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Full Length Research Paper

Determination of toxicity and chromatographic analysis of spilanthol content in *in vitro* culture of *Spilanthes oleracea* Jacq.

Viviane Gesteira[1], Naomi Kato Simas[2], Morgana, Castelo T. L. Branco[3], Alexandre dos S. Pyrrho[2] and Alice Sato[1,4*]

[1]Programa de Pós- Graduação em Biotecnologia Vegetal, Universidade Federal do Rio de Janeiro, CCS, Bloco K, Cidade Universitária, Ilha do Fundão, 21952-590 Rio de Janeiro - RJ, Brazil.
[2]Faculdade de Farmácia - Universidade Federal do Rio de Janeiro, CCS, Bloco A, Cidade Universitária, Ilha do Fundão, 21949-590 Rio de Janeiro - RJ, Brazil.
[3]Instituto de Ciências Biomédicas- Universidade Federal do Rio de Janeiro, CCS, Bloco B, Cidade Universitária, Ilha do Fundão, 21949-590 Rio de Janeiro - RJ, Brazil.
[4]Universidade Federal do Estado do Rio de Janeiro – UNIRIO, Departamento de Botanica, Avenida Pasteur 458, 22290-040 Rio de Janeiro – RJ, Brazil.

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***Spilanthes oleracea* Jacq. has been extensively used due to its pharmacological activities, and it has been considered as a promising vegetable crop. Nodal-segment cultures of *S. oleracea* were established in order to analyze the content of alkamide spilanthol. On average, 11 buds/nodal segment were developed after 30 days on MS basal salts liquid medium containing 2.22 µM benzyladenine (BA). Regenerated shoots formed complete plantlets in medium without growth regulators, and 80.2% of the regenerated plantlets survived in field conditions. Higher production of spilanthol (58.5% relative area) was obtained from the leaves and stems of plants grown in liquid medium up to 90 days, than in field-grown plants. Addition of BA to the culture medium significantly affected spilanthol accumulation. The production of spilanthol was also detected on the shoot basal callus. The spilanthol content varied with the plant organ in micropropagated plants. Ethanolic extract of leaves from field-grown plants showed no acute toxicology in Swiss mice at a dosage of 3 g/kg.**

Key words: *Spilanthes oleracea*, toxicity, plant regeneration, calli, spilanthol, gas chromatography.

INTRODUCTION

Spilanthes oleracea Jacq. is a plant of the Amazon region that is commonly utilized in local cuisine and folk medicine. The entire plant is claimed to have medicinal properties (Dubey et al., 2013). The leaves are eaten raw or as a vegetable by many tribes in India (Chakraborty et al., 2004) and also in the Amazon region. It is commonly

*Corresponding author: E-mail: alicesato@unirio.br.

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known as “toothache plant” or “Paracress” (Wyk and Wink, 2009). The alkamide spilanthol, which occurs in several members of Asteraceae including *S. oleracea*, causes a pronounced tingling and mouthwatering effect upon ingestion. Industrial applications of this substance include oral care (Hirayama and Ikenishi, 2010), and as a flavoring and preservative in food (Miyazawa and Yamaguchi, 2010; Tanaka and Yagi, 2009). In cosmetics, it has been recently been employed as an anti-ageing ingredient, among other applications (Demarne and Passaro, 2008). Besides these applications, the leaf extract showed larvicidal activity against *Aedes aegypti*, increasing the possibility that it could be used as an important tool in the control of dengue (Ramsewak et al., 1999; Pandey et al., 2007, 2011).

The content of spilanthol is generally higher in the field-harvested flower heads, as also reported for *Echinacea* and *Spilanthes* (Perry et al., 1997; Nayak and Chand, 2002), than in other tissues, showing a tissue-specific distribution. Plant tissue culture can be used to induce quantitative and qualitative modifications in the production of plant secondary metabolites, by changing nutrient and hormone contents in the culture medium (Abyari et al., 2016; Collin, 2001). In addition, tissue culture eliminates the effect of climate conditions and diseases to which field-grown plants are subject.

Earlier studies of *in vitro* culture of different species showed that the accumulation of different secondary metabolites can be efficiently induced by various elicitors. Abiyari et al. (2016) demonstrated that the application of casein hydrolysate and L-phenylalanine is effective for productions of scopoletin. Alkamides were induced by methyl jasmonate (MeJa) in *Echinacea pallida* (Binns, 2001). Romero et al. (2009) demonstrated the efficacy of *E. pallida, Echinacea purpurea* and *Echinacea angustifolia* hairy root cultures in the *in vitro* production of alkamides. Moreover, *in vivo* elicitation through foliar application of elicitors [acetylsalicylic acid, salicylic acid, and methyl salicylate; as well as the metal elicitor (titanium (IV) ascorbate)] on *E. purpurea* increased the phenolic content up to 10 times as compared to the control, and also increased the biomass yield (Kuzel et al., 2009). Co-culture of different organs/species together also has been attempted since the co-culture provides the opportunity for metabolites produced by one organ/species to be excreted into medium and taken up by another organ. Sidwa-Goricka et al. (2003) have established co-culture of hairy roots of *Ammi majas* and cell-shoot suspension culture of *Ruta graveolens* to investigate possible interaction of metabolic pathways of coumarins, whereas, Wu et al. (2008) established the co-culture of ginseng (*Panax ginseng*) and *E. purpurea* adventitious roots for the productions of secondary metabolites.

This study aimed to assess spilanthol production in *S. oleracea in vitro* culture under the influence of benzyladenine (BA) and methyl jasmonate (MeJa), and evaluate the co-cultivation effect and acute toxicity of the crude extract from field-grown plants.

MATERIALS AND METHODS

Plant material and tissue culture

Seeds of *S. oleracea* Jacq. were collected in Belém, State of Pará, Brazil, and were identified by Dr. Ricardo Secco of the Emílio Goeldi Museum, Belém. Voucher specimens were deposited in the Emílio Goeldi Museum under catalogue number Herbarium MG 156.773. Four-week-old *in vitro* germinated seedlings were used as a source of explants for initiation of cultures in MS medium (Murashige and Skoog, 1962). Nodal segments excised from *in vitro*-cultured seedlings were inoculated in different treatments: culturing in the presence or absence of agar (30 and 90 days), under the influence of BA (2.22 and 4.44 µM).

The interaction with elicitors (2.0 µL salicylic acid and 45 µL methyl salicylate (Sigma Aldrich) added to ethanol, with a final concentration of 100 ppm (Binns, 2001) was put on cotton pieces that were put with the plants (with 60 days of culture on growth regulator-free MS medium). In addition, co-cultivation of nodal segments of *S. oleracea* and *Polygala paniculata* L. was performed. *P. paniculata* L. *in vitro* culture demonstrated production and release of methyl salicylate according to Victorio et al. (2011). The cultures were maintained in a growth chamber under cool-white fluorescent lighting tubes (1.6 W m^{-2}, 23 µmol.m^{-2} s^{-1} and daily photoperiod of 16 h at 25 ± 2°C).

Spilanthol extraction and GC analysis

Freeze-dried leaves, shoots and calli were macerated for 2 days in 90% chloroform (p/v) according to Simas (2003). The extract was then filtered and the solvent volume was reduced in a rotary evaporator. The resulting dry residue was weighed to determine the yield of crude extract for each treatment (Table 1).

GC-FID

Quantitative analysis of the extracts (30 mg/mL) was performed in a gas-chromatography system (Shimadzu GC-17A) equipped with a flame ionization detector (FID) and DB-5 capillary column (30 m, 0.32 mm, 0.25 µm), and 1 µL of each sample was injected with a split-mode injector (1: 6) into a flow of hydrogen gas held constant at 1 mL/min flow rate. The oven temperature was programmed for an initial temperature at 100°C, increasing at 10°C/min up to 200°C, held for 20 min, then a second ramp-up of temperature at 3°C/min to a final temperature of 250°C, and held for 5 min. The temperatures of the injector and detector were held at 250 and 200°C, respectively. The percentage content of spilanthol was calculated by integrating the areas of the corresponding signals.

Qualitative analysis was performed in a mass spectrometer (Hewlett-Packard, model HP-5971 A) coupled to a gas chromatograph, model HP-5890 A, Series II, equipped with a DB-5 capillary column (30 m, 0.32 mm, 0.25 µm). Experimental conditions were: ionization by electron impact at 70 eV, helium as carrier gas at flow rate of 1 mL/min. The National Institute of Standards and Technology (NIST, 1990) database was used for comparison of mass spectra.

Acute toxicology assay

Thirty female albino Swiss mice (25-30 g), two months old, were obtained from the central animal house of the Microbiology Institute/UFRJ. They were housed in standard polypropylene

Table 1. Yield of dried chloroform extracts (%, m/DM) obtained from dry mass *in vitro* plants of *S. oleracea* for different treatments.

Treatments	Leaf	Shoot	Total
MS/30 days	10.5	10.5	21.0
MS/90 days	4.9	2.0	6.9
2.2 µM BA/30 days	9.0	10.0	19.0
4.4 µM BA/30 days	20.0	7.6	27.6
Co-culture/30 days	14.2	6.2	18.4
Acclimatized plants/30 days	7.7	4.6	12.3

m/DM- Total extract mass/total dried plant mass.

Table 2. *Spilanthes oleracea in vitro* development. Effect of liquid and solid media, BA concentrations and co-culturing with *Polygala paniculata.*

Solid medium	MS 30 days	MS 90 days	Co-culture MS 30 days	2.22 µM BA 30 days	4.44 µM BA 30 days
No. of shoots	1.0±0.2	1.7±1.1		1.6±0.6*	1.5±0.7*
No. of nodal segment	3.1±0.7	12.7±4.6		4.4±1.3*	4.5±2.1*
No. of buds	6.2±1.4	26.8±9.1		8.9±2.6*	9.2±3.9*
Elongation (cm)	3.0±0.7	16.1±3.0		2.1±0.8*	2.1±1.0*
Rooting (%)	100	100		70*	13.3*
Líquid medium					
No. of shoots	1.0±0	1.5±0.8	1.0±0	2.0±0.7*	1.3±0.5*
No. of nodal segment	3.9±0.5	12.2±3.0	3.9±0.4	5.7±1.7*	3.9±1.9
No. of buds	5.8±1.0	23.5±6.1	7.9±0.7	11.3±3.3*	7.7±3.6*
Elongation (cm)	2.9±0.9	16.6±0	4.9±1.1	2.4±0.6	1.9±1.0*
Rooting (%)	100	100	100	73.3	56.7*

Mean ± standard error (n=30) * p≤0.05 in relation to control at 30 days. Kruskal-Wallis test.

cages, five per cage, and kept under controlled room temperature (28 ± 2°C; relative humidity 50-55%) in a 12-h light cycle. The mice were given a standard laboratory diet and water *ad libitum*. Food was withdrawn 12 h before and during the experimental period. The leaf ethanolic extract from field-grown plant was dissolved in 10% DMSO and administered to the animals orally by gavage. Two doses of the ethanolic extract (300 and 3000 mg/kg) were tested through intraperitoneal administration. On the first day of treatment, the animals were observed for 3 h, for any behavioral changes or deaths. After ten days of treatment, the animals were anesthetized lightly with ether and killed by cardiac puncture, and their body and organs (liver and spleen) were weighed. All experimental protocols were approved by the institutional Animal Ethics Committee (IBCCF/UFRJ 036).

Statistical analysis

All experiments used a fully randomized block design. Each experiment consisted of 5 nodal segments/vessel and 6 replicates per treatment (plant growth regulator, elicitors). All the experiments were repeated three times. The data were subjected to one-way ANOVA, and mean values were compared by Dunnett's multiple comparison test or by the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test as post-test, using the software GraphPad InStat, version 6.01. Rooting percent data were tested for the significance of the difference between two percentages, at the 5% significance level, using the software Statistica for Windows, version 5.0. For the quantitative analysis of phytochemical compounds, the data were collected from two independent experiments, and are presented as the mean values.

RESULTS AND DISCUSSION

In vitro culture of *S. oleracea*

Liquid or low-agar concentration media increase the growth of certain species cultured *in vitro* (Casanova et al., 2008; Abdoli et al., 2007), which is caused by greater availability of water and nutrients (Debergh, 1983); however, *S. oleracea* showed no difference in *in vitro* shoot development on liquid or solid media (Table 2). Liquid cultures are suitable for bud development and shoot multiplication, with BA added to the culture medium. Consequently, liquid medium was used throughout this study.

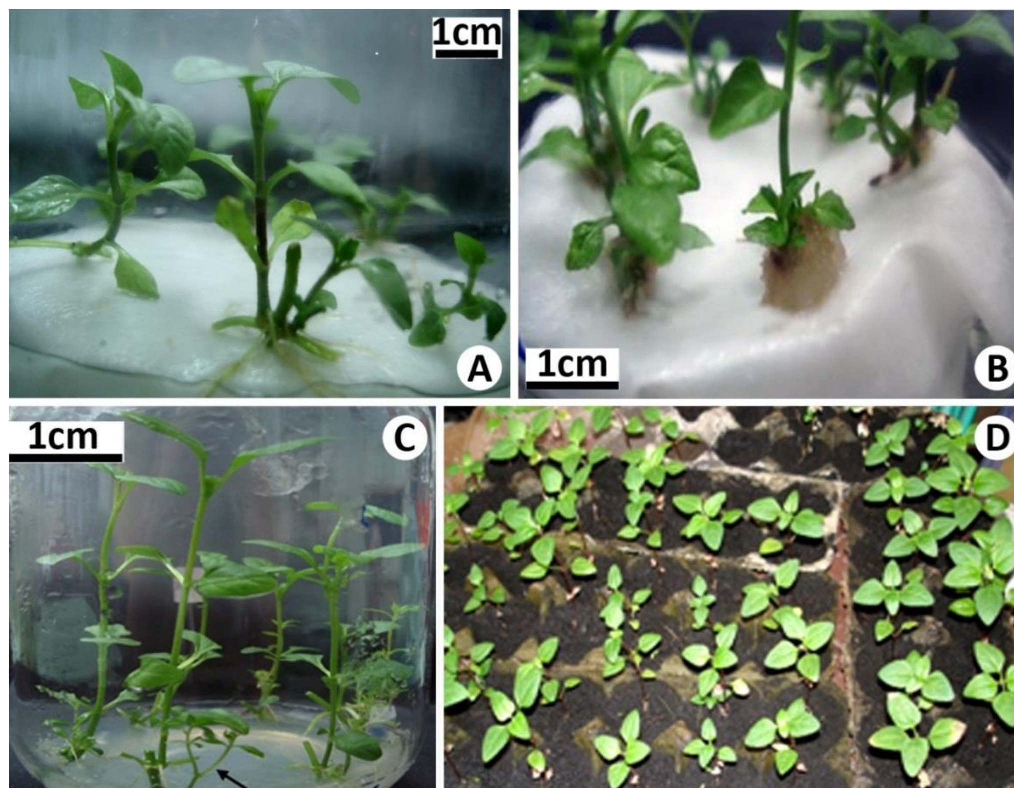

Figure 1. Plant regeneration from nodal segments of *S. oleracea***.** a- Shoot development from axillary buds induced by 2.22 μM BA liquid after 30 days. b- Basal and friable calli development in 4.44 μM BA liquid medium after 30 days. c- Co-culture of *Spilanthes oleracea* and *Polygala paniculata* (arrow); d- Acclimatized plants.

The growth ability of *S. oleracea* nodal explants was improved with the additional BA source (Figure 1a, b and Table 2) as previously reported (Haw and Keng, 2003; Saritha et al., 2002; Bais et al., 2002; Deka and Kalita, 2005; Saritha and Naidu, 2007). Furthermore, the assayed concentrations (2.22 and 4.44 μM) did not show significant differences in the explant responses except in relation to the root development. BA increased the multiplication rate (numbers of buds and nodal segments); however, rooting of BA-grown plantlets was inhibited, with no significant changes in shoot length. Basal-shoot calli were induced to the detriment of root development (Figure 1b). At the end of the cycle (multiplication phase), 70% of the plantlets had rooted. Auxin supplementation was not necessary for root formation.

The co-cultivation with *P. paniculata* (Figure 1c) resulted in an increase of the height of the plants, accompanied by increased bud neoformation. The significant difference in plant height between the co-culture and control reinforces the hypothesis that the methyl salicylate produced by *P. paniculata* may act as an allelopathic compound, since co-culturing stimulated the growth of *S. oleracea*. All plantlets acclimatized well, and the establishment of micropropagated plants occurred at a high rate (96%) (Figure 1d).

Gas chromatographic analysis of spilanthol content in micropropagated plants

Spilanthol content was determined in different parts of *in vitro* grown plants and in each tissue type analyzed (Table 3, Figure 2). The roots from *in vitro* plants, including acclimatized plants, contained no spilanthol (Table 3). Franca et al. (2016) determined the spilanthol content in all organs of *in vitro* culture of *Acmella oleracea* using Murashige and Skoog as basal medium and phytagel as the gelling agent. The aerial parts (leaves and stems) of plants exposed to 100 ppm salicylic acid and methyl salicylate did not contain spilanthol. However, the plants co-cultivated with *P. paniculata* maintained their capacity to synthesize spilanthol, with about 11.9% relative abundance (RA) (Table 3). Possibly, the concentration of elicitors may be related to the difference in responses between experiments involving methyl salicylate alone and methyl salicylate released from *P. paniculata.*

In order to increase the levels of spilanthol *in vitro*, Binns (2001), using elicitors such as jasmonates, found

Table 3. Values of relative area (%) of spilanthol content from plant organ extract for each treatment.

Treatments	Relative area (%)				
	Leaf	Stem	Root	Callus	Total
MS/30 days	8.3	10.7	0	-	19.0
MS/90 days	24.0	34.5	0	-	58.5
2.2 µM BA/30 days	14.7	20.5	0	2.1	37.3
4.44 µM BA/30 days	20.7	12.4	0	6.0	39.1
Co-culture/30 days	8.2	3.7	0	-	11.9
Acclimatized plants/30 days	2.4	1.7	0	-	3.9
100 ppm salicylic acid/24 h	0	0	0	-	0
100 ppm Methyl Salicylate/24h	0	0	0	-	0

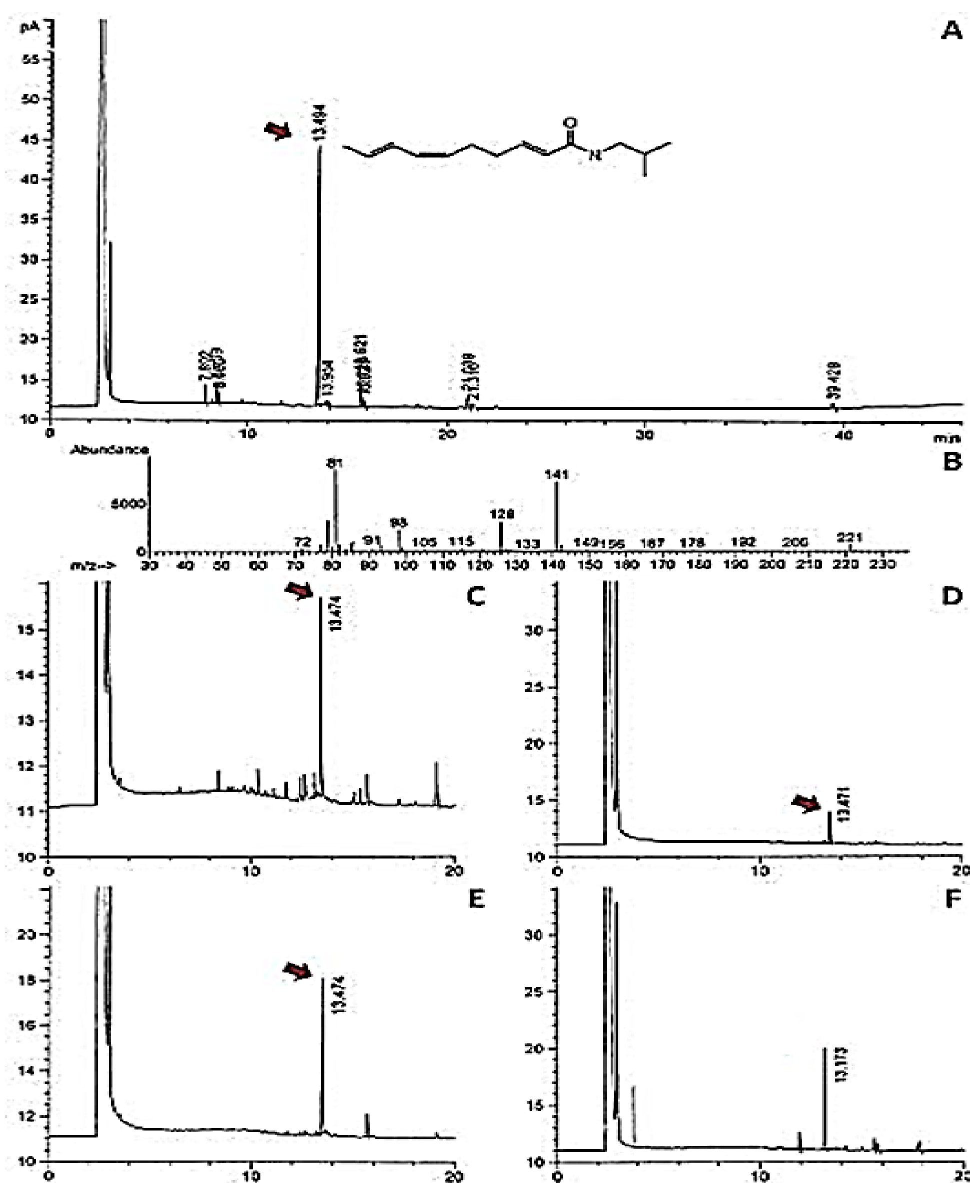

Figure 2. GC fingerprint of plant materials of S. oleracea. A- Chromatographic profile of field grown flowers of S. oleracea. B- Spilanthol fragmentation pattern. C to F- chloroformic extracts of samples from in vitro culture. C- leaves, D- Calli obtained from 4.4 µM BA, E- Shoot, F- Root.

Table 4. Mean weights (g) of mouse organs obtained from animals used (n = 5/treatment) for acute toxicology testing. The inoculum applied was an ethanol extract of aerial parts of field-grown plants of *S. oleracea*.

Inoculum	Spleen	Liver
	Median±standard deviation	
Control	0.145 ± 0.04	1.140 ± 0.10
DMSO (control)	0.164 ± 0.02	1.168 ± 0.30
Field plants 300 mg/kg	0.146 ± 0.05	1.202 ± 0.30
Field plants 3000 mg/kg	0.153 ± 0.04	1.295 ± 0.10

that methyl jasmonate increases the production of some alkamides in *Echinacea pallida*. However, in this study, the MeSa and SA treatments showed an inhibitory effect on spilanthol content at the concentrations assayed. The aerial parts of plants exposed to 2.22 and 4.44 μM BA had total spilanthol contents of 37.3 and 39.1% RA, respectively (Table 3). Basal calli grown in the medium exhibited spilanthol contents increasing from 2.1 to 6.0% RA in 4.44 μM BA (Figures 1c and 2). The highest spilanthol accumulation was found in MS control plants after 90 days of culture, reaching 58.5% RA of total content, against 19.0% in a 30-day culture (Table 3). However, when these highest-accumulating spilanthol plants were transferred to field conditions, the spilanthol content was low (4.0%) (Table 3).

Extracts from flowers enriched with spilanthol contained 1.2% in the pentane extract, 6.17% in the methanol extract, and up to 17% in the CO_2 supercritical fluid extract in different species of field-grown *Spilanthes* (Stashenko et al., 1996, Ramsewak et al., 1999). Data for spilanthol content specifically in leaves and stems are lacking, and the existing reports emphasize the contents in flowers or homogenized aerial parts of *S. oleracea*. However, Stashenko et al. (1996) calculated a spilanthol content of 21% as measured by area percent of gas chromatogram in a CO_2 supercritical fluid in an extract from leaves of *S. americana*.

The *in vitro* culture of *S. oleracea* showed the influence of nutrient-enriched Murashige and Skoog medium cultures on spilanthol accumulation. This accumulation increased in the time-consuming medium at 90 days of culture, and organ-specific production of spilanthol in the leaves, stems and even in the callus was observed. The results show the importance of *in vitro* MS culture, as compared to field culture, which did not result in spilanthol accumulation. In *in vitro* culture, *S. oleracea* had high spilanthol content in three months, from small amounts of *in vitro* plants extracted by maceration in chloroform solvent.

Acute toxicology assay

After ten days of the experiment, all animals treated intraperitoneally with the ethanol extracts of *S. oleracea* leaves (300 and 3000 mg/kg) were alive, with no external physical abnormalities. No notable changes were observed in the weights of liver and spleen from animals treated with the extract, in any dose tested, indicating no acute toxicity (Table 4). Chakraborty et al. (2004) conducted experiments with intraperitoneal administration of an aqueous extract of aerial parts (100, 200 and 400 mg/kg) for four hours, to evaluate the analgesic activity in albino mice. At the same time, the authors evaluated the acute toxicity of an aqueous extract of *S. acmella,* and observed no adverse effect or mortality in albino mice that ingested up to 3 g/kg *p. o.* during a 24-h observation period.

Spilanthol is one of several compounds that are extractable by ethanol from *S. oleracea* leaves. This possibility was demonstrated by Molina-Torres et al. (1999) in their spilanthol extraction and purification procedure from an ethanol extract. Ethanol, as used in this study, proved to produce a *Spilanthes* extract, which proved to have a safe composition in the animal-model tests. The absence of subchronic toxicology was observed in other studies, as related by Zuluaga et al. (2008) who administered an ethanolic extract of *Spilanthes americana* in Swiss albino mice, and by Ekor et al. (2005) who evaluated an aqueous extract of *Spilanthes filicaulis*.

Conclusion

The *in vitro* propagated *S. oleracea* in liquid-medium culture maintained the capacity to synthesize spilanthol in the leaves, stems and calli. The roots of *in vitro*-grown plantlets contained no spilanthol. Spilanthol was present in the regenerated plants; however, in much lower amounts than in *in vitro* culture. In this study, the plant organ-specific biosynthesis of spilanthol and *in vitro* culture proved to be an efficient method to obtain spilanthol in a liquid medium, without the need for the time-consuming addition of a growth regulator. The ethanolic extract obtained from field-grown leaves was proven to be safe, by an acute test in mice. The regeneration protocol and GC analysis developed here provide a new approach towards quality control of micropropagated plants. This method of producing

secondary metabolites has significant implications for the production of standardized-quality phytopharmaceuticals through mass production and analysis of the active ingredients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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