International Journal of Industrial Electronics and Control.
ISSN 0974-2220 Volume 3, Number 2 (2011), pp. 103-109

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In Vitro and *In Vivo* Comparative Study of Primary Metabolites and Antioxidant Activity in *Spilanthes Acmella* Murr.

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Abstract

Spilanthes acemella (Compositae) known as toothache plant promising secondary metabolites, has successfully been cultured *in vitro*. We have raised callus from leaf explants. Among the various concentrations 2, 4-D at 6.78µM/liter found best for callus induction. Callus and plant parts (root, stem, leaf) used for quantitative estimation of primary metabolites and antioxidant activity. Maximum soluble sugars (51±0.84mg/gdw) in callus, starch (30±1.64mg/gdw) in stem, protein (25±1.02mg/gdw) and phenolic contents (52.3±1.6mg/gdw) in leaves and lipids (80±0.71mg/gdw) in roots were estimated. Methanolic extract of stem showed highest superoxide radical scavenging activity (39.54±1.41%) while leaves have showed maximum (76.42±1.67%) 2, 2-diphenyl-1-picryhydrazyl (DPPH) scavenging activity than other plant parts and callus.

Keywords: *Spilanthes acemella,* DPPH, Superoxide radical scavenging activity, Callus culture.

Introduction

The importance of reactive oxygen species and free radicals has attracted increasing attention over the past decade. Reactive oxygen species (ROS) include free radicals such as superoxide anion radicals ($O_2^{\cdot -}$) and hydroxyl radicals ($OH^{\cdot}$). These molecules are exacerbating factors in cellular injury and aging process[1, 2, 3]. Therefore, there is a growing interest on natural additives as potential antioxidants [2, 4, 5, 6].

Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites[7]. Cell and organ cultures have a higher rate of metabolism than

field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organs and to a condensed biosynthetic cycle[8].

Spilanthes acmella (L.) Murr. belongs to the family Asteraceae and is an important medicinal plant grown in tropics and subtropics. *Spilanthes acmella* is an acutely threatened plant species[9]. It has been well documented for its uses as antibacterial, antifungal, and antimalarial activity. Traditionally, plant is also used in treatment of toothache, flue, cough, and tuberculosis[10]. The antimicrobial activity is mainly due to the presence of an alkaloid spilanthol (N-isobutyl-2, 6, 8-decatrienamide[11, 12]. During past years, considerable efforts have been made for *in vitro* plant regeneration of this important plant through organogenesis [10, 12, 13, 14]. However, there is no report on studies of primary metabolites and antioxidant activity in callus.

Material and methodology

Plant material: Healthy plants of *Spilanthes acmella* Murr. were collected from pot cultivated plants from University of Rajasthan and authenticated by Herbarium, University of Rajasthan, Jaipur, Rajasthan, India.

Chemicals: All the chemicals and growth regulators were used are analytical grade and purchased from Hi Media Pvt. Ltd., Mumbai, India.

Callus induction: Explants (leaf and nodal segments) were surface sterilized by 1 % *Teepol* for 15 min followed by immersion in 70 % ethanol for 1 min and in 0.1 % mercuric chloride for 10 min, and then rinsed thoroughly with sterile distilled water. The explants were inoculated in the MS medium[15] fortified with different concentrations of 2, 4-D and IAA. The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg cm-2 and 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25 ± 2 °C, relative humidity 55 ± 5%, and 16-h photoperiod. 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals.

Primary metabolite estimation

Callus, root, stem and leaf parts of *Spilanthes acmella* were evaluated quantitatively to estimate the total levels of soluble sugars, starch, proteins, lipids and phenols following the established methods for the sugars, starch[16], lipid[17], protein[18]and phenol [19]. All experiments were repeated five times for precision and values were expressed in mean ± standard deviation in terms of shade dried material.

Antioxidative assay:

The antioxidative activity of the extracts was elucidated by the DPPH radical scavenging assay [20]. Experiments were initiated by preparing a 0.1 mM solution of DPPH in methanol. Two ml of this solution was added to a sample solution (0.1 ml, 1 mg/ml; dissolved in methanol). After 30 min, absorbance at 515 nm was measured and the percentage of radical scavenging activity was calculated from the following equation:

$$\% \text{ Radical scavenging} = (1\text{-Abs.sample/Abs.control}) \times 100$$

Abs. control is the absorbance of the DPPH solution without sample and Abs. sample is the absorbance of the tested sample.

The superoxide radical scavenging capacity of plant extract was analyzed using a modified method of Beauchamp and Fridovich (1971)[21], as described by Zhishen *et al* (1999)[22]. The 2 ml of reaction mixture containing $3x10^{-6}$ mol/l riboflavin, $1x10^{-2}$ mol/l methionine and $1x10^{-4}$ mol/l nitrobluetetrazolium (NBT) in 0.05 M phosphate buffer (pH 7.8) was illuminated with two 20W fluorescent lamps at 25ºC for 25min in an aluminium foil-lined box. The photochemically reduced riboflavin generated O^{2-} which reduced NBT to blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance (A) was measured at 560nm. The plant extracts (0.2ml, 1 mg/ml; dissolved in methanol) were added to the reaction mixture, which scavenged O^{2-} generation, thereby inhibiting the NBT reduction. Absorbance (A_1) was measured and the decrease in O^{2-} was calculated by $A–A_1$. The degree of the scavenging was calculated by the following equation:

$$\text{Scavenging (\%)} = (A - A_1 / A) \times 100\%$$

Butyl Hydroxy Anisole (BHT) 0.1mg/ml was used as standard antioxidant compound.

Results

Callus induction

MS medium supplemented with different concentrations of 2, 4- D and IAA for callus induction. Leaf explants showed maximum callus formation on 2, 4- D at the concentration 6.78µM/liter. Callus was fragile and yellowish green colored. However, IAA showed direct root induction from leaf and nodal explants at all the concentrations used. Callus obtained from 2, 4- D (6.78µM/liter) was further evaluated for primary metabolite estimation and antioxidant activity. (Shown in Table I and Figure I)

Figure I, Induction and proliferation of adventitious roots and callus from leaf explants of *Spilanthus acemella* A,B Initiation of adventitious roots from leaf and nodal explants (on MS medium supplemented with 5.71 and 8.56µM IAA). **C, D** Callus formation from leaf explants (on MS medium supplemented with 6.78 µM 2, 4-D) after 2 weeks and after 6 weeks

Table (I) Percentage of the morphogenic induced responses in explants from immature *Spilanthus acemella* Murr leaves under different levels of 2,4-D and IAA after 6 weeks of culture .

S. No	Growth regulators	Concentration (µM/liter)	Morphogenic response induced % (after 30 days of inoculation)		Nature of callus
			Callus	Direct rooting	
1	2,4-D	2.26	0.0±0.0	0.0±0.0	-
		4.52	0.0±0.0	0.0±0.0	-
		6.78	75±1.1	0.0±0.0	Yellowish green, fragile
		9.05	65±0.9	0.0±0.0	Yellowish green, fragile
2	IAA	2.85	0.0±0.0	12.0±1.7	-
		5.71	0.0±0.0	55.0±1.2	-
		8.56	0.0±0.0	79.6 ± 1.9	-
		11.41	0.0±0.0	26±1.7	-

Data are presented as mean ± S.E.M (n=20)

Primary metabolites

Root, stem, leaf and callus showed all the primary metabolites at various concentrations depend upon their metabolic and physiological state.

Maximum level of soluble sugars (51±0.84mg/gdw) was found in callus and minimum level of soluble sugars (26±1.41mg/gdw) was found in leaves. Maximum starch (30±1.64mg/gdw) was found in stem and minimum starch was found in (21±0.49 mg/gdw). Highest amount of protein (25±1.02mg/gdw) was estimated in leaf and lowest amount (17±0.49 mg/gdw) in stem. Maximum lipid (80±0.45mg/gdw) was found in root and leaf and minimum lipid (40±0.71mg/gdw) was found in callus. Highest phenolic contents (52.3±1.6mg/gdw) were estimated in leaves and lowest amount of phenolic contents was estimated in callus. (Shown in Table II and graph I)

Table (II): Concentration of primary metabolites in callus and different plant parts of *Spilanthus acemella* Murr. (mg/gdw)*

Experiments	**Root**	**Stem**	**Leaf**	**Callus**
Sugars	33±1.14	48±1.58	26±1.41	51±0.84
Starch	25±1.3	30±1.64	22±1.09	21±0.49
Lipids	80±0.71	70±0.84	80±0.45	40±0.71
Proteins	21±1.41	17±0.49	25±1.02	23±1.41
Phenolic contents	32±0.75	38±1.67	52.3±1.6	29±1.09

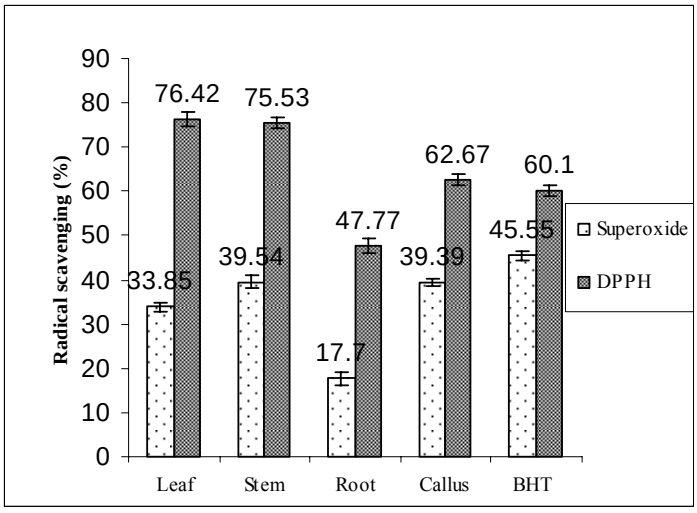
*mg/ gdw- milligram per gram dry weight

Data are presented as mean ± S.E.M (n=3)

Antioxidant activity

The antioxidant activity of *Spilanthes acmella* Murr. methanolic extract was measured using DPPH and superoxide radical scavenging assays. The results showed that all the parts extracts exhibited antioxidative activity comparable to standard Butyl Hydroxy Anisole (BHA). In superoxide radical scavenging assay at 0.1mg/ml concentration highest radical scavenging activity (39.54±1.41% and 39.39±0.79%) was observed in stem and callus respectively, while minimum superoxide radical scavenging activity (17.7±1.58%) was found in roots.

In DPPH radical scavenging activity was found maximum (76.42±1.67%) in leaf and minimum in (47.77±1.64%) in root. Callus showed significant (62.67±1.31%) DPPH radical scavenging activity.



Graph (I) Antioxidant activity of callus and other plant parts of *Spilanthus acemella* Murr.

Discussion

In vitro raised callus are being popularly used for metabolites production. Biochemical studies have been conducted in callus culture of *Arachis hypogea*[23]. Biochemical changes in leaf callus of *Zamia furfuracea* L.[24]. In our studies yellowish green callus was raised from leaf explants on MS media supplemented with 2, 4-D at 6.78µM/liter concentration. It contains higher soluble sugars in callus, starch in stem, lipid in leaf and root, total phenolic content in leaves than other plant parts. Callus showed significant antioxidant activities it may be due to its sugar contents. Many polysaccharides with antioxidant activities have been isolated [25, 26] in different plant species. It was concluded that *Spilanthes acmella* Murr possessed a strong antioxidant activity that is comparable to that of reference compound Butyl Hydroxy Anisole (BHA). It will lead to use *in vitro* raised cells for herbal antioxidant drug formulation, which in turn help to save this threatened species.

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