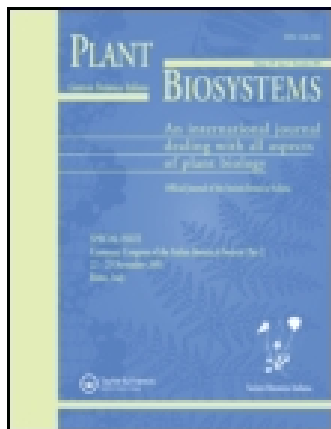


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### Extracts of dedifferentiated cultures of *Spilanthes acmella* Murr. possess antioxidant and anthelmintic properties and hold promise as an alternative source of herbal medicine

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## Extracts of dedifferentiated cultures of *Spilanthes acmella* Murr. possess antioxidant and anthelmintic properties and hold promise as an alternative source of herbal medicine

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### Abstract

This study reports the antioxidant and anthelmintic activities of *Spilanthes* cell cultures. Murashige and Skoog medium supplemented with 5.0  $\mu\text{M}$  *N*<sup>6</sup>-benzylaminopurine, 1.0  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid and 1.0  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid was found to be the best medium to induce dedifferentiation from the leaf cells and for further maintenance of the callus cultures. These cell cultures and *in vivo* leaves were subjected to solvent extraction by hexane, ethyl acetate, methanol and water, and their extraction yield and total phenolic contents were evaluated. The antioxidant activity of extracts was determined by using 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay. The methanol extracts of *in vitro* callus and *in vivo* leaf showed an apparent antioxidant activity and their IC<sub>50</sub> values were 1342.9 and 1085.1  $\mu\text{g/ml}$ , respectively. The anthelmintic efficacy of the extracts was also tested using live trematode (flake) parasites of cattle as the model test material. The aqueous extract of dedifferentiated callus showed a strong anthelmintic activity, which was higher than the activity of the water extract of the field-grown plant. The results of this study verify the potential of *Spilanthes* as a reservoir of bioactive agents and also substantiate the value of callus cultures as a new source of anthelmintic and antioxidant compounds.

**Keywords:** Asteraceae, amphistomes, antioxidant, anthelmintic, phenolics, *Spilanthes acmella* Murr

**Abbreviations:** BAP, *N*<sup>6</sup>-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; Kinetin, *N*<sup>6</sup>-furfuryladenine; NAA,  $\alpha$ -naphthaleneacetic acid; pCPA, para-chlorophenoxyacetic acid; TDZ, thidiazuron

### Introduction

Medicinal plants have always had an important place in the therapeutic arsenal of mankind (Ishtiaq et al. 2010) because they are able to synthesize an array of bioactive compounds. At present, 80% of the populations in developing countries are completely dependent on plants for their primary healthcare systems (Bannerman 1983). In spite of enormous progress in medicinal chemistry, over one-fourth of all prescribed pharmaceuticals in developed countries originate directly or indirectly from plants (Newman et al. 2000). Furthermore, out of 252 drugs considered indispensable by the World Health Organisation, 11% are mainly derived from flowering plants and 28% of synthetic drugs are obtained from natural precursors (Namdeo 2007). Large-scale evaluation of the plants used in traditional medicine

for various biological activities is a crucial first step in the isolation and characterization of the active principle and further investigations leading to drug development. In view of this, *Spilanthes acmella* Murr., an important medicinal plant of the Asteraceae family, was selected for this study. The plant has been used as folklore medicine since ancient times to cure severe toothache, infections of throat and gums, stomatitis, paralysis of tongue and psoriasis (Anonymous 1989). In the Amazon basin, *Spilanthes* has been used for the treatment of tuberculosis by laymen (Storey & Salem 1997). In addition, it is accredited with antiseptic, analgesic, diuretic, immune-modulatory, antioxidant and insecticidal biological properties (Jondiko 1986; Ramsewak et al. 1999; Guiotto et al. 2008; Matthias et al. 2008; Pandey & Agrawal 2009; Prachayasittikul et al. 2009). The plant's virtues are, to a large extent,

attributable to its secondary metabolites such as alkylamides, phenolics, coumarin and triterpenoids which are therapeutic and account for its use in folk medicines all over the world, since time immemorial (Prachayasittikul et al. 2009).

Despite the widespread use of this plant in traditional medicine, there are very few reports that support the biological properties of this plant scientifically. Although the antioxidant property of *Spilanthes* has been reported by two workers (Wongsawatkul et al. 2008; Prachayasittikul et al. 2009), there is no information available on the anthelmintic property of this plant. Moreover, all bioassays carried out, so far, have been done on plants growing in the wild. As environmental fluxes are known to cause alterations in type and quantity of metabolites produced (Minutolo et al. 2012), establishment of *in vitro* cultures will help to utilize the biomass and nullify the effect of seasonal variation on important metabolite content.

The aims of this study were (1) to establish the *in vitro* cultures of *S. acmella*, (2) to compare the antioxidant activities of *in vitro* culture extracts against field-grown extracts and (3) to assess the anthelmintic activities of both field-grown and *in vitro* culture-derived extracts.

## Materials and methods

### Establishment of aseptic cultures

Healthy leaves of *Spilanthes* were collected from the campus of the Indian Institute of Technology Guwahati. Leaves were surface sterilized with 1% (v/v) savlon (Johnson and Johnson, India) for 20 min and with 0.1% (w/v) mercuric chloride (HgCl<sub>2</sub>) for 10 min, followed by three rinses in sterile water. Leaf-disc explants were prepared by punching the sterilized leaves with 5-mm sized cork-borer before being cultured with their abaxial side in contact with the media. Leaf discs were incubated on MS (Murashige & Skoog 1962) basal medium supplemented with varying combinations and concentrations of auxins and cytokinins including  $\alpha$ -naphthaleneacetic acid (NAA), indole-3-acetic acid, para-chlorophenoxyacetic acid (pCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), N<sup>6</sup>-benzylaminopurine (BAP), thidiazuron and N<sup>6</sup>-furfuryladenine (Kinetin). Unless mentioned otherwise, all media contained 30 g/l sucrose and were solidified with 0.8% agar (Hi Media Laboratories, Mumbai, India). The pH of the medium was adjusted to 5.8 before the agar was added. The cultures were maintained at 25  $\pm$  2°C temperature with 50–60% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (1000–2000 lux). At least 24 cultures were raised for each treatment and each experiment was repeated at least three times.

Observations were recorded at weekly intervals and subcultured regularly into fresh medium at 5-week intervals.

### Preparation of samples

To prepare samples, cultured plant cells were harvested, washed with distilled water and filtered under vacuum. Thereafter, washed cell lines and leaves from *in vivo* grown plants were dried separately in an oven at 30  $\pm$  2°C until a constant weight was achieved. The drying temperature was kept low to prevent thermal decomposition of metabolites. Then, dried powdered cell mass and leaf samples were separately soaked in different solvents (hexane, ethyl acetate, methanol and water) for 12 h. The samples were then centrifuged in a high-speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5000 rpm for 10 min. The supernatant was transferred into a new tube and the residue was re-extracted thrice with 10 ml solvent. Thereafter, the residue was discarded and the supernatant was pooled, filtered and evaporated to dryness in a rotatory evaporator (Buchi Rotavapor R-200, Tokyo, Japan) at 40°C. The water extracts were lyophilized in a freeze dryer and used for further studies. The percentage yield of extracts was calculated by using the following formula: extraction yield(%) = [weight of dried extract (g)/weight of dried callus or leaves (g)]  $\times$  100.

### DPPH radical scavenging activity

Free radical scavenging activity of the plant extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described by Mensor et al. (2001) with a slight modification. Stock solutions (10 mg/ml each) of the extracts were prepared in methanol. About 0.5 mg/ml solution of DPPH in methanol was prepared and 500  $\mu$ l of this solution was added to 2.0 ml of extract solution. Thirty minutes later, the absorbance of this solution was recorded on an ultraviolet and visible (UV-vis) spectrophotometer (Cary 100, Middelburg, the Netherlands) at 517 nm using a blank containing the same concentration of DPPH radicals. A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage (I%) of DPPH free radical by the sample and was calculated using the following formula:

$$I\% = [(A \text{ blank} - A \text{ sample}) / A \text{ blank}] \times 100,$$

where *A* blank is the absorbance value of the control reaction (containing all reagents except the extract) and *A* sample is the absorbance values of the extract.



Figure 1. Establishment of cultures of *S. acmella*. (A) Leaf disc at culture ( $\times 7.8$ ), (B) dedifferentiating leaf-disc explant of *Spilanthes* after 2 weeks of culture ( $\times 7.4$ ), (C) an 8-week-old culture showing soft, friable and brownish callus ( $\times 2.66$ ) and (D) a 15-week-old culture showing healthy, fresh, friable and light green calli ready for analysis after three passages ( $\times 2.40$ ).

Ascorbic and vanillic acids (Sigma, St. Louis, MO, USA) were used as reference compounds.

#### Assay for total phenolics

Total phenolic constituents of extracts were determined by methods involving Folin–Ciocalteu reagent and gallic acid standard (Djeridane et al. 2006). Solutions of each extract (200  $\mu$ l; 5 mg/ml) were taken individually in test tubes. To this solution, 500  $\mu$ l each of distilled water and Folin–Ciocalteu reagent was added, and the flasks were thoroughly shaken. After 1 min, 800  $\mu$ l of 7.5%  $\text{Na}_2\text{CO}_3$  solution was added and the mixtures were allowed to stand for 30 min with intermittent shaking. Absorbances were taken at 760 nm. The same procedure was repeated for all the standard gallic acid solutions, and a standard curve was obtained. Total phenols of the extract, as gallic acid equivalents, were determined by using the absorbance of the extract measured at 760 nm as input to the standard equation. All tests were carried out in triplicate, and phenolic contents as gallic acid equivalents were reported.

#### Anthelmintic assay

Live trematodes (*Gastrothylax cruminefer*, flukes commonly parasitizing the rumen of cattle livestock) were collected in 0.9% phosphate buffered saline

(PBS; 8 g NaCl, 0.34 g  $\text{KH}_2\text{PO}_4$  and 1.21 g  $\text{K}_2\text{HPO}_4$  in 1 l of distilled water, pH  $7 \pm 0.3$ ) from freshly slaughtered cattle (*Bos indicus*) at local abattoirs. The flukes were incubated at  $37 \pm 1^\circ\text{C}$  in PBS supplemented with 1% Dimethyl sulfoxide containing no extract (control) or crude extract at 10, 20 and 30 mg/ml. The time required for complete inactiveness or paralysis and death of the parasite was recorded. For each set of treatment, five replicates were used. Distocide (Chandra Bhagat Pharma, Mumbai, India) was used as the reference drug.

#### Statistical analyses

All experiments were carried out in triplicate along with proper controls. The significance of results was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and by Student's *t*-test. The results were analysed statistically using the SPSS (version 16) software and significance was determined at  $p < 0.05$ . Data were expressed as mean  $\pm$  standard deviation. To minimize experimental error, exponentially growing cells of the same passage were used.

## Results

#### Dedifferentiation from leaf-disc cultures

Leaf-disc explants of 5 mm size were cultured on MS basal medium or basal medium supplemented with growth regulators. Leaf-disc cultures showed no response in the absence of growth regulators. On few combinations, such as MS + NAA (5.0  $\mu\text{M}$ ), MS + pCPA (5.0  $\mu\text{M}$ ), MS + BAP (7.0  $\mu\text{M}$ ) and MS + 2,4-D (5.0  $\mu\text{M}$ ), cultures responded initially with callus formation, but the calli did not survive after the first subculture. The best treatment in terms of number of explants showing callus, the degree of callusing and sustained growth of calli was the combination of MS + BAP (5.0  $\mu\text{M}$ ) + NAA (1.0  $\mu\text{M}$ ) + 2,4-D (1.0  $\mu\text{M}$ ). On this medium, after 2 weeks, friable, light brown calli began to develop from 100% of leaf-discs. Although callus proliferation increased with subsequent subcultures but remained brown in colour till 10th week, by the 11th week, vigorously growing light green and healthy callus was obtained (Figure 1(A)–(D)), which remained in an unorganized (dedifferentiated) state. The calli were subdivided and transferred to fresh medium of the same composition as the initial medium at 5-week intervals.

#### Yield of extracts

Selection of solvent is an important step for obtaining extracts with acceptable yields and strong antiox-

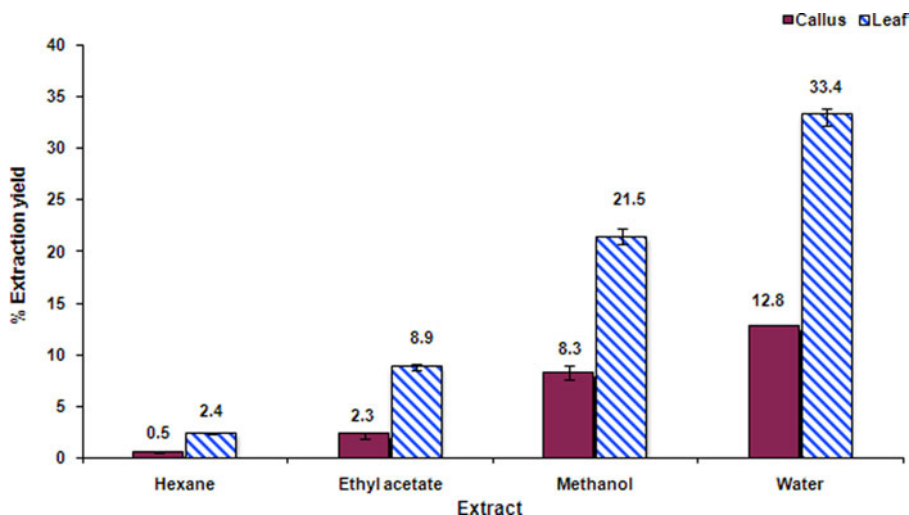


Figure 2. Yield of extracts from callus and *in vivo* leaves. Data are means ( $n = 3$ )  $\pm$  standard deviation ( $n = 3$ ),  $p < 0.05$ .

identant activity. The results on yield of extracts of *in vivo* leaves and calli from various organic solvents are shown in Figure 2. The yields of extracts from different solvents were obtained in the following order: water > methanol > ethyl acetate > hexane. Extraction yields varied from 2.4% to 33.4% for leaf extracts and from 0.5% to 12.8% for callus extracts. The highest and the lowest yields were obtained from water and hexane extracts, respectively, in both *in vivo* leaves and *in vitro* callus cultures (Figure 2).

#### DPPH radical scavenging activity of various extracts

The results of the antioxidant assay revealed that *in vivo* leaf and callus extracts possessed a significant variability in their inhibitory activity against DPPH radical. The radical scavenging activity of each extract is indicated as the percentage of reduction of the initial DPPH absorbance.  $IC_{50}$  values for the extracts studied were also calculated. A noticeable

effect of the extract on radical scavenging activity was observed at a concentration range of 250–2000  $\mu\text{g}/\text{ml}$  and the effect was found to be dose dependent. The highest radical scavenging activity was detected in *in vivo* leaf methanolic extract followed by dedifferentiated callus methanolic extract. At 1000  $\mu\text{g}/\text{ml}$  concentration, methanolic extracts of calli and leaf exhibited 46.2% and 52.2% DPPH inhibition and their  $IC_{50}$  values were 1342.9 and 1085.1  $\mu\text{g}/\text{ml}$ , respectively (Table I). The water, ethyl acetate and hexane extracts of *in vivo* leaf and callus showed no radical scavenging activity with DPPH.

In order to understand the free radical scavenging capacities of sample extracts, it is important to compare their activities with the relative activities of standard antioxidant compounds. For this study, ascorbic and vanillic acids were used as the standard antioxidant compounds. Ascorbic acid showed excellent radical scavenging activity with  $IC_{50}$  value

Table I. DPPH radical scavenging activity of *S. acmella* methanolic extracts of *in vivo* leaf and dedifferentiated callus, after 30 min.

Serial no.	Sample	Extract concentration ( $\mu\text{g}/\text{ml}$ )	% DPPH inhibition	$IC_{50}$
1.	<i>In vivo</i> leaf methanolic extract	0.0	$0.0 \pm 0.0f$	1342.9
		250.0	$18.6 \pm 1.2e$	
		500.0	$30.6 \pm 2.4d$	
		1000.0	$52.2 \pm 4.1c$	
		1500.0	$67.6 \pm 3.5b$	
		2000.0	$84.9 \pm 1.0a$	
2.	Dedifferentiated callus methanolic extract	0.0	$0.0 \pm 0.0e$	1085.1
		250.0	$10.6 \pm 0.3d$	
		500.0	$28.0 \pm 2.7c$	
		1000.0	$46.2 \pm 2.5b$	
		1500.0	$61.3 \pm 2.9a$	
		2000.0	$64.9 \pm 3.1a$	

Data are means ( $n = 3$ )  $\pm$  standard deviation ( $n = 3$ ). Mean values sharing the same letter do not differ significantly ( $p < 0.05$ ) according to Tukey's test.

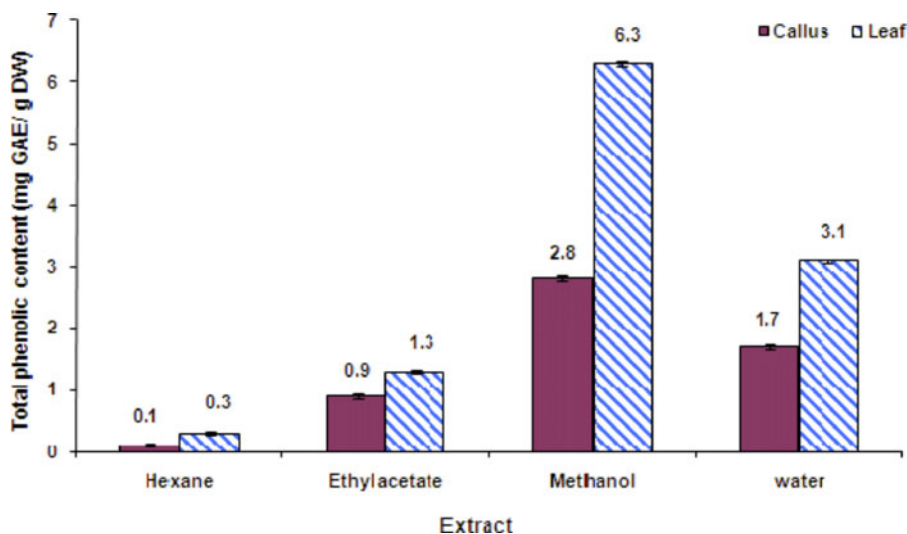


Figure 3. Total phenolic content in callus and *in vivo* leaf extracts. Data are means ( $n = 3$ )  $\pm$  standard deviation ( $n = 3$ ),  $p < 0.05$ .

of 14.36  $\mu\text{g/ml}$ . It has 93.5-fold and 75.6-fold more antioxidant activity than methanolic extracts of calli and leaves, respectively. Whereas vanillic acid showed very low radical activity with  $\text{IC}_{50}$  values as 3394.43  $\mu\text{g/ml}$ . Both leaf and callus methanolic extracts possess greater, 2.5-fold and 3.1-fold, respectively, antioxidant activity than vanillic acid extracts. Thus, the sample extracts exhibited relatively appreciable antioxidant activities with reasonable  $\text{IC}_{50}$  values.

The content of total phenolic compounds in the *Spilanthes in vivo* leaf and *in vitro* callus extracts was determined through a linear gallic acid standard curve  $y = 0.0022x + 0.0679$ ;  $R^2 = 0.9673$ , and the results are shown in Figure 3. The total phenolic content varied from 0.3 to 6.3 mg Gallic Acid Equivalents (GAE)/g Dry Weight (DW) for leaf extract and from 0.1 to 2.8 mg GAE/g DW for callus extract. The highest value of total phenolic compounds was

detected in the methanolic extracts of both leaf and callus, whereas the lowest content was obtained in the hexane extracts.

#### Anthelmintic assay

The results of the anthelmintic assay revealed that aqueous and methanolic extracts exhibited varying degrees of activity against tested parasite and caused paralysis followed by death at all tested concentrations. ANOVA followed by Tukey's test indicates that both the water and methanol extracts had significant ( $p < 0.05$ ) dose-dependent activity on trematode (Tables II and III). However, water extracts showed more potent activity than methanolic extracts at lower concentrations (5 and 10 mg/ml). At 5 mg/ml concentration, the water extract of callus caused onset of paralysis in 45.7 min and death in 87 min, whereas its methanolic extract showed paralysis and death in 83

Table II. ANOVA of callus extracts effect on trematode survivability.

Extracts	Response (min)		Sum of squares	df	Mean square	F	Sig.
Water	Paralysis Time	Between groups	924.222	2	462.111	26.660	.001
		Within groups	104.000	6	17.333		
		Total	1028.222	8			
	Death Time	Between groups	2804.222	2	1402.111	27.613	.001
		Within groups	304.667	6	50.778		
		Total	3108.889	8			
Methanol	Paralysis Time	Between groups	5297.556	2	2648.778	83.063	.000
		Within groups	191.333	6	31.889		
		Total	5488.889	8			
	Death Time	Between groups	8490.889	2	4245.444	35.543	.000
		Within groups	716.667	6	119.444		
		Total	9207.556	8			

Table III. ANOVA of leaf extracts effect on trematode survivability.

Extracts	Response (min)		Sum of squares	df	Mean square	F	Sig.
Water	Paralysis Time	Between groups	2033.556	2	1016.778	56.839	.000
		Within groups	107.333	6	17.889		
		Total	2140.889	8			
	Death Time	Between groups	33,326.000	2	16,663.000	154.287	.000
		Within groups	648.000	6	108.000		
		Total	33,974.000	8			
Methanol	Paralysis Time	Between groups	10,848.667	2	5424.333	272.732	.000
		Within groups	119.333	6	19.889		
		Total	10,968.000	8			
	Death Time	Between groups	50,882.000	2	25,441.000	257.848	.000
		Within groups	592.000	6	98.667		
		Total	51,474.000	8			

and 126 min, respectively (Figure 4). These results indicate that higher concentration of each extract produced a paralytic effect much earlier and the time to death was shorter. Extract derived from *in vivo* leaves also showed similar activity against the flukes (Figure 5), in which water extracts showed the best activity at all tested concentrations in the range of 5–30 mg/ml. Distocide, the reference drug, caused paralysis and death of parasites at 0.01 mg/ml concentration in 10 and 20 min, respectively.

## Discussion

In spite of being a plant of potential medicinal interest, there are very few reports that document the properties of *Spilanthes* scientifically. Therefore, in this study, cell cultures and *in vivo* leaf extracts of *S. acmella* were investigated for antioxidant and anthelmintic properties.

Among different media combinations, maximum callus formation (100%) was observed on MS + BAP

(5.0  $\mu$ M) + NAA (1.0  $\mu$ M) + 2,4-D (1.0  $\mu$ M). However, establishing the fresh, friable and constantly growing cell lines of *Spilanthes* was a difficult task to accomplish because of browning of the callus. Many workers have discussed the phenomenon of browning of cultures due to phenol exudation (Naik et al. 1999; Wu & du Toit 2004). It has been recommended that antioxidants such as ascorbic acid, citric acid, polyvinylpyrrolidone and polyvinylpolypyrrolidone can be used to surmount culture browning and death. Besides this, recurrent subculturing at regular intervals is also an efficient and alternative approach to overcome browning of cultures (Rout et al. 1999; Srivastava et al. 2010). In this study, the browning problem was avoided by subculturing of calli. Use of antioxidants was avoided to prevent changes in the secondary metabolite spectrum of cultured cells.

It has been well known that under appropriate culture conditions, *in vitro* grown cells/tissue genetically inherits the biosynthetic potential of *in situ* cells and can produce the range of metabolites found in

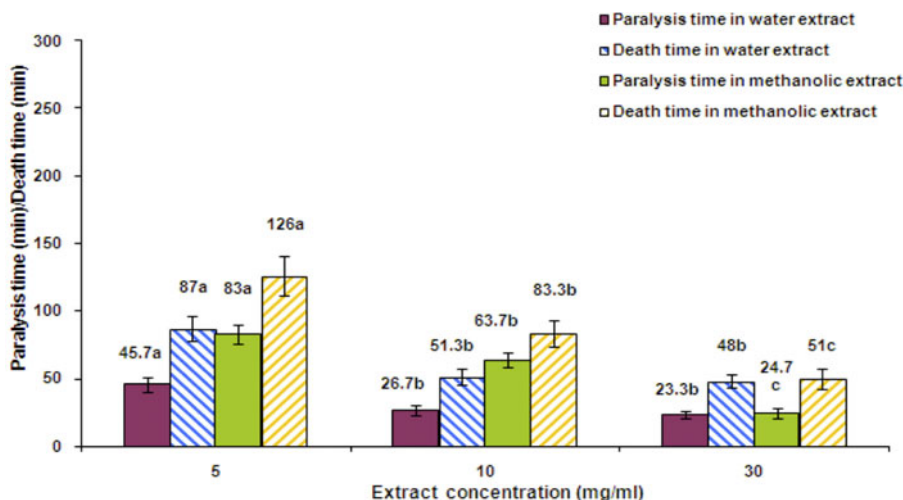


Figure 4. Effect of water and methanolic extracts of callus on trematode survivability. Data are means ( $n = 3$ )  $\pm$  standard deviation ( $n = 3$ ). Values in a column followed by different letters are significantly ( $p < 0.05$ ) different according to Tukey's test.

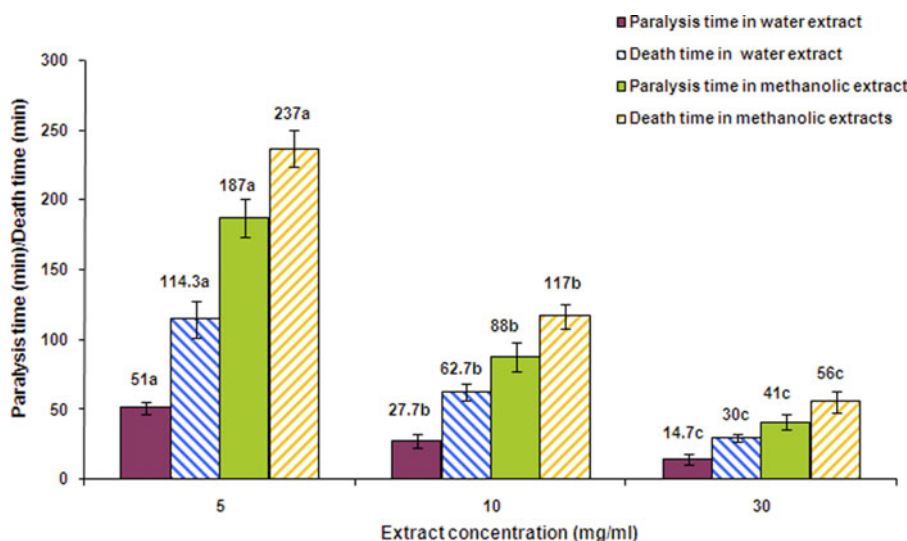


Figure 5. Effect of water and methanolic extracts of leaf on trematode survivability. Data are means ( $n = 3$ )  $\pm$  standard deviation ( $n = 3$ ). Values in a column followed by different letters are significantly ( $p < 0.05$ ) different according to Tukey's test.

the parent plant (Rao & Ravishankar 2002). The importance of *in vitro* culture establishment in *Spilanthes* is due to the uniformity and stability of metabolites produced *in vitro*. In addition, they produce metabolites of interest in a relatively non-contaminated background, unaffected by seasonal variations. In addition, *in vitro* cultures can provide a vehicle, which can be used to investigate the metabolic pathways and basic physiology of the plant.

Antioxidant activity of *Spilanthes* extracts was evaluated by the DPPH method. DPPH is a purple-coloured stable free radical, which is reduced to  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazine (yellow coloured) by accepting an electron or hydrogen radical from an antioxidant (Soares et al. 1997; Hossain & Shah 2011). So far, there are two reports on the antioxidant activity of *Spilanthes* extracts (Wongsawatkul et al. 2008; Prachayasittikul et al. 2009). However, in both these reports, crude extracts obtained from the plants growing in the wild were used for the assay. In this study, for the first time, *Spilanthes in vitro* cultures were examined for antioxidant property. Results of the DPPH assay showed that methanolic extracts of both callus and leaf have higher antioxidant potential than water, ethyl acetate and hexane extracts. Both callus and leaf methanolic extracts showed DPPH scavenging activities in a dose-dependent manner and their  $IC_{50}$  values were 1342.9 and 1085.1  $\mu\text{g/ml}$ , respectively. Earlier reports have also suggested that methanolic extracts show relatively higher antioxidant activity than the other solvents including acetone, diethyl ether, ethyl acetate and water (Zieliński & Kozłowska 2000; Oki et al. 2002).

Recently, Leng et al. (2011) have reported the production of butylated hydroxytoluene, an antiox-

idant compound, from *Spilanthes in vitro* cultures, which can be responsible for the *Spilanthes* callus antioxidant property. However, further detailed study is needed to explore *Spilanthes in vitro* cultures as a source of natural antioxidants and nutraceuticals to enhance health benefits.

Production of antioxidant compounds from *Spilanthes* cell culture will offer many benefits over field-grown plants. It is not subjected to the limitations of seasonal growth, genetic, geographical and climatic variations. Moreover, cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. As a result, metabolite production can take place within a short cultivation time (about 2–5 weeks) with an added advantage of tunability. In addition to this, interfering compounds that occur in field-grown plants can be avoided in cell cultures.

It was generally assumed that dedifferentiated cultures proved to be less active as antioxidants (Grzegorzczak et al. 2007). However, the present results indicated that dedifferentiated callus extracts showed significant antioxidant potential. Similar to this study, dedifferentiated cell lines of *Rosmarinus officinalis* (Yesil-Celiktas et al. 2007), *Harpagophytum procumbens* (Georgiev et al. 2010) and *Psoralea corylifolia* (Shinde et al. 2010) have shown promising antioxidant activity using the DPPH assay. Antioxidant activity of dedifferentiated cultures can be further improved by using different strategies such as screening of cell lines, media optimization and elicitation.

Several studies have revealed that phenolic contents in plants are associated with their antiox-



idant activities, probably due to their redox properties, that allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Chang et al. 2001). Therefore, the content of total phenolic compounds in the *Spilanthes* callus and *in vivo* leaf extracts was determined through a linear gallic acid standard curve.

For estimation of total phenolic content of plant samples, several methods have been reported that are based on spectroscopy chemiluminescence (Wang et al. 2007), spectrophotometry (Leamsomrong et al. 2009) and chromatography (Ng et al. 2000). In spite of the fact that many methods are available, most of them lack versatility, simplicity and suitability for large-scale analysis. Among all, the spectrophotometric-based Folin–Ciocalteu method is the most commonly used for rapid determination of total phenolic compounds in different samples (Singleton et al. 1999). Its popularity over other methods is due to the fact that it has the advantage of a fairly equivalent response to different phenols. Moreover, it is an affordable technique with lower expenses and requires lower reagent consumption. The only limitation of this method is that it lacks specificity and is influenced by interference from reducing agents such as sugars, sulphites and protein (Singleton et al. 1999).

In this study, the Folin–Ciocalteu method has been used for comparative analysis of different extracts. The highest total phenolic compounds were detected in the methanolic extracts of both callus and leaf, whereas the lowest content was obtained in their hexane extracts. These findings clearly demonstrate the influence of the solvent on the extractability of phenolics. The findings of this study are in agreement with previous reports which suggested that the nature of solvent exerts a great influence on the extraction of phenolics from the plant (Akowuah et al. 2005; Turkmen et al. 2006).

In recent years, development of multiple drug/chemical resistance in pathogens due to continuing and indiscriminate use of commercial drugs has been perceived as a serious problem impeding therapeutic control strategies. So, exploiting traditional herbal medicines for therapeutic use has become a necessary undertaking. In this study, anthelmintic properties of *Spilanthes* were evaluated by using common trematode parasite of ruminant hosts. Helminthes cause serious health problems in both animals and humans worldwide. The test parasites used in this study are known to cause haemorrhages at the site of attachment, vacuolar degeneration in the liver and hyperplasia in the bile duct, thus seriously affecting the health of infected animals (Iqbal et al. 2001).

To date, this is the first scientific evidence that *Spilanthes* extracts possess anthelmintic properties; moreover, we observed that tissue culture extracts possess more anthelmintic activity than leaf extracts. This result suggests that *in vitro*-cultured tissues or

cell cultures of *Spilanthes* can be a better source of effective natural anthelmintic agents than field-grown plants. This might be due to enhanced production of some bioactive compounds in *in vitro* cultures. Nonetheless, presence and interference of other compounds cannot be ruled out in *in vitro* conditions which might be responsible for the observed anthelmintic activity. Further study is required to find out the active anthelmintic principle from the callus extracts and to carry out pharmaceutical studies on the same.

## Conclusion

In the present investigation, an efficient and reproducible protocol for establishing *in vitro* cell cultures from leaves of *Spilanthes* has been developed. Furthermore, for the first time, the antioxidant and anthelmintic activities of *Spilanthes* cell culture extracts have been demonstrated. The aqueous extract isolated from *in vitro*-derived cell cultures exhibited promising anthelmintic activity, which was much higher than the activity of the field-grown parent plant extract. The study confirms the significance of callus cultures as a source of high-value metabolites and will help to move a step forward in the search for novel antioxidant and anthelmintic agents of plant origin.

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