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EVALUATION OF *IN VITRO* ANTIOXIDANT PROPERTIES OF CALLUS CULTURES OF AN ENDANGERED MEDICINAL TREE SPECIES, *HILDEGARDIA POPULIFOLIA* (ROXB.) SCHOTT & ENDL.

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ABSTRACT: Protocol for efficient callogenesis in *Hildegardia populifolia* has been developed by using leaf, node, internode and petiole explants. Preliminary phytochemical analysis and quantitative estimation of flavonoids, phenols and tannins were made along with the evaluation of antioxidant properties by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays in *in vitro* callus cultures of the same species. It is an indigenous endangered multipurpose medium sized tree being used for traditional medical practices in Tamil Nadu and Andra Pradesh. Among the explants, the efficient callogenesis was accelerated within 10-19 days in leaf explant on Murashige and Skoog (MS) medium supplemented with 2, 4-D alone at 2 mg/L. Effective calli were produced from node (BAP and IAA at 2 mg/L and 0.4 mg/L respectively in MS medium), internode (2,4-D at 2 mg/L in MS medium) and petiole (2,4-D at 2 mg/L in MS medium) explants. The phytochemical study revealed that phenolics and tannin content were higher in 60 days old leaf callus cultures. Flavonoid content was more in 60 days old internode derived callus culture. Evaluation of antioxidant property established that $DPPH^{\bullet}$ radical scavenging activity was relatively higher in leaf derived callus (86.24 mg/g) and $ABTS^{\bullet+}$ activity was higher in internode derived callus (3077.98 µmol TE/g extract). This study had shown that plant tissue culture offers itself as a highly efficient method for mass propagation and conservation of *H. populifolia* and also helps to identify the unique *in vitro* culture with natural antioxidant potential to get health care benefits.

INTRODUCTION: *Hildegardia populifolia*, an endangered, medium sized tree belongs to the family Sterculiaceae is found in few tropical deciduous forests of Tamil Nadu and Andhra Pradesh in India. Various parts of this species are being prescribed by the local healers of southern India for various ailments.

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It is reported to have antimicrobial activity [1-3], antioxidant activity [4] and antiinflammatory activity [5]. It is used to treat malaria and dog bite also [6]. The fibre extracted from the bark is used for domestic purposes and the leaf extract is prescribed in traditional medical practices of Tamil Nadu and Andhra Pradesh [7].

As per the information given in the Red data Book of Indian plants [8], this narrow endemic species is under great threat due to both extrinsic and intrinsic factors. It is an enigmatic species in that its conservation status has been variously assessed as critically endangered [9].

Therefore, it is the species of conservation priority, need modern propagation strategies for enhancing its population [10] to prevent extinction.

Vegetative propagation by means of cell and tissue culture techniques is a powerful tool for plant conservation and hence reforestation [11, 12]. Exploiting callus cultures for therapeutic uses in some species is another method of conservation of wilds to some extent [13, 14]. However, prior to large scale application, evaluation of therapeutic properties must be important along with the estimation of phytochemicals in the callus of respective species. Hence, in the present study, development of protocol for callus cultures was made to estimate flavonoids, phenols and tannins and to evaluate antioxidant properties to confirm the medicinal properties of callus of the species, *H. populifolia.*

MATERIALS AND METHODS

Collection of plant material: Plants of *H. populifolia* collected from their natural habitat were grown and maintained in the garden of the Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India for the source of explants.

Surface sterilization: Different parts *viz*., nodes, internodes, petioles and young healthy leaves were used as explants. The explants were first thoroughly washed under running tap water for 15-20 minutes and then treated with liquid detergent (Tween – 20) for 5-10 minutes. The explants were dipped in a systemic fungicide solution of 1% Bavastin (Carbendezin 50% WP) for 2 minutes. Later, these explants were washed with double distilled water for 5 minutes. Subsequently, explants were immersed in 70% (v/v) ethanol for 2-3 minutes and washed with sterile glass double distilled water for 2-3 times. Eventually, the explants were treated with aqueous solution of 0.1% (w/v) $HgCl_2$ for 1-2 minutes and rinsed with sterile glass double distilled water for 2-3 times to remove traces of mercuric chloride. The sterilized explants were aseptically inoculated onto MS medium with different combinations and concentrations of hormones.

Culture media and growth condition: MS medium with 3% (w/v) sucrose and pH 5.6-5.8 solidified with 0.8% agar prior to autoclave at 121˚C at 15 lbs pressure for 15-20 minutes was used. Inoculation was done under aseptic conditions in a laminar air flow cabinet, where culture bottles containing 35-40 mL medium kept under UV light for sterilization before inoculation for 30 minutes. All culture bottles were incubated in a controlled environmental in growth chamber at 25˚C±2˚C under 16-18 hrs photoperiod at 3000 lux light intensity (40w white fluorescent tubes, Philips, India) and with 55-60% relative humidity.

Preparation of Extract: Calli derived from the leaf, node, internode and petiole were collected and dried in an oven at 40±1˚C for 5 h. Dried calli were homogenized to a fine powder and stored in airtight bottles. 50g of calli powder was extracted with 250 mL of solvents *viz*., petroleum ether, chloroform and methanol for 24 h separately by using Soxhlet apparatus. The extracts were lyophilized after solvent removal under reduced pressure. This crude callus extract was used for the determination of phytochemicals and antioxidant activity.

Preliminary phytochemical analysis: Phyto-chemical screening of the extract was carried out to identify the secondary metabolites such as alkaloids (Mayer's and Dragendorff's test), flavonoids (Shinoda test), terpenoids (Salkowski test), tannins (Ferric chloride test), saponins (Frothing test), cardiac glycosides (Keller-Killiani test), steroids and triterpenoids (Liebermann-Burchard test) and phenols (Ellagic acid test) according to standard phytochemical methods as described by Trease and Evans [15], Mojab *et al* [16], Sofowora [17] and Harbone [18].

Estimation of Phytochemicals:

Determination of Total Flavonoids: The total flavonoid content of samples was determined by following the modified colorimetric method of Zhishen *et al*[19]. 0.5 mL extract was mixed with 2 mL of distilled water and subsequently with 0.15 mL of 5% $NaNO_2$ solution. After 6 min, 0.15 mL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Immediately distilled water was added to bring the final volume to 5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was recorded at 510nm versus prepared water blank.

Rutin was used as standard for the quantification of total flavonoid. All the values were expressed as milligram of rutin equivalent (RE) per gram of extract.

Determination of Total Phenolics and Tannins: The total phenolic content was determined according to the method described by Siddhuraju and Becker[20]. Aliquots of each extract were taken in test tubes and made up to the volume of 1 mL with distilled water. Then 0.5 mL of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents (GAE).

Using the same extract, the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP). Hundred mg of PVPP was weighed in a 100 × 12 mm test tube and to this 1.0 mL of distilled water and then 1.0 mL of tannin containing phenolic extract were added. The content was vortexed and kept in the test tube at 4ºC for 4 h. Then the sample was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured, as monitored above and expressed as the content of non-tannin phenolics on dry matter. From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Free phenolics (%).

***In vitro* anti-oxidant activity :**

1. **DPPH$^{\bullet}$ radical scavenging activity:** The 2, 2-diphenyl-picryl-1-picryl-hydrazyl radical (DPPH) scavenging activity was measured according to the method of Blois[21]. Methanol extract of callus samples at various concentrations was added separately to each 5mL of 0.1mM methanolic solution of DPPH and allowed to stand for 20min. Absorbance at 517nm using spectrophotometer was measured. Rutin and Quercetin were used as standard. The corresponding blank reading was also taken and DPPH radical scavenging activity was calculated by using the following formula:

DPPH radical scavenging activity (%) = Control OD-Sample OD/Control OD×100

IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical / OH^{+} radical which has been determined by using the software SPSS v.16.

2. **ABTS$^{\bullet+}$ assay:** The total antioxidant activity of the samples was measured by $ABTS^{\cdot+}$ [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation decolorization assay according to the method of Re *et al*[22]. $ABTS^{\bullet+}$ was produced by reacting 7mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30˚C to give an absorbance of 0.700 ± 0.02 at 734 nm. The stock solution of the sample extracts was diluted such that after introduction of 10 µL aliquots into the assay, which have been produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS solution to 10 µL of sample or trolox standards (final concentration 0–15 µM) in ethanol, absorbance was measured at 30˚C exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated from the blank absorbance at 734 nm and then it was plotted as a function of trolox concentration. The unit of total antioxidant activity (TAA) is defined as the concentration of trolox having equivalent antioxidant activity expressed as µmol/g sample extract on dry matter.

Statistical analysis: All tests were conducted in triplicate. Data were reported as means ± standard deviation (SD) and were subjected to one way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's Multiple Range Test ($P<0.05$) using the statistical software Inc., Tulsa, OK, USA.

RESULTS AND DISCUSSION: The response of various explants for callus formation to different plant growth hormones (PGRs) in MS medium *viz*., BAP, 2,4-D, NAA and IAA in various combinations and concentrations was varied widely for the study species, *H. populifolia* (**Tables 1 and 2**). Callus formation was observed to be started at the margins in explants. Further, no callus formation was observed during the culture period in the control (MS medium without growth regulators) in all explants.

In the case of solo application of PGRs, 2, 4-D and BAP in combination with 2, 4-D produced friable, embryonic and pale yellow calli. Greater number of explants *viz*., leaf, internode and petiole responded rapidly in MS medium fortified with 2,4-D at 2.0 mg/L for callus formation (**Fig. 1a, c, d**). Similar kind of higher response of leaf explant to 2,4-D at 0.5 mg/L for callus formation was noted for the medicinal herb, *Ionidium suffruticosum* by Arunkumar and Jayaraj [23]. The time required for callus formation was also varied from 12 to 19 days across the explants used (Table 1).

Callus fresh weight produced by the leaf explant was higher (26.28 g/explant) than that of other explants used. Mohajer *et al* [24] investigated that generally the leaf explants of many plant species produced higher fresh weight of callus than any other explants attempted.

After 15 weeks of inoculation, it was noted that calli were turned to light brownish (**Fig. 1e**) due to the synthesis of phenolic compounds. The transfer of calli to fresh medium was found to be effective in reducing the browning due to phenol exudation [25]. In many species *viz*., *Ephorbia lathyris* [26], *Pisonia alba* [27], *Bambusa ventricosa* [28] and *Tecomella undulate* [29] this method of transfer of calli to fresh medium was used to control the browning of callus. Embryoid initiation was noted during subculturing of callus on MS medium with different combinations and concentrations of PGRs. It is reported that variations in hormonal combinations may be one of the effective methods for embryoids initiation from undifferentiated callus [30-34].

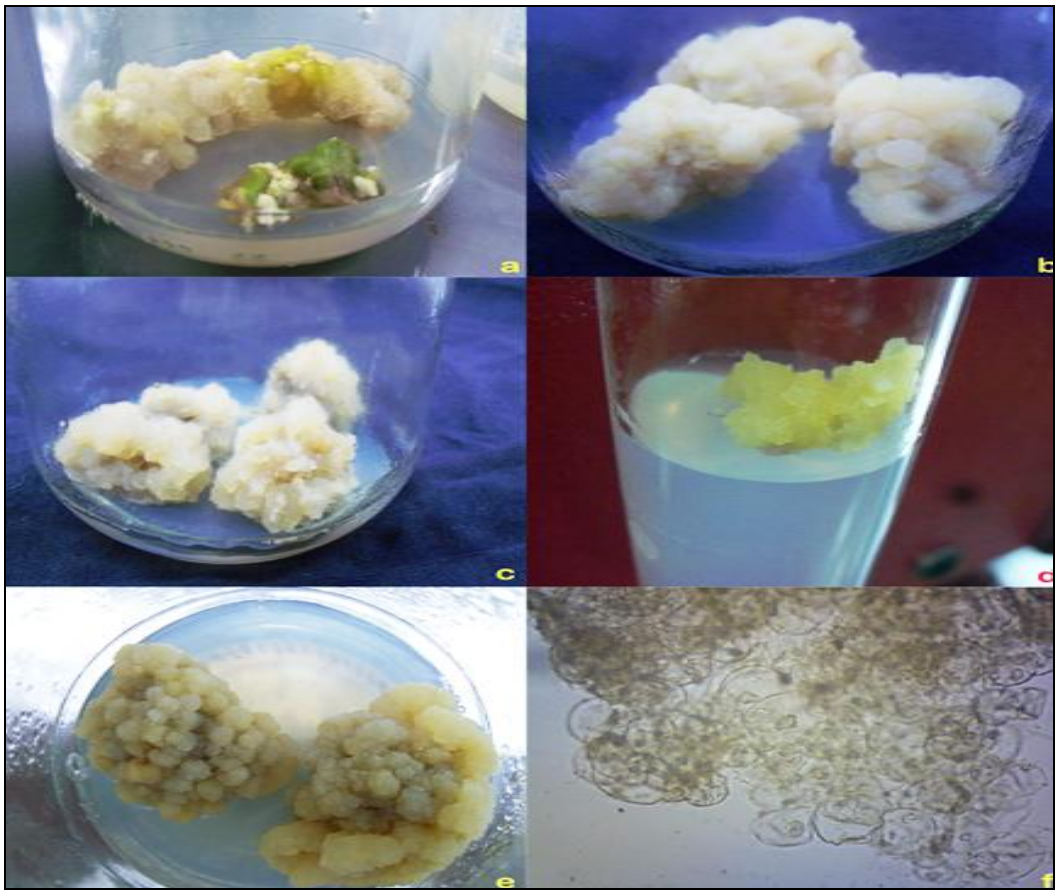

FIG. 1A: CALLUS DEVELOPED FROM LEAF EXPLANTS CULTURED ON TO THE MS MEDIUM FORTIFIED WITH 2,4-D AT 2mg/L, (B) FORMATION OF NODE CALLUS ON MS MEDIUM SUPPLEMENTED WITH BAP AND IAA AT 2.0 AND 4.0 mg/L, (C AND D) INTERNODE AND PETIOLE CALLUS ON MS MEDIUM SUPPLEMENTED WITH 2,4-D AT 2mg/L RESPECTIVELY, (E) BROWNING OF CALLUS AFTER 15 WEEKS, (F) MICROSCOPIC VIEW OF THE CALLUS CELL

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TABLE 1: EFFECT OF DIFFERENT COMBINATIONS AND CONCENTRATIONS OF GROWTH REGULATORS ON PER CENT CALLUS INDUCTION FROM LEAF AND NODE EXPLANTS OF THE SPECIES, *HILDEGARDIA POPULIFOLIA*

Growth regulators (mg/L)				Days required for callus formation after inoculation		Callus formation (%)		Fresh weight of callus (g/explant)	
				Explant		Explant		Explant	
BAP	2, 4-D	NAA	IAA	Leaf	Node	Leaf	Node	Leaf	Node
0.0	0.5	0.0	0.0	18	26	$63.23^{fg} \pm 0.73$	$26.21^{h} \pm 0.27$	$2.85^{m} \pm 0.32$	$3.94^{g} \pm 0.64$
0.0	1.0	0.0	0.0	19	28	$57.36^{hi} \pm 0.24$	$34.62^{fg} \pm 0.32$	$5.93^{kl} \pm 0.37$	$2.39^{fg} \pm 0.92$
0.0	1.5	0.0	0.0	21	23	$73.41^{c} \pm 0.25$	$31.37^{gh} \pm 0.42$	$8.35^{de} \pm 0.61$	$1.10^{hi} \pm 0.35$
0.0	2.0	0.0	0.0	12	27	$89.62^{a} \pm 0.27$	$42.61^{de} \pm 0.55$	$16.28^{a} \pm 0.22$	$4.30^{fg} \pm 0.20$
0.0	2.5	0.0	0.0	20	25	$81.47^{b} \pm 0.14$	$22.05^{ij} \pm 0.23$	$7.43^{b} \pm 0.63$	$3.21^{h} \pm 0.45$
0.0	3.0	0.0	0.0	25	27	$61.82^{g} \pm 0.92$	$40.13^{e} \pm 0.16$	$6.94^{f} \pm 1.65$	$6.01^{c} \pm 1.30$
0.5	0.0	0.0	0.1	28	24	$64.33^{f} \pm 0.04$	$43.34^{d} \pm 0.85$	$10.45^{cd} \pm 0.65$	$3.20^{bc} \pm 1.84$
1.0	0.0	0.0	0.2	23	20	$61.27^{g} \pm 0.15$	$24.41^{i} \pm 0.58$	$5.03^{g} \pm 0.58$	$4.34^{e} \pm 0.69$
1.5	0.0	0.0	0.3	27	18	$65.16^{ef} \pm 0.54$	$31.15^{gh} \pm 0.76$	$11.39^{e} \pm 0.56$	$5.43^{d} \pm 1.82$
2.0	0.0	0.0	0.4	26	15	$67.34^{e} \pm 0.57$	$61.16^{a} \pm 0.74$	$3.75^{lm} \pm 0.20$	$10.00^{b} \pm 2.64$
2.5	0.0	0.0	0.5	21	16	$69.42^{cde} \pm 0.86$	$53.58^{b} \pm 0.95$	$4.93^{l} \pm 0.64$	$12.49^{a} \pm 2.10$
3.0	0.0	0.0	0.6	22	19	$53.41^{ij} \pm 0.75$	$36.24^{f} \pm 0.35$	$5.39^{kl} \pm 0.58$	$7.00^{fg} \pm 0.67$
1.0	0.5	0.0	0.0	20	20	$65.32^{ef} \pm 0.83$	$32.81^{g} \pm 0.58$	$7.33^{ij} \pm 0.37$	$6.51^{ef} \pm 0.59$
1.5	0.5	0.0	0.0	22	23	$64.51^{f} \pm 0.65$	$31.49^{gh} \pm 0.25$	$6.29^{k} \pm 0.19$	$6.15^{g} \pm 0.91$
2.0	0.5	0.0	0.0	19	27	$78.47^{bc} \pm 0.47$	$40.37^{e} \pm 0.75$	$14.92^{c} \pm 0.65$	$5.37^{e} \pm 0.55$
2.5	0.5	0.0	0.0	24	30	$64.42^{f} \pm 0.23$	$42.31^{de} \pm 0.83$	$12.28^{d} \pm 1.94$	$4.52^{c} \pm 0.64$
3.0	0.5	0.0	0.0	27	-	$68.25^{de} \pm 0.24$	$46.35^{c} \pm 0.75$	$11.38^{gh} \pm 0.67$	$3.35^{d} \pm 0.28$
0.0	0.0	0.5	0.0	20	21	$55.43^{i} \pm 0.54$	$32.46^{g} \pm 0.23$	$10.49^{h} \pm 0.25$	$7.21^{f} \pm 0.31$
0.0	0.0	1.0	0.0	28	27	$58.27^{h} \pm 0.67$	$25.39^{hi} \pm 0.15$	$7.83^{f} \pm 0.46$	3.64 ± 0.82
0.0	0.0	1.5	0.0	26	26	$67.34^{e} \pm 0.26$	$22.19^{ij} \pm 0.27$	$8.39^{i} \pm 0.79$	$5.00^{h} \pm 0.37$
0.0	0.0	2.0	0.0	21	29	$71.26^{cd} \pm 0.14$	$31.29^{gh} \pm 0.25$	$4.00^{f} \pm 0.64$	$3.59^{d} \pm 0.18$
0.0	0.0	2.5	0.0	22	25	$59.61^{gh} \pm 0.78$	$37.38^{ef} \pm 0.19$	$10.92^{h} \pm 0.41$	$3.61^{i} \pm 0.30$

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT.

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TABLE 2: EFFECT OF DIFFERENT COMBINATIONS AND CONCENTRATIONS OF GROWTH REGULATORS ON PER CENT CALLUS INDUCTION FROM INTERNODE AND PETIOLE EXPLANTS OF THE SPECIES, *HILDEGARDIA POPULIFOLIA*

Growth regulators (mg/L)				Days required for callus formation after inoculation		Callus formation (%)		Fresh weight of callus	
				Explant		Explant		Explant	
BAP	2, 4-D	NAA	IAA	Internode	Petiole	Internode	Petiole	Internode	Petiole
0.0	0.5	0.0	0.0	19	22	46.28^{c} ±0.32	48.09^{b} ±0.38	5.34^{b}±1.67	2.55^{cd}±0.31
0.0	1.0	0.0	0.0	20	23	37.41^{de} ±0.50	32.61^{h} 0.49	4.62^{de}±0.51	3.37^{ef}±0.15
0.0	1.5	0.0	0.0	22	20	47.34^{bc} ±0.13	46.84^{c} ±0.38	6.45^{c}±0.38	6.60^{b}±1.37
0.0	2.0	0.0	0.0	17	18	50.08^{a} ±0.32	51.51^{a} ±0.48	10.34^{a}±1.56	10.41^{a}±1.64
0.0	2.5	0.0	0.0	21	21	46.36^{c} ±0.54	49.50^{ab}±0..57	5.50^{d} ±1.34	10.48^{c}±0.38
0.0	3.0	0.0	0.0	28	27	47.06^{bc}±0.56	35.76^{g} ±0.86	6.65^{e} ±0.69	6.38^{f}±0.19
0.5	0.0	0.0	0.1	29	-	45.06^{bcd} ±0.45	-	7.46^{f} ±0.45	-
1.0	0.0	0.0	0.2	26	27	26.61^{fg} ±0.96	37.01^{fg}±0.56	2.38^{gh} ±0.37	5.64^{de}±0.67
1.5	0.0	0.0	0.3	-	29	-	23.97^{jk}±0.68	-	3.30^{ef}±0.15
2.0	0.0	0.0	0.4	21	24	29.36^{f}±0.23	20.05^{k} ±0.46	3.58±0.12	2.42^{e}±0.64
2.5	0.0	0.0	0.5	-	-	-	-	-	-
3.0	0.0	0.0	0.6	28	25	40.18^{d}±0.42	41.56^{e} ±0.10	5.54^{g}±0.38	4.51^{d}±0.34
1.0	0.5	0.0	0.0	27	23	35.05^{e} ±0.75	29.50^{i} ±0.43	4.80^{f}±0.61	2.37^{f}±0.25
1.5	0.5	0.0	0.0	25	21	49.31^{ab}±0.45	43.28^{d} ±0.21	7.02^{f}±0.24	3.54^{de}±0.67
2.0	0.5	0.0	0.0	-	20	-	44.38^{cd} ±0.19	-	5.36^{cd}±0.19
2.5	0.5	0.0	0.0	27	22	49.25^{ab}±0.65	-	3.34^{d} ±1.27	-
3.0	0.5	0.0	0.0	30	27	48.24^{b} ±0.45	38.09^{f} ±0.19	2.00^{e} ±0.38	1.30^{ef}±0.46
0.0	0.0	0.5	0.0	-	28	19.82^{h} ±0.38	27.45^{ij} ±0.54	1.81^{gh}±0.91	2.61^{fg}±0.38
0.0	0.0	1.0	0.0	30	30	16.38^{i} ±0.25	18.47^{l} ±0.25	0.90^{ef}±0.28	1.30^{g}±0.12
0.0	0.0	1.5	0.0	28	-	34.64^{ef} ±0.45	-	1.36^{f} ±0.64	-
0.0	0.0	2.0	0.0	27	29	29.38^{f} ±0.34	25.05^{j} ±0.34	2.21^{gh}±0.37	3.40^{fg}±0.27
0.0	0.0	2.5	0.0	26	-	25.61^{g} ±0.53	-	6.44^{g}±0.71	-

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT.

Results of the preliminary phytochemical studies are presented in **Table 3**. The data revealed the presence of alkaloids, phenols, steroids, flavonoids and terpenoids in all the calli derived from different explants. However, triterpenoid was present in higher quantity only in leaf callus and totally absent in petiole callus. Cardiac glycoside was observed to be present in leaf and internode calli only and the tannin was present with high quantity in internode callus. Saponins were not detected in any callus of various explants. The secondary metabolites investigated to be present in various calli are reported to have various medicinal properties. Addae-Mensah [35] reported that the alkaloids and tannins are used in traditional herbal preparations against various common ailments in central African countries. Phenolic compounds including flavonoids possess diverse biological properties like antiinflammatory, anticarcinogenic, antidiabetic and antioxidant activities [36-38].

TABLE 3: THE DEGREE OF PRESENCE OR ABSENCE OF SECONDARY METABOLITES IN THE CALLI DERIVED FROM DIFFERENT EXPLANTS OF *HILDEGARDIA POPULIFOLIA*

S. No.	Test	Callus explants			
		Leaf	Node	Internode	Petiole
1	Alkaloids	+++	++	+++	++
2	Flavonoids	+++	+	+++	+
3	Phenols	+++	+++	+++	++
4	Steroids	+++	+	++	+
5	Terpenoids	+++	++	++	+
6	Triterpenoids	+++	+	+	-
7	Cardiac glycosides	++	-	+++	-
8	Saponins	-	-	-	-
9	Tannins	+	+	++	-

+, ++, +++ and – indicate the tracer, little and higher amount of respective secondary metabolite and – shows the absence of respective secondary metabolites.

Quantitative analysis of total flavonoids, phenols and tannins content showed differences among the calli derived from different explants and different days after inoculation for the study species, *Hildegardia populifolia* (**Table 4**). The results of the study exhibited that considerable amount of flavonoids were present in the calli of all explants. It was higher (13.35 mg RE/g extract) in 60 days old internodal callus followed by 40 days old callus of the same explant (12.101 mg RE/g extract) and 60 days old callus of leaf explant (10.28 mg RE/g extract). Flavonoids are naturally occurring phenolic compounds which have a widespread distribution in intact plants and have been found in many *in vitro* cultures also [39].

TABLE 4: QUANTITATIVE ESTIMATION OF TOTAL PHENOLS, FLAVONOIDS AND TANNINS IN METHANOLIC EXTRACTS OF CALLI DERIVED FROM DIFFERENT EXPLANT OF *HILDEGARDIA POPULIFOLIA*

S. No.	Explant	Age of callus (days)	Content of secondary metabolite in callus		
			Total flavonoids (mg RE/g extract)	Total Phenols (mg GAE/g extract)	Total tannins (mg GAE/g extract)
1	Leaf	20	07.43±0.91^{e}	09.97±0.45^{e}	14.59±1.85^{g}
		40	07.92±0.23^{e}	13.65±1.30^{c}	21.35±1.48^{c}
		60	10.28±1.14^{c}	18.20±0.87^{a}	25.45±1.43^{a}
2	Node	20	04.92±0.14^{g}	04.39±0.19^{h}	07.45±0.86^{i}
		40	05.39±0.74^{f}	07.34±0.34^{f}	09.34±0.64^{h}
		60	09.23±0.53^{d}	11.21±0.65^{d}	15.97±1.23^{f}
3	Internode	20	07.53±0.78^{e}	11.32±0.45^{d}	15.53±2.28^{e}
		40	12.10±0.71^{b}	13.99±0.68^{c}	20.22±1.96^{d}
		60	13.35±0.29^{a}	16.38±0.21^{b}	23.73±1.34^{b}
4	Petiole	20	02.40±0.09^{h}	03.35±0.23^{i}	02.68±0.32^{l}
		40	02.93±0.21^{h}	06.97±0.61^{g}	05.07±0.44^{k}
		60	04.35±0.43^{g}	07.55±0.94^{f}	06.35±0.85^{j}

Values are expressed as mean±SD (n=6). Values within the same column not sharing common superscript letters (a-l) differ significantly at $p<0.05$ by DMRT.

Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of biological system may contribute directly to their antioxidant action[40]. The total phenolics content in the 60 days old leaf callus culture was estimated to be higher (18.20 mg GAE/g extract) than in the other calli derived from other explants (Table 4). 60 days old internodal callus with 16.38 mg GAE/g extract ranked second for phenolics contents.

Similar to phenolics, the higher content of tannins was estimated in 60 days old leaf callus of *H. populifolia* (25.45 mg GAE/g extract) followed by 60 days old intermodal callus (23.73 mg GAE/g extract) and 40 days old leaf and intermodal callus (20.35 and 20.22 mg GAE/g extract respectively). Tanaka *et al* [41] reported that the presence of tannins such as gallic acid, catechins and tannin-glycosides in callus cultures of the woody species, *Quercus acutissima* added the value of callus of this species for antioxidant properties.

All the major secondary metabolites identified were higher in 60 days old callus cultures than that of the 40 and 20 days old cultures. It is explained that some secondary plant products were synthesized in higher quantity by differentiated tissues by accumulation in specialized tissues or organs. Therefore, it is indispensable to sustain differentiated and organized tissues for the preparation of drugs [39, 42].

DPPH scavenging activity of methanolic extracts of various age old series of callus culture of leaf, node, internode and petiole showed greater variation (**Table 5**). 60 days old leaf derived callus culture had relatively higher DPPH radical scavenging activity with the IC_{50} value, 86.24 mg/g than that of the node, internode and petiole callus cultures. However, it was not comparable to that of the standards, rutin and quercetin which were with the IC_{50} values 56.24 and 47.05 mg/g respectively. These results were in similar trend of that obtained by Farouk *et al* [43] who reported that leaf derived callus contained relatively higher radical scavenging activity in the species, *Citrullus colocynthis*.

TABLE 5: DPPH• RADICAL SCAVENGING ACTIVITY AND ABTS•+ ACTIVITY IN DIFFERENT AGE OLD SERIES OF CALLI DERIVED FROM VARIOUS EXPLANTS OF *HILDEGARDIA POPULIFOLIA*

S. No.	Explant	Age of callus (days)	DPPH• (IC_{50}) µg/mL	ABTS•+(µmol TE/g extract)
1	Leaf	20	122.56±6.03^{fg}	1309±132.3^{f}
		40	94.80±3.54^{d}	1893±103.4^{de}
		60	86.24±4.20^{c}	2109.36±201.1^{c}
2	Node	20	206.27±11.93^{k}	982±38.92^{h}
		40	174.24±3.67^{j}	1583±94.35^{e}
		60	120.43±8.34^{f}	2090.90±95.1^{b}
3	Internode	20	164.54±5.44^{i}	1943±69.24^{d}
		40	137.92±9.22^{h}	2694±124.46^{b}
		60	98.43±5.37^{e}	3077.98±241.9^{a}
4	Petiole	20	407.43±13.09^{n}	735±25.65^{i}
		40	366.42±12.93^{m}	957±67.94^{hi}
		60	323.95±15.45^{l}	1034.32±104.8^{g}
5	Rutin		56.24±2.90^{b}	-
6	Quercetin		47.05±1.83^{a}	-

Values are expressed as mean±SD (n=6). Values within the same column not sharing common superscript letters (a-n) differ significantly at $p<0.05$ by DMRT.

Total antioxidant activity was measured by $ABTS^{+}$ method. It was found that the 60 days old internode derived callus culture has higher antioxidant activity (3077.98 µmol TE/g extract) followed by 60 days old leaf derived callus (2109.36 µmol TE/g extract), 60 days old node derived callus (2090.90 µmol TE/g extract) and 60 days old petiole derived callus (1034.32 µmol TE/g extract).

The values were expressed as trolox equivalents. Renuka *et al.*[44] investigated that the *in vitro* callus culture of the species, *Ruta graveolens* showed prominent antioxidant activity, which may be attributed to higher content of phenolics found in the callus of this species.

The present study concluded that the MS medium supplemented with 2,4-D produced callus from higher percentage of most of the explants used. Moreover, the leaf and intermodal callus contained greater amount of phenolic compounds and hence the antioxidant activity. As the study species, *Hildegardia populifolia* is a critically endangered species, priority must be given to conserve the wilds. However, to meet the demand, *in vitro* culture sources particularly the callus derived from leaf and internodal explants can be exploited for antioxidant property.

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REFERENCES:

1. Sunilbabu K, Ammani K, and Varaprasad B: Selected plant extracts as biocontrol agents against the management of Chilli (*Capsicum annuum*) diseases. Journal of Pharmacy Research 2010; 3(12): 3143-3146.
2. Sunilbabu K, Ammani K, and Varaprasad B: Phytochemical screening and antibacterial properties of *Hildegardia populifolia* (Roxb.) Schott & Endl. Journal of Pharmacy Research 2011; 4(3): 907-909.
3. Saradha M and Paulsamy S: Antibacterial activity of leaf and stem bark extracts of the endangered tree species, *Hildegardia populifolia* (Roxb.) Schott and Endl. (Sterculiaceae). Journal of Research in Antimicrobials 2012a; 1: 023-027.
4. Saradha M and Paulsamy S: *In vitro* antioxidant activity and polyphenol estimation of methanolic extract of endangered medicinal tree species, *Hildegardia populifolia* (Roxb.) Schott & Endl. International Journal of Phytomedicine. 2012b; 4: 362-368.
5. Saradha M and Paulsamy S: Antinociceptive and antiinflammatory activities of stem bark of an endangered medicinal plant, *Hildegardia populifolia* (Roxb.) Schott and Endl. International Journal of Pharma and Bio Sciences 2013; 4(3): 30-36.
6. Varaprasad B, Katikala PK, Naidu KC, and Penumajji S: Antifungal activity of selected plant extracts against pytopathogenic fungi *Aspergillus niger*. Indian Journal of Science and Technology 2009; 2(4):87-90.
7. Anuradha T and Pullaiah T: Effect of hormones on the organogenesis and the somatic embryogenesis of an endangered tropical forest tree *Hildegardia populifolia* (Roxb.) Schott. & Endl. Taiwania 2001; 46(1): 62-74.
8. Nayar MP and Sastry ARK: Red Data Book of Indian Plants-Volume 3, Botanical Survey of India, Calcutta, 1990; 251-253.
9. Sarcar and Sarcar: Status, botanical description, natural distribution zone, propagation practices and conservation efforts of *Hildegardia populifolia* (Roxb.) Schott & Endl.: A Threatened tree species of dry tropical forests in India. The Indian Forester 2002; 128(7): 757-770.
10. Saradha M and Paulsamy S: Clonal propagation, an effective alternative propagation method for the endangered medicinal plant, *Hildegardia populifolia* (Roxb.) Schott & Endl. (Sterculiaceae). International Journal of Biology, Pharmacy and Allied Sciences 2012c; 1(8): 1145-1152.
11. Biondi S and Thorpe TA: Clonal propagation of forest tree species. In: Rao AN (Ed.) Proceedings COSTED Symposium on tissue culture of economically important plants. Forest Res.Inst. Malaysia, 1982; 197-204.
12. Cruz-Cruz CA, González-Arnao MT, and Engelmann F: Biotechnology and Conservation of Plant Biodiversity. Resources 2013; 2: 73-95
13. Saikia M, Srivastava K and Sureshkumar singh S: Effect of Culture Media and Growth Hormones on Callus Induction in *Aquilaria malaccensis* Lam., a Medicinally and Commercially Important Tree Species of North East India. Asian Journal of Biological Scinces 2013; 6: 96-105.
14. Verma P, Mathur AK and Jain SP: *In vitro* conservation of twenty three over-exploited medicinal plants belonging to the Indian sub-continent. The Scientific World Journal 2012; 1-14.
15. Trease GE and Evans WC: Pharmacognosy 17th edn., Bahiv Tinal, London. 1985; p.149.
16. Mojab F, Kamalinejad M, Ghaderi N, and Vahidipour HR: Phytochemical Screening of flavonoids and tannins. IV Daru 1992; 2: 2813 291.
17. Sofowora AA: Medicinal plants and Traditional Medicines in Africa. Spectrum Books Ltd., Ibadan, Nigeria 1993; 2: 81-85.
18. Harborne JB: (eds.). Phytochemical Methods: A guide to modern techniques of plant analysis. 3rd ed. Chapman and Hall, London ISBN: 0341235727032, 1998; 302.
19. Zhishen J, Mengcheng T, and Jianming W: Research on antioxidant activity of flavonoids from natural materials. Food Chemistry 1999; 64: 555-559.
20. Siddhuraju P and Becker K: Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of Drumstick tree (*Moringa oleifera* Lam.) leaves. Journal of Agricultural and Food Chemistry. 2003; 51(8): 2144 – 2155.
21. Blois MS: Antioxidants determination by the use of a stable free radical. Nature 1958; 26: 1199-1200.
22. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice Evans C: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26: 1231-1237.
23. Arunkumar BS and Jayaraj M: Rapid *in vitro* callogenesis and phytochemical screening of leaf and leaf callus of *Ionidium suffruticosum*, Ging-A seasonal multipotent medicinal herb. World Journal of Agricultural Science 2011a; 7(1): 55-61.
24. Mohajer S, Mat Taha R, Khorasani A, and Yaacob JS: Induction of different types of callus and somatic embryogenesis in various explants of Sainfoin (*Onobrychis sativa*). Australian Journal of Crop Science, 2012; 6(8): 1305-1313.
25. Khosroushahi AY, Manesh HN, and Simonsen HT: Effect of antioxidants and carbohydrates in callus cultures of *Taxus brevifolia*: evaluation of browning, callus growth, total phenolics and paclitaxel production. BioImpacts 2011; 1(1): 37-45.
26. Ripley KP and Preece JE: Micropropagation of *Euphorbia lathyris* L. Plant cell, Tissue and Organ Culture 1986; 5: 213-218.
27. Jagadish Chandra KS, Rachappaji S, Gowdu KRD, and Thara Saraswathi KJ: *In vitro* propagation of *Pisonia alba* (Linn.) Sapanogae (Lettuce tree), A threatened species, *Phytomorphology* 1999; 49: 43-47.

28. Kheng TC and Lee CC: Somatic embryogenesis from mature *Bambusa ventricosa.* College of Tropical Agriculture and Human Resource. Bio 2011; 11: 1-5.
29. Danya U, Udhayasankar MR, Punitha D, Arumugasamy K and Suresh SN: *In vitro* regeneration of *Tecomella undulata* (Sm.) Seem- an endangered medicinal plant. International Journal of Plant, Animal and Environmental Sciences 2012; 2(4): 44-49.
30. Charndra Indrani and Bhanja P: Study of organogenesis *in vitro* from callus tissue of *Flacourtia jangomas* (Lour.) Raeusch. Through scanning electron microscopy. Current Science 2002; 83(4): 476-479.
31. Ghosh KC and Bannerjee N: Influence of plant growth regulators on *in vitro* propagation of *Rauwolfia tetraphylla* L. Phytomorphology 2003; 53: 11-19.
32. Kaur P and Kothari SL: Embryogenic callus induction and efficient plant regeneration from root cultures of Kodo Millet. Phytomorphology 2003; 53: 49-56.
33. Arunkumar SB and Jayaraj M: Effect of auxins and cytokinins on *in vitro* propagation of *Ionidium suffruticosum* Ging -a seasonal multipotent medicinal herb. International Journal of Research in Ayurveda and Pharmacy 2011b; 2(1):198-203.
34. Suresh K and Aniket K: Direct somatic embryogenesis and plant regeneration from leaf and stem explants of *Nothapodytes foetida*: a critically endangered plant species. International Journal of Plant, Animal and Environmental Science 2013; 3(1): 257-0264.
35. Addae-Mensah I: 1992. Towards a rational scientific basis for herbal medicine – A phytochemist's two decades contribution. An inaugural lecture delivered at the University of Ghana, Legon, Ghana Universities Press, Accra, 63.
36. Chung K, Wong T, Huang TY, and Lin YW: Tannins and human health: A review. Critical Reviews in Food Science and Nutrition 1998; 38: 421-464.
37. Rani U, Verma RN and Batra A: Total phenolic (flavonoids) contents and antioxidant capacity of different *Murraya koenigii* (L.) Spreng. Extracts. International Journal of Medicinal Plant Research 2012; 1(7): 123-128.
38. Terron MP, Garrido M, and Rodríguez AB: Beneficial Effects of Melatonin-Rich and Melatonin-Enriched Foods on Health. International Journal of Health and Nutrition 2013; 4(1): 1-14.
39. Mala A and Raka K: *In vitro* Clonal Propagation and Phytochemical Analysis of *Momordica charantia* .Linn. Journal of Pharmacognosy and Phytochemistry 2013; 2(1): 66-77.
40. Tosun M, Ercisli S, Sengul M, Ozer H, and Polat T: Antioxidant properties and total phenolic content of eight *Salvia* species from Turkey. Biological Research 2009; 41: 175-181.
41. Tanaka N, Shimomura K, and Ishimaru K: Tannin production in callus cultures of *Quercus acutissima*. Phytochemistry 1995; 40(4): 1151-1154.
42. Samariya K and Sarin R: Isolation and identification of flavonoids from *Cyperus rotundus* linn. *In vivo* and *in vitro.* Journal of Drug Delivery & Therapeutic 2013; 3(2): 109-113.
43. Farouk KE, Amal AM and Sami IA: Callus formation, phenolics content and related antioxidant activities in tissue culture of a medicinal plant colocynth (*Citrullus colocynthis*). Nova Biotechnologica 2010; 10(2): 79-94.
44. Renuka D, Amit S and Nutan M: Phytochemical composition and antioxidant potential of *Ruta graveolens* L. *In vitro* culture lines. Journal of Botany 2012; 1-6.

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