaneta Americana* L. Pesti. sci. 25, 329-335.
76. Knobloch, K.H., Berlin, J. 1980. Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. Zeitschriftfuer Naturforschung c. 35, 551-556.
77. Kochan, E., Chmiel, A. 2011. Dynamics of ginsenoside biosynthesis in suspension culture of *Panax quinquefolium*. Acta physiol plant. 33, 911-915.
78. Kossel, A. 1891. On the chemical composition of the cell. Arch physiol. German. 81–86.
79. Kretzschmar, F.S., Oliveira, Jr.C.J., Braga, M,R. 2007. Differential sugar uptake by cell suspension cultures of *Rudgeajasminoides*, a tropical woody Rubiaceae. *In vitro* Cell. Dev. Biol - plant. 43, 71-78.
80. Krogstad, D.J. 1999. In: Guerrant RL, Walker DH,Weller PF. (Eds.), Malaria, in tropical infectious diseases. Churchill Livingstone. pp. 736–766.
81. Kumar, A., Paliwal, D., Saini, D., Thakur, A., Aggarwal, S., Kaushik, D. 2014. A comprehensive review on synthetic approach for antimalarial agents. European j med chem. 85, 147-178.
82. Lattoo, S.K., Bamotra, S., Dhar, R.S., Khan, S., Dhar, A.K., 2006. Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker–an endangered medicnal herb. Plant cell rep. 25:499-506.
83. Lemos, T.L.G., Pessoa, O.P.L, Matos, F.J.A., Alencar, J.W., Craveiro, A.A. 1991. The essential oil of *Spilanthes acmella* (Murr.). J essentoil res. 3, 369–370.
84. Leng, T.C., Haw, A.B., Lai-Keng, C. 2004. Effect of reduced N6-Benzyladenine, explant type, explant orientation, culture temperature and culture vessel type on regeneration of adventitious shoot and *in vitro* plantlets of *Spilanthes acmella*. J. Plant Biol. 47, 15-20.
85. Leng, T.C., Ping, N.S., Lim, B.P., Keng, C.L. 2011. Detection of bioactive compounds from *Spilanthes acmella* (L.) plants and its various *in vitro* culture products. J. Med. Plants Res. 5:371-8.
86. Li, G.P., Shen, B.C., Zhao, J.F., Yang, X.D., Li, L. 2007. Two new alkamides from *Spilanthes callimorpha*. J. Integr. Plant Biol. 49, 1608-1610.

TH-1738_10610620

87. Lian, M.L., Chakrabarty, D., Paek, K.Y. 2002. Effect of plant growth regulators and medium composition on cell growth and saponin production during cell-suspension culture of mountain ginseng (*Panax ginseng* CA Mayer). J. Plant boil. 45, 201-206.
88. Liu, K.C.S., Yang, S.L., Roberts, M.F., Phillipson, J.D. 1990. Production of canthin-6-one alkaloids by cell suspension cultures of *Bruceajavanica* (L.) Merr. Plant Cell Rep. 9, 261-263.
89. Lucchesini, M., Bertoli, A., Mensuali-Sodi, A., Pistelli, L. 2009. Establishment of *in vitro* tissue cultures from *Echinacea angustifolia* DC adult plants for the production of phytochemical compounds. Sci. Hort. 122, 484-490.
90. Łuczkiewicz, M., Głód, D. 2005. Morphogenesis-dependent accumulation of phytoestrogenes in *Genistatinctoria in vitro* cultures. Plant Sci. 168, 967-979.
91. Majumder, A., Goyal, A. 2008. Enhanced production of exocellular glucan sucrase from *Leuconostocdextranicum* NRRL B-1146 using response surface method. Bioresource technol. 99, 3685-3691.
92. Marschner, H. 1986.Mineral nutrition of higher plants. Academic press. London.
93. Masuda, H., Komiyama, S., Sugawara, S., 1988. Extraction of enzymes from cell walls of sugar beet cells grown in suspension culture. Plant Cell Physiol. 29, 623-627.
94. Mbeunkui, F., Grace, M.H., Lategan, C., Smith, P.J., Raskin, I., Lila, M.A. 2011. Isolation and identification of anti-plasmodial *N*-alkylamides from *Spilanthes acmella* flowers using centrifugal partition chromatography and ESI-IT-TOF-MS. J. Chromatogr. B. 879, 1886-1892.
95. Meyers, R.H., Montgomery, D.C. 2002. Response surface methodology. Process and Product Optimisation Using Design Experiments, second ed, Willey, New York.
96. Misra, A.K., Bhatnagar, S.P., 1995. Direct shoot regeneration from the leaf explant of cucumber (*Cucumissativus* L.). Phytomorphology. 45, 45-55.
97. Molinatorres, J., Salgado-Garciglia, R., Ramirez-Chavez, E., Delrio, R.E., 1996. Purely olefinic alkamides in *Heliopsis longipes* and *Acmella (Spilanthes) opposotifolia.* Biochem. Syst. Ecol., 24, 43-47.
98. Mondal, A.K., Parui, S., Mandal, S., 1998. Analysis of the free amino acid content in pollen of nine asteraceae species of known allergenic activity. Ann. Agric. Environ. Med., 5, 17-20.
99. Montgomery, D.C. 1991. Design and analysis of experiments, 3rd edn. Wiley, New York.

TH-1738_10610620

100. Moreno, P.R.H., Van der Heijden, R., Verpoorte, R. 1995. Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: A literaturesurvey II. Updating from 1988 to 1993. Plant Cell Tiss. Org Cult. 42, 1–25.
101. Mukherjee, S., Das, P., Sivapathasekaran, C., Sen, R. 2008. Enhanced production of biosurfactant by a marine bacterium on statistical screening of nutritional parameters. Biochem. Eng. J. 42, 254-260.
102. Murashige ,T., Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum. 15, 473-497.
103. Nagella, P., Murthy, H.N. 2010. Establishment of cell suspension cultures of *Withania somnifera* for the production of withanolide A. Biores. Technol. 101, 6735-6739.
104. Naik, P.M, Manohar, S.H., Praveen, N., Murthy, H.N. 2010 Effects of sucrose and pH levels on *in vitro* shoot regeneration from leaf explants of *Bacopa monnieri* and accumulation of bacoside A in regenerated shoots. Plant Cell Tiss. Org. Cult. 100, 235-239.
105. Naik, S.K., Pattnaik, S., Chand, P.K. 1999. *In vitro* propagation of pomegranate (*Punicagranatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Sci. Hort. 79, 175-183.
106. Nakatani, N., Nagashima, M. 1992. Pungent alkamides from *Spilanthes acmella* L. var. oleracea Clarke. Biosci. Biotech. Biochem. 56, 759-762.
107. Narang, S., Sahai, V., Bisaria, V.S. 2001. Optimization of xylanase production by *Melanocarpusalbomyces* IIS68 in solid state fermentation using response surface methodology. J. Biosci. Bioeng. 91, 425-427.
108. Natarajan, U., Periyanan, P.R., Yang, S.H. 2011. Multiple-response optimization for micro-endmilling process using response surface methodology. Int. J. Adv. Manuf. Tech. 56, 177-185.
109. Niratker, C., Singh, M., Preeti. 2014. Callus induction and plant regeneration from leaf explants of *Spilanthes acmella* Murr. : An endangered medicinal plant. Ann. Biol. Res. 5, 66-71.
110. Ouyang, J., Wang, X.D., Zhao, B., Wang, Y.C. 2005. Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of *Cistanche deserticola*. Process Biochem. 40, 3480-3484.
111. Pandey, V., Agrawal, V., Raghavendra, K., Dash, A.P. 2007. Strong larvicidal activity of three species of *Spilanthes* (Akarakara) against malaria (*Anopheles*

TH-1738_10610620

stephensi Liston, Anopheles culicifacies, species C) and filarial vector (*Culexquinquefaciatus*Say). Parasitol Res. 102, 171-174.

112. Pandey, V., Agrawal, V. 2009. Efficient micropropagation protocol of *Spilanthes acmella* L. possessing strong antimalarial activity. *In vitro* Cell Dev. Biol-plant. 45, 491-499.
113. Parveen, A., Chakraborty, A., Konreddy, A.K., Chakravarty, H., Sharon, A., Trivedi, V., Bal, C. 2013. Skeletal hybridization and PfRIO-2 kinase modeling for synthesis of α-pyrone analogs as anti-malarial agent. European J. Med. Chem. 70, 607-612.
114. Payne, G.F., Bringi, V., Prince, C., Shuler, M.L. 1991. Suspension culture, In: Payne, G.F., Bringi, V., Prince, C., Shuler, M.L. (Eds.), Plant cell and tissue culture in liquid systems, Hanser. pp. 145–176.
115. Perez-Paron, M.A., Gonzalez-Benito, M.E., Perez, C. 1994. Micropropagation of *Fraxinus angustifolia* from mature and juvenile plant material. Plant Cell Tiss. Org. Cult. 37, 297-302.
116. Pezzuto, J.M. 1995. Natural product cancer chemoprotective agents. In: Arnason JT, Mata R, Romeo JT, (Eds.), Recent advances in phytochemistry. Vol. 29. Phytochemistry of medicinal plants. New York: Plenum. pp 19–45.
117. Plackett, R.L., Burman, J.P. 1946. The design of optimum multifactorial experiments. Oxfors University Press, Biometrika.
118. Prachayasittikul, S., Suphapong, S., Worachartcheewan, A., Lawung, R., Ruchirawat, S., Prachayasittikul, V. 2009. Bioactive metabolites from *Spilanthes acmella* Murr. Molecules. 14, 850-867.
119. Prakash, G., Srivastava, A.K. 2005. Statistical media optimization for cell growth and azadirachtin production in *Azadirachtaindica* (A. Juss) suspension cultures. Process Biochem. 40,3795-3800.
120. Prathibha, K.P., Raghavendra, B.S., Vijayan, V.A. 2014. Larvicidal, ovicidal, and oviposition-deterrent activities of four plant extracts against three mosquito species. Environ. Sci. Poll Res. 21, 6736-6743.
121. Puri, M., Sharma, D., Barrow, C.J., Tiwary, A.K. 2012. Optimization of novel method for the extraction of steviosides from *Stevia rebaudiana* leaves. Food chem. 132, 1113-1120.
122. Raghuvanshi, V.S., Singh, J.K., Singh, M., Paul, R., Rana, K.S. 2010. Insecticidal activity of *Spilanthes acmella* Murr. against*Chilopartellus* (Swinhoe.). Int. J. Entomol. 1, 13–18.

TH-1738_10610620

123. Ramsewak, R.S., Erickson, A.J., Nair, M.G. 1999. Bioactive N-isobutylamides from the flower buds of *Spilanthes acmella*. Phytochemistry. 51,729-732.

124. Raner, G.M., Cornelious, S., Moulick, K., Wang, Y., Mortenson, A., Cech, N.B. 2007. Effects of herbal products and their constituents on human cytochrome P450 2E1 activity. Food Chem. Toxicol. 45, 2359-2365.

125. Rao, K.J., Kim, C.H., Rhee, S.K. 2000. Statistical optimization of medium for the production of recombinanthirudin from *Saccharomyces cerevisiae* using response surface methodology. Process Biochem. 35, 639-647.

126. Rao, S.R., Ravishankar, G.A. 2002. Plant cell cultures: chemical factories of secondary metabolites. Biotech. Adv. 20, 101-53.

127. Rastogi, B.N. 1993. Compendium of Indian Medicinal Plants. Vol. II, CDRI Lucknow, New Delhi publication and Information Directorate.

128. Ratnam, B.V.V., Rao, S.S., Rao, M.D., Rao, M.N., Ayyanna, C. 2005. Optimization of medium constituents and fermentation conditions for the production of ethanol from palmyra jiggery using response surface methodology, World J. Microb. Biotechnol. 21, 399-404.

129. Ratnasooriya, W.D., Pieris, K.P. 2005. Attenuation of persistent pain and hyperalgesia by *Spilanthus acmella*. flowers in rats. Pharma. Biol. 43, 614-619.

130. Razaq, M., Heikrujam, M., Chetri, S.K., Agrawal, V. 2013. *In vitro* clonal propagation and genetic fidelity of the regenerants of *Spilanthes calva* DC. using RAPD and ISSR marker. Physiol Mol Biol Plants. 19, 251-260.

131. Renaudin, J.P., Guern, J. 1990. Transport and vacuolar storage of secondary metabolites in plant cell cultures. Secondary products from Plant Tissues Culture, In: Chrwood, B.V., Rhodes, M.J.C. (Eds.), Secondary products from plant tissues culture. Oxford science publications. pp, 59-78.

132. Rios, M.Y. 2012.Natural alkamides: pharmacology, chemistry and distribution. In: Vallisuta, O., Suleiman M.O. (Eds.), Drug Discovery Research in Pharmacognosy, 1st ed. I-tech education and publishing. pp. 107-144.

133. Roseiro, J.C., Esgalhado, M.E., Collaco, M.A., Emery, A.N. 1992. Medium development for xanthan production. Process Biochem. 27, 167-175.

134. Rout, G.R., Samantaray, S., Das, P. 2000. *In vitro* manipulation and propagation of medicinal plants. Biotech. Adv. 18, 91-120.

TH-1738_10610620

135. Saleh, G., Soave, R., Lo Presti, L., Destro, R. 2013. Progress in the understanding of the key pharmacophoric features of the antimalarial drug dihydroartemisinin: An experimental and theoretical charge density study. Chemeur J. 19, 3490-3503.

136. Sana, H., Rani, A.S., Sulakshana, G. 2014. Determination of Antioxidant Potential in *Spilanthes acmella* using DPPH assay. Int. J. Curr. Microb. App. Sci. 3, 219-223.

137. Sanjeeviroyar, A., Rajendran, A., Muthuraj, M., Basha, K.M., Thangavelu, V. 2010. Sequential optimization and kinetic modeling of L-asparaginase production by *Pectobacterium carotovorum* in submerged fermentation. Asia-pacific J. Chem. Eng. 5, 743-755.

138. Santesson, C.G. 1926. Some drugs from the Cameroon area and their native uses. Archieves furdie Botany A, German. 20, 1–34.

139. Saraf, D.K., Dixit, V.K. 2002, *Spilanthes acmella* Murr.: study on its extract spilanthol as larvicidal compound. Asian J. Exp. Sci. 16, 9-19.

140. Saritha, K.V., Prakash, E., Ramamurthy, N., Naidu, C.V. 2002. Micropropagation of *Spilanthes acmella* Murr. Biol. Plantarum. 45, 581–584.

141. Saritha, K.V., Naidu, C.V. 2008. Direct shoot regeneration from leaf explants of *Spilanthes acmella*. Biol Plantarum. 52, 334-338.

142. Savadi, R.V., Yadav, R., Yadav, N. 2010. Study on immunomodulatory activity of ethanolic extract of *Spilanthes acmella* Murr. leaves. Ind. J. Nat. Prod. Res. 1, 204-207.

143. Service, M., Townson, H., 2002. The Anopheles vector. In: Warrell, D., Gilles, H., (Eds.), Essential Malariology. 4th ed. Arnold publishers. pp. 85–106.

144. Sharma, G., Gupta, V., Sharma, S., Shrivastava, B., Bairva, R. 2010. Toothache plant *Spilanthes acmella* Murr.: a review. J. Nat. Conscientia. 1, 135-142.

145. Sharma, S., Shahzad, A. 2013. Efficient Micropropagation of *Spilanthes acmella* (L.) Murr.: A threatened medicinal herb. British Biotechnol. J. 3, 405-415.

146. Sharma, V., Boonen, J., Chauhan, N.S., Thakur, M., De Spiegeleer, B., Dixit, V.K. 2011. *Spilanthes acmella* ethanolic flower extract: LC–MS alkylamide profiling and its effects on sexual behavior in male rats. Phytomedicine. 18, 1161-1169.

147. Shimada, T., Gomi, T. 1995.Spilanthol-rich essential oils for manufacturing toothpastes or other oral compositions. JP Pat. 7090294.

148. Sidhu, O.P., Annarao, S., Chatterjee, S., Tuli, R., Roy, R., Khetrapal, C.L. 2011. Metabolic alterations of *Withania somnifera* (L.) Dunal fruits at different developmental stages by NMR spectroscopy. Phytochem. Anal. 22, 492-502.

TH-1738_10610620

149. Singh, M., Chaturvedi, R. 2010. Improved clonal propagation of *Spilanthes acmella* Murr. for production of scopoletin. Plant Cell Tiss. Org. Cult. 103, 243-253.

150. Singh, M., Chaturvedi, R. 2012a. Screening and quantification of an antiseptic alkylamide, spilanthol from *in vitro* cell and tissue cultures of *Spilanthes acmella* Murr. Ind. Crop Prod. 36, 321-328.

151. Singh, M., Chaturvedi, R. 2012b. 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.

152. Singh, M., Chaturvedi, R. 2012. Statistical optimization of media for enhanced azadirachtin production from redifferentiated zygotic embryo cultures of neem (*Azadirachtaindica* A. Juss.). *In vitro* Cell Dev. Biol-plant. 48, 92-98.

153. Singh, M., Roy, B., Tandon, V., Chaturvedi, R. 2014. Extracts of dedifferentiated cultures of *Spilanthes acmella* Murr. possess antioxidant and anthelmintic properties and hold promise as an alternative source of herbal medicine. Plant Biosyst. 148,259-267.

154. Singh, K.P., Gupta, S., Singh, A.K., Sinha, S. 2010. Experimental design and response surface modeling for optimization of Rhodamine B removal from water by magnetic nanocomposite. Chemical Eng. J. 165, 151-160.

155. Singh, S.K., Rai, M.K., Asthana, P., Pandey, S., Jaiswal, V.S., Jaiswal U. 2009a. Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* (L.) Murr., a medicinally important and herbal pesticidal plant species. Acta Physiol. Plant. 31, 649-653.

156. Singh, S.K., Rai, M.K., Asthana, P., Sahoo, L. 2009b. An improved micropropagation of Spilanthes acmella L. through transverse thin cell layer culture. Acta Physiol. Plant. 31, 693-698.

157. Sivanandhan, G., Dev, G.K., Jeyaraj, M., Rajesh, M., Muthuselvam, M., Selvaraj, N., Manickavasagam, M., Ganapathi, A. 2013. A promising approach on biomass accumulation and withanolides production in cell suspension culture of *Withaniasomnifera* (L.) Dunal. Protoplasma. 250, 885-898.

158. Sivanandhan, G., Selvaraj, N., Ganapathi, A., Manickavasagam, M. 2014. An efficient hairy root culture system for *Withania somnifera*. African J. Biotechnol.13, 4141–4147.

159. Spelman, K., Depoix, D., Mccray, M., Mouray, E., Grellier, P. 2011. The traditional medicine *Spilanthes acmella,* and the alkylamides spilanthol and undeca-2*E*-ene-8,10-

TH-1738_10610620

diynoic acid isobutylamide, demonstrate *in vitro* and *in vivo* anti-malarial activity. Phytothera Res. 25, 1098-1101.

160. Srivastava, P., Sisodia, V., Chaturvedi, R. 2011. Effect of culture conditions on synthesis of triterpenoids in suspension cultures of *Lantana camara* L. Bioprocess Biosyst. Eng. 34, 75-80.
161. Stashenko, E.E., Puertas, M.A., Combariza, M.Y. 1996. Volatile secondary metabolites from *Spilanthes americana* obtained by simultaneous steam distillation-solvent extraction and supercritical fluid extraction. J Chromatogr. A. 752, 223-232.
162. Storey, C., Salem, J.I. 1997. Lay use of Amazonian plants for the treatment of tuberculosis. Acta Amazonica. 27, 175-82.
163. Stroescu, M., Stoica-Guzun, A., Ghergu, S., Chira, N., Jipa, I. 2013. Optimization of fatty acids extraction from *Portulaca oleracea* seed using response surface methodology. Ind. Crop Prod. 43, 405-411.
164. Su, W.W. 1995. Bioprocessing technology for plant cell suspension cultures. Appl. Biochem. Biotech. 50, 189–230.
165. Sundaram, U., Anupama, V., Gurumoorthi, P. 2013. Optimization of pH and sucrose in the callus culture for the micro propagation of *Mucuna pruriens* using response surface methodology. Int. J. Pharm. Pharmaceut. Sci. 5, 420-426.
166. Suvarnalatha, G., Chand, N., Ravishankar, G.A., Venkatarama, L.V., 1993. Computer aided Modelling and Optimization for capsaicinoid production by immobilized *Capsicum frutescens* cells. Enzyme. Microb. Technol. 15, 710-715.
167. Tanwer, B.S., Choudhary, R. Vijayvergia, R. 2010. *In vitro* and *in vivo* comparative study of primary metabolites and antioxidant activity in *Spilanthes acmella* Murr. Int. J. Biotechnol. Biochem. 6, 819-825.
168. Tiwari, K.L., Jadhav, S.K., Joshi, V. 2011. An updated review on medicinal herb genus *Spilanthes*. Zhong Xi Yi Jie He XueBao. 9, 1170-1178.
169. Trivedi, V., Chand, P., Srivastava, k., Puri, S.K. Maulik, P.R. Bandyopadhyay, U. 2005. Clotrimazole inhibits hemoperoxidase of *Plasmodium falciparum* and induces oxidative stress proposed antimalarial mechanism of clotrimazole, J. Biol. Chem. 280, 41129-41136.
170. Tyagi, A., Delanty, N. 2003. Herbal remedies, dietary supplements, and seizures. Epilepsia. 44, 228-235.
171. Urankar, M., Desai, A., Bhat, R. 2013. A review on medicinal herb genus *Spilanthes* and its applications in oral hygiene. Universal journals publications. 2, 25-33.

TH-1738_10610620

172. vanUden, W., Pras, N., Maingré, T.M. 1990. The accumulation of podophyllotoxin-β-D-glucoside by cell suspension cultures derived from the conifer *Callitrisdrummondii*. Plant Cell Rep. 9, 257-60.

173. Varsha, N.N., Ghulam, Y.M. 2013. Callus culture of *Spilanthes paniculata* (DC) Jansen-An aromatic medicinal plant. Int. J. Sci Res. 4, 3013-3015.

174. Veryser, L., Wynendaele, E., Taevernier, L., Verbeke, F., Joshib, T., Tatke, P., De Spiegeleer, B. 2014. *N*-alkylamides: from plant to brain. Functional foods in health and disease. 4, 264-275.

175. Vyas, Y.K., Chandrashekhra, K.T., Bhatnagar, M., Sharma, K. 2009. Effect of *Spilanthes calva* DC. root extract, herbal and synthetic dentifrices on oral microflora using *in vitro* human tooth model. J. Herb Med. Toxicol. 3, 141-148.

176. Wang, J.P., Chen, Y.Z., Wang, Y., Yuan, S.J., Yu, H.Q. 2011. Optimization of the coagulation-flocculation process for pulp mill wastewater treatment using a combination of uniform design and response surface methodology. Water Res. 45, 5633- 5640.

177. Wen, Z.Y., Zhong, J.J. 1997. Effects of initial phosphate concentration on physiological aspects of suspension cultures of rice cells: A kinetic study. J. Ferment. Bioeng. 83, 381-385.

178. Wongsawatkul, O., Prachayasittikul, S., Isarankura-Na-Ayudhya, C., Satayavivad, J., Ruchirawat, S., Prachayasittikul, V. 2008. Vasorelaxant and antioxidant activities of *Spilanthes acmella* Murr. Int. J. Mol. Sci. 9, 2724-2744.

179. WHO. World Malaria Report. WHO/HTM/GMP/2008. 2008.

180. World Health Organization. World Malaria Report, 2012. Geneva: WHO, 2012.

181. Wink, M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor. Appl. Gen. 75, 225-233.

182. Winslow, L.C., Kroll, D.J. 1998. Herbs as medicines. Arch. Int. Med. 158, 2192-2199.

183. Wu, H.C., Du Toit, E.S. 2004 Reducing oxidative browning during *in vitro* establishment of *Proteacynaroides*. Sci. Hort. 100, 355-358.

184. Wu, Y., Cui, S.W., Tang, J., Gu, X. 2007. Optimization of extraction process of crude polysaccharides from boat-fruited sterculia seeds by response surface methodology. Food Chem. 105, 1599-1605.

185. Wu, L.C., Fan, N.C., Lin, M.H., Chu, I.R., Huang, S,J., Hu, C.Y., Han, S.Y. 2008. Anti-inflammatory effect of spilanthol from Spilanthes acmella on murine

TH-1738_10610620

macrophage by down-regulating LPS-induced inflammatory mediators. J. Agri. Food Chem. 56, 2341-2349.

186. Yadav, K., Singh, N. 2010. Micropropagation of *Spilanthes acmella* Murr.–An important medicinal plant. Nat. Sci. 8, 5-11.
187. Yadav, K., Singh, N., Aggarwal, A. 2012. Arbuscularmycorrhizal (AM) technology for the growth enhancement of micropropagated *Spilanthes acmella* Murr. Plant Protect. Sci. 48, 31-36.
188. Yadav, R.A., Yadav, N.I., Kharya, M.D., Savadi, R.U. 2011. Preliminary studies on diuretic effect of *Spilanthes acmella* leaves extracts in rats. Int. J. Pharm. Sci. 3, 245-247.
189. Yasuda, I., Takeya, K., Itokawa, H. 1980. The geometric structure of spilanthol. Chem. Phar. Bull. 28, 2251-2253.
190. Yoganarasimhan, S.N. 1996. Medicinal Plants of India. Vol. II. Interline publishing pvt ltd. Bangalore.
191. Yotsawimonwat, S., Rattanadechsakul, J., Rattanadechsakul, P., Okonogi, S. 2010. Skin improvement and stability of *Echinacea purpurea* dermatological formulations. Int. J. Cosmet. Sci. 32, 340-346.
192. Zeng, F., Wang, W., Zhan, Y., Xin, Y. 2009. Establishment of the callus and cell suspension culture of *Elaeagnusangustifolia* for the production of condensed tannins. African J. Biotechnol. 8, 5005-5010.
193. Zenk, M.H., El-Shagi, H., Schulte, U. 1975. Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta Med. Suppl. 28, 79-101.
194. Zhang, Y.H., Zhong, J.J., Yu, J.T. 1996. Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng*: manipulation of medium sucrose. J. Biotechnol. 51, 49-56.

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APPENDIX

Spilanthes paniculata Wall. EX. DC

Kingdom	-	Plantae	-	plant
Subkingdom	-	Tracheobioanta	-	Vascular plant
Super division	-	Spermatophyta	-	Seed plant
Division	-	Magnoliophyta	-	Flowering plants
Class	-	Magnoliopsida	-	Dicotyledonous
Order			-	Asterales
Family			-	Compositae
Genus			-	*Spilanthes*
Species			-	*paniculata*

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Indian Institute of Technology Guwahati

List of Publications

In International Journals

1. Rajendran R, Chaturvedi R. Screening and optimizing media constituents for enhanced production of medicinal *N*-alkylamide Deca-2*E*, 6*Z*, 8*E*-trienoic acid isobutylamide from dedifferentiated in vitro cell lines of *Spilanthes paniculata,* 2017. *Biocatalysis and Agricultural Biotechnology*. 9:95-102.

2. Rajendran R, Narashimman BS, Trivedi V, Chaturvedi R. Isolation and quantification of antimalarial *N*-alkylamides from flower-head derived in vitro callus cultures of *Spilanthes paniculata,* 2017. *Journal of Bioscience and Bioengineering*. Mar 31.

Book Chapter

3. **Radhika Rajendran** and Chaturvedi Rakhi*. Plant Cell Cultures: A Promising Biometabolite Reservoir. In: Mala Trivedi and Rachana Singh (Editors) Biotechnology Trends and Applications. Studium Press LLC, USA, pp. 49-77, 2016.

Papers in National Conferences/Seminars

4. **Radhika Rajendran** and Chaturvedi Rakhi*. Establishment of callus and cell suspension cultures from leaf explants of *Spilanthes acmella* – A medicinal plant. In: National Seminar (DBT, Govt. of India Sponsored) on Prospects of Biotechnological Applications for Sustainable Agriculture, January 30-31, 2012. B.B.K. College, Nagaon, Barpeta, Assam, India. Page No. 34, 2012.

5. **Radhika Rajendran** and Chaturvedi Rakhi*. Micropropagation and establishment of callus and suspension cultures of *Spilanthes acmella* Murr. - A medicinal plant. In: National Conference on Exploring Strategies for Enhancement of Secondary Metabolites in Medicinal Plants, February 13-14, 2013. University of Pune, Pune, India. Page No. 34, 2013.

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Papers in International Conferences

6. **Radhika Rajendran** and Chaturvedi Rakhi*. The Elevated production of spilanthol from *in vitro* cultures of *Spilanthes acmella* Murr. by optimization of media via response surface methodology. In: 2nd International Conference on Agricultural & Horticultural Sciences, February 2-5, 2014, 167th Omics Group Conferences, Hyderabad, Page No. 193, 2014.

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Figure 1

Establishment of leaf-disc callus cultures from *S. paniculata*

A. One-day-old leaf-disc culture on callus induction medium (bar = 0.15 cm)

B. Same as A, 4-week-old flower head callus culture (bar = 0.21 cm)

C. Same B, an 8-week-old brown, friable, undifferentiated calli on MS + BAP (5 µM) + 2, 4-D (1 µM) + NAA (1 µM) (x0.16 cm),

D. Same as C, 15-week-old healthy, fresh, friable massively growing calli (0.15 cm).

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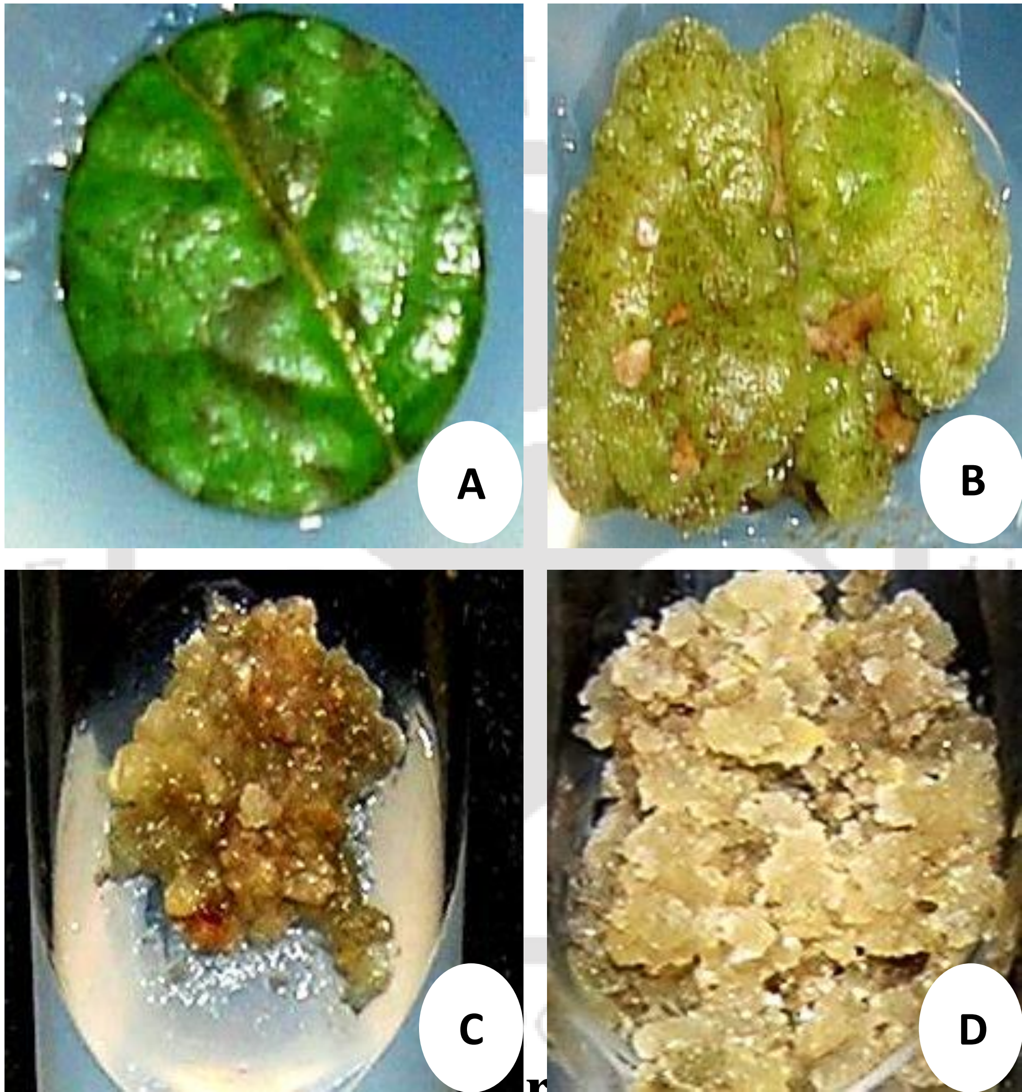

Figure 1

Figure 2

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 (bar = 0.18 cm)

B. Same as A, after 4 weeks of inoculation showing callus initiation from individual flowers in the flower-head (bar = 0.11 cm)

(C) Same as B, after 8 weeks of inoculation showing brown and wet undifferentiated calli (bar = 0.17)

(D) Same as C, after 12weeks showing profusely growing, healthy, fresh, cream and friable calli (bar = 0.72 cm)

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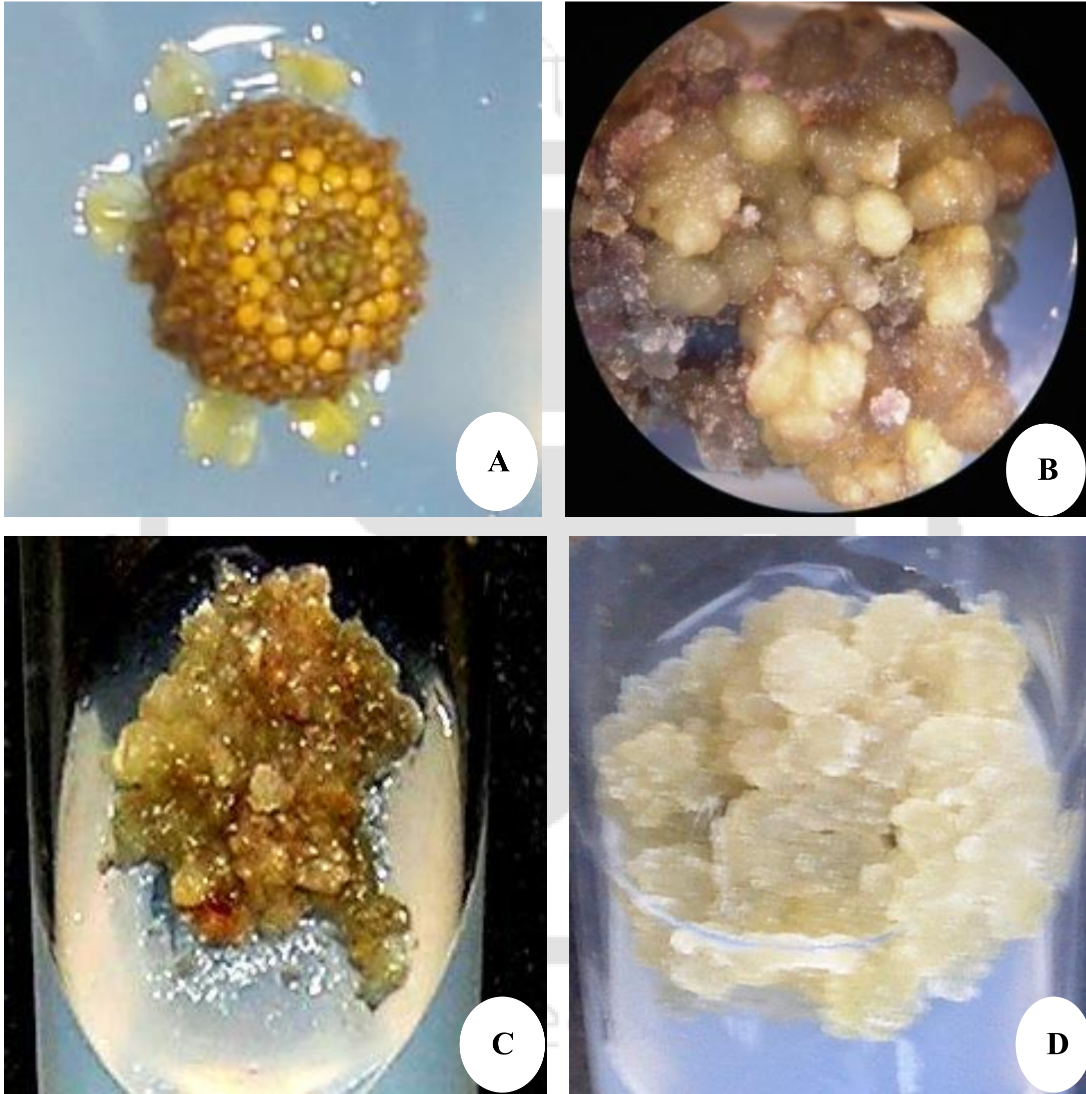


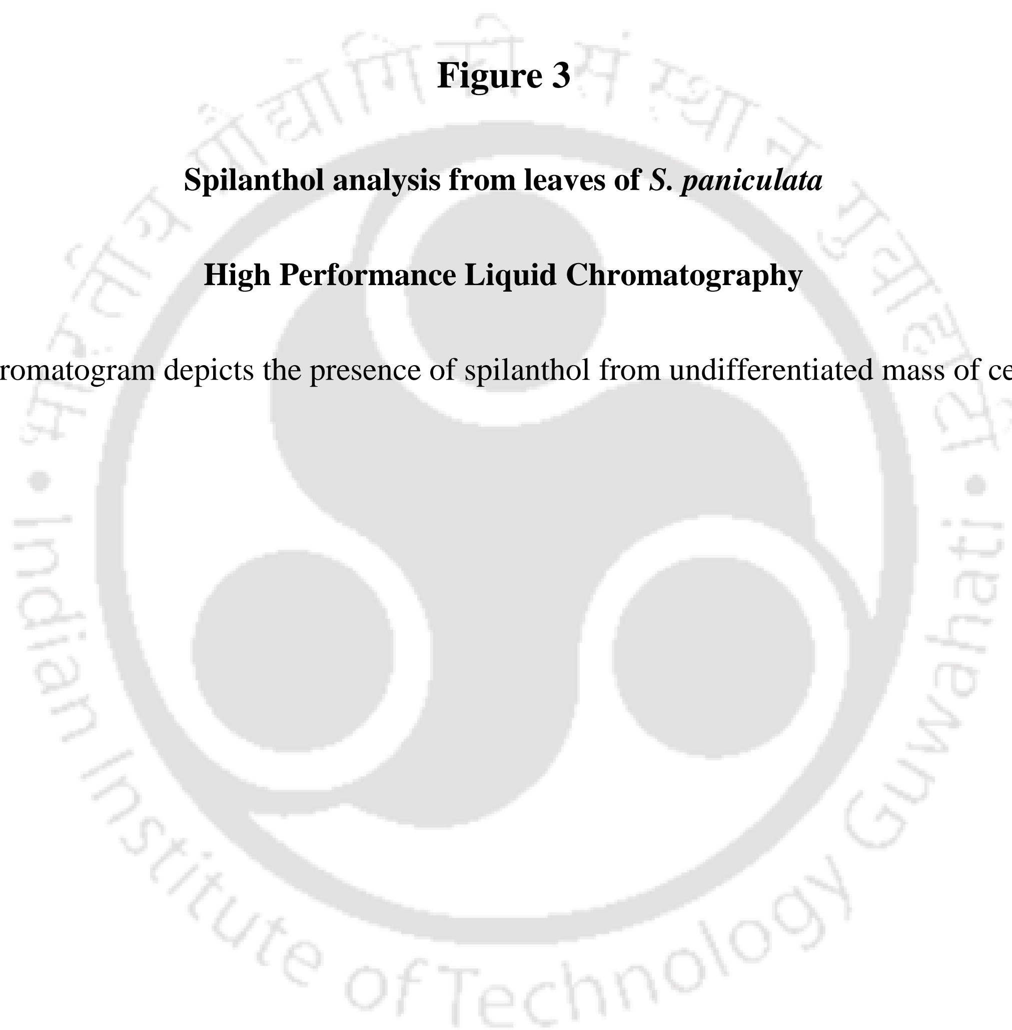
Figure 2

Figure 3

Spilanthol analysis from leaves of *S. paniculata*

High Performance Liquid Chromatography

HPLC chromatogram depicts the presence of spilanthol from undifferentiated mass of cells.



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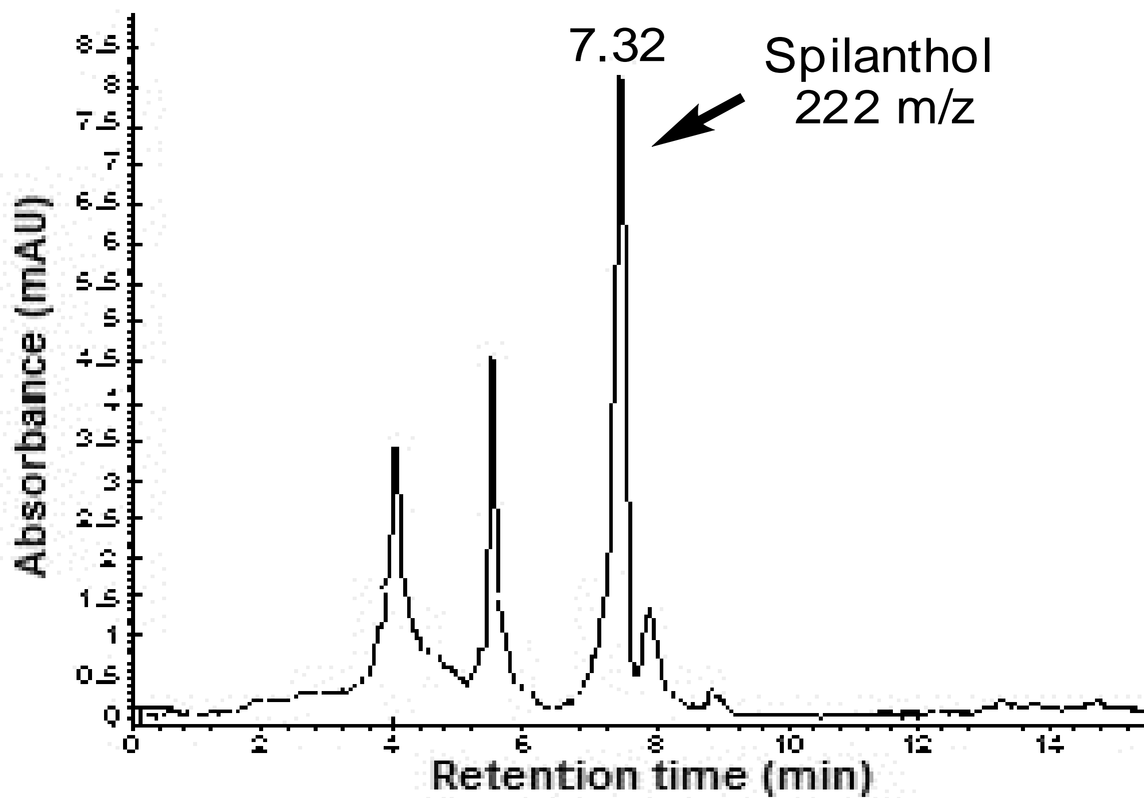

Figure 3

Figure 4

Mass spectrometry through electron spray inonization (ESI)

Positive electron spray ionization mass spectrum of (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) at 221 m/z and (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) at 230 m/z in semi-purified PTLC extracts

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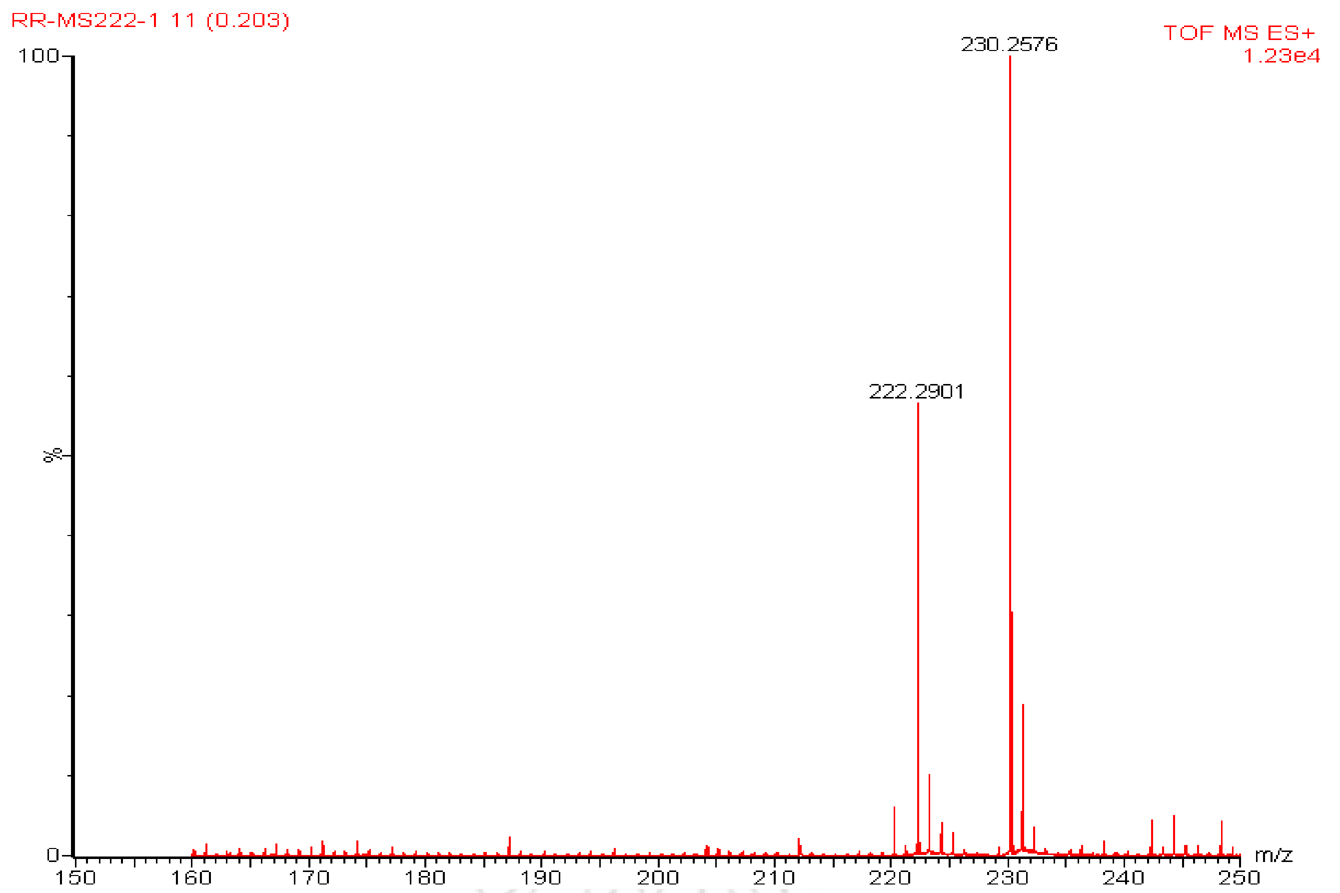

Figure 4

Figure 5

Analysis of *N*-alkylamides from flower-heads *in vitro* cell cultures of *S. paniculata*

High performance liquid chromatography

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)

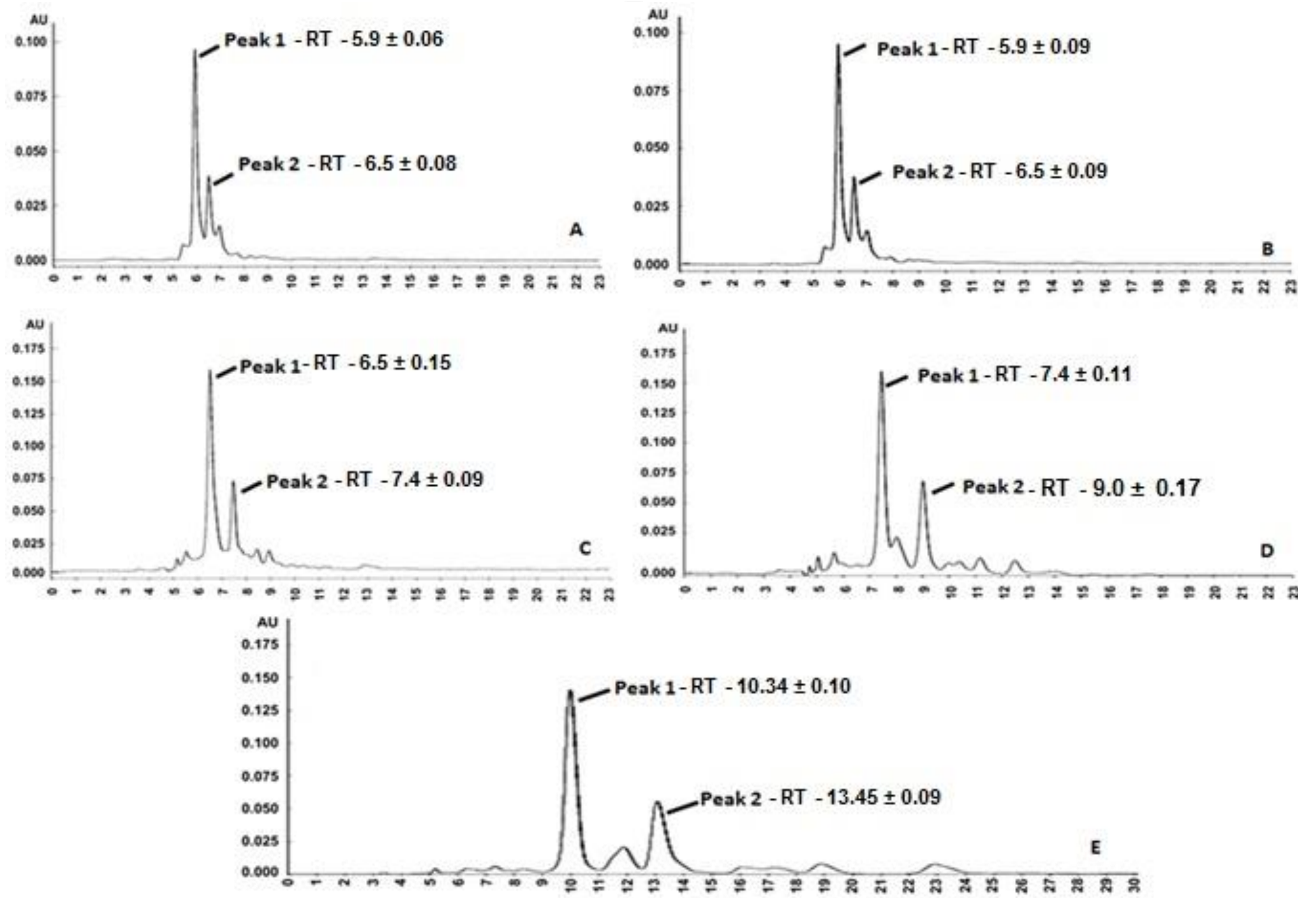

Figure 5

Figure 6

Analysis of (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide from flower-heads *in vitro* cell cultures of *S. paniculata*

High performance liquid chromatography

Isolation of (2*E*,*4Z)*-N-isobutyl-2,4-undecadiene-8,10-diynamide (UDA) at retention time of 10.34 ± 0.10 minute with m/z 230

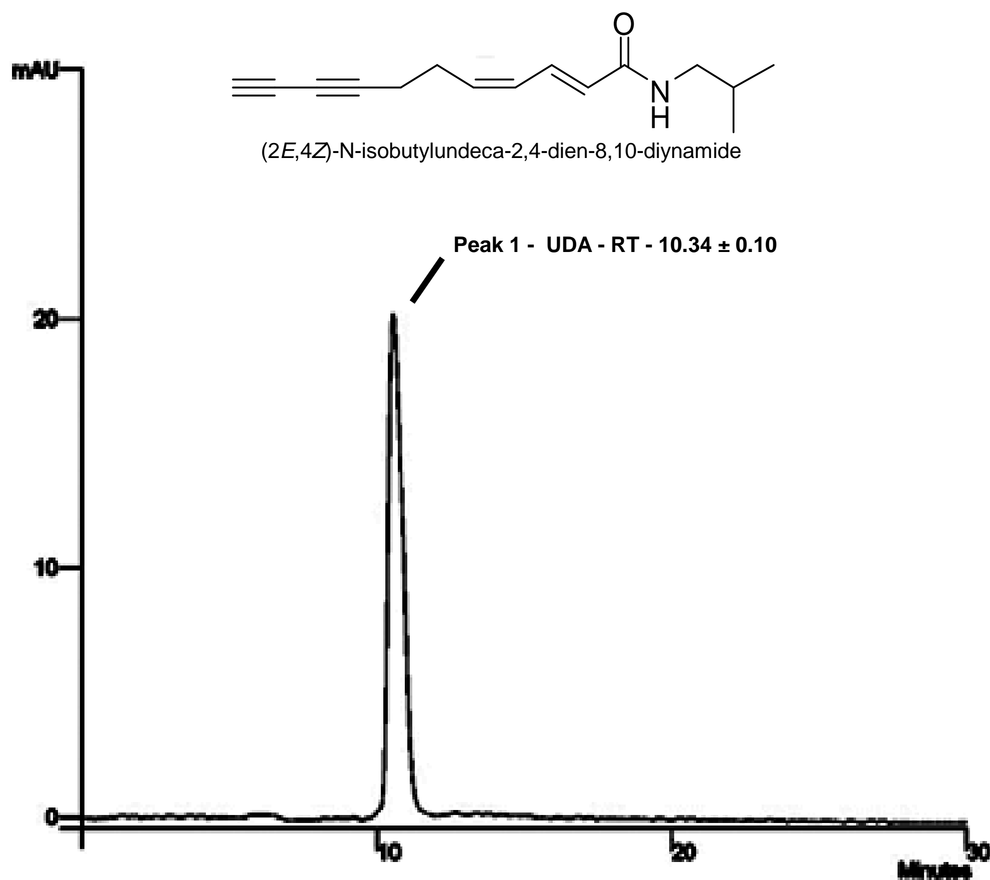

Figure 6

Figure 7

Analysis of *N*-alkylamide spilanthol

High Performance Liquid Chromatography

Isolation of (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol) at the retention time of 13.45 ± 0.09 minute with m/z 222

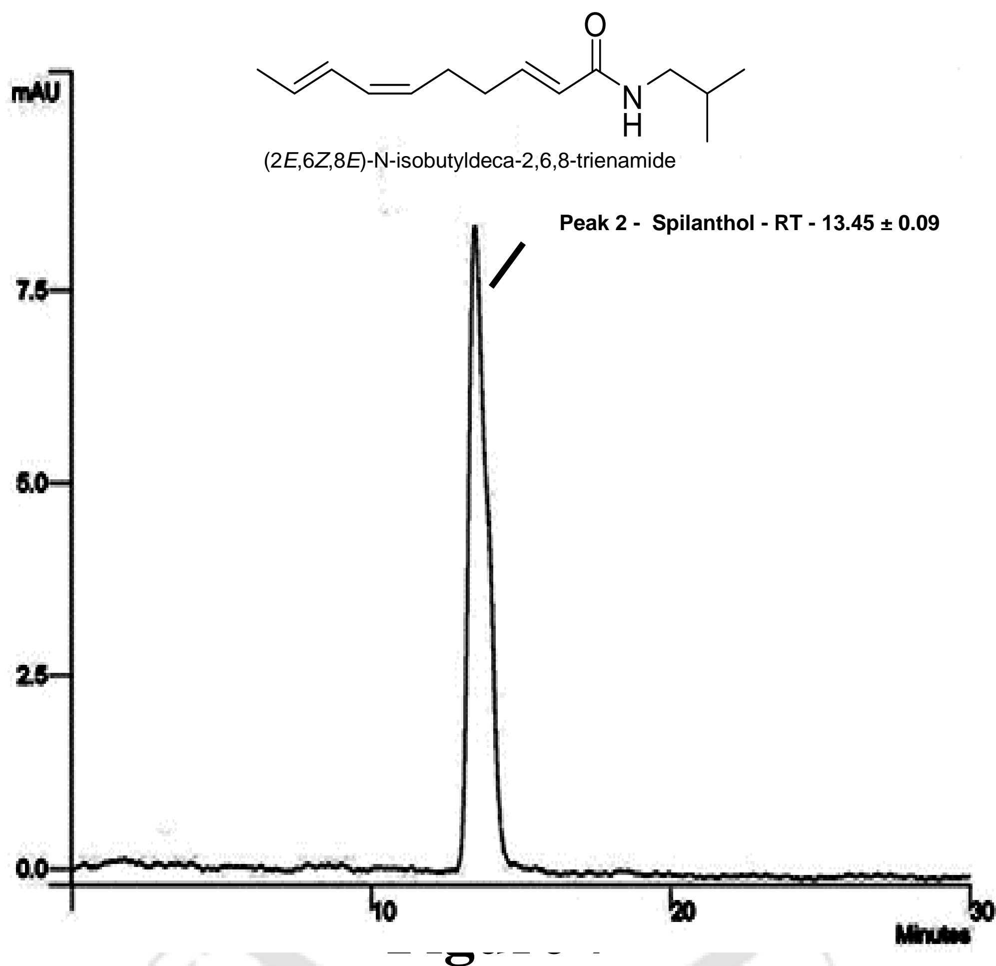

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Figure 7

Figure 8

Analysis of *N*-alkylamide spilanthol

Mass spectrometry

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

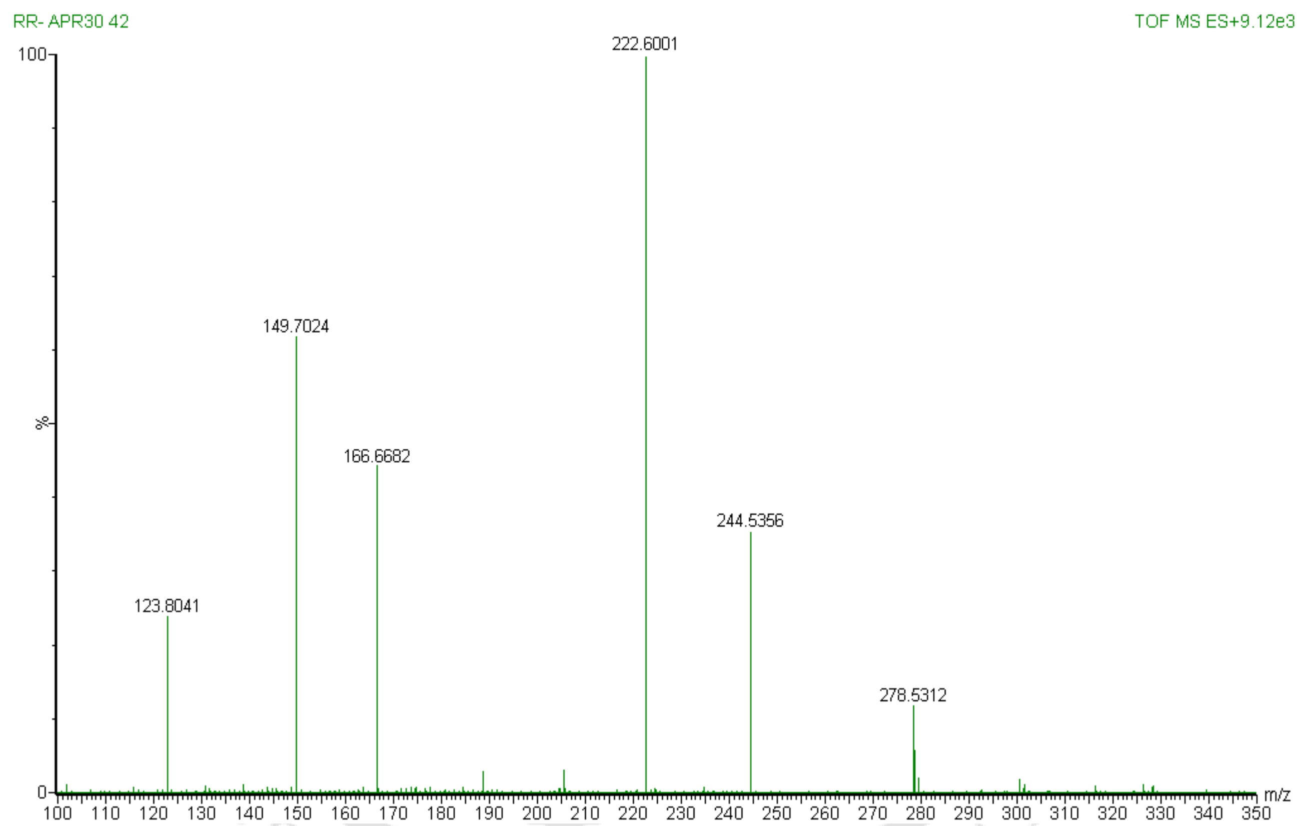

Figure 8

Figure 9

Analysis of (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide

Mass spectrometry

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 $\pm$ 0.10

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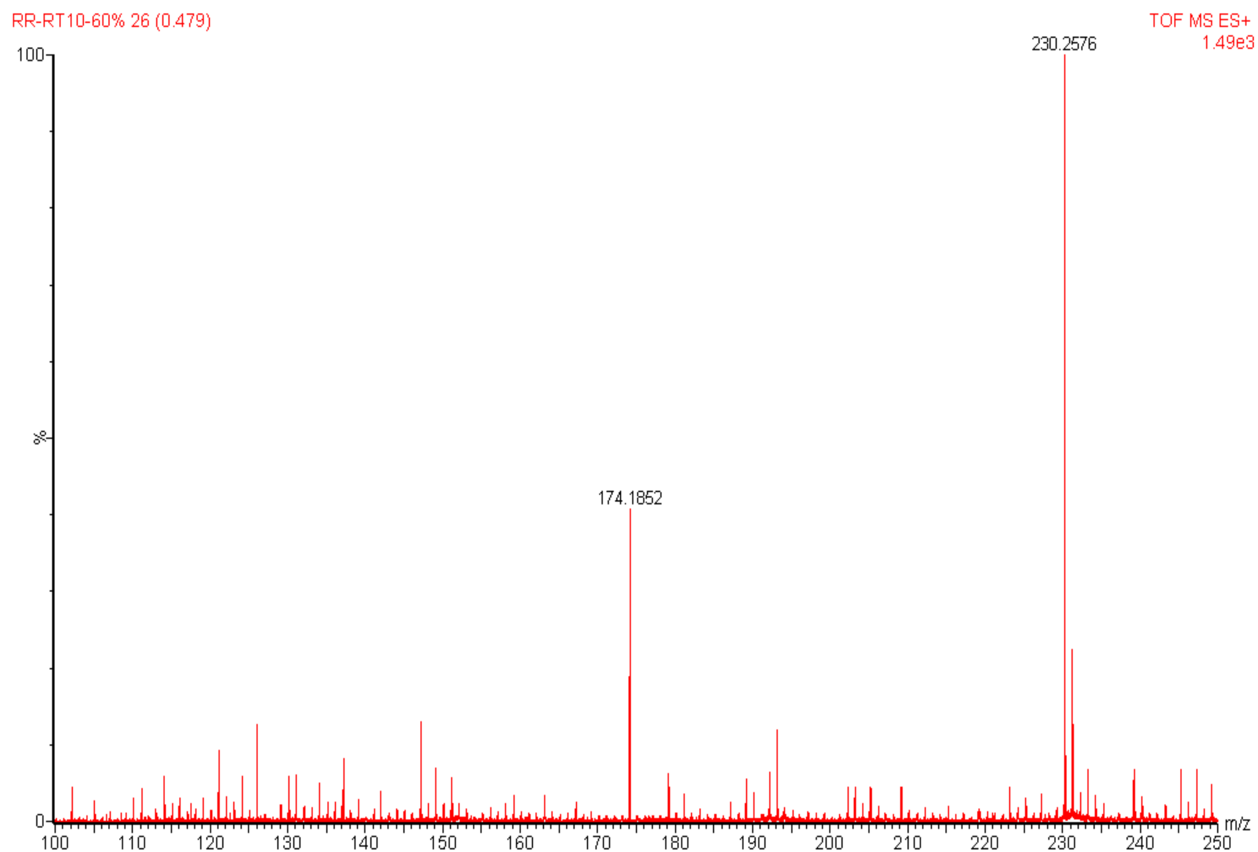

Figure 9

Figure 10

Analysis of *N*-alkylamide spilanthol

Infra-red spectroscopy

Infra-red 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

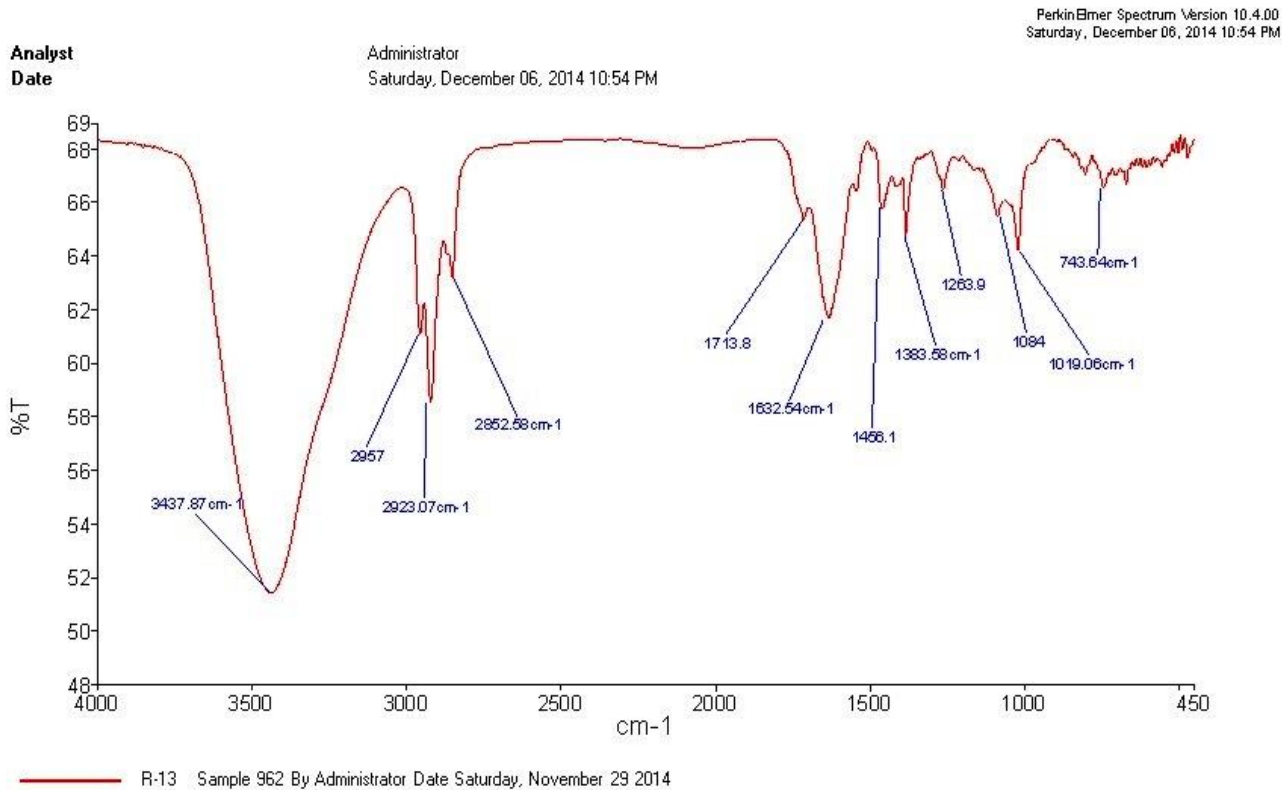

Figure 10

Figure 11

Analysis of (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide

Infra-red spectroscopy

Infra-red spectra of purified (2*E*,4*Z*)-*N*-isobutyl-2,4-undeca-8,10-diynamide (UDA) eluted at retention time of 10.34 ± 0.10

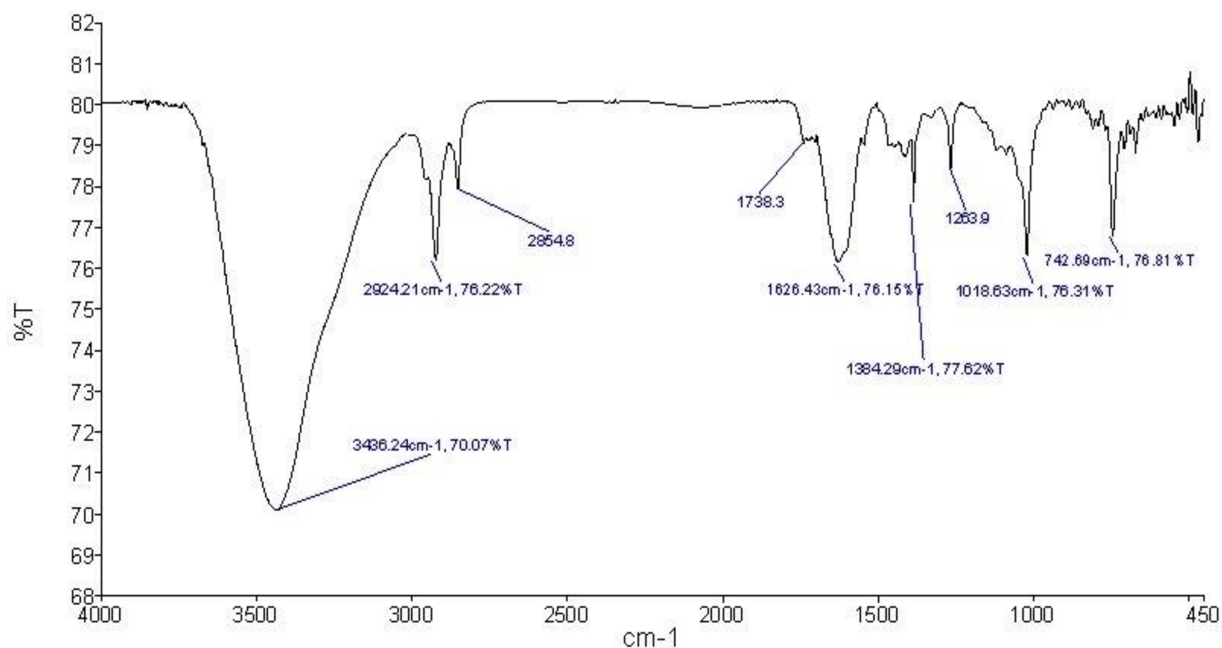

Figure 11

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Figure 12

Shake flask culture

Effect of agitation speed on cells viability

The suspended cells stained with 1% fluorescein diacetate solution

(A) The clumped cells fluorescing green at 60 rpm

(B) Cells maintained at 90 rpm showing loosely attached cellular clump

(C) Free, live and stained cells are obtained at the cells maintained at 120 rpm

(D&E) Showing sheared and clumped cells maintained at 150 & 180 rpm.

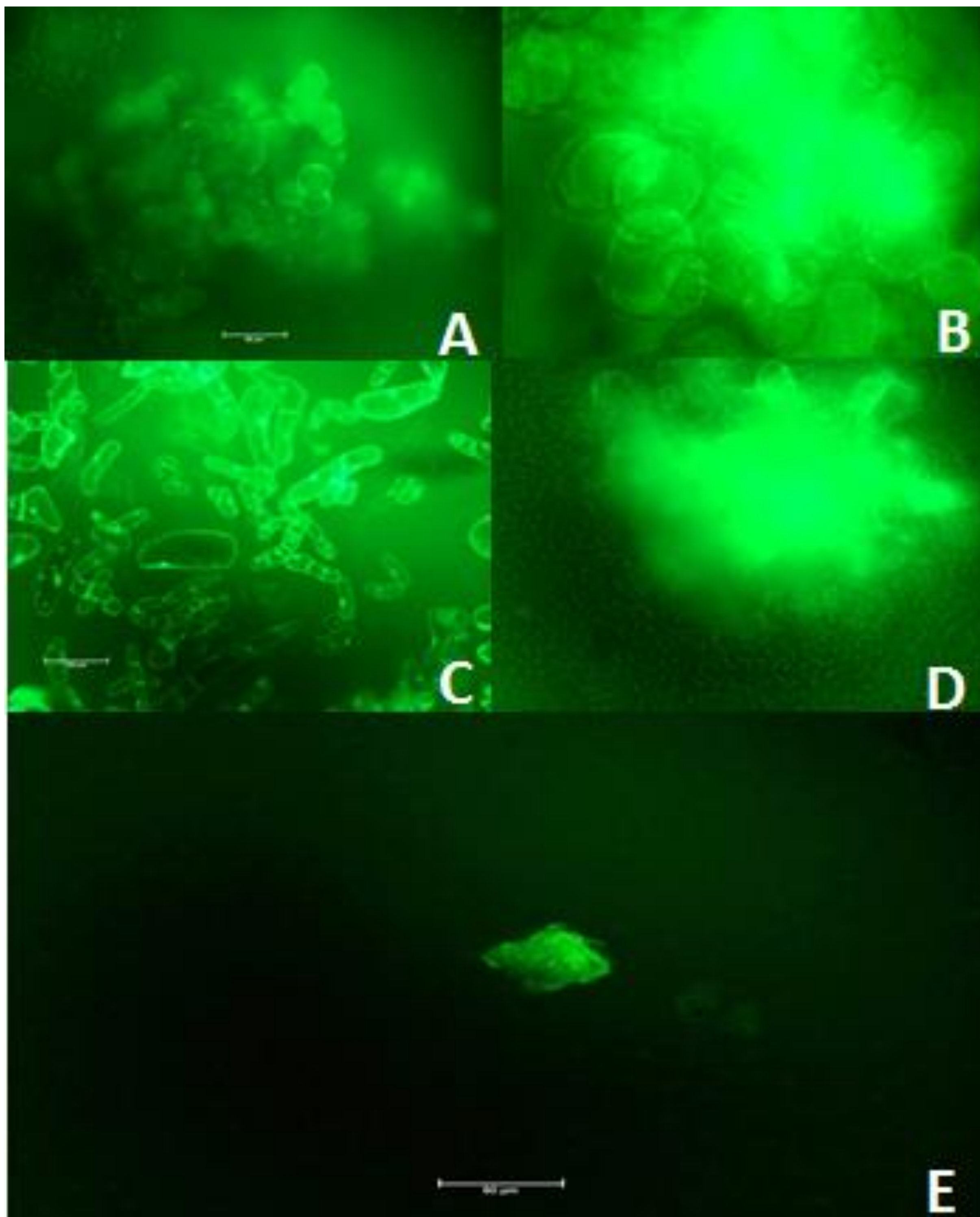

Figure 12

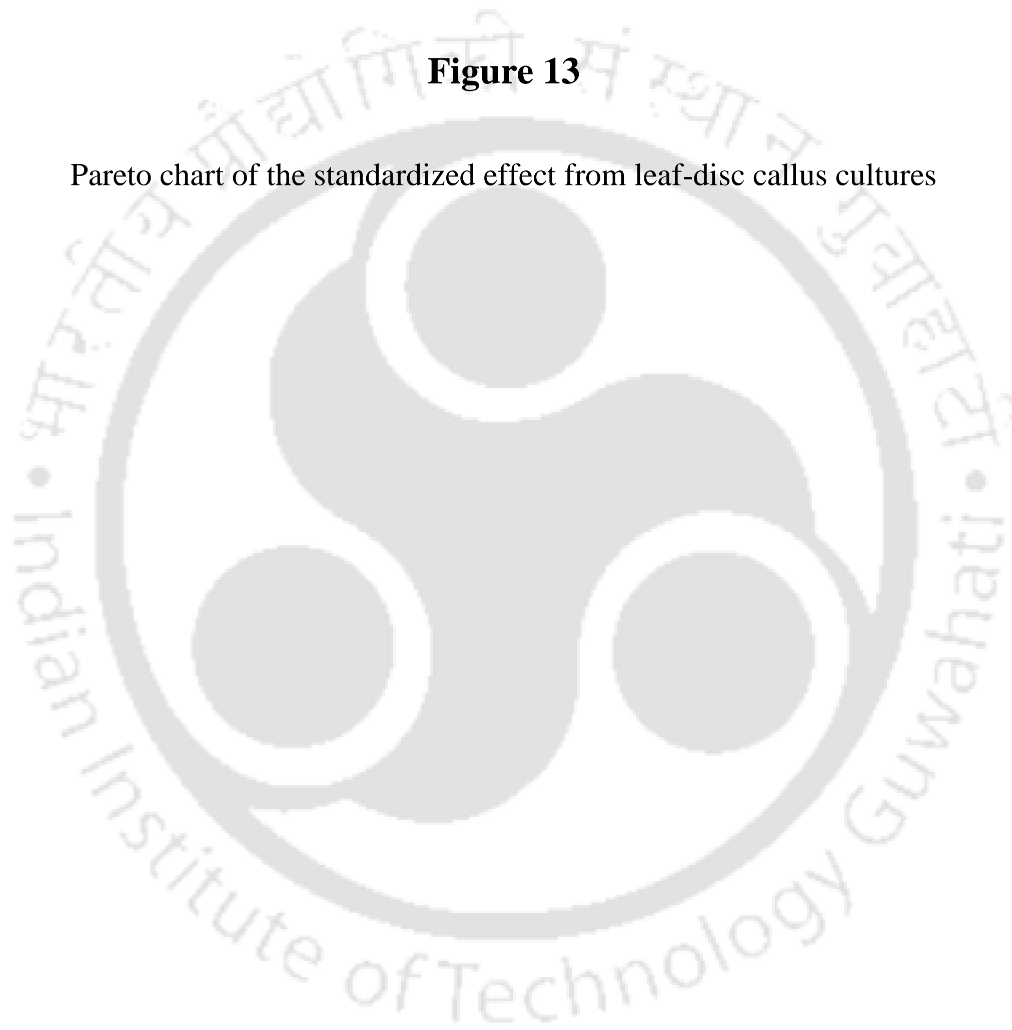

Figure 13

Pareto chart of the standardized effect from leaf-disc callus cultures

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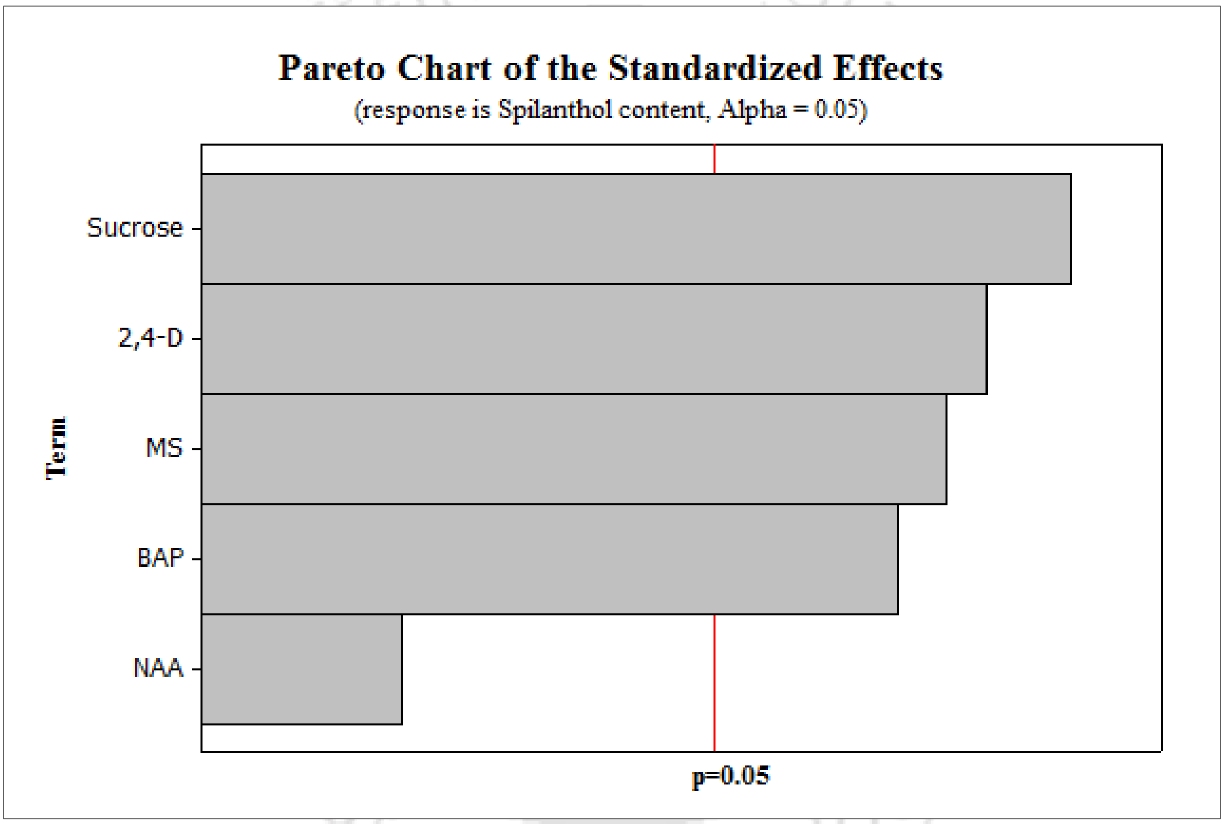

Figure 13

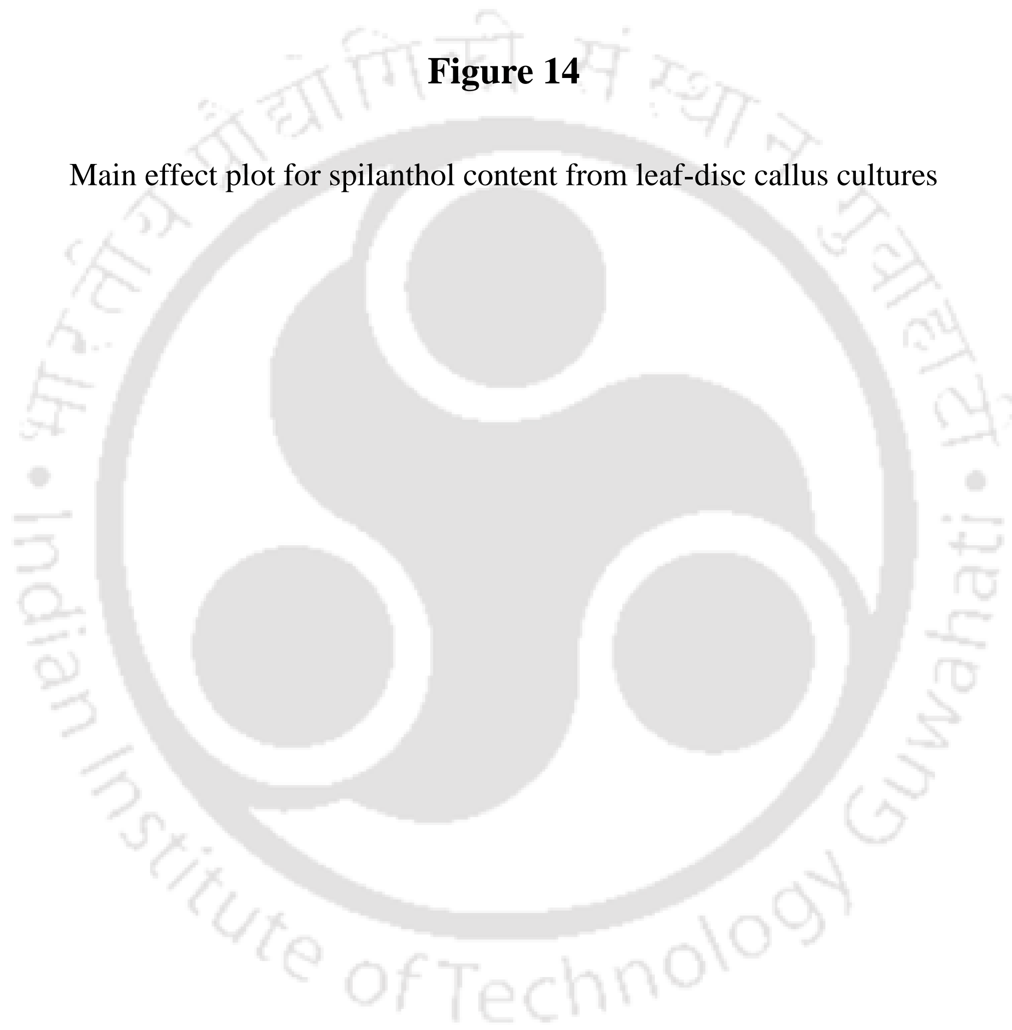

Figure 14

Main effect plot for spilanthol content from leaf-disc callus cultures

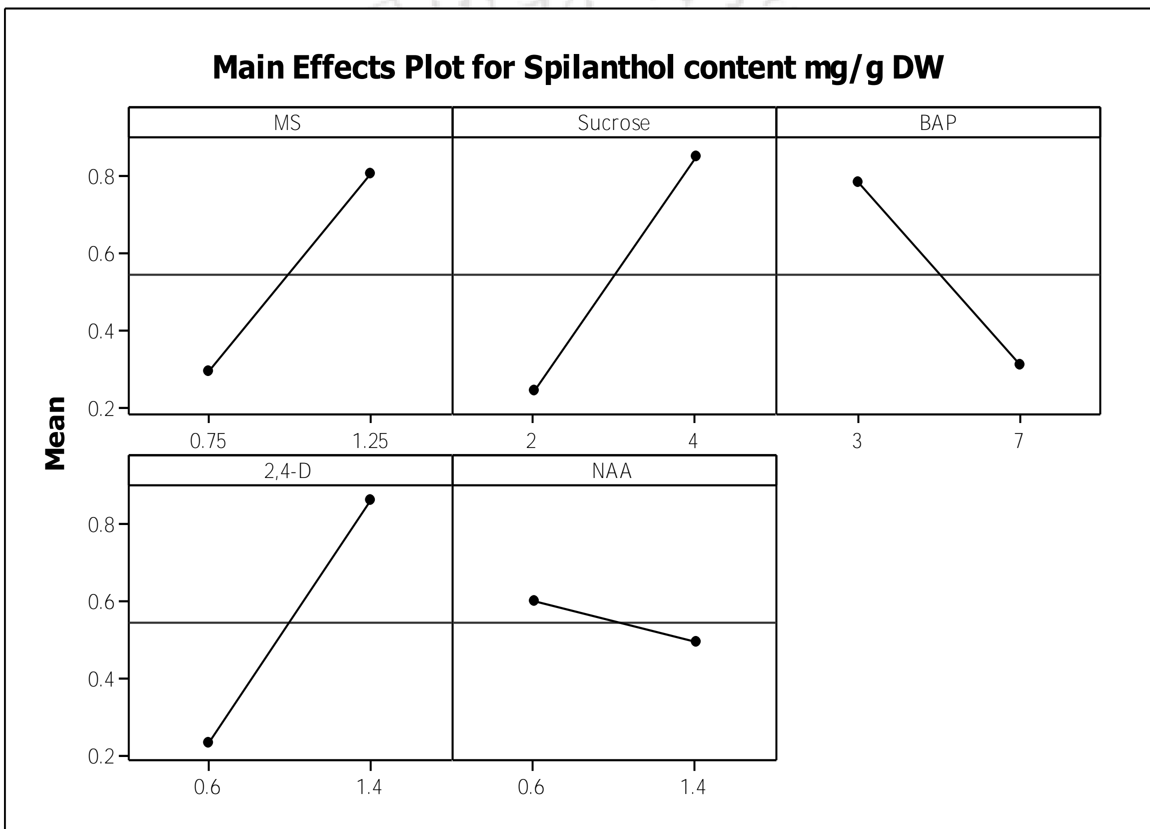

Figure 14

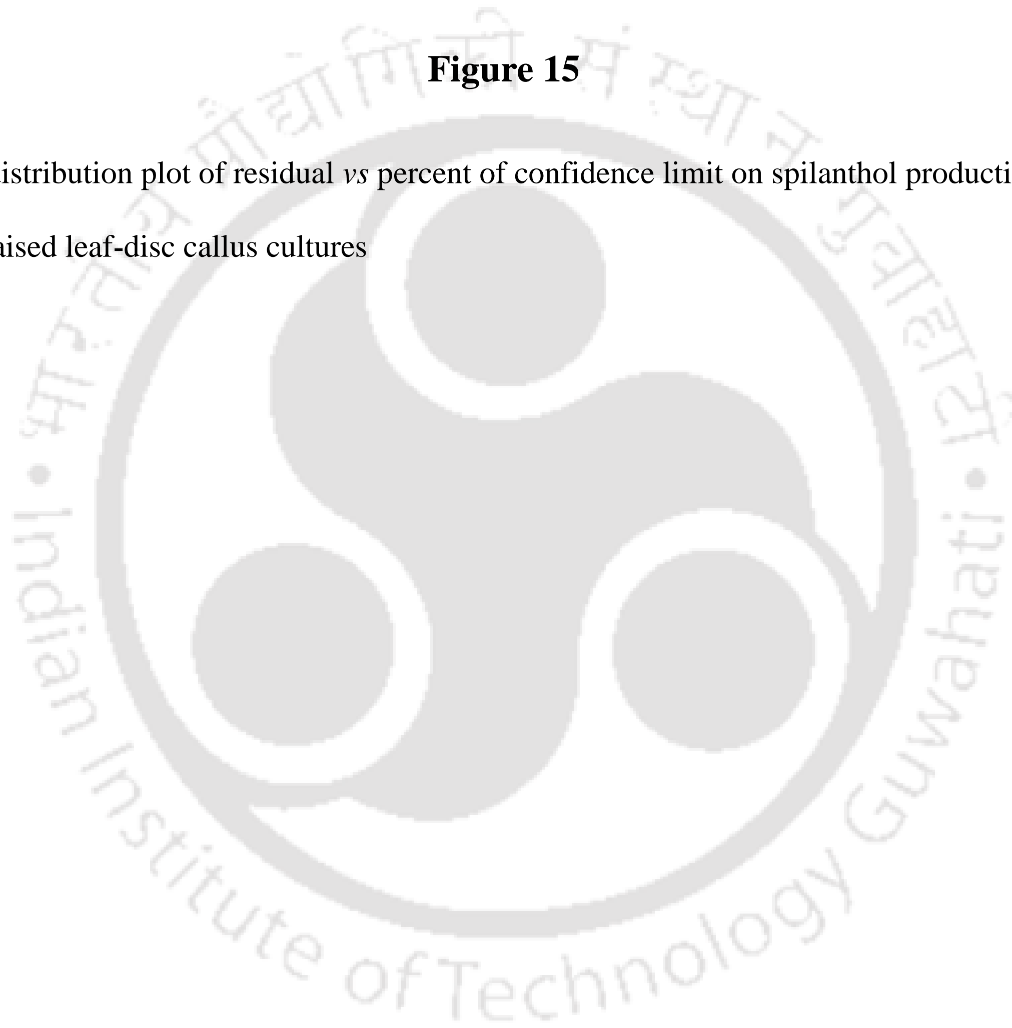

Figure 15

Normal distribution plot of residual *vs* percent of confidence limit on spilanthol production from *in vitro* raised leaf-disc callus cultures

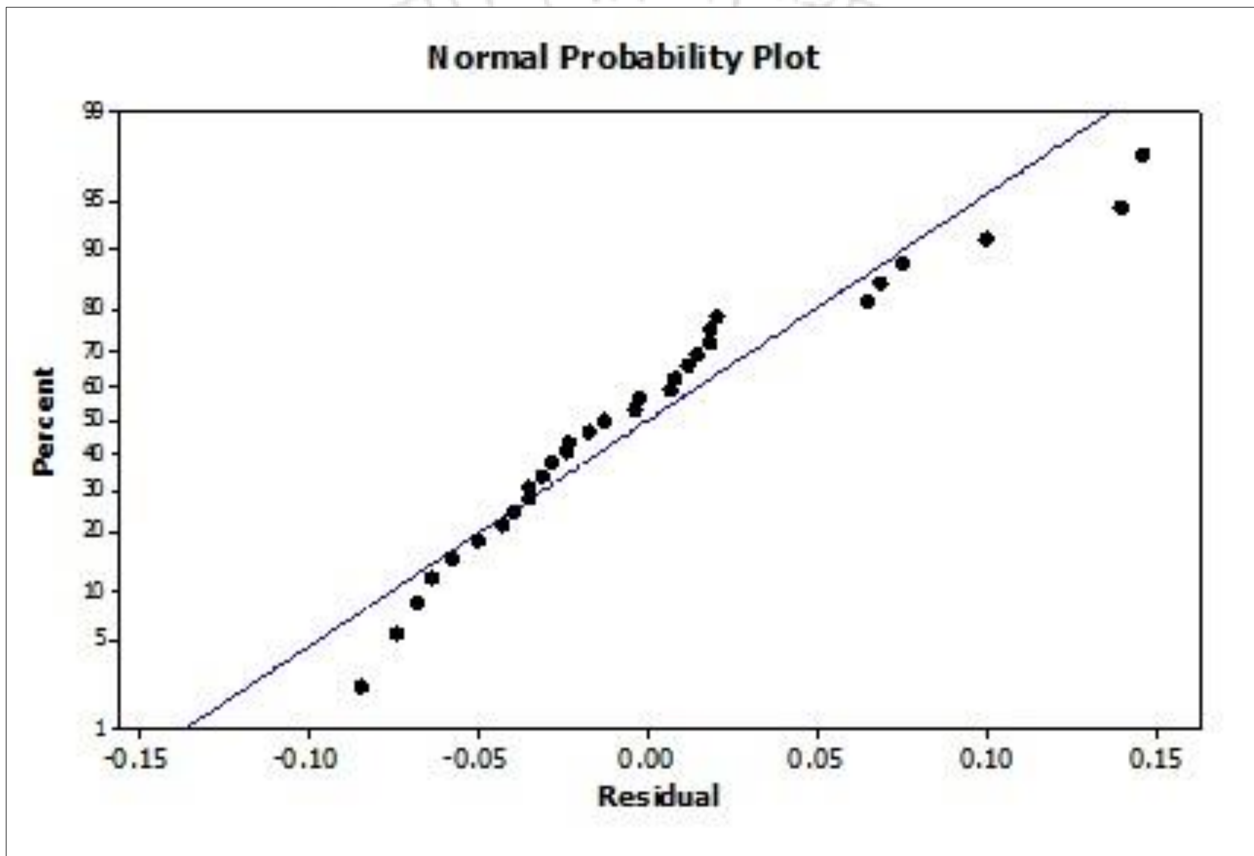

Figure 15

Figure 16

Response surface plots by the interaction of two variables from leaf-disc callus cultures

A. MS and Sucrose, fixed level: BAP and 2,4-D; Response: Spilanthol content mg/g DW

B. MS and 2,4-D, fixed level: Sucrose and BAP; Response: Spilanthol content mg/g DW

C. Sucrose and 2,4-D, fixed level: MS and BAP; Response: Spilanthol content mg/g DW

D. Sucrose and BAP, fixed level: MS and BAP; Response: Spilanthol content mg/g DW

E. MS and BAP fixed level: Sucrose and 2,4-D; Response: Spilanthol content mg/g DW

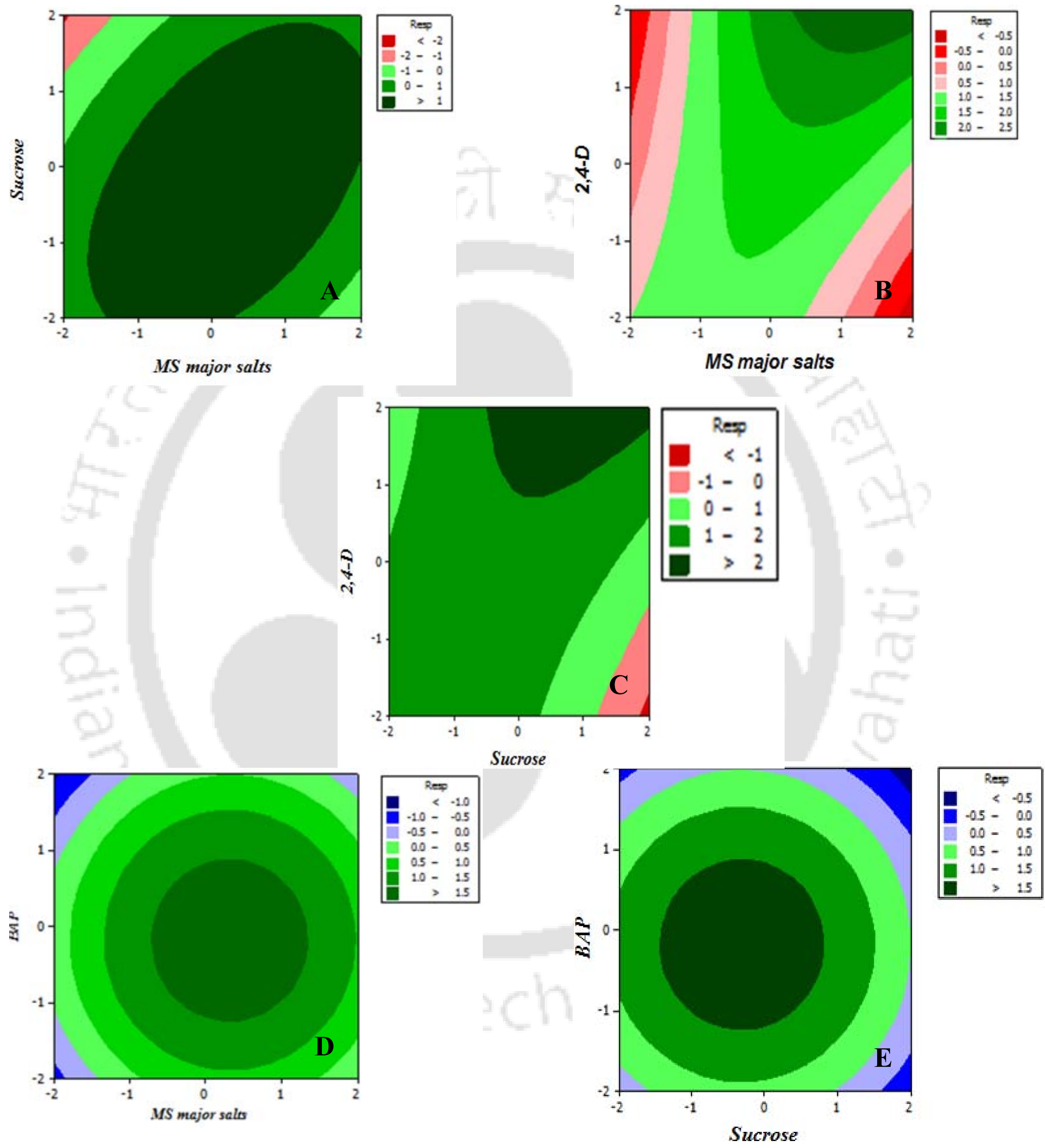

Figure 16

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Figure 17

Main effect plot for spilanthol content from flower-head callus cultures

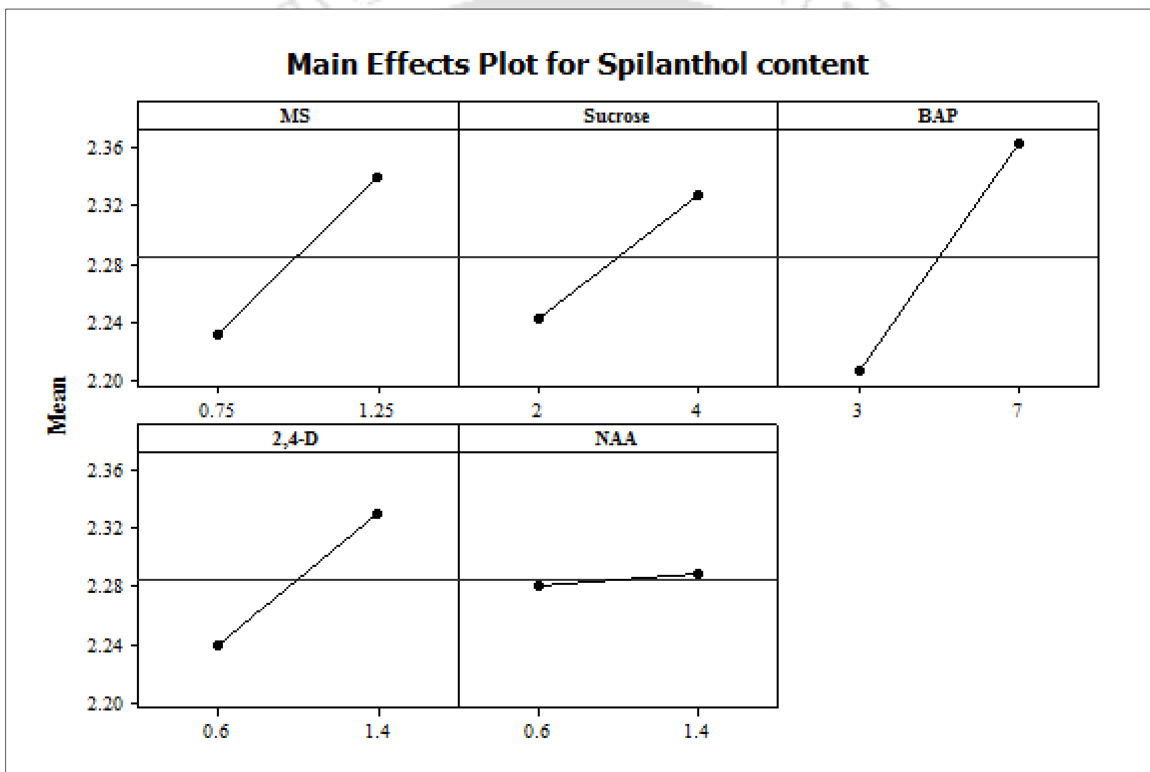


Figure 17

TH-1738_10610620

Figure 18

Pareto chart of the standardized effect from flower-head callus cultures

TH-1738_10610620

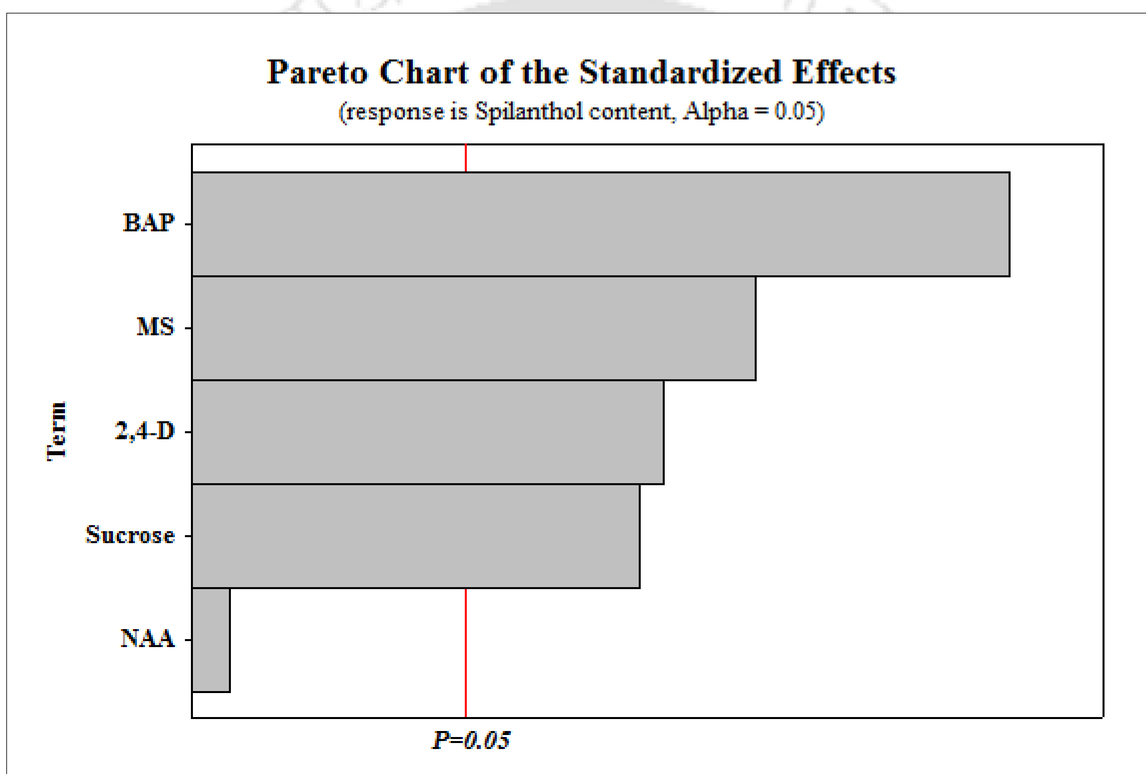


Figure 18

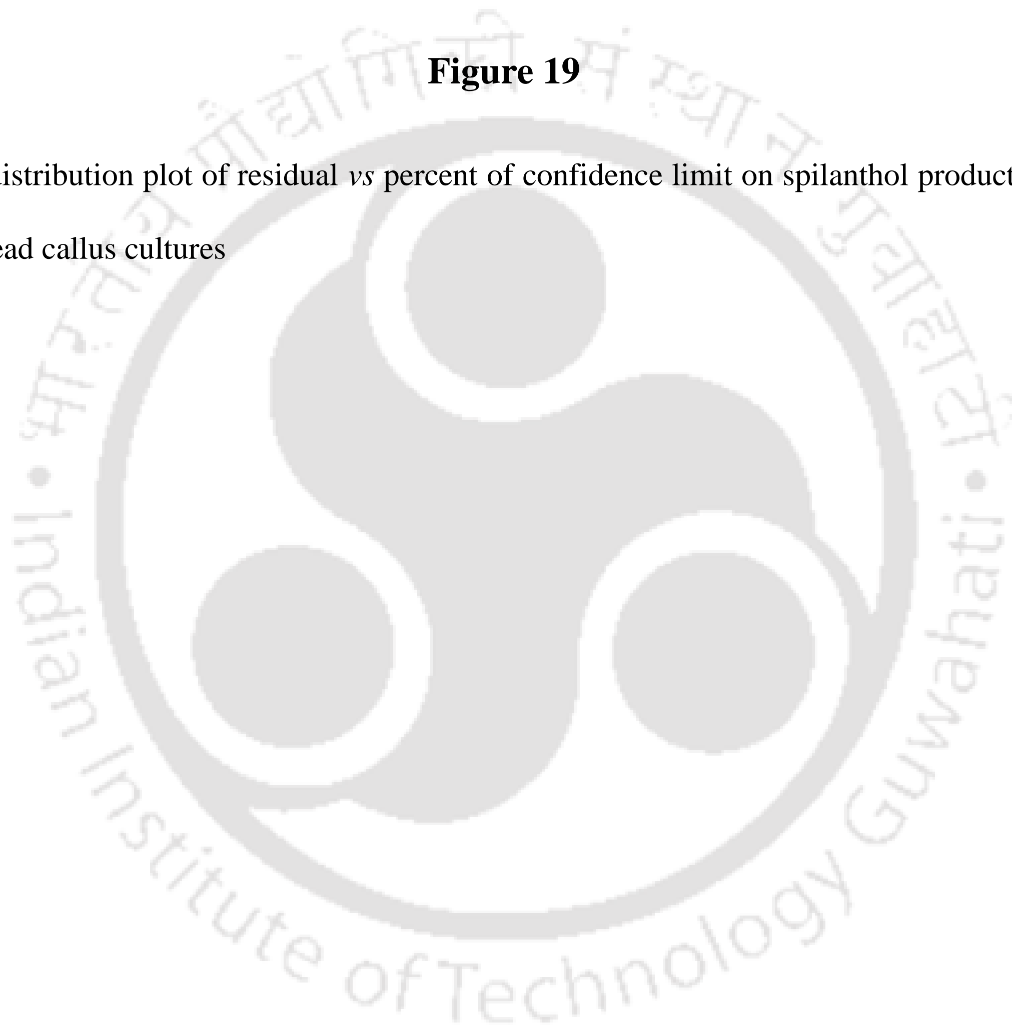


Figure 19

Normal distribution plot of residual *vs* percent of confidence limit on spilanthol production from flower-head callus cultures

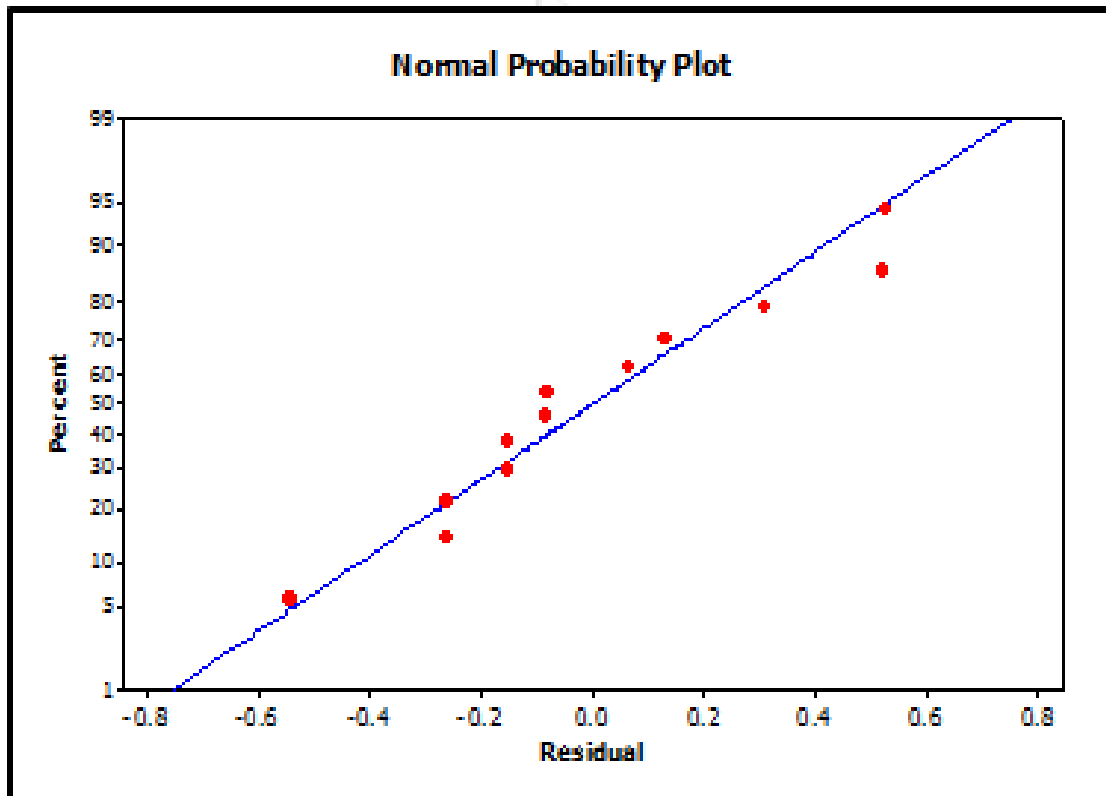


Figure 19

Figure 20

Response surface plots by the interaction of two variables from callus cultures of flower-head callus cultures

A. MS and Sucrose, fixed level: BAP and 2,4-D; Response: Spilanthol content mg/g DW

B. MS and BAP, fixed level: Sucrose and 2,4-D; Response: Spilanthol content mg/g DW

C. MS and 2,4-D, fixed level: Sucrose and BAP; Response: Spilanthol content mg/g DW

D. Sucrose and BAP, fixed level: MS and 2,4-D; Response: Spilanthol content mg/g DW

E. Sucrose and 2,4-D, fixed level: MS and BAP; Response: Spilanthol content mg/g DW

F. BAP and 2.4-D fixed level: MS and Sucrose; Response: Spilanthol content mg/g DW

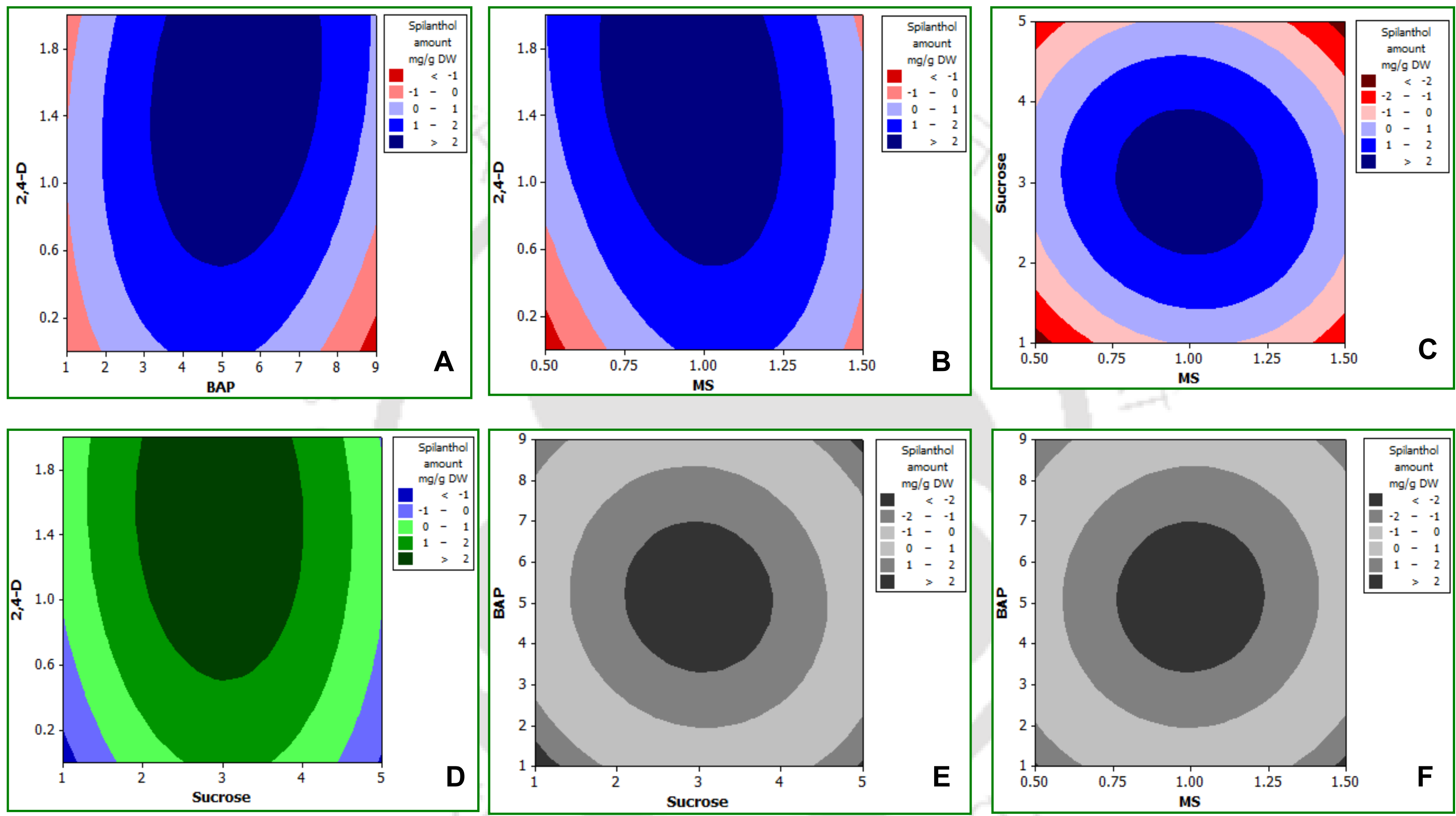


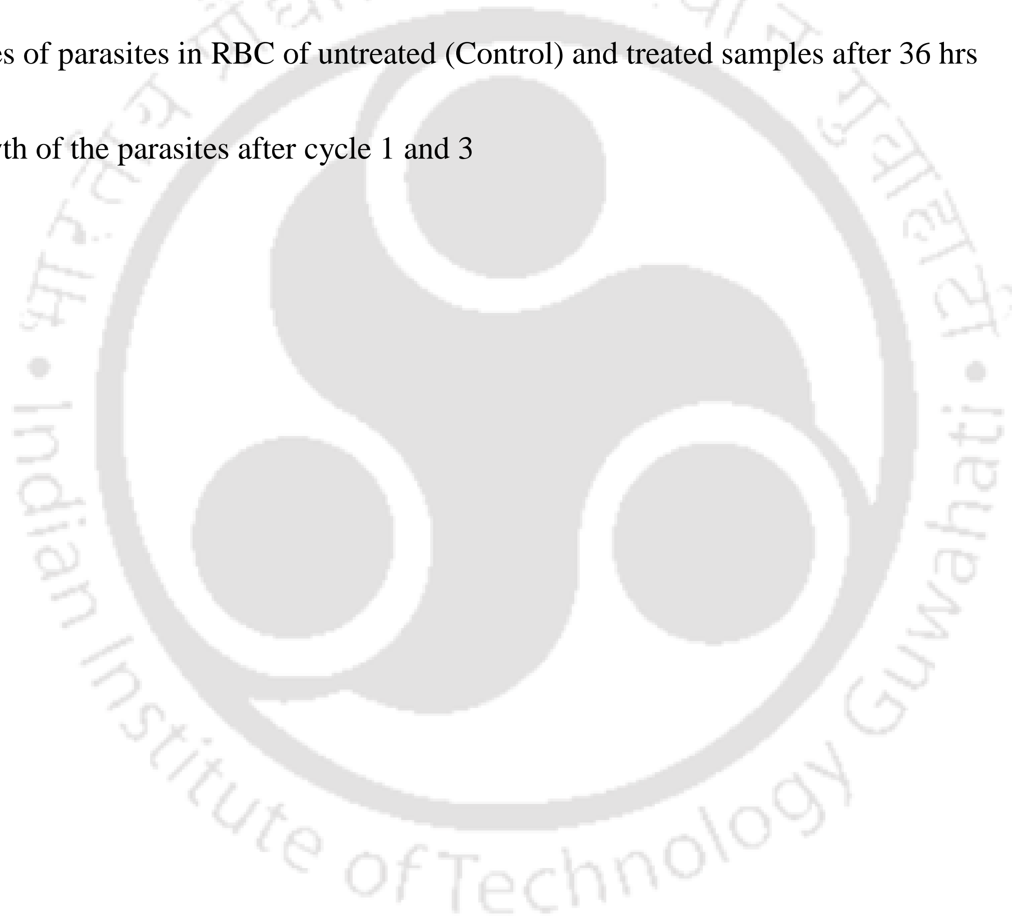
Figure 20

Figure 21

Microscopic evidence of erythrocytic antimalarial activity of *Spilanthes paniculata*

(A) Stages of parasites in RBC of untreated (Control) and treated samples after 36 hrs

(B) Growth of the parasites after cycle 1 and 3



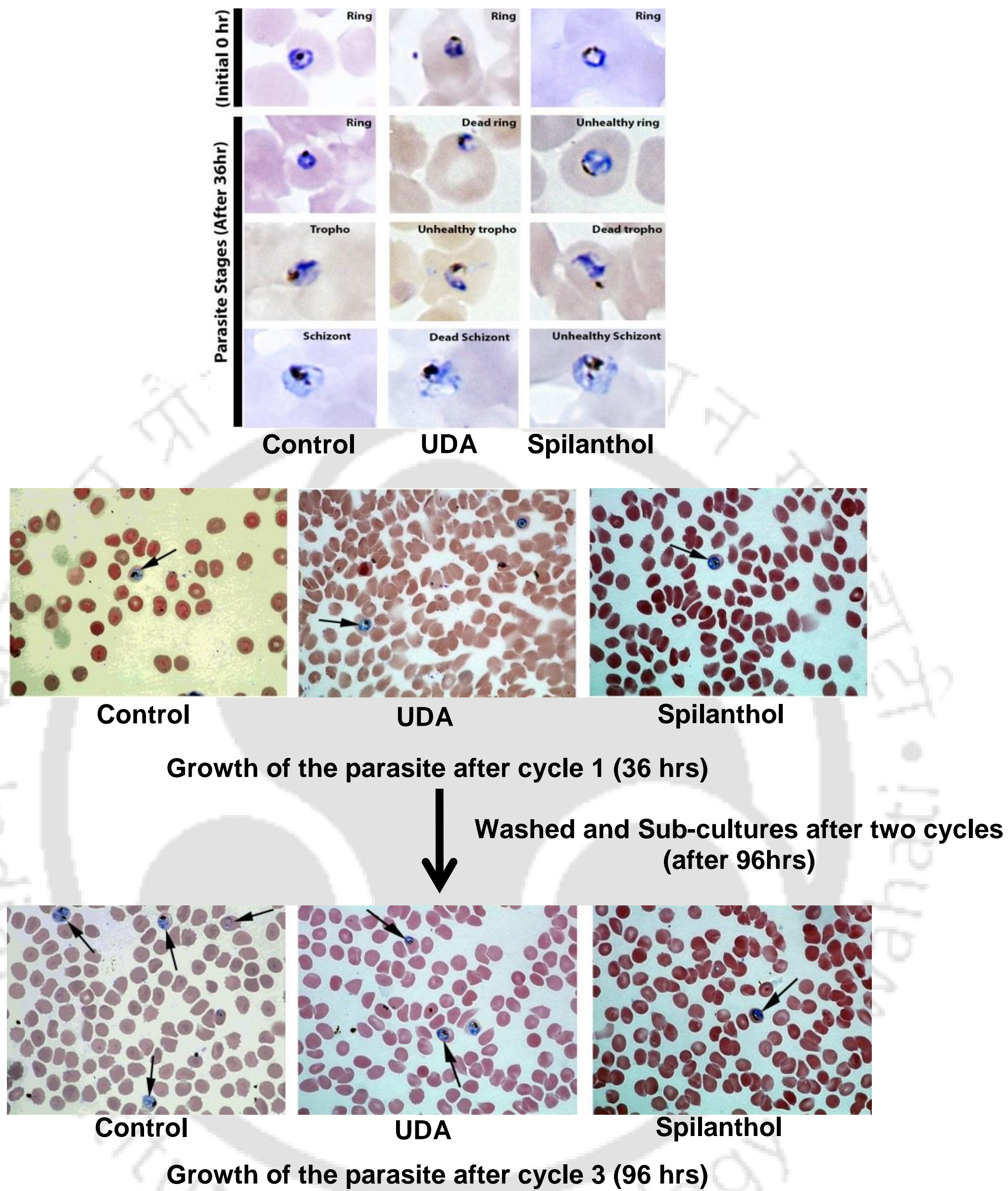


Figure 21

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