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EFFECTS OF SECONDARY METABOLITE PRODUCTION AND ANTIOXIDANT ENZYME RESPONSE OF SELECTED MEDICINAL PLANTS LEAF CELL CULTURES

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ABSTRACT

Leaf explants of three medicinally important plants viz., *Convolvulus microphyllus* Seib., *Peltophorum pterocarpum* (DC.) Baker ex. K. Heyne and *Pergularia daemia* (Forsk.) Chiov. were cultured in Murashige and Skoog's medium with different concentrations of auxins and cytokinins. Callus of each of the plant species was dried, extracted in alcohol and estimated for total levels of phenolics and flavonoids. Antioxidative enzymes such as catalase (CAT) and ascorbate peroxidase (APX), which play important role in plant defense systems, also calculated. The data revealed that with age of the callus, total levels of phenolics and flavonoids increased. Among the test extracts, 8 weeks-old callus of *C. microphyllus* demonstrated higher levels of total phenolics (20.36 ± 6.67 mg GAE g⁻¹ dw), while flavonoids were higher in 8 weeks-old callus of *P. daemia* (23.80 ± 0.05 mg QE g⁻¹ dw). However, ascorbate peroxidase and catalase activities decreased with the age of the callus levels of phenolics and flavonoids were found to be higher. The results indicate that, with the age of cultures generated stress leading to the production of secondary metabolites. It is known that, production of phenolics and flavonoids suppresses CAT and APX activities, critical factor for the damage of oxidative stress.

Keywords: Cell cultures, Phenolics, Flavonoids, Antioxidants, cardiovascular disease, *Peltophorum pterocarpum*, *Pergularia daemia*.

Abbreviations

APX	Ascorbate peroxidase	BA	6-Benzylaminopurine	CAT	Catalase
2,4-D	2,4-dichlorophenoxyacetic acid	GAE	Gallic acid equivalent	Kn	Kinetin
NAA	Naphthalene acetic acid	QE	Quercetin equivalent		

Introduction

Neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, Creutzfeldt–Jacob disease and meningoencephalitis (Greco *et al.*, 2000). Other diseases include highly disabling vascular pathologies like cardiovascular disease and cardiac failure (Jha *et al.*, 1995), alcohol-induced liver disease (Arteel, 2003) and ulcerative colitis (Ramakrishna *et al.*, 1997), and cancer caused by a complex of different causes, of which (reactive nitrogen species) free radical is a component. In plants, reactive oxygen species (ROS) can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles (Stewart and Bewley, 1980; Karabal *et al.*, 2003). Peroxidation of plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular cell damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplast (Scandalios, 1993)

Enzymatic antioxidant defenses such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), ascorbate peroxidase (APX) etc. and non-enzymatic antioxidants as ascorbic acid (Vitamin C), α -tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids act by one or more of the mechanisms like reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen (Stanner *et al.*, 2004).

Convolvulus microphyllus Seib. belong to (Fam. Convolvulaceae), is a prostrate, sub-erect, spreading, hairy, perennial herb. Scopoletin, kaempferol-3-glucoside and 3,4 dihydroxycinnamic acid (Singh and Bhandari, 2000) has been reported from this plant species. Upadhyay (1986) studied the therapeutic role of this Ayurvedic herb, in mental disorders and classified as brain tonic with antidiabetic (Alam *et al.*, 1990), antianxiety (Dandiya, 1990), tranquilising (Handa, 1994) and antiulcer (Sairam *et al.*, 2001) hypolipidaemic activities (Chaturvedi *et al.*, 1995).

Peltophorum pterocarpum (DC.) Baker ex. K. Heyne (Fam. Caesalpinaceae) is a deciduous tree growing up to 25-25 m. The bark of tree is used in dysentery, for gargles, tooth powders and externally in eye diseases, muscular pains and sores as lotions (Deshaprabhu, 1966). From its flowers a crystalline compound peltophorin which was later called as "berginin", along with β -sitosterol, lupeol and a flavonoid glycoside naringenin-7-glucoside (Joshi and Kamat, 1969; Rahman *et al.*, 1969; Rao 1965; Varshney and Dubey, 1969) have been isolated. Callus induction by cotyledon, nodal, leaf segments has been reported (Uddin *et al.*, 2005).

Pergularia daemia (Forsk.) Chiov. (Fam. Asclepiadaceae) has traditionally been used as an anthelmintic, laxative, antipyretic, expectorant and also to treat infantile diarrhea (Warrier *et al.*, 1995; Kirtikar and Basu, 1999; Nadkarni, 2002). From its aerial parts various pharmacological activities viz., hepatoprotection (Sureshkumar and Mishra, 2006, 2008), antifertility (Golam Sadik *et al.*, 2001), antidiabetic (Wahi *et al.*, 2002), analgesic, antipyretic and anti-inflammatory (Jain *et al.*, 1998) has been reported. *In vitro* plant propagation was carried out using shoot tip, nodal segment and cotyledonary nodes (Kiranmai *et al.*, 2008)

However, there is no report on growth, secondary metabolites production and antioxidant enzyme activities of cell cultures of these selected plants species. Therefore, the objective of the study was to gain new insights of secondary metabolites production in cell cultures of three different plant species and their role in regulating antioxidant enzyme activities, acting as effective free radical scavengers.

Material and Methods

Plant material

Young leaf explants (from 2 months-old green house grown plants) were pre-sterilised with 70% ethanol for two min and washed with sterile distilled water. Each of the explant was surface-sterilized with 0.1% (v/v) HgCl_2 , subsequently washed thoroughly (4-5 times) with sterile distilled water inside the laminar flow cabinet, until the trace of HgCl_2 was removed. Finally, the leaf explants were inoculated into culture flasks containing 20 ml Murashige and Skoog (MS) medium.

As the plant belongs to diverse families different combinations of growth hormones were used for the callus induction. Using previously described hormone concentrations, *C. microphyllus* on 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 mg l⁻¹); *P. pterocarpum* on benzene-6-aminopurine (BAP; 2 mg l⁻¹), naphthalene acetic acid (NAA; 0.5 mg l⁻¹) and *P. daemia* were inoculated on MS supplemented with 2,4-D (2 mg l⁻¹) and kinetin (0.5 mg l⁻¹).

Subsequently these callus were subculture after 6 weeks of inoculation. Callus at different growth stages (2, 4, 6 and 8 weeks-old) was harvested, estimated for their growth indices (GI= Final weight of the callus-initial weight of the callus/Initial weight of the callus).

Extract Preparation

For total phytochemical estimation 20 g of dried callus of each age group was powdered and extracted in ethanol (3 x 18 hr). Resultant extracts were filtered, concentrated under reduced pressure and stored at 4°C for further study.

Total phenolics content

Total phenolics were measured by following the method of Wu *et al.*, (2004). To 1 ml of test extract was added to 2.5 ml of deionized water and 0.1 ml (2N) Folin-Ciocalteu reagent, allowed to stand for 6 min. Later, 0.5 ml of 20% sodium carbonate solution was added, incubated (30 min) and absorbance was taken at 750 nm using UV-Vis spectrophotometer. Optical density was compared with standard regression curve of standard gallic acid and expressed as mg gallic acid equivalents (GAE g⁻¹ dry weight).

Total flavonoids content

Total flavonoids content was measured by the aluminum chloride colorimetric assay (Zhishen *et al.*, 1999). 1 ml of test extract was added to 4 ml of distilled water, 0.3 ml 5% NaNO_2 , 0.3 ml 10% AlCl_3 (after 5 min) and 2 ml of 1 M NaOH (after 1 min) in sequence. Total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against the prepared blank reagent at 510 nm. Total flavanoids levels were expressed as mg quercetin equivalents (mg QE g⁻¹ dry weight).

Compound isolation

In *C. microphyllus*, alcoholic extract was used for the detection of the phenolic compounds. Extracts were spotted on Silica gel G plates using ethyl acetate-toluene-acetic acid (5:4:2) solvent system. The developed plates were air-dried and sprayed with freshly prepared vanillin sulfuric acid reagent, heated to 100°C for 10-15 min until dark blue color developed and their R_f values were calculated.

In *P. pterocarpum*, is known for presence of flavonoid compounds, ethanol extract was applied on TLC plates and developed using ethyl acetate-methanol, (4:1) as solvent system. Each of the developed chromatograms was examined under UV light and in presence of I₂ vapors. Later, plates were sprayed with ceric ammonium sulphate and heated at 100°C for 10 min.

P. daemia cell cultures were purple pigmented showing the presence of anthocyanins; cells were harvested, extracted in 1% HCl-MeOH and concentrated. The dried extract was applied on silica gel TLC plates using butanol: acetic acid: water (4:1:5) solvent system. Positive spots for anthocyanins appeared purple-blue against blue background under UV. Later, plates were sprayed with *p*-anisaldehyde reagent.

For the isolation of compounds PTLC, using etanolic extracts of whole plant along with their cell cultures were applied on activated silica gel G plates, developed on selected solvent system and visualized under UV light. The fluorescent spots were marked, scrapped, eluted in methanol dried and crystallized using benzene methanol mixture. Later, these were subjected to m.p. and IR studies.

Quantitative analysis

The total levels of phenolic acids were determined using previously established methods. For preparative TLC 1 g of ethanolic callus extract of each age group was applied on plates and developed in suitable solvent (ethyl acetate-toluene-acetic acid 5:4:2 for *C. microphyllus*; ethyl acetate-methanol 4:1 for *P. pterocarpum* and butanol: acetic acid: water 4:1:5 for *P. daemia*). These plates were air-dried and visualized in I₂ vapors. Spots coinciding to the standards

were marked, collected in separate test tubes, eluted with methanol, filtered and dried.

In *C. microphyllus* and *P. pterocarpum*, 100 µl of the concentrated isolates was mixed in 2.5 ml of deionized water, followed by the addition of 0.1 ml (2N) Folin-Ciocalteu reagent and sodium carbonate solution (0.5 ml of 20%). OD was taken after 30 min at 760 nm using UV-Vis spectrophotometer. Similarly, standard curves of gallic acid and bergenin were prepared using 1000-62.5 µg concentration and isolated compounds were computed from the respective standard regression curve (µg/g dw).

Total cyanidin-3 glucoside concentration in the extract was measured using extinction coefficient ($E_{1\%}^{1\text{cm}} = 98.2$ at 535 nm) for red onion anthocyanin extract (Fossen *et al.*, 2003; Shariff *et al.*, 2010). Fresh callus was extracted in 0.1% HCl-methanol for 15 min on magnetic stirrer (40 rpm at room temperature). Supernatant was centrifuged (10,000 g) for 5 min and the absorbance of the supernatant was measured at 535 nm. Similarly, standard curve of cyanidin-3-glucoside was prepared using red onion (0.25-3 g/10 ml concentrations) and anthocyanin content (mg/g fresh weight) was calculated.

Sample collection for enzyme assay

Fresh calli were harvested and washed immediately with double distilled water. Callus samples (1 g) were homogenized in 10 ml ice-cold 0.1M phosphate buffer (pH 7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuged tubes and centrifuged at 4°C in refrigerated centrifuge for 15 min at 15000×g and the supernatants were used for assay (Esfandiari *et al.*, 2007).

Different dilutions of bovine serum albumin solutions (BSA) were prepared by mixing 0.05-1 mg ml⁻¹ concentration in water. The final volume in each of the test tubes was made up to 5 ml. From each of the test tube, 2 ml of alkaline copper sulphate reagent was added. The solution was mixed well and incubated at room temperature for 10 min. Later, 0.2 ml of Folin-Ciocalteu solution was added to each tube and incubated for 30 min using a colorimeter where the optical density (OD) was measured at 660 nm against blank.

Assay of enzymes

Ascorbate peroxidase assay

Ascorbate peroxidase activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada (1984). The reaction mixture consisted of 0.01 ml of enzyme extract (100, 200, 300, 400 and 500 µl concentration), 0.01 ml of 0.5 mM ascorbic acid, 0.01 ml of 30% H₂O₂ and 2.97 ml of sodium phosphate buffer (pH 7.2). Rate of ascorbate oxidation was measured at 290 nm.

Catalase assay

Catalase activity was assayed as described by Chance and Machly (1995). Enzyme extract (100, 200, 300, 400 and 500 µl) was added to 3 ml of 30% H₂O₂ in potassium phosphate buffer (0.4 ml of 30% H₂O₂ to 100 ml of phosphate buffer, pH 7) and breakdown of H₂O₂ was measured at 240 nm in spectrophotometer. An equivalent amount of buffer containing H₂O₂ was used as reference.

Enzyme activity was computed by calculating the amount of H₂O₂ decomposed. The soluble protein content was expressed in terms of units (µmol⁻¹) per mg of protein. A protein standard curve was prepared according to the method of Lowry (1976) using BSA as a standard.

For all the experiments five replicates were used. Data were subjected to a one-way analysis of variance (ANOVA) and calculated using SSPS software (Windows, 2010).

Results and Discussion

Cell cultures were obtained after 6 weeks of inoculation. Growth indices (GI) were documented in Table 1 where *P. daemia*, cell cultures demonstrated maximum GI (6.68 on 8 weeks-old age). On the basis of m.p. and ir analysis gallic acid (Rf 0.96, m.p. 195-198°C) from *C. microphyllus*, bergenin (Rf 0.50, m.p. 146-147°C) from *P. pterocarpum* and cyanidin-3-glucoside were isolated and confirmed from *P. daemia* (pigmented callus showed absorption maxima (λ_{max}) at 535 nm, overlap the characteristic peak absorbance of cyanidin-3-glucoside).

On phytochemical estimation of *C. microphyllus* total phenolics and flavonoids were reported higher in 8 weeks-old callus (20.36 ± 6.67 mg GAE g⁻¹ dw and 8.62 ± 0.03 mg QE g⁻¹ dw; Fig. 1), followed by 6 weeks-old callus (19.00 ± 12.26 mg GAE g⁻¹ dw and 7.73 ± 0.64 mg QE g⁻¹ dw extract for total phenolics and total flavonoids level respectively). On quantification gallic acid estimated higher in 8 weeks-old callus extract i.e. 502.66 ± 1.76 µg ml⁻¹

In *P. pterocarpum*, total phenolics and flavonoids were higher in 8 weeks-old callus (12.40 ± 1.44 mg GAE g⁻¹ dw and 22.5 ± 0.07 mg QE g⁻¹ dw respectively) followed by 6 weeks-old callus (11.80 ± 1.40 mg GAE g⁻¹ dw and 19.50 ± 0.08 mg QE g⁻¹ dw respectively; Fig. 2) as bergenin levels were also higher in 8 weeks-old callus followed by 6 weeks-old callus (616.85 ± 0.07 and 313.01 ± 0.05 µg ml⁻¹ respectively).

In *P. daemia* higher levels of phenolics were reported in 4 weeks-old callus (12.86 ± 0.61 mg GAE g⁻¹ dw) whereas flavonoids in 8 weeks-old callus 23.80 ± 0.05 mg QE g⁻¹ dw, higher levels of Cyanidin-3 glycoside documented in 4 weeks-old callus (314.52 ± 0.05 µg ml⁻¹)

In the present experiment, all the experimental plants demonstrated appreciable levels of phenolic and flavonoid levels. In general, total levels of phenolics and flavonoids increases with the age of callus, but higher levels of anthocyanins were observed in 4 weeks-old callus.

Phenolics and flavonoids compounds are considered to be secondary metabolites that are synthesized in plants through the phenylpropanoid pathway and function as a defense mechanism that reacts to various biotic and abiotic stress conditions (Dixon and Paiva, 1995). In addition to this, oxidative stress also plays important role in the production of secondary metabolites in plants. A cellular content of H₂O₂ reflects cellular damage resulting from oxidative stress (Dhindsa *et al.*, 1981; Ozden *et al.*, 2009). It has been demonstrated that the plants activate their defense systems by altering antioxidant molecule levels and including antioxidative enzymes to combat oxidative stress (Noctor and Foyer, 1998). Antioxidants compounds donate a hydrogen atom rapidly to a lipid radical, forming a new radical, which is more stable (Pryor 1994; Esterbauer 1995) and stop chain

reactions. Some antioxidants react with the initiating radicals, or reduce the oxygen level. In present experiment with the age of callus total phenolic and flavonoids levels increased reducing the levels of free H₂O₂.

A significant decrease in antioxidant enzyme activities was observed in the callus with the increase of age. In *C. microphyllus* maximum APX levels were recorded in 2 weeks-old callus and minimum in 8 weeks-old (1.15 ± 0.16 and 0.19 ± 1.41 units g⁻¹ protein at 100 µl and 500 µl concentration respectively; Table 2; Fig. 3). In CAT activity, lower levels were estimated at 8 weeks-old age (0.34 ± 9.16 units g⁻¹ protein at 500 µl concentration; Table 3; Fig. 4).

In *P. pterocarpum*, continuous decline in antioxidant enzyme activity was observed with the age of the callus. APX activity on 100 µl concentration was 2.82 ± 0.29 units g⁻¹ proteins with the lower profile of 0.92 ± 0.14 units g⁻¹ proteins on 8 weeks-old callus. Subsequently, on 500 µl concentration antioxidant capacity was 0.94 ± 0.12 and 0.39 ± 3.05 units g⁻¹ protein on 2 and 8 weeks-old callus respectively. CAT levels also followed similar pattern where 2 weeks-old age demonstrated 3.94 ± 3.80 units g⁻¹ proteins on 100 µl concentration of enzyme extract. In higher callus age, lower proteins profile was estimated (0.84 ± 5.03 , 0.40 ± 0.20 units g⁻¹ proteins on 100 and 500 µl concentration).

In *P. daemia* APX activity, 2 weeks-old callus the levels were higher (1.01 ± 0.99 units g⁻¹ proteins on 100 µl concentration), and levels in 8 weeks-old age callus (500 µl concentration 0.07 ± 4.64 units g⁻¹ proteins). In CAT activity, similar results were obtained with 2.14 ± 0.44 units g⁻¹ proteins in 2 weeks-callus at 100 µl concentration and progressively declined towards 500 µl concentration (1.01 ± 2.45 units g⁻¹ protein), followed by 4 weeks-old callus 1.24 ± 0.99 units g⁻¹ protein at 100 µl concentration.

H₂O₂ is known to be the signal that induces antioxidant defense systems in plants in response to biotic and abiotic stresses (Neil *et al.*, 2002a, b) and its accumulation induces the production of polyphenolic compounds. Antioxidant defense enzymes such as APX, CAT are systems that are designed to minimize the concentrations of ROS. It is known

that H₂O₂ is eliminated by CAT (Mittler, 2002) as CAT dismutates H₂O₂ into water. Increase CAT activity is responsible for the removal of excess H₂O₂.

In early growth stages, reactive oxygen species damages membranes increasing protein levels, oxidative stress and enzyme activities as a result produce signals to induce phenols and flavonoids synthesis. Higher activity of ascorbate oxidase and catalase decreased H₂O₂ levels in cells and increased the stability of membranes and CO₂ fixation because several enzymes of the Calvin cycle within chloroplast are extremely sensitive to H₂O₂. However, higher level of H₂O₂ inhibits CO₂ fixation (Yamazaki *et al.*, 2003). These results were in harmony with total phenolics and flavonoids levels previously described. Further, as the levels of total phenolics and flavonoids increases stability of membrane but and reduces ascorbate oxidase and catalase activities.

As evidenced from the results, lower levels indicated higher secondary metabolite production and lesser oxidative damage. Among the tested extracts, *P. daemia* cell cultures demonstrated higher levels of flavonoids and anthocyanins in 8 weeks-old cultures exhibited higher with GI and lower APX and CAT activity. Similarly, *C. microphyllus* cell cultures exhibited appreciable levels of phenolics, gallic acid and reduced APX and CAT activities.

Conclusion

Conclusively, it can be stated that cell cultures of selected plants possess potentialities of producing useful bioactive compounds viz., gallic acid, bergenin and anthocyanin. Also, the production and accumulation of secondary metabolites increase with the age of the callus has been reported further, enhancement of phenolics and flavonoids associated with the peroxide detoxification as a result of physiological process. Activities of APX and CAT were recorded higher in the cell cultures of 2 and 4 weeks, which reduce significantly as the age progresses. It is proved that, among the three plant species, *P. daemia* cell cultures demonstrated better defense against oxidative stresses.

Table 1 : Effect of age of the callus growth, total levels of phenolic and flavonoid contents and on isolated compounds in selected medicinal plants.

Plants used	Age in weeks	GI ⁺	Total phenolics (mg GAEg ⁻¹ dw) ^a	Total flavonoid (mg QE g ⁻¹ dw) ^b	*Isolated compounds (µg ml ⁻¹)
<i>A. C. microphyllus</i>	2	3.20	15.23 ± 4.18	4.90 ± 0.09	444.33 ± 1.20
	4	3.65	17.90 ± 12.59	5.09 ± 0.15	445.66 ± 0.26
	6	3.79	19.00 ± 12.26	7.73 ± 0.64	451.00 ± 1.15
	8	4.35	20.36 ± 6.67	8.62 ± 0.03	502.66 ± 1.76
<i>B. P. pterocarpum</i>	2	1.07	9.73 ± 1.27	10.3 ± 0.07	303.33 ± 0.05
	4	1.22	10.86 ± 0.80	13.5 ± 0.00	310.20 ± 0.10
	6	1.38	11.80 ± 1.40	19.5 ± 0.08	313.01 ± 0.05
	8	1.28	12.40 ± 1.44	22.5 ± 0.08	616.85 ± 0.07
<i>C. P. daemia</i>	2	1.29	10.86 ± 2.13	14.0 ± 0.08	274.33 ± 0.11
	4	2.31	12.86 ± 0.61	17.1 ± 0.10	314.52 ± 0.05
	6	4.38	10.60 ± 1.03	9.50 ± 0.05	74.23 ± 0.02
	8	6.68	10.26 ± 1.21	23.8 ± 0.05	52.69 ± 0.08

Data were statistically analyzed and representing mean ± standard deviation of three replicates per experiment(s).

⁺GI= Final weight of the callus-Initial weight of the callus/Initial weight of the callus.

^aTotal phenolics content in mg gallic acid equivalent g⁻¹ dry weight (GAE g⁻¹ dw);

^bTotal flavonoids content in mg quercetin equivalent/g dry weight (QE g⁻¹ dw)

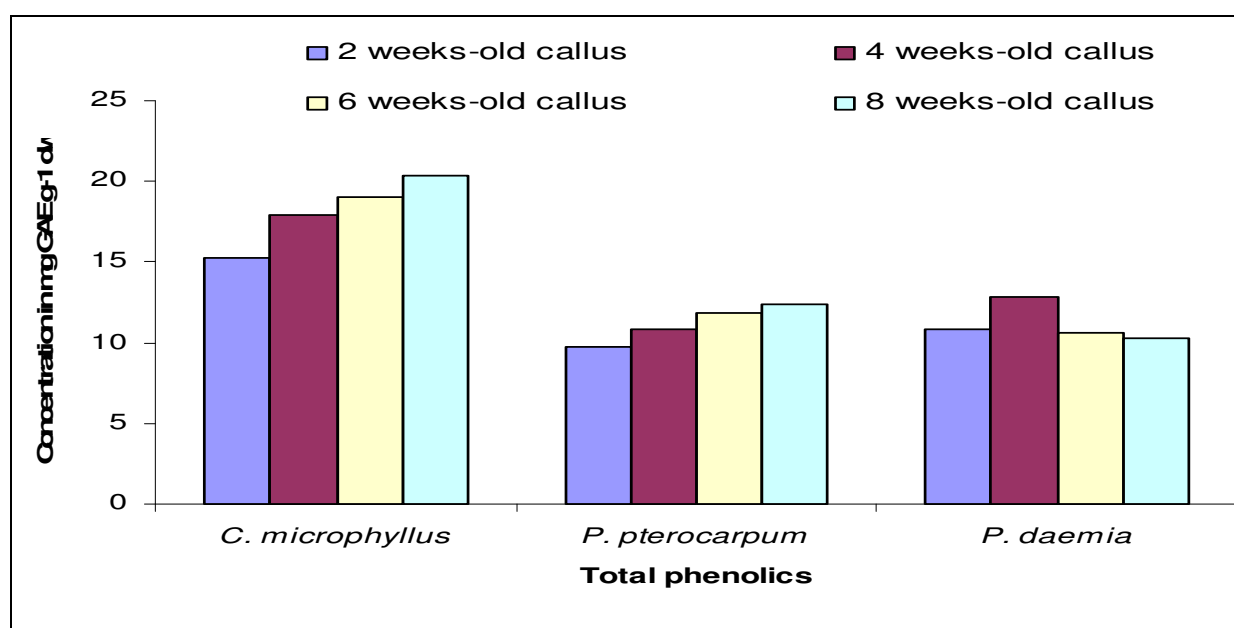
* Dry weight of isolated compounds-gallic acid for *C. microphyllus*, bergenin for *P. pterocarpum* and Cyanidin-3-glucoside in case of *P. daemia*.

Table 2 : Antioxidant activity by Ascorbate oxidase method (in unit g⁻¹ protein)

Age of callus (in weeks)	Concentration of callus enzyme in $\mu\text{l ml}^{-1}$				
	*100 μl	200 μl	300 μl	400 μl	500 μl
A. C. microphyllus					
2	1.15 ± 0.16	0.94 ± 0.20	0.72 ± 0.00	0.58 ± 3.46	0.32 ± 6.96
4	0.98 ± 4.61	0.85 ± 8.08	0.76 ± 8.32	0.55 ± 0.15	0.46 ± 0.00
6	0.87 ± 6.11	0.70 ± 4.16	0.67 ± 4.16	0.67 ± 1.16	0.35 ± 5.71
8	0.54 ± 0.16	0.46 ± 4.00	0.29 ± 6.11	0.21 ± 6.11	0.19 ± 1.41
B. P. pterocarpum					
2	2.82 ± 0.29	2.38 ± 3.40	2.02 ± 0.16	1.51 ± 9.71	0.94 ± 0.12
4	2.32 ± 0.10	2.05 ± 0.12	1.58 ± 4.16	1.71 ± 3.21	0.87 ± 0.14
6	0.96 ± 0.00	0.82 ± 0.72	0.65 ± 8.32	0.52 ± 2.30	0.34 ± 3.46
8	0.92 ± 0.14	0.77 ± 6.11	0.66 ± 4.61	0.50 ± 5.29	0.39 ± 3.05
C. P. daemia					
2	1.01 ± 0.99	0.95 ± 1.24	0.90 ± 1.33	0.87 ± 1.44	0.74 ± 3.97
4	0.55 ± 1.15	0.45 ± 7.52	0.45 ± 0.20	0.37 ± 0.23	0.27 ± 3.05
6	0.33 ± 0.11	0.34 ± 0.00	0.25 ± 4.16	0.21 ± 2.30	0.14 ± 5.29
8	0.27 ± 5.77	0.32 ± 5.29	0.20 ± 1.15	0.12 ± 0.00	0.07 ± 4.64

Table 3 : Antioxidant activity by Catalase method (in unit g⁻¹ protein).

Age of callus (in weeks)	Concentration of callus enzyme in $\mu\text{l ml}^{-1}$				
	*100 μl	200 μl	300 μl	400 μl	500 μl
A. C. microphyllus					
2	2.86 ± 0.19	2.14 ± 9.23	2.01 ± 6.11	1.43 ± 0.11	0.95 ± 4.61
4	1.98 ± 4.61	1.85 ± 8.08	1.76 ± 8.32	1.25 ± 1.15	1.45 ± 0.00
6	1.38 ± 5.29	1.21 ± 2.28	0.94 ± 4.16	0.85 ± 8.00	0.51 ± 4.16
8	1.36 ± 0.14	1.01 ± 6.11	0.82 ± 7.02	0.61 ± 9.45	0.34 ± 9.16
B. P. pterocarpum					
2	3.94 ± 3.80	3.79 ± 0.37	2.39 ± 3.05	2.07 ± 8.08	1.88 ± 0.11
4	2.30 ± 8.71	2.10 ± 0.12	2.94 ± 0.11	1.75 ± 4.16	1.56 ± 7.20
6	0.96 ± 0.00	0.84 ± 7.57	1.44 ± 4.61	0.17 ± 0.39	0.70 ± 0.17
8	0.84 ± 5.03	0.76 ± 4.00	0.64 ± 0.13	0.46 ± 4.16	0.40 ± 0.20
C. P. daemia					
2	2.14 ± 0.44	2.03 ± 2.59	1.44 ± 0.19	1.09 ± 5.44	1.01 ± 2.45
4	1.24 ± 0.13	1.04 ± 0.11	0.75 ± 5.03	0.57 ± 2.30	0.44 ± 2.30
6	0.60 ± 0.00	0.52 ± 5.77	0.44 ± 2.30	0.34 ± 0.77	0.26 ± 2.30
8	0.47 ± 0.08	0.42 ± 0.26	0.22 ± 1.58	0.20 ± 0.21	0.20 ± 1.15

**Fig. 1 :** Total phenolics levels in selected plant species.

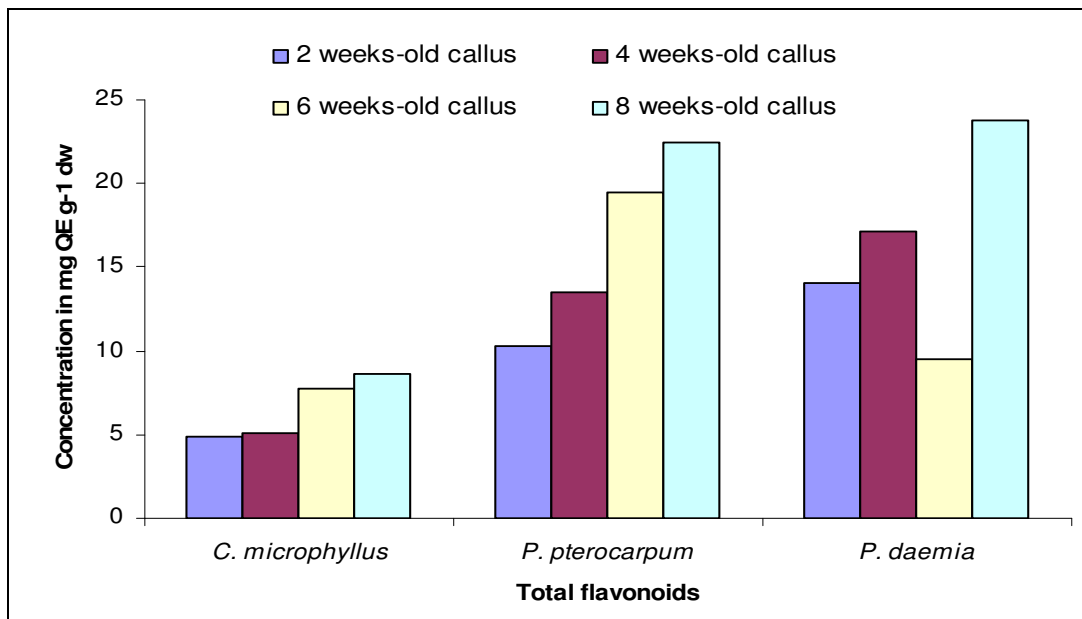


Fig. 2 : Total flavonoids levels in selected plant species.

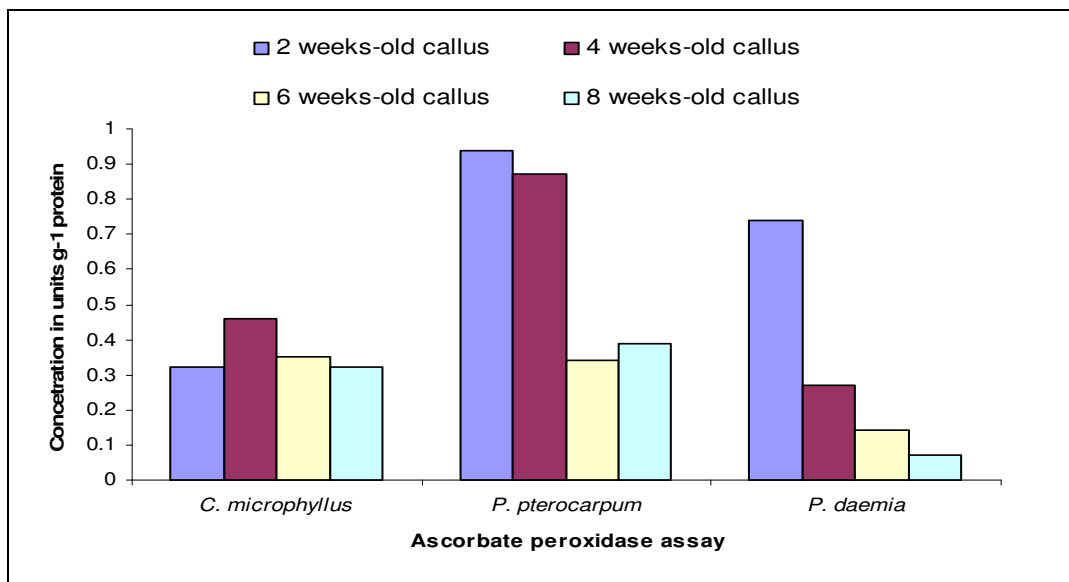


Fig. 3 : Activities of Ascorbate peroxidase in selected plant species with respect to their relative transfer ages.

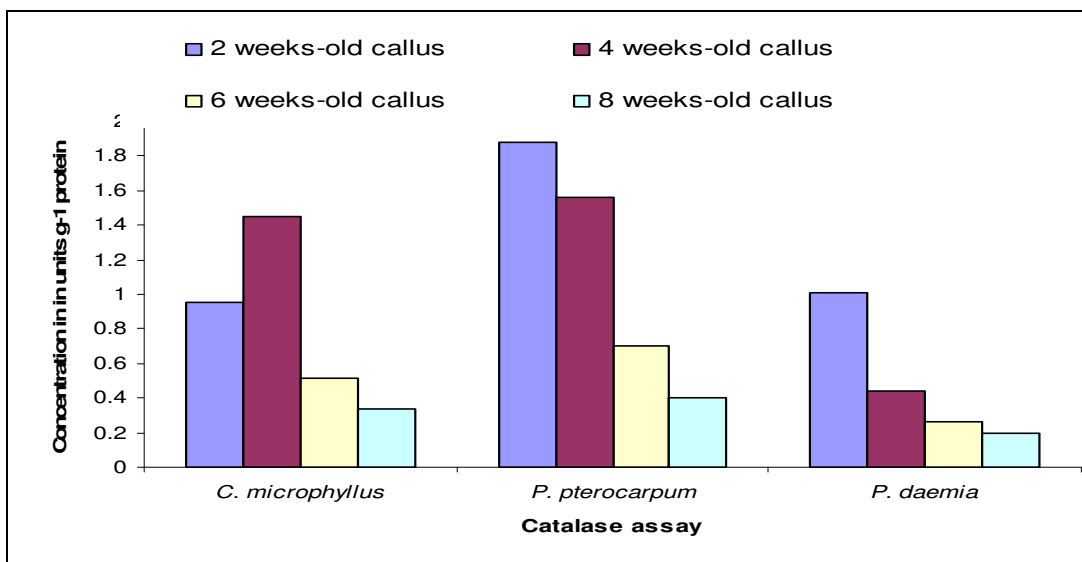


Fig. 4 : Activities of Catalase in selected plant species with respect to their relative transfer ages.

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