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REVIEW ARTICLE

In vitro production of spilanthol from *Spilanthes acmella* Murr.: State of the art and future prospect

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Abstract

Spilanthes acmella Murr., commonly known as toothache plant, is a well-known traditional plant of India. It has been demonstrated for a wide array of biological activities such as antifungal, antipyretic, bio-insecticide, antioxidant, aphrodisiac, analgesic, pancreatic lipase inhibitor, antimicrobial, diuretic, vasorelaxant and anti-inflammatory effects. These attributes are mainly due to the presence of an alkylamide, spilanthol. With increase in awareness about spilanthol therapeutic properties, industrial demand for this compound has also increased. Currently, spilanthol based industrial products are produced by collecting field grown plants. The utilization of huge quantities of whole plant parts is alarming as it can reduce local plant populations and erode genetic diversity. Moreover, the plants growing in wild undergo various climatic and environmental fluxes that may lead to change in their chemical profile. *In vitro* cultures have been viewed as promising alternatives to whole plant extraction for obtaining spilanthol, irrespective of seasons and regions, offering stable and consistent production. However, the research on *in vitro* spilanthol production is in its infancy and requires culture and process optimization for the development of a commercially feasible process. This review states the present status and future challenges of plant tissue culture for spilanthol production.

INTRODUCTION

Spilanthes acmella Murr. (Common names: Akarkara and Toothache plant) is an indigenous species that belongs to daisy family, Asteraceae. Five species of *Spilanthes* viz. *S. acmella* Murr., *S. acmella* L. var *oleraceae* clarke, *S. calva* L., *S. paniculata* L. and *S. mauritiana* L. are reported from India (Anonymous, 1989). Among these *Spilanthes* species, *S. acmella* Murr. and *S. acmella* L. var *oleraceae* Clarke are rare in occurrence. It is grown as perennial plant throughout the tropics and subtropics, and can be found in damp pastures, at swamp margins, on rocks near the sea and as a weed of roadsides. *Spilanthes* is a hairy herb upto 30-60 cm tall, with numerous prostrate or ascending branched cylindrical hairy stem and simple ovate opposite leaves without stipules.The flowers are yellow, non-fragrant with five petals on long glabrous peduncles. Many small, bright yellow colour flowers are aggregated into capitulum (flower head) which make them attractive to insects, thus, paving the way for entomophily. The plant is conventionally propagated through seeds that lose their viability within a short period of time and show slow germination rates (Pati et al., 2006; Dobránszki and da Silva, 2010). Vegetative means too have not shown successful results in propagating *Spilanthes* (Tiwari et al., 2011).

Spilanthes is a well-known plant in Indian traditional system of medicine with multiple pharmacological actions. It is reported to have immune-modulatory, antioxidant and insecticidal biological properties (Ramsewak et al., 1999; Pandey and Agrawal, 2009; Guiotto et al., 2008; Matthias et al., 2008; Prachayasittikul, 2009). Extracts of the plant have been used as a spice for appetizers and as folk medicines for stammering, toothache, stomatitis and throat complains (Nakatani and Nagashima, 1992; Ramsewak et al., 1999). *Spilanthes* is one of the main constituent of compositions for acute- or long-term cure of microbial infections (Adler, 2006). It has been reported in being effective against blood parasites and is poisonous to most invertebrates whereas harmless to the vertebrates (Watt and Brayer-Brandwijk, 1962). In addition, its extract is an active constituent of beauty care cosmetics such as for a fast acting muscle relaxant to accelerate repair of functional wrinkles (Belfer, 2007). The plant extract is also used as a nutritional supplement for taste improvement as a sweetener with high sweetness devoid of distasteful savour that does not affect the taste or odor of foods or drinks (Miyazawa et al., 2006).

The plant contains several secondary metabolites such as alkylamide, phenolics, coumarin and triterpenoids which are therapeutic and account for its use in traditional medicines all over the world, since long (Prachayasittikul, 2009). Among all spilanthol, N-isobutylamide, is one of the most sought after compound of the plant. Spilanthol has a strong pungent taste; it may produce local astringency and anaesthetic effects. In small amount spilanthol is present in all parts of plant but its highest concentration is present in flower (Dias et al., 2012). All commercial spilanthol formulations contains spilanthol which is extracted from the flowers of naturally grown whole plant, this approach has the disadvatages of heterogenecity in spilanthol content depending upon the plant genotype and environment. Total chemical synthesis could be another route to obtain the required amount of this compound. Although the synthetic route of spilanthol is established, but synthetic products due to detrimental side effects are not preferred by industries as well as by consumers.

Spilanthol is a high value bioactive molecule and as the awareness towards more safe, natural compounds are increasing, its demand is also increasing continuously. Current supply of spilanthol from *Spilanthes* plant will not meet the increasing demand if the extraction from flowers remains the only source hence there is need for the development of commercially viable alternatives for its production. In this respect plant cell and organ cultures offer an attractive alternative, for homogeneous, controlled production of spilanthol, throughout the year, especially when commercial demand is taken into account. This method not only facilitate the de novo synthesis of novel compounds, but are able to produce metabolites, sometimes even in higher amounts than the intact plants. Production of spilanthol from plant cell culture can be an area of active research and development, however most of the data available on spilanthol is regarding its chemistry, biological effects and extraction and analysis from field growing plants. Till date, only a few attempts have been made on tissue culture aspects of spilanthol production. This review deals with the current state of the art and future challenges of plant cell culture technology for spilanthol production.

Importance of Spilanthol

Spilanthol obtained from *Spilanthes* species is one of the most important compound currently in use. This bioactive molecule is responsible for many pharmacological activities of the plant specifically its antimalarial property (Khadir et al., 1989). Scientists have demonstrated its *in vivo* and *in vitro* antimalarial activity (Spelman et al., 2011). It has shown good activity against the vector for malaria (various *Aedes* species) and filaria (*Culex quinquefasciatus*). It is also claimed to have insecticidal properties that support its use against bedbuds and cockroaches. This alkylamide has been found harmless to majority of vertebrates and lethal to invertebrates (Watt and Brayer-Brandwijk, 1962). Additionally, spilanthol has an efficient analgesic, anti-inflammatory and antimicrobial properties. This makes spilanthol well suited for use in the treatment of painful conditions such as trauma and insect bites. The analgesic activity of spilanthol has been attributed to an increased gamma-aminobutyric acid (GABA) release in the temporal cerebral cortex (Rios et al., 2007). Interestingly, spilanthol has been demonstrated to inhibit nitric oxide (NO) production in a murine macrophage cell line, to efficiently down-regulate the production of inflammatory mediators interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), and to attenuate the expression of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) (Wu et al., 2008). Other investigations have also confirmed the down regulation of some pro-inflammatory cytokines by bioactive alkylamides under various experimental conditions (Cech et al., 2006; Guiotto et al., 2008; Wang et al., 2008). These findings confirm spilanthol anti-inflammatory property and suggest that it can be used as a potential new lead compound for COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs).

Spilanthol based local buccal mucosa preparations are used for painful mouth tissues and small mouth ulcer. Spilanthol can readily permeate the buccal (oral cavity) mucosa and skin surface, which indicates good bioavailability and explains the fairly rapid efficacy of the remedy (Boonen et al., 2010b).

Spilanthol clinical trial has also been done for anti-aging property. It stimulates, reorganize and strengthen the collagen network, notably those of the face, and can be used as an anti-wrinkle product. It has been reported that spilanthol increase the permeation properties of the skin towards other compounds (De Spiegeleer et al., 2012).

Chemistry, Extraction and Analysis Methods

Chemically spilanthol is (2E,6Z,8E)-N-isobutylamide-2,6,8-decatrienamide having molecular formula $C_{14}H_{23}NO$ (**Fig. 1**.). Spilanthol was first isolated in 1945 from ethanol extract of *S. acmella* flower head. The literature suggests that spilanthol can be extracted by using simple maceration, supercritical fluid extraction, solid phase extraction and microwave assisted methods (Dias et al., 2012; Singh and Chaturvedi, 2012a; Costa et al., 2014). In most of the studies hexane, ethanol, methanol and hydroethanolic solvents were used for spilanthol extraction **(Table 1)**. Among all the investigated extraction techniques, supercritical fluid extraction has been proven to be the most effective extraction process for spilanthol from all parts of *S. acmella* (e.g. flowers, leaves and stems). The main benefit of this technique is that by this ready-to-use extract can be obtained. Moreover this technique is solvent independent (Dias et al., 2012). In other plant species such as *S. americana* (Stashenko et al., 1996) and *Echinacea angustifolia* (Sun et al., 2002) supercritical fluid extraction method has been validated. It is reported that this method is efficient for selective extraction of spilanthol from *S. americana* flowers and leaves. Centrifugal partition chromatography (CPC) is another technique used for quantitative isolation of N-alkylamides from *S. acmella* methanolic flower extract (Mbeunkui et al., 2011). The CPC offered high recovery of the target compounds and high throughput as compared with other traditional separation methods, for example, column chromatography and thin layer chromatography. Mbeunkui et al. (2011) demonstrated the potential of CPC for large-scale isolation of major N-alkylamides from *S. acmella*.

So far, various analytical techniques such as ^{1}H and ^{13}C nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (HPLC-MS) has been used for spilanthol detection and quantification (Nakatani and Nagashima, 1992; Ramsewak et al., 1999; Leng et al., 2011; Singh and Chaturvedi, 2012b; Mbeunkui et al., 2011; Costa et el., 2014) (**Table 1**).

Nakatani and Nagashima (1992) used NMR and high pressure liquid chromatography-mass spectrometry (HPLC-MS) with atmospheric pressure chemical ionization and electron impact ionization to determine structure of spilanthol in extracts of *S. acmella*. Bae et al. (2010) used high pressure liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) for rapid identification and quantification of spilanthol from *S. acmella* (e.g. whole plants, leaves, flowers, stems and roots) ethanol extracts. Mbeunkui et al. (2011) identified Spilanthes alkylamide by electrospray ionization-ion trap-time of flight mass spectrometry (ESI-IT-TOFMS) and validated by ^{1}H-and$1^{3}C$-NMR analysis. Leng et al. (2011) employed GC-MS to detect spilanthol present in mother plant, flower heads and *in vitro* plantlets of *S. acmella*. The comparison of the GC-MS masss pectrum with the NIST database library gave more than 90% match as well as a confirmatory compound structure match. Recently, Singh and Chaturvedi (2012a) used HPLC and then MS with positive ESI for identification and quantification of spilanthol present in *in vivo* and *in vitro* plants. For HPLC analysis, acetonitrile and water at the ratio of 93:7 as the mobile phase was found to be appropriate for satisfactory separation of compounds at a flow rate of 0.5 ml min^{-1}. For quantification of spilanthol an external standard viz. dodeca-2(E), 4(E)-dienoic acid isobutylamide has been used. For spilanthol characterization, all peaks eluted from HPLC, were collected, concentrated and analyzed by mass spectrometry. Samples were analyzed in both positive and negative electrospray ionization mode but, the sensitivity and reproducibility of the dominant ions in the positive electrospray ionization mode was found to be better than in negative ionization mode. Therefore, they analysed all HPLC peaks in positive mode. Spilanthol was identified by its fragmentation profile which was further confirmed with literature data. In their study mass spectrum of spilanthol has a base peak at m/z 222 corresponding to the protonated $[M+H]^+$ molecular ion. The ion at m/z 244 was generated due to sodium ion adduct formation $[M+Na]^+$. The characteristic fragment at m/z 149 was formed due to the dissociation of the C–N bond. Another fragment seen at the m/z 123 can be attributed to the $[MH-C_5H_9NO]^+$. The loss of a fragment with specific m/z 99 from the protonated $[M+H]^+$ molecular ion (m/z 222) confirmed that spilanthol contains isobutylamide group (**Fig. 2**).

***In Vitro* Production of Spilanthol**

In spite of advancement in synthetic organic chemistry, plant kingdom still contributes significantly in both quantity and product range to the specialty chemicals used by a number of industries because their complex structure cannot be synthesize by these industrial processes. Moreover, with increase awareness about side effects of synthetic products, interest has considerably increased towards natural products specifically towards plant based natural products. As field grown plants are limited, the major limitation to the commercial use of potential metabolites is their very scarce supply. Production of useful metabolites from plant cell culture has been regarded as a potential

solution to this supply constraint. Plant cells are biosynthetically totipotent which means that each cell in culture retains the complete genetic information to produce the range of chemicals found in the parent plant. These systems possess a number of advantages, for example, they are not subjected to the limitation of soil, water, season and environment conditions and the cells can grow at a relatively fast rate. Moreover, production can be more reliable, simpler, and more predictable and the compounds that interfere in field grown plant can be avoided in cell cultures. To date, only a few plant metabolites have been produced via cell culture techniques at industrial scale. The reason for the lower concentrations is not fully understood, but it may be due to the development of secondary metabolites in plants is connected with the development process of organs (roots, stem, leaves) and the concentrations in cultures of undifferentiated cells (callus culture, suspension) are therefore low. In a few cases, however, cell cultures have been found to produce higher levels of secondary metabolites than the differentiated mother plant itself. Examples are the production of anti-inflammatory naphthoquinone shikonin from *Lithospermum erythrorhizon* and antiseptic alkaloid berberine from *Coptis Japonica*. If the level of metabolites is to be increased, culture conditions need to be optimized and accurate information is required about even low concentrations of the metabolites present. This, in turn, demands much more sensitive and accurate analytical methods than those employed in measuring the larger concentrations in naturally grown plants.

So far, a few reports have described chemical constituents of *Spilanthes* growing in the wild, but the field grown plant is not desirable for constant metabolite production at the commercial level because of environmental fluctuation, climatic and geographical variations, diseases and pathogen attack. In contrast to this, plant cell culture technology shows promise for the large-scale production of high-value secondary metabolites. This technique offers uniform secondary product synthesis by eliminating effect of unforeseen climatic conditions and diseases as observed in field grown plants. In *Spilanthes*, so far, only two reports are available on *in vitro* spilanthol production. First of all, Singh and Chaturvedi (2012a) reported spilanthol accumulation in leaves of *in vitro* plants. They have developed an efficient HPLC-MS method for the identification and quantitative estimation of spilanthol in plants of *S. acmella*. This method can detect even a low concentration of spilanthol and could be easily implemented in routine practice. Interestingly, they noticed significantly ($p<0.05$) higher spilanthol production (3294.36 ± 12.4 µg/g DW) from leaf disc derived plants than from field grown plants. In the same study, callus cultures established from leaf disc accumulated low amount of spilanthol (998.03 ± 15.6 µg/g DW). The study confirms the other reports which suggested that differentiated (organized and redifferentiated) cells and specialized organs generally produce most secondary products compared to dedifferentiated (unorganized) cells in cultures (Rao and Ravishankar, 2002; Tang et al., 2010; Singh and Chaturvedi, 2013).

Singh and Chaturvedi (2012b) also established suspension cultures from the leaf-disc derived callus. The best treatment in terms of sustained growth of calli, percent explants callused and the degree of callusing, was the combination of MS + BAP (5.0 µM) + NAA (1.0 µM) + 2,4-D (1.0 µM). The same growth regulator combination, MS + BAP (5.0 µM) + NAA (1.0 µM) + 2,4-D (1.0 µM), which was used for callus culture, worked for cell suspension cultures as well. However, the cell growth was faster in liquid medium than in semi-solid medium. It took only 18 days for the cell cultures to complete the growth cycle in comparison with calli on semisolid medium which needed at least 5 weeks to attain maximum growth.

In suspension cultures spilanthol production started at the end of the lag phase (6th day). During exponential phase spilanthol content increased and was found to be growth associated and showed an increase with the increase in biomass until 15th day. Thereafter, spilanthol production declined rapidly and dramatically due to nutrient depletion and cell death (nutrient depletion followed by cell death). In this report effect of carbon source on spilanthol content and biomass has also been studied. They found that, among the tested carbon sources, the highest production of spilanthol as 91.4 µg/g DW was in the medium supplemented with sucrose, followed by glucose which produced 56.8 µg/g DW of spilanthol. Spilanthol could not be detected in fructose containing medium. The results of this study, thus, form a backdrop for future studies related to large-scale production of spilanthol in bioreactors.

Possibilities and Challenges

The increasing demand of spilanthol and its acceptance in the market because of its potent pharmacological property and therapeutic value have been proving to be real blessing to the people. In the last decade, a lot of work has been done to scientifically document its pharmacological properties. Concerted efforts have been made to characterize biomolecules present in the *Spilanthes* and more focus has been given to the spilanthol. Spilanthol has been identified as a key potent molecule of the plant which is responsible for most of the therapeutic properties of the plant. It will not be exaggerating to say that *Spilanthes* importance is mainly due to the presence of Spilanthol. With increased awareness about this molecule, exploitation of the plant has increased at a very high rate which necessitates the development of an alternative method for the production of this high value alkylamide. Though in recent years, a few scientists have worked on establishment of its *in vitro* culture and *in vitro* metabolite production

but still these studies are far from large scale production of spilanthol. To achieve this objective a much more intensive study is needed.

Plant secondary metabolite is known to vary according to genotype and environment conditions. For the development of efficient culture system for spilanthol production screening and establishment of productive cell line is a prerequisite. Cell line screening is needed to be performed by comparative studies of different cell lines on the growth and spilanthol yield i.e. derivations of cell strain with inherent capacity to produce increased yield and further selection for better yielding cell lines.Spilanthol producing callus or cell lines may also be induced from the crown gall of different parts of plant infected with *Agrobacterium tumefaciens*.

The productivity of cell line is greatly influenced by the culture conditions of which the culture medium is most important. No extensive study is performed to analyze the effect of different plant growth regulators and their concentration, nitrogen and phosphorus concentration and NH_3: NO_3- ratio on biomass yield and spilanthol production. There is only a single report (Singh and Chaturvedi, 2012a) available in which effect of different carbon source on spilanthol production has been studied. These preliminary studies on spilanthol production strongly suggest the need of optimization of different media for enhanced growth and product response.

Very low yield of spilanthol in cell culture may be due to the lack of optimization of medium components therefore the optimization of media components and cultural conditions holds a good promise for attempting enhancement of spilanthol production in plant cell culture. At an early stage of media optimization, statistical techniques such as Plackett–Burman and response surface methodology (RSM) are promising tools for growth and secondary metabolite production in cell culture (Singh and Chaturvedi, 2011). RSM integrates the interaction of various parameters, generally resulting in higher production yields and limiting the number of experiments. In addition to analyzing the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process. In addition to this, there exists the fairly good possibility of increasing the spilanthol yield of *Spilanthes acmella* tissue culture through metabolic regulation i.e. with the use of elicitors and addition of precursors of spilanthol synthesis in culture medium. Different biotic and abiotic compounds are known to trigger the synthesis and accumulation of secondary metabolite in many cell cultures.

Scale-up of suspension culture in a bioreactor that provides the best conditions possible for growth and product formation is necessary for mass production of any secondary metabolites. Engineering challenge for *in vitro* metabolite production lies in the scale-up production process. Extensive literature survey reveals the non-availability of any report on the large-scale production of spilanthol under controlled conditions in bioreactor. It would be interesting to study the cultivation of *Spilanthes* in bioreactor to enhance the production of spilanthol. Different reactor configurations have been reviewed for plant cell cultures (Georgiev and Weber, 2014). The suitability of particular reactor type, impeller type and choice between batch, fed-batch or continuous operation condition needed investigation based on the dynamics of culture of *Spilanthes* in suspension culture.

Concluding Remarks

Since last few years, spilanthol demand has increased all over the world due to its potential application in pharmaceutical, food and cosmetic industries. The production of spilanthol from field grown plant is a labour intensive and expensive process.To fulfill the increasing demand of spilanthol other alternatives need to be investigated. At present, the research into spilanthol production through cell tissue culture is in its very initial state and there is long way to go before the establishment of an economical viable process. To develop feasible efficient culture technology for spilanthol production, optimization of all physiological, environmental and bioengineering aspects of plant tissue culture is highly warranted.

Table 1: Spilanthol extraction, detection and content in different *in vivo* and *in vitro* systems.

Samples	**Extraction method**	**Solvent Used**	**Separation and Detection method**	**Quantification**	**Reference**
Flower	Maceration	Hexane	GC-MS, NMR	-	Ramsewak et al., 1999
Whole plant	Maceration, Solid phase extraction	95% Ethanol	HPLC/ESI-MS	-	Bae et al., 2010
			LC-MS		Boonen et al.,

					2010
Flower, Mother plant, In vitro Plantlets	Maceration	Methanol and Hexane	GC-MS	-	Leng et al., 2011
In vivo and In vitro leaves, callus	Maceration	Methanol	HPLC-MS	In vivo leaves-2703 µg/g DW In vitro leave-3294.36 µg/g DW Callus-998.03 µg/g DW	Singh and Chaturvedi, 2012a
Suspension culture	Maceration	Methanol	HPLC-MS	91.4 µg/g DW	Singh and Chaturvedi, 2012b
Flower, leaves, stems	Supercritical fluid extraction	Carbon dioxide		-	Dias et al., 2012
Flower	Microwave extraction	Ethanol: Hexane (3:7)	GC	-	Costa et al., 2014

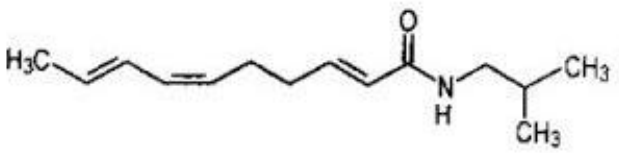

Fig. 1. Chemical structure of spilanthol

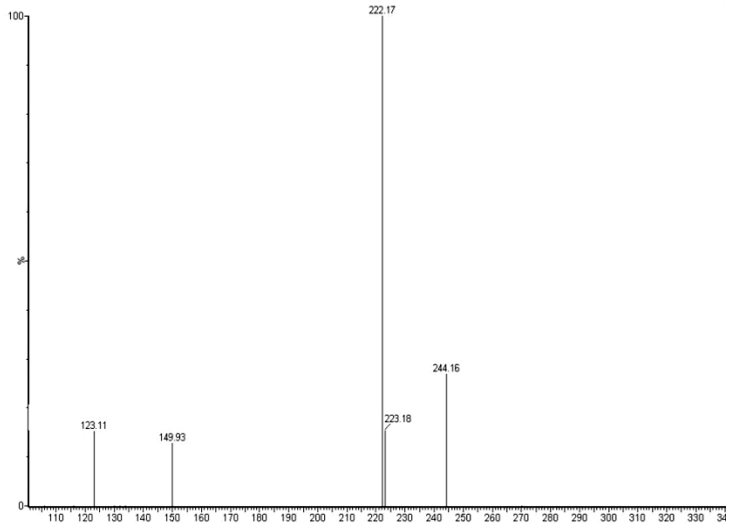

Fig. 2. Positive electrospray ionization mass spectra of spilanthol
(Source: After Singh and Chaturvedi 2012a)

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