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RESEARCH ARTICLE

In Vitro Micropropagation and Comparative Free Radical Scavenging Activity of Wild Plant and Callus Extract of *Leucas aspera*

M. Priya Dharishini[1], A. Radha[2], K. Balasubramanian[1,3*], S. Dinesh[4] and M. Narasimman[4]
[1]Plant Tissue Culture Laboratory, National Agro Foundation, Chennai; [2]Dept. of Botany, Bharathi Women's College, Chennai-108;
[3]Dept. of Chemistry, Sri Chandrasekharendra Swaraswathi Mahavidyalaya (SCSVMV) University, Enathur, Chennai;
[4]Dept. of Biotechnology, Sree Sastha Institute of Engineering and Technology, Chennai, India
dr.kbala@gmail.com*; +91 9962842704

Abstract

Morphogenetic response of nodal explants of *Leucas aspera* was observed in Murashige and Skoog (MS) medium supplemented with different hormones (Auxin, cytokinin) and concentrations of sucrose (30 g/L and 35 g/L). Direct shoot induction was observed when amended with IAA (0.1 mg/L), BA (0.2 mg/L), sucrose (30 g/L) with a growth response of 25% along with formation of callus and 16.67% of direct induction of shoots with IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L). Callus induction was also observed in medium containing NAA (0.1 mg/L), BA (0.2 mg/L), sucrose (35 g/L) and NAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (35 g/L) with a growth response of 44.44% and 36.84% respectively. *In vitro* generated leaf explants in medium containing IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L) exhibited 75% of callus induction. Shoot multiplication was observed for *in vitro* generated nodal callus in medium with IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L) with a record of 12 shoots in 34 d. The wild leaf and callus methanol extract when screened for phytochemical constituents, antioxidant activity and total phenolic content revealed significant amounts of flavonoids, phenols, steroids, saponins and glycosides. Wild leaf extract showed better antioxidant activity than the callus extract. The total phenolic content of the leaf and callus methanol extract (145 mg/g and 25 mg/g) was expressed in terms of gallic acid equivalent.

Keywords: *Leucas aspera*, micropropagation, callus, shoot multiplication, phytochemicals, antioxidant.

Introduction

Leucas aspera (Willd.) Linn. (Family: Lamiaceae), commonly known as 'Thumbai', is distributed throughout India from the Himalayas down to Ceylon. The plant is used traditionally as an antipyretic and insecticide (Anand *et al.,* 2011). Bruised leaves are applied locally in snake bites (Srinivasan *et al.,* 2011). *Leucas aspera* is reported to have antifungal, prostaglandin inhibitory, antioxidant, antimicrobial, antinociceptive and cytotoxic activities (Prajapati *et al.*, 2010). Hot water extract of *L. aspera* is used orally as stimulant, anthelmintic, laxative and diaphoretic (Balunas and Kinghorn, 2005). It is also used orally for the treatment of headache, asthma and bronchitis (Reddy *et al*., 1993). Entire plant extract is used orally to treat scabies, psoriasis and snake bite (Pushpangadan and Atal, 1984). A variety of phytoconstituents have been isolated from *Leucas* spp., which include lignans, flavonoids, coumarins, steroids, terpenes, fatty acids and aliphatic long-chain compounds (Das *et al*., 2012). Methanol and ethanol extracts of *Leucas* revealed the presence of tannins, alkaloids and glycosides (Ramya *et al.,* 2012). Plant tissue culture has paved way for the production of both pathogen-free plants and secondary metabolites in large scale. This study was aimed to develop an efficient protocol for micropropagation of *Leucas aspera* using nodal explants. A comparative account on free radical scavenging activity using DPPH, total phenolic content and the profile of phytochemicals of wild leaf and callus methanol extract was also evaluated. The above mentioned studies on *Leucas aspera* are reported for the first time.

Materials and methods

Chemicals: Analytical grade chemicals from Hi-Media, Merck and Sigma were used throughout the study. Analytical grade 1,1-diphenyl2,picrylhydrazyl (DPPH) was purchased from Merck. Ascorbic acid (Vitamin C) purchased from Sigma chemicals was used as the standard for antioxidant activity.

Collection of plant material: Healthy and disease free plant was selected from the area of Anna University campus, Chennai, Tamil Nadu, India in the month of January 2014. For the comparative biological studies, leaves were collected in the same area and 45 d old callus after tissue culture were collected for the study.

Micropropagation and plant regeneration: Laboratory techniques recommended by Purvis *et al.* (1966) were followed for the preparation of media, inoculation and maintenance of cultures.

Shoot induction: Well grown nodes were collected and washed under running tap water for 10 min without damage to the tissues and washed with 0.1% Tween-20 followed by immersion for 2-3 min in sodium hypochlorite (0.5%) and 1-2 min in mercuric chloride (0.5%) solution. The explants were finally washed 5 times with sterile distilled water, cut into small pieces (0.5-1.0 cm) and inoculated on MS medium supplemented with IAA (0.1 mg/L) + BA (0.2 mg/L) + sucrose (30 g/L) and IAA (0.1 mg/L) + BA (0.3 mg /L) + sucrose (30 g/L), incubated under dark condition at 25°C for 3 d and later transferred to 16/8 h light dark period for direct shoot induction.

Callus induction: For callus induction, sterilized explants were inoculated on MS medium supplemented with NAA (0.1 mg/L), BA (0.2 mg/L), sucrose (35 g/L) and NAA (0.1 mg/L), BA (0.3 mg/L), sucrose (35 g/L). *In vitro* generated leaf explants were directly subcultured on the solid MS medium supplemented with IAA (0.1 mg/L), BA (0.3 mg/L), sucrose (30 g/L) and incubated under dark condition at 25ºC for 3 d and then transferred to 16/8 h light/dark period. The leaf callus was harvested for comparative studies.

Shoot multiplication: For shoot multiplication, 1.0 g of 25 d old callus from IAA (0.1 mg/L), BA (0.2 mg/L), sucrose (30 g/L) were subcultured on the MS medium supplemented with IAA (0.1 mg/L), BA (0.3 mg/L), sucrose (30 g/L) and incubated under light condition at 25ºC. The number of shoots produced from the callus was counted after 3-4 weeks culture.

Solvent extract: Air-dried powdered leaves and callus were cold macerated with methanol for 3 d with occasional stirring. The extract was then filtered through Whatmann filter paper (No.1) and the solvent was removed at low temperature (40-50ºC) under reduced pressure in a rotary evaporator.

DPPH radical scavenging activity: Free radical scavenging activity by DPPH was determined according to Vinayagam and Sudha (2011). Various concentrations (200, 400, 600, 800 and 1000 µg/mL) of methanol leaf and callus extract was mixed with of 0.1 mM DPPH solution in methanol. The mixture was shaken vigorously and incubated in dark for 30 min at room temperature. Absorbance was read at 517 nm using spectrophotometer. The control was prepared as above without the compound and methanol. Low absorbance indicated high free radical scavenging activity. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the following equation:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test activity}}{\text{Absorbance of control}} \times 100$$

The extract concentration providing 50% inhibition (IC50) was calculated from the values and graphs of percentage scavenging activity against concentration of samples. Ascorbic acid was used as a reference standard.

Qualitative phytochemical analysis: Tests for screening of chemical constituents present in the methanol extract of leaves and callus of *Leucas aspera* were carried out according to Antariksh *et al.* (2010) and Latha *et al.* (2013). Compounds were detected with the respective reagents and chemicals: alkaloids with Mayer's reagent and iodine, flavonoids with alkaline reagent and ammonia, phenols with ferric chloride, steroids with salkowaski reaction, saponins with foam test and glycosides with Keller-killiani and Baljiet test.

Quantitative phytochemical estimation: the content of total phenolic compound was determined by Folin-Ciocalteau method (Woraratphoku *et al*., 2007) with slight modifications. The extract samples (1 mL of different dilutions) were mixed with Folin Ciocalteu reagent (1 mL, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (1 mL, 1 M) was then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (mg/g of dry mass), a common reference compound which was used to prepare the standard curve.

Results and discussion

Direct shoot induction: The nodal explants of *Leucas aspera* were inoculated in MS medium supplemented with auxins and cytokinins for direct shoot and callus induction. It was observed that combination of IAA (0.1 mg/L), BA (0.2 mg/L) and sucrose (30 g/L) induced 25% shoot production when compared to 16.67% growth response in combinations of IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L). Samantaray *et al.* (2013) has reported highest shoot formation in medium containing IAA and BAP in *Vitex trifolia,* a member of Lamiaceae*.* Direct shoot induction was observed in MS medium supplemented with IAA (0.1 mg/L), BA (0.2 mg/L), sucrose (30 g/L) and IAA (0.1 mg/L), BA (0.3 mg/L), sucrose (30 g/L) (Table 1).

Callus induction: Maximum response of callus induction (75%) was observed in *in vitro* generated leaf explants in media containing IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L) (Fig. 1) than from nodal explants (44.44 and 36.84%), when inoculated in MS medium supplemented with NAA (0.1 mg/L), BA (0.2 mg/L) and NAA (0.1 mg/L), BA (0.3 mg/L) respectively with an increase in sucrose concentration (35 g/L) (Table 2).

Shoot multiplication: Maximum shoot multiplication was observed from 25 d old nodal callus in IAA (0.1 mg/L), BA (0.3 mg/L) and sucrose (30 g/L) where maximum of 12 shoots were induced within 34 d after inoculation (Fig. 1 and 2). The results are summarized in Table 3.

Table 1. Effect of plant growth hormones on direct shoot formation.

Explant type	Media conditions (MS media)				Shoot response %	No of shoots induced	Nature of morphogenetic response
	Hormone (mg/L)		Sucrose (g/L)	pH			
	IAA	BA					
Node	0.1	0.2	30	5.7	25	1	Shoot along with callus (0.9 g) was also developed.
Node	0.1	0.3	30	5.61	16.67	2	Shoot was alone induced.

Table 2. Effect of plant growth hormones on callus induction.

Explants type	Media conditions (MS media)				callus response %	Weight of callus (g)	Nature of morphogenetic response
	Hormone (mg/L)		Sucrose (g/L)	pH			
	NAA	BA					
Node	0.1	0.2	35	5.66	44.44	0.85	Proliferation of callus light yellow colour.
Node	0.1	0.3	35	5.62	36.84	0.5	Callus was induced 10 d after Inoculation.
In vitro Generated leaf	IAA	BA	30	5.64	75	0.667	Callus was completely developed 34 d after inoculation.
	0.1	0.3					

Table 3. Effect of plant growth hormones on shoot multiplication from 25 d old nodal callus.

Day of observation after inoculation	Media conditions (MS media)				No of shoots induced
	Hormones (mg/L)		Sucrose (g/L)	pH	
	IAA	BA			
13	0.1	0.3	30	5.6	4
24	0.1	0.3	30	5.6	8
34	0.1	0.3	30	5.6	12

Fig. 1. Callus from *in vitro* generated leaf explant IAA:BA(1:3).

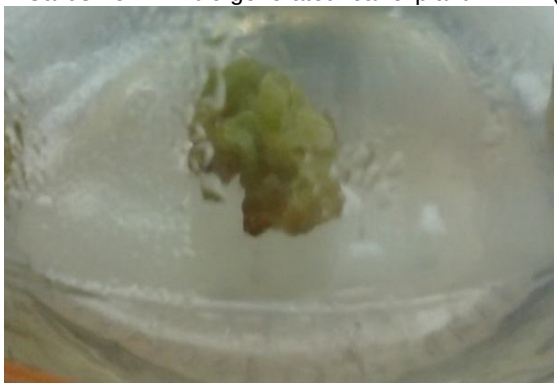


Fig. 2. Shoot multiplication.

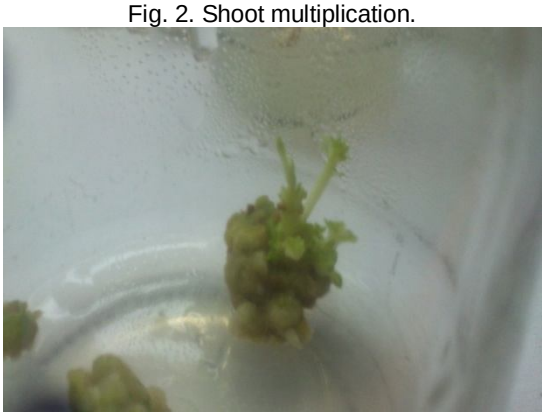
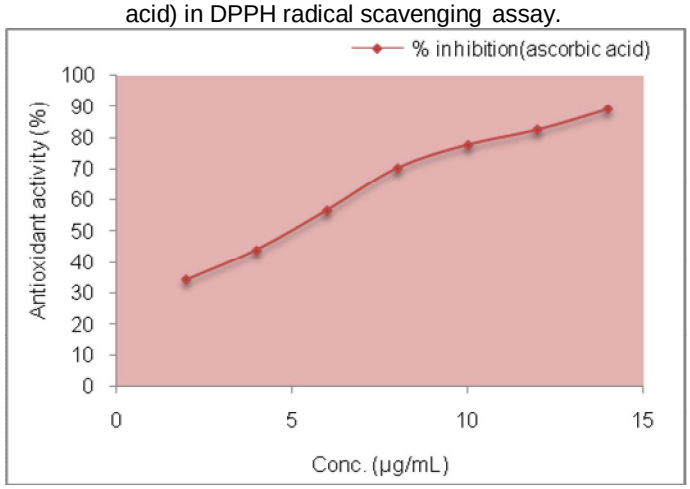


Fig. 3. Effect of different concentrations of standard (ascorbic acid) in DPPH radical scavenging assay.



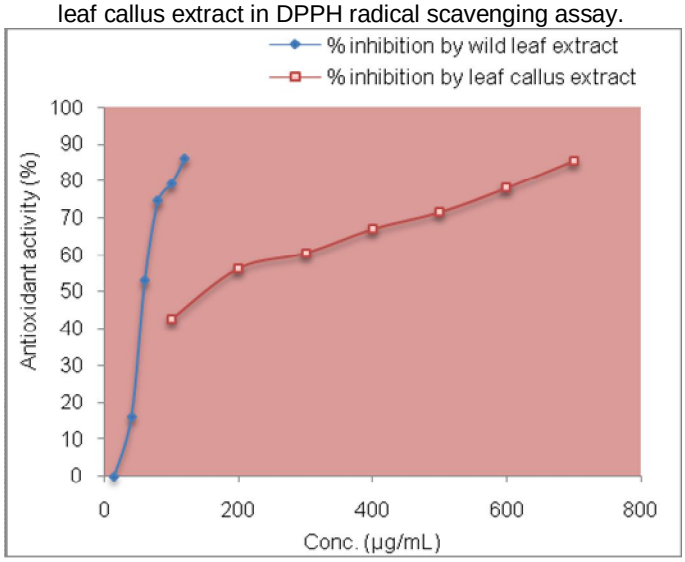
DPPH radical scavenging activity: The response ratio of radical scavenging activity of ascorbic acid, wild leaf extract and leaf callus extract has been compared. Wild leaf and leaf callus methanol extract exhibited high percentage of inhibition (86.06 and 85.49%) at higher concentrations (120 and 700 µg/ mL) with IC50 value of 57 and 178 µg/mL respectively, when compared to ascorbic acid with 89% inhibition at 14 µg/mL and IC50 value of 5 µg/mL (Fig. 3 and 4).

Table 4. Phytochemical analysis of wild leaf and leaf callus extract.

Phytochemical compounds	Test performed	Inference	Wild leaf extract	Callus leaf extract
Alkaloids	Mayer's test	Formation of creamy white precipitate	-	-
	Iodine test	Blue colour formation	-	-
Flavonoids	Alkaline reagent test	Intense yellow colour formation	+++	++
	Ammonia test	Strips turn yellow	++	++
Phenols	Ferric chloride test	Dark green colour formation	+++	+++
Steroids	Salkowaski reaction test	Formation of red colour in $CHCl_3$ layer	+++	++
Saponins	Foam test	Honey comb like froth formation	+++	++
Glycosides	Keller-killiani test	Reddish-brown colour ring formation	+++	+++
	Baljiet test	Stable orange colour formation	+++	+++

'-' = no reaction, '+++' = intensity of reaction is high, '++ ' = intensity of reaction is moderate, '+' = intensity of reaction is too low.

Fig. 4. Effect of different concentrations of wild leaf extract and leaf callus extract in DPPH radical scavenging assay.



The scavenging effect increased with increase in concentration of the extract. Methanol extract of the whole plant of *Leucas mollissima* showed insignificant free radical and superoxide anion scavenging activity (Shyur *et al*., 2005). However, significant activity was found in the ethanolic extract of *Leucas aspera* root (IC50 of 7.5 µg/mL) (Rahman *et al.,* 2007). Antioxidant activity of *Leucas indica* has also been reported by Vinayagam and Sudha (2011).

Phytochemical analysis: Preliminary phytochemical screening of methanolic leaf extract of *Leucas aspera* revealed significant amounts of flavonoids, phenols, steroids, saponins and glycosides in higher concentrations, whereas in the leaf callus extract, phenols and glycosides were significantly predominant and the rest were moderately present (Table 4).

Total phenolic content: Phenolics, a diverse group of compounds, demand much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelators. In phytochemical screening of methanolic extract of *Leucas aspera,* the predominant compounds present were phenolics.

The total phenolic content of the wild leaf methanol extract was high (145 mg/g) than in leaf callus extract (25 mg/g) and was compared with gallic acid, a reference standard (Fig. 5, Table 5).

Fig. 5. Callibration curve of gallic acid.

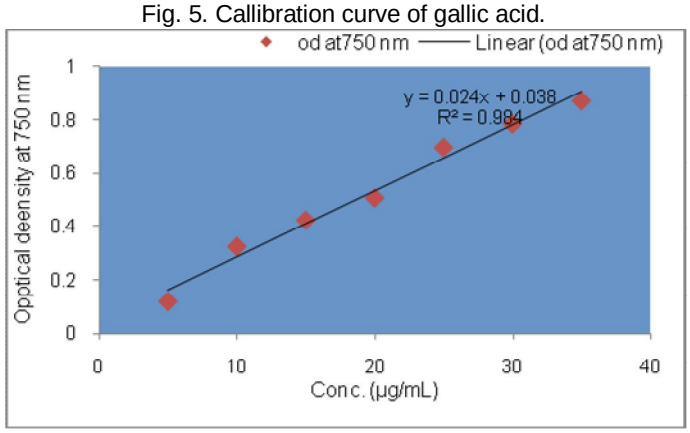


Table 5. Total phenolic content of plant extracts.

Extract	Total phenol content (mg/g) of plant extract in GAE.
Wild leaf extract	145
Leaf callus extract	25

Conclusion

Efficient micropropagation of *Leucas aspera* using nodal explants was developed and observed for morphogenetic response in MS medium supplemented with various concentrations of auxins and cytokinins. A comparative study of wild leaf and *in vitro* generated leaf callus methanolic extract revealed antioxidant activity moderately comparable to ascorbic acid and phytochemical analysis detected the presence of flavonoids, phenols, steroids, saponins, glycosides in significant amounts. Total phenolic content of wild leaf and leaf callus methanol extract was estimated, compared and expressed as gallic acid equivalent. This study will help in bioactivity guided isolation and purification of lead antioxidant molecules from the wild leaf and *in vitro* generated leaf callus of *Leucas aspera* which may have great potential in pharmacology.

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