Year:**2013**, Volume: **1**, Issue: **3**
First page: (**135**) Last page: (**141**)
ISSN: **2321-2187**
Online Available at **www.florajournal.com**

International Journal of Herbal Medicine

Determination of cytotoxicity and chemical identities in natural plants and callus cultures of *Spilanthes paniculata* Wall. ex DC.

Kulathilaka PS[1], Senarath WTPSK[1*]

1. Department of Botany, University of Sri Jayewardenepura, Nugegoda, Sri Lanka
[Email: wtpsk2011@yahoo.com]

Plants have been extensively used for medicinal purposes and human disease management since early ages, due to presence of valuable chemical identities. Present study was carried out to identify chemical identities and the cytotoxicity of *Spilanthes paniculata* Wall. ex DC. which belongs to family Asteraceae. This plant is commonly known as 'Tooth-ache plant' due to the presence of anesthetic properties. A mother stock has been maintained for experiments. Leaves obtained from green-house grown plants and *in vitro* callus cultures of 3-weeks-old were used in this study. *In vitro* callus induction was optimized with leaf disc explants grown on MS medium supplemented with 0.15 mg/L of NAA and 1.50 mg/L of BA. GC-MS analysis of ethyl acetate extracts of leaf and callus samples confirmed the existence of the key chemical compound spilanthol in both samples, with significantly higher amount in fresh leaves. Cytotoxicity tests confirmed the toxicity of hexane extracts of leaf and callus samples against late third/ early fourth instar larvae of *Aedes aegypti* and nuaplii of *Artemia salina* (100% mortality with 800 µg/mL). Leaf extracts were found to be highly toxic (70% mortality with 100 ppm for *A. aegypti* and 100% mortality with 800 µg/mL for nuaplii of *A. salina*) to both test organisms than callus extract.

Keyword: Callus, cytotoxicity, GC-MS analysis, leaf extracts, larvicide, *Spilanthes paniculata.*

1. Introduction

Spilanthes paniculata Wall. ex DC., commonly known as "Acmella" or "Toothache plant", is a medicinal plant with immense properties of importance not only in indigenous medicine but also in Western medicine, food, health and body care product industries. This is an annual herb belongs to family Asteraceae. Different species of the genus *Spilanthes* are widely distributed in tropical and subtropical regions of the world [1] In Sri Lanka, only three *Spilanthes* species are recorded, *S. iabadicensis*, *S. calva* and *S. paniculata* [2].
The genus is well documented for its uses as a spice, antiseptic and antimicrobial agent, a remedy for toothache, cough, throat and gum infections, larvicide, insecticide and many more [3]. Extracts of this plant proved to possess anti-nociceptive activity against continuous inflammatory pain and anti-hyperalgesic activity, possibly by inhibiting prostaglandin synthesis [4]. Among all phytochemicals present in the plant; N-Isobutyl-2(E),6(Z),8(E)-decatrienamide - an alkylamide – which is commonly named as spilanthol is the key chemical compound [5].

2. Materials and methods

2.1 Establishment mother stock and callus cultures

Mother stock of *S. paniculata* was maintained at the Botanical Garden in a green-house. Leaf disc explants from second

or third fully opened leaves were obtained. They were kept under running tap water for 1 h. Then, transferred to a mixture of 5 drops of Tween 20, Captan® (2.0 g/L) and a drop of Teepol for 10 min. for further washing. After that, leaves were transferred into a solution of 10% Clorox for 5 min. and 70% alcohol for 30 sec. each followed by three successive washings in sterile distilled water for surface sterilization. Leaf discs of 5 mm diameter were obtained and cultured on Murashige and Skoog medium (MS) [6] supplemented with different combinations of NAA (0.10 - 0.20 mg/L) and BA (1.00 - 2.00 mg/L). Growth regulator free MS medium was used as the control and there were 20 replicates in each treatment. Completely randomized design was used in the experiment and the data obtained were analyzed using Minitab statistical package. Cultures were incubated at 25±1^{0}C under completely dark conditions. Callus diameter was measured in 7 day intervals. Cultures were sub cultures after 3 weeks.

2.2 Gas Chromatogram-Mass Spectrometry (GC-MS) of callus and leaves

Healthy leaves from the 2-months-old mother plants were collected and washed under running tap water for 30 min. Callus samples were collected from 3-4 weeks-old cultures and traces of the agar were removed carefully. The initial weight of both leaf and callus sample was recorded. Samples were air dried for few hours and then oven dried at 60^0 C for 48 h to get a constant dry weight. Dried samples were ground and the fine powder of the ground sample was collected using a clean dry tea strainer. Powdered leaf and callus samples (0.5 g each) were placed separately in 270.0 mL of ethyl acetate and extracted using the Soxhlet Extractor at a temperature range of 45-85 ^{0}C. The procedure was repeated 5 times. Then the samples were evaporated to dryness using the rotary evaporator at 45^0 C with pressure reduction starting from 300 mbar. The solvent-free extracts were dissolved in 9.0 mL of 80% methanol and then 3.0 mL of hexane was added to it and mixed on a magnetic stirrer for 45 min. After that solvent mixture was left for about 10-15 min. to separate the organic solvents into two layers. The top hexane fraction was carefully pipetted out. The process was repeated for three times with 9.0 mL of hexane introduced in splits of 3.0 mL. Methanol fractions collected were evaporated to dryness and dissolved in 2.0 mL of HPLC-Grade absolute methanol and a pinch of sodium anhydrous to remove any trace of water from the samples. Methanol extract (2.0 µL) was injected to the GC-MS machine (7890A) with reference library. Chromatograms were observed and compared the chemical identities present in fresh leaves and callus samples.

2.3 Larvicidal effect and cytotoxicity of callus and leaf extracts

Procedure adopted by Pandey and Agrawal (2009) [1] was modified and used in this experiment. Extracts of leaf and callus were prepared by dispensing 0.5 g of the powdered samples separately into 10.0 mL of the hexane. Toxicity of the extracts was determined against two animal models - late third/ early fourth instar larvae of *Aedes aegypti* (WHO, 1981) [7] and newly hatched nauplii of *Artemia salina* (brine shrimp).

Larvae of *A. aegypti* were introduced to clean containers, 10 larvae in each with 100.0 mL of water mixed with triton (10.0 µL/L of water). Different concentrations (5.0, 10.0, 20.0, 50.0 and 100.0 ppm) of extracts of leaf and callus samples were introduced separately into the containers. A mixture of water and hexane (10.0 ppm) was used as the solvent control and distilled water alone as a control. Number of dead larvae was counted after 24 h.

Artificial sea water (1.0 L) was prepared by dissolving 20.0 g of table salt in tap water. Newly-hatched nauplii of *A. salina* were introduced into containers (30 nauplii in each container) with 20.0 mL of artificial sea water. Hexane extracts of leaf and callus samples were introduced to the containers in different concentrations (25.0, 50.0, 100.0, 200.0, 400.0 and 800.0 µg/mL). DMSO (10.0 µg/mL) was used as the negative control while Standard Colchicine (10.0 µg/mL) was used as the positive control. Hexane (100.0 µL/mL) was used as the solvent control. Mortality of nauplii was taken after 24 h counting the number of dead nauplii.

3. Results and Discussion

3.1 Establishment mother stock and callus cultures

Initiation of light green dense callus from leaf disc explants was observed after 4 days of incubation and the culture vessel was entirely covered by the callus after 3 weeks. At the beginning a swollen appearance was observed on the margins of leaf discs. Cultures were subcultures in every 3 weeks interval as callus started turning into brown. Although it has been cited as callus started differentiation into shoot bud in the same medium after 30 days [8] in present study, the whole callus turned brown after 30 days. Callus growth over the time is given in Table 1. There was a significant difference in callus induction between tested treatments. Leaf disc explants cultured on growth regulator free MS medium (control) did not show any growth of callus when compared to the rest of the treatments.

Table 1: Mean callus diameter in MS medium supplemented with different concentrations of NAA and BA over a period of 3 weeks. There were 20 replicates per treatment.

Concentration of NAA : BA	Mean callus diameter (mm) ± SE		
	7 days	**14 days**	**21 days**
0.0 : 0.0 (Control)	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
0.10 : 1.00	7.10 ± 0.60	9.70 ± 0.74	11.70 ± 0.54
0.15 : 1.00	7.10 ± 0.25	10.90 ± 0.700	13.00 ± 0.32
0.20 : 1.00	8.30 ± 0.44	12.10 ± 0.33	13.60 ± 0.33
0.10 : 1.50	8.40 ± 0.19	11.30 ± 0.44	13.50 ± 0.16
0.15 : 1.50	**8.50 ± 0.27**	**12.60 ± 0.46**	**15.70 ± 0.30**
0.20 : 1.50	7.70 ± 0.34	8.80 ± 0.44	9.70 ± 0.44
0.10 : 2.00	7.90 ± 0.25	10.90 ± 0.43	12.50 ± 0.27
0.15 : 2.00	7.90 ± 0.29	11.70 ± 0.26	13.40 ± 0.19
0.20 : 2.00	8.20 ± 0.26	11.70 ± 0.68	13.00 ± 0.52
LSD 5%	0.01	0.01	0.01

LSD- Least Standard Deviation at 5% confidence intervals**, SE-** Standard Error

Among treatments, MS medium supplemented with 0.15 mg/L of NAA and 1.50 mg/L of BA found to be the best treatment for callus induction, which gave the highest mean callus diameter in 7 day (8.50 ± 0.27 mm), 14 day (12.60 ± 0.46 mm) after 21 day (15.70 ± 0.30 mm) of incubation. Callus produced in this medium was light yellowish green in color. Presence of 0.20 mg/L NAA and 1.00 mg/L BA could be considered as the second best medium which was significantly different from the best treatment. MS medium supplemented with 0.20 mg/L NAA and 1.50 mg/L BA showed the poorest callus growth (9.70 ± 0.436 mm) even after 21 days. Mucilaginous fragile callus (embryonic callus) was produced in MS medium supplemented with 2.25 mg/L BA and 1.0 mg/L 2,4-D[8] indicating the presence of higher level of

BA and 2, 4-D induces the embryonic callus but not the vegetative callus. MS medium supplemented with 10.0 mg/L BA induced callus formation and resulted in the eventual death of explants [3]. The results of the present study indicated that the presence of lower concentrations of BA (1.50 mg/L) would promote the callus growth although very high concentrations of BA (10.0 mg/L) causes the death of calli after induction.

3.2 Gas Chromatogram-Mass Spectrometry (GC-MS) of callus and leaves

Chromatograms and mass spectrum obtained from GC-MS analysis of leaf and callus samples are illustrated in Figure 1.

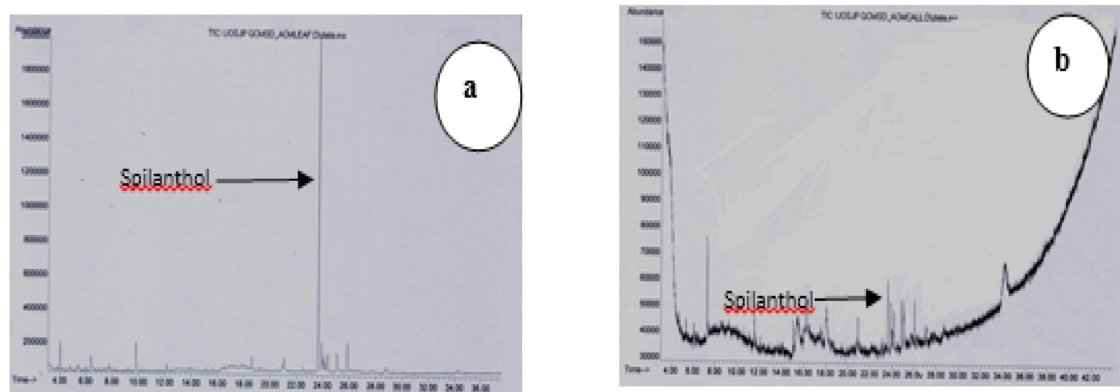

Fig 1: Gas chromatograms of leaf and callus extracts (ethyl acetate) of *S. paniculata*: a) leaf sample and b) callus sample

Distinctive peaks in each chromatogram were identified by the chemical name using the software of compound library Accordingly six compounds in leaf sample and four compounds in callus sample were identified (Table 2).

Table 2: Chemical compounds identified in leaf and callus samples by GC-MS analysis

Sample	Compound	Molecular weight (g/mol)	Similarity (%)
Leaf	n-Hexadecanoic acid	256	99
Leaf	N-(2-methylbutyl)-(2E,6Z,8E)-decatrienamide	235	90
Leaf	2-Methoxy-4-vinylphenol	150	93
Leaf	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276	87
Leaf	Methyl, 8,11,14-heptadecatrienamide	278	91
Leaf/ Callus	N-Isobutyl-2(E),6(Z),8(E)-decatrienamide (**spilanthol**)	221	90
Callus	N-isobutyl-2(E), 4(Z), 8(Z), 10(E)- dodecatetraenamide	247.3	91
Callus	5-(hydroxymethyl)- 2-furancarboxaldehyde	126	87
Callus	2,5-bis(1,1-dimethylethyl)-phenol	206	86
Callus	3-Acetylphenanthrene	220	87

Analysis of the active chemical compounds of *in vitro* plantlets of *S. acmella* using GC-MS revealed that the naturally occurring insecticide, - spilanthol - was present in mother plant, flower heads and *in vitro* plantlets with similar retention time (43.18 to 43.21 min.) [9] and also the presence of spilanthol in callus cultures was confirmed[5]. In the present study with leaf samples and callus of *S. paniculata* it was identified that peaks eluted at retention time of 23.652 min (leaf) and 23.628 min (callus) are the key chemical compound spilanthol (N-Isobutyl-2(E), 6(Z), 8(E)-decatrienamide) with molecular weight of 221.0 g/mol indicating that *in vitro* produced callus also has the same key chemical compounds as leaf samples. Even though the chromatograms of both leaf and callus sample showed number of peak they could not be identified by the chemical name as the similarity was below 80%. As depicted in Figure 1, the abundance of spilanthol in leaf sample is much higher than that of callus sample. The concentration of spilanthol is higher in field grown natural plants than *in vitro* callus cultures [10] which confirms the results of the present study. Thus, it can be suggested, that *in vitro* propagation of *S. paniculata* does not eliminate the ability of plant cells to produce the key chemical compound spilanthol, however the results indicated significant reduction in the production of spilanthol in callus cultures.

All the chemical compounds given in the Table 2 are identified by name as they showed more than 80 % similarity when compared with the compounds in the reference library. Total of ten compounds were identified by name and only key compound found in both callus and fresh leaves was spilanthol. Presence of four alkylamides in *S. acmella*, namely spilanthol, (2E)-N-(2-methylbutyl)-undecene-8,10-diynamide, (2E,7Z)-N-Isobutyl-2,7-tridecadiene-10,12-diynamide and (7Z)-N-Idobutyl-7-tridecene-10,12-diynamide have been reported [11]. N-isobutyl-2(E), 4(Z), 8(Z), 10(E)-dodecatetraenamide -a potent mosquito larvicide - was only detected in the *in vitro* plantlets of *S. acmella* [9] and in the present study also it was observed that the same compound is present only in callus cultures but not in leaf samples tested. Fatty acids (n-Hexadecanoic acid and tetradecanoic acid) could be obtained from all the sample extracts of mother plant, flower heads, *in vitro* plantlets, callus, air-dried cells, freeze-dried cells and fresh cells [9] however, in the present study it was observed that only n-Hexadecanoic acid is present in leaf samples but not in callus and also no tetradecanoic acid even in leaves (Table 2). Those differences may due to differences in the species studied - *S. acmella* has been used in one of the studies [9] while in the present study *S. paniculata* has been used.

3.3 Larvicidal effect and cytotoxicity of callus and leaf extracts

The hexane extracts of both leaf and callus extracts used in different dilutions have indicated variable mortality against the late third/early fourth instar larvae of *A. aegypti*. Percentage mortality at each dilution is given in Table 3.

According to the results obtained, 70 % mortality of late third/early fourth instar larvae of *A. aegypti* has been obtained with 100.0 ppm of leaf extract. Callus extracts showed less effectiveness which gives only 20% mortality with the same dilution (100.0 ppm). Furthermore, the results revealed that at least 71.4 ppm of the phytochemical extract is needed for LD_{50}.

Table 3: Percentage mortality of mosquito larvae at different dilutions of leaf and callus extracts

Treatment code	Dilution of the extract (ppm)	Percentage mortality (%)	
		Leaf	Callus
T0[1] (hexane only)	10.0	0.0	0.0
T0[2] (water only)	0.0	0.0	0.0
T1	5.0	0.0	0.0
T2	10.0	10.0	0.0
T3	20.0	10.0	0.0
T4	50.0	30.0	0.0
T5	100.0	70.0	20.0

[1] = Solvent control, [2] = Negative control

Mortality of *A. salina* nauplii in each dilution of hexane extracts of leaf and callus samples are given below in Table 4. According to the results obtained it could be suggested that the mortality is totally due to the effect of the extracts, as the solvent (hexane) does not pose mortality. Highest cytotoxicity of hexane extracts of *S. paniculata* on nauplii with 24 h of exposure time was observed when 800.0 μg/mL concentration of extracts has been used giving 100% mortality with leaf extracts but the mortality rate was only 16.67% with callus sample.

Table 4: Percentage mortality of brine shrimp nauplii at different dilutions of leaf and callus extracts after 24 h.

Treatment code	Concentration of the extracts (μg/mL)	Percentage mortality (%)	
		Leaf extract	Callus extract
T0[1] (DMSO)	10.0	0.00	0.00
T0[2] (Colchicine)	10.0	0.00	0.00
T0[3] (Hexane)	100.0	3.33	3.33
T1	25.0	10.00	0.00
T2	50.0	16.67	0.00
T3	100.0	30.00	0.00
T4	200.0	43.33	6.67
T5	400.0	66.67	13.33
T6	800.0	100.00	16.67

[1]DMSO = (-) control, [2]Colchisine = (+) control, [3]Hexane = solvent control

However, as hexane only was use as solvent control 3.33% of mortality of brine shrimp nauplii was observed indicating that there is an effect of the solvent too. When standard Colchicine was used as the positive control 100% mortality was observed [12] but the results of the present study no mortality has been observed in the presence of Colchicine. The possible reason for such observation could be the problem with Colchicine used.

4. Conclusions

The results of the present study revealed that, the initiation of callus from leaf discs explants of *S. paniculata* is possible within 4 days of incubation in MS medium supplemented with NAA (0.15 mg/L) and BA (1.50 mg/L) in dark. GC- MS analysis confirmed the presence of spilanthol in both fresh leaves and 3-weeks-old callus samples. Callus samples contain lower amount of spilanthol compared to leaf samples. However, GC-MS analysis of the present study revealed that *in vitro* culture of *S. paniculata* does not eliminate the ability of plant cells to produce spilanthol. Therefore, either by improving the culture conditions or optimizing extraction procedure by

partitioning with different organic solvents, which will provide appropriate separation of the chemicals better results could be obtained. Cytotoxicity studies confirmed the toxicity of the hexane extracts of leaf and callus samples against late third/early fourth instar larvae of *A. aegypti* and nauplii of *A. salina* indicating the possibility of using *S. paniculata* as potential agent in controlling mosquito larvae in small scale water-logs such as vases, flower pots etc. however not in open areas as they have potential cytotoxicity against other organisms too.

Furthermore, finding of the present study could be manipulated with improved technologies to characterize the chemical properties of various extracts of *S. paniculata* and to develop their formulations to be incorporated in novel medicines and health/body care products.

5. Acknowledgements

Dr. SDM Chinthaka from the Department of Chemistry, University of Sri Jayewardenepura, Sri Lanka is highly acknowledged for his unlimited support in GC-MS analysis and confirming the results. And also Dr. C. Wijesundara, Director, Royal Botanical Gardens, Sri Lanka for helping in authentication of the plant species.

6. References

1. Pandey V, Agrawal V. Efficient micropropagation protocol of *Spilanthes acmella* L. possessing strong anti-malarial activity, *In Vitro* Cellular Developmental Biology-Plant 2009 45; 491–499.
2. Senarathna LK. A check list of flowering plants of Sri Lanka 2001, National Science Foundation, Sri Lanka, 59.
3. Haw AB, Kena CL. Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots, Journal Applied Horticulture 2003 5(2):65-68.
4. Dias AMA, Santos P, Seabra IJ, Junior RNC, Braga MEM, de Sousa HC. Spilanthol from *Spilanthes acmella* flowers leaves and stems obtained by selective supercritical carbon dioxide extraction, Journal of Supercritical Fluids 2011; 2303:1-9.
5. Saraf DK, Dixit VK. *Spilanthes acmella* Murr. : Study on Its Extract Spilanthol as Larvicidal Compound, Asian Journal of Experimental Sciences 2002; 16:9-19.
6. Murashige T, Skoog FA. revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 1962 15:473-497.
7. World Health Organization. Instruction for determining the susceptibility or resistance of mosquito larvae to insecticides 1981. WHO/VBC/81.807. WHO, Geneva.
8. Hewage S, Senarath WTPSK. *In vitro* callus induction of *Spilanthes calva* DC [*Spilanthes acmella* auct. non L.] (Maha Akmella), Proceedings of the 11th Annual Forestry and Environment Symposium 2006, 25.
9. Leng TC, Ping, NS, Lim, BP, Keng CL. Detection of bioactive compounds from *Spilanthes acmella* (L.) plants and its various *in vitro* culture products. Journal of Medicinal Plants Research 2011; 5(3): 371-378.
10. Singh M, Chaturvedi R. Screening and quantification of an antiseptic alkylamide, spilanthol from *in vitro* cell and tissue cultures of *Spilanthes acmella* Murr. Industrial Crops and Products 2012; 36: 321–328.
11. Nakatani N, Nagashima M. Pungent Alkylamides from *Spilanthes acmella* L.var. *oleracea* Clarke, Japan Society for Bioscience- Biotechnology and Agrochemistry 1992; 56(5):759-762.
12. Morshed MA, Uddin A, Rahman A, Hasan T, Roy S, Amin AA *et al*. *In vitro* antimicrobial and cytotoxicity screening of *Terminalia arjuna* ethanol extract. International Journal of Biosciences 2011; 1(2):32–38.