Antioxidant Activity of Callus Culture of *Vigna unguiculata* (L.) Walp.

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Abstract: Tissue culture of *Vigna unguiculata* (L.) Walp. was done on MS medium supplemented with various concentrations of auxins and cytokinins. Maximum callusing was observed in basal MS medium containing 5 ppm Kn and 1 ppm NAA. Methanolic extract of callus was successively partitioned with n-hexane, chloroform and ethyl acetate. Maximum phenolic content and antioxidant activity was observed in ethyl acetate fraction and minimum activity in n-hexane. The results provide callus culture as an alternative source of natural antioxidant.

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1. Introduction

Free radicals are produced in our body as a by product of many reactions. However, improper life style, ill eating habit and stress enhance the production of free radicals. These free radicals initiate a chain reaction which makes other molecules unstable thereby adversely affecting the metabolism. Human system is equipped to combat these free radical through antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase and prevent oxidative stress. Nevertheless, external supplementation of antioxidants is necessary. There are synthetic antioxidants available but search for natural antioxidants is the need of the hour as whole world has now realized the significance of alternative and complementary medicines. Plants and plant products form best sources of natural antioxidants and possess various bioactivities (Mathur *et al*, 2007; Bhatia *et al*, 2008). Several plants have been explored for its antioxidative potential (Miguel, 2010).

Overexploitation of plants for therapeutic purpose has led to biodiversity problems. There is need to search for alternative sources for the production of natural antioxidants. Plant tissue culture is an important venture in this regard. There is production of useful compounds under controlled conditions free from environmental constraints. Moreover, production can be more reliable, simpler and rather predictable.

In the present study *Vigna unguiculata* L. (Walp.) was explored for its tissue culture and antioxidant potential. *V. unguiculata* (Family: Fabaceae), commonly called as Cowpea is an annual plant. It is a grain legume which forms an important source of dietary protein in many parts of the world. There are few reports on tissue culture of *Vigna* sps. Micropropagation of cowpea has been successfully attempted (Odutayo *et al*, 2005; Diallo *et al*, 2008). Plant regeneration from cotyledonary node in *Vigna radiata* has been also been reported (Gulati and Jaiwal 1994). There are scanty reports on antioxidant activity of Cowpea (Siddhuraju and Becker, 2006; Nair *et al*, 2007). However, there are probably no reports on antioxidant activity of *in vitro* culture of *V. unguiculata* to the author's knowledge.

2. Materials and Methods

Tissue culture

The seeds of *V. unguiculata* were washed under running water for 20-25 min and then treated with 0.1% $HgCl_2$ (Mercuric chloride) for 6 minutes in laminar flow hood, finally washed with sterile distilled water for 3 times. Hypocotyl was used as the explant from *in vitro* grown seedling. The explants were then inoculated in MS media (Murashige and Skoog 1962) supplemented with various concentrations of auxins viz., 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and NAA (Naphthalene Acetic Acid), and cytokinin Kn (Kinetin) and BAP (Benzylaminopurine). All cultures were maintained at 25±2ºC under light intensity (1200 lux) for photoperiod of 16h light and 70±10 relative humidity. The cultures were maintained for about 6 months by periodic subculturing of 6-8 weeks time interval.

Extraction

Callus was dried in oven at 50°C till constant weight was achieved. It was then powdered and extracted with methanol in shaker at 30°C for 48 h. Methanol was evaporated and redissolved in water to obtain crude extract. This crude extract was then partitioned with hexane (H) and then successively partitioned with chloroform (CF) and ethyl acetate (EA). Every fraction was dried, redissolved in

methanol and filtered for further biochemical analysis.

Total phenolic content

The total phenolics were determined colorimetrically according to the Folin- Ciocalteaeu method (Sharma *et al*, 2009). Briefly, 0.5 mL of water and 0.125 mL of the methanolic extract of various fractions were added to a test tube. Folin-Ciocalteu reagent (0.125 mL), 1.25 mL of the sodium carbonate solution and 3 mL of water was added successively and allowed to stand for 90 minutes. The absorbance was measured at 760nm. Total phenol content was expressed as gallic acid equivalents (GAE) in (mg GAE/g dry weight of sample) dry material. Values are expressed as Mean ± S.D

DPPH free radical scavenging activity

Different fractions (1ml) were mixed with 1ml of 0.3mM. DPPH reagent and allowed to stand at room temperature for 30 minutes in dark. The absorbance was taken at 517nm. Radical scavenging activity was calculated according to the following formula and IC_{50} value was evaluated and expressed as Mean ± S.D.

% DPPH radical scavenging=

$$\frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

FRAP assay

Acetate buffer, 300mmol/l, 10 mmol/l 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mmol/l HCl and 20 mmol/l $FeCl_3$ x $6H_2O$ in distilled water was prepared. 25ml of acetate buffer, 2.5ml TPTZ solution and 2.5ml $FeCl_3$ x $6H_20$ solution was mixed to make working solution. 50µl of sample extract was mixed with 1.5ml of FRAP reagent and monitored up to 5 min at 593nm. Absorbance was compared with calibration curve of aqueous solution of known Fe (II) concentration (µM/l). Values are expressed as Mean ± S.D.

3. Results and discussion

Basal MS medium was used for *in vitro* germination of the seeds of *V. unguiculata*. Germination started after 2-3 days. Hyocotyl was found to be more responsive to initiate callus as compared to other parts of seedling. Initially the cut ends of the explants showed swelling which later turned into callus. The callus was fragile and light brown in colour. Maximum % induction of explant (90%) and callusing was observed in MS + Kn (5ppm) + NAA (1ppm). Similar observations were made, where higher cytokinin concentration induced callus at the basal end of explants (Diallo *et al, 2008***)**. Minimum % induction of explant (70%) and callusing was observed in MS + 2, 4-D (1 ppm). Other combination of hormones showed moderate callusing (Table-1). The media in which maximum amount of callusing was observed were sub-cultured periodically after 6 weeks and maintained further and analysis (Figure 1). Callus in Cowpea was observed in MS medium supplemented with 1 µM NAA and BAP (Odutayo *et al*, 2005). Amitha and Reddy (1996) reported smooth nodular callus from zygotic embryos.

The dried and powered callus was extracted in methanol and subjected to fractionation. Percentage yield of fractions in hexane, chloroform and ethyl acetate was 4.2 ± 1.2, 5.6 ± 0.9 and 9.8 ± 1.6, respectively. The EA fraction showed maximum (Figure 2) phenolic content (60 ± 0.64 mg/g GAE) and minimum content in H fraction (6 ± 0.72 mg/g GAE). This suggests that phenols are mostly soluble in ethyl acetate fraction. EA fraction possessed highest DPPH radical scavenging activity as it had minimum IC_{50} value (80 ± 1.2 µg/ml) whereas in H fraction IC_{50} value was maximum (640 ± 1.9 µg/ml). The FRAP value exhibited similar results like DPPH (Figure 2). The maximum ferric reduction activity was observed in EA fraction (812 ± 2.1 µM/l) and minimum in H fraction (245 ± 1.8 µM/l). Since EA fraction had maximum phenolic content this might be the reason for highest scavenging activity. Phenols have –OH group which donate H to form DPPH-H leading to higher antioxidant potential. Moreover phenolic compounds are known to inhibit the oxidation activity of free radicals and enhance activity of antioxidative enzymes. Phenolic compounds include flavonoids which have diverse therapeutic potential mainly due to their antioxidant potential (Pietta, 2000; Vats, 2009). Dietary flavonoids are recommended by many dieticians for healthy living.

Table1: Effect of different concentrations of phytohormones on % induction and callusing of *V. unguiculata*

2,4D	Kn	NAA	BAP	Callusing	% induction
1ppm	-	-	-	+ +	70±1.0
1ppm	0.1ppm	-	-	+ + +	81±1.2
-	5ppm	1ppm	-	+ + + +	90±0.08
0.1ppm	-	-	1ppm	+ + +	85±1.4

Values are Mean ± S.D (++++ = very good; +++ = good; ++ = moderate)

Figure 1: Callus induction in MS + Kn (5ppm) + NAA (1ppm)

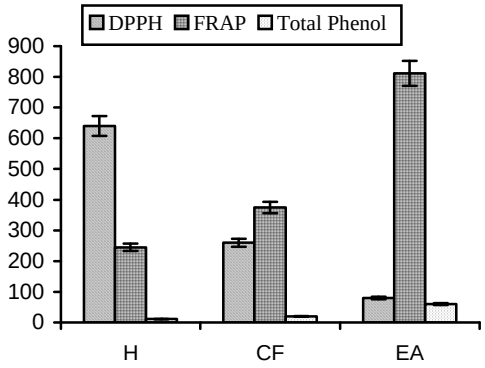

Figure. 2: DPPH (µg/ml), FRAP (µM/l) and total phenol (mg/g GAE) values of fractions. H- hexane, CF- Chloroform, EA-Ethyl Acetate.

Conclusion

The results confirm the antioxidant potential of *V. unguiculata* and provide callus culture as an alternative source of natural antioxidant.

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