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IN VITRO CALLUS INDUCTION AND SHOOT REGENERATION OF *DECALEPIS ARAYALPATHRA* (JOSEPH AND CHANDRAS) VENTER

***Raveesha H R and Ashalatha K S**

Department of Botany, Bangalore University, Jnanabharathi Campus, Bengaluru – 560 056 Karnataka

***Corresponding Author Email: hrraveesh74@gmail.com**

ABSTRACT

The aim of the present study was to develop an efficient protocol for callus induction, shoot regeneration, biomass and antioxidant activity of Decalepis arayalpathra. The different explants were cultured on MS medium supplemented with different concentration of auxins and cytokinins. The total phenolic content and antioxidant activities were determined by standard procedures. Callus induction was higher from leaf explants on media supplemented with individual growth regulator (2 mg/L of 2, 4-D, and 1 mg/L of BAP). However, combination of BAP (2 mg/L) + 2, 4-D (1 mg/L) and BAP (1 mg/L) + NAA (0.5 mg/L) resulted in maximum callus induction. The callus biomass was significantly higher in medium supplemented with BAP (2 mg/L) + NAA (1 mg/L). Shoot regeneration was higher from shoot tip and nodal explants on medium fortified with BAP (1.0 mg/L) + NAA (0.2 mg/L) & IBA (0.5 mg/L). Root induction was maximum on half strength MS medium supplemented with IBA (0.5 mg/L) and NAA (0.2 mg/L). The plants were subjected to hardening and showed around 70% of survival after acclimatization. The total phenolic content and antioxidant activities were significantly higher in methanolic extract of leaf compared to callus. Our results demonstrated that leaf and nodal explants are the good source for the callus induction and shoot regeneration, which might be helpful in mass propagation and conservation of this medicinally important plant.

KEY WORDS

Auxins, antioxidants, callus induction, cytokinins, Decalepis arayalpathra.

INTRODUCTION

Medicinal plants are the local heritage of the tribal communities for their livelihood, food, medicine and shelter. In India, herbal medicines have been the basis of treatment and cure for various diseases in traditional medicine such as Ayurveda, Unani and Siddha [1]. Rapid industrialization, urbanization and over exploitation of economically valuable natural flora have imposed a serious threat to biodiversity all over the globe. The application of plant tissue culture in *ex-situ* conservation of rare and endangered plant taxa has long been recognized [2]. *Decalepis arayalpathra* (*D. arayalpathra*) is one such medicinal plant belongs to the family Periplocaceae has been used for treating various diseases such as peptic ulcers, cancer like affliction, skin diseases etc [3-5].

Roots of *D. arayalpathra* are a rich source of polyphenols such as α-amyrinacetate, 4-methoxy salicylaldehydes, magnificol, β-sitosterol, naringenin and aromadendrin [6]. It is used as flavouring agent, pickles, appetizer, blood purifier and as a cool drink [7-9]. The antioxidant potential of the polyphenols is due to the redox properties of their phenolic compounds which quench free radicals in biological system. This species was assessed as critically endangered species due to the occurrence of small populations in the crevices of the rocks and its narrow distribution [10, 11]. The National biodiversity authority of India also

declared it as a species of high conservation concern [12].

Conventional propagation of *D. arayalpathra* is at stake with several factors like poor fruit set, seed germination and rooting on stem cutting [13]. Development of biotechnological methods such as micropropagation, cell/root and hairy root cultures is one of major solutions for several problem [14]. Limited studies have been carried out on *in vitro* regeneration of shoot from different explants [2, 15]. Advances in plant tissue culture are helpful for increasing the production of secondary metabolites from callus culture [16]. Therefore, the present study was aimed to develop an efficient protocol for callus induction and *in vitro* shoot regeneration of *D. arayalpathra,* and to determine their phenolic content and antioxidant activity.

MATERIALS AND METHODS

Collection and sterilization of plant material

D. arayalpathra seeds and seedling were collected from Trivandrum, Kerala. The plant material was authenticated in the Botanical Survey of India, Western Regional Centre, Pune. A voucher specimen is deposited in the herbarium (No. BSI/WRC/100-1/DEN. CER. /2018/76). Seedlings were maintained in the greenhouse condition. The young and healthy explants were excised from the one-y ear old plant, washed thoroughly under running water with Tween-20 for 30 min. Then surface sterilized with bavastin (0.5%) a fungicide for 30 min. Finally, the explants were sterilized with 0.1% mercuric chloride for 2-3 min followed by washing with double distilled water (d.w) for four times.

Culture medium

Murashige & Skoog (MS) medium supplemented with 3% (w/v) sucrose, 0.8% agar (Himedia, Mumbai) was used for all experiments [17]. The plant growth regulators 2, 4-D (2, 4-dichlorophenoxyacetic acid), NAA (*α*-naphthalene acetic acid), IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), BAP (6-benzyl amino purine), KIN (6-furfuryl amino purine) or (Kinetin) and Zeatin were procured from Sigma-Aldrich (St. Louis, MO, USA). The pH of the medium was maintained at 5.8 and autoclaved for 15 min 109 Kpa. The cultures were incubated at 25 ± 2 ^{o}C under white florescent light for 16:8 h photoperiod.

Callus induction

Young and healthy leaves (1 cm^2) were cultured on MS media fortified with 0.5 - 4.0 mg/L of auxins (2, 4-D, NAA, IAA and IBA) and cytokinins (BAP, KIN and Zeatin) individually and in combination.

Shoot regeneration and root induction

Shoot tips and nodes were cultured on shoot regeneration medium supplemented with various concentrations and combination of BAP (0.5 - 4.0 mg/L) + NAA and IBA (0.1 - 1.0 mg/L). The well-developed regenerated shoots were transferred to half strength MS medium fortified with 0.1 - 1.0 mg/L of NAA and IBA individually for root induction.

Acclimatization

Rooted plants were recovered from the culture tubes and gently washed by using d.w thoroughly to remove agar residues. Then the plants were transferred to sterilized pots and maintained in culture room for 2-3 w, further the plants were transferred to pots containing sand, soil and manure in 2:1:1 ratio and kept in greenhouse condition.

Callus biomass

The callus biomass was determined individually and in combination of growth regulators. After 12 w of inoculation, callus was transferred into Petri dish to weigh the fresh weight, then stored in oven at 60 °C for 1 to 2 d and dry weight was recorded.

Preparation for plant extracts

Dry leaf and callus were collected, shade dried and powdered. 10 g of powder was extracted with methanol in soxlet apparatus for about 12 -14 h. After extraction the residues were collected and concentrated by evaporation and stored at 4 ^{o}C for further analysis.

Total phenolic contents

The total phenolic content in methanolic extracts of leaf and callus were determined by using Folin Ciocalteu (FC) method [18]. 0.5 ml of leaf and callus extract (1 mg/ml) was mixed with 0.5 ml of FC reagent and allowed to stand for room temperature (RT) for 5 min. Following this, 1 ml of 7% sodium carbonate was added and the final volume was made up to 5 ml with d.w. After 90 min of incubation at room temperature in dark, the absorbance was read at 725 nm using UV visible spectrophotometer in triplicates. Gallic acid was used for calibration of standard curve. The results were expressed as mg of Gallic acid equivalent (mg GAE/g) of dry weight of plant material.

Determination of DPPH radical scavenging assay

The free radical scavenging activity was measured by DPPH assay [19, 20] with minor modification. Briefly, different concentration of methanolic extracts of leaf

and callus (10-100 µg/ml) was added to 3 ml of 0.25 mM (95% methanol) of DPPH solution. After 30 min of incubation at RT in dark, the absorbance was measured at 517 nm in triplicates. BHT (Butylatedhydroxytoluene) was used as a standard. DPPH radical scavenging activity was calculated by the following formula.

$$\text{Percentage inhibition} = \frac{\text{Absorbance control - Absorbance sample}}{\text{Absorbance control}} \times 100$$

The IC_{50} value is the concentration of the plant extract required to scavenge 50% of the total DPPH radicals available.

Determination of reducing power

The reducing power assay was carried out according to the procedure of Kuda *et al.* [21]. Briefly, 0.5 ml of sample solution (leaf and callus) was mixed with 2 ml of phosphate buffer (0.1M pH 7) and 2 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 ^{o}C for 20 min. After incubation 2 ml of 10% TCA (Trichloroacetic acid) was added and the mixture was a centrifuged at 3000 rpm for 10 min. 1 ml of sample was mixed with same volume of d.w and 0.5 ml of ferric chloride solution (0.1%) and the absorbance was measured at 700 nm using ascorbic acid as a standard.

Statistical analysis

The results were expressed as Mean ± standard deviation (SD). All the experiments were repeated thrice, and data were analyzed by one-way analysis of variance followed by Duncan's multiple range tests using SPSS software. Probability values *P<0.05* were considered significant.

RESULTS AND DISCUSSION

***In vitro* callus induction from leaf explants**

The initiation of callus was observed from leaf explants within two weeks of inoculation on MS media supplemented with various plant growth regulators. Maximum callus induction was observed after eight weeks of culture on MS media supplemented with 2, 4-D (2 mg/L) and BAP (1 mg/L) individually. Whereas, combination of BAP (0.5 mg/L) + 2, 4-D (1 mg/L) and BAP (1 mg/L) + NAA (0.5 mg/L) also showed higher callus induction. Increase in the growth hormone concentration resulted in poor callus induction. The morphology of the callus was varied with different growth regulators, creamish callus was observed in 2, 4-D. While, in combination resulted in the formation of green friable and profuse callus (**Table. 1-3; Fig.1 a-f**). Similar results were observed in *Decalepis hamiltonii* (*D. hamiltonii*), where in maximum callus induction was observed in combination of BAP and NAA from cotyledonary explants [22]. Studies by Ahmad *et al*. also reported higher callus induction from leaf explants of *Ruta graveolens* on MS media fortified with individual growth regulators as well as combination [23].

Table.1: Effect of growth hormones on callus induction from leaf explants of *D. arayalpathra*

Growth hormones	Concentration mg/L	Number of explants	Mean ± SD	Nature of callus
2, 4-D	0.5	20	16.00±1.00^{b}	Creamish callus
	1.0	20	18.00±1.00^{c}	Creamish callus
	2.0	20	19.33±0.58^{d}	Creamish yellow callus
	3.0	20	17.00±1.00^{b}	Creamish yellow callus
	4.0	20	11.00±1.00^{a}	Creamish yellow callus
NAA	0.5	20	13.00±1.00^{b}	Creamish callus
	1.0	20	16.00±1.00^{c}	Creamish callus
	2.0	20	17.00±1.00^{d}	Creamish callus
	3.0	20	13.00±1.00^{b}	Creamish yellow callus
	4.0	20	11.00±1.00^{a}	Creamish yellow callus
BAP	0.5	20	18.00±1.00^{b}	Greenish callus
	1.0	20	19.33±0.58^{c}	Greenish callus
	2.0	20	17.67±0.58^{c}	Greenish callus
	3.0	20	15.67±0.58^{bc}	Creamish green callus
	4.0	20	13.00±1.00^{bc}	Creamish callus
	0.5	20	07.67±8.41^{b}	Creamish callus

KIN	1.0	20	13.67±0.58^{c}	Creamish green callus
	2.0	20	16.00±1.00^{c}	Greenish callus
	3.0	20	16.33±0.58^{bc}	Greenish callus
	4.0	20	14.67±0.58^{bc}	Creamish green callus

Values represent the Mean ± SD in triplicates. Means with the different letters in columns indicate significant differences at 5% level.

Table.2: Effect of combination of auxins and cytokinins on callus induction from leaf explants of *D. arayalpathra*

Growth hormones	Concentration mg/L	Number of explants	Mean ± SD	Nature of callus
BAP+2, 4-D	0.5+0.5	20	15.75±1.71^{ab}	Creamish callus
	0.5+1	20	19.50±0.71^{c}	Creamish callus
	0.5+2	20	17.00±1.00^{bc}	Creamish callus
	0.5+3	20	14.00±1.00^{a}	Creamish yellow callus
	0.5+4	20	14.00±1.00^{a}	Creamish yellow callus
2, 4-D+BAP	0.5+0.5	20	16.00±1.00^{ab}	Creamish callus
	0.5+1	20	17.00±1.00^{c}	Creamish yellow callus
	0.5+2	20	19.00±1.00^{bc}	Creamish green callus
	0.5+3	20	19.00±1.00^{a}	Creamish green callus
	0.5+4	20	19.00±1.00^{a}	Creamish green callus
	1+2	20	19.67±0.58^{c}	Creamish green callus
	2+1	20	19.00±1.00^{a}	Creamish green callus
BAP+NAA	0.5+0.5	20	19.00±1.00^{ab}	Friable green callus
	0.5+1	20	18.00±1.00^{c}	Creamish green callus
	0.5+2	20	16.00±1.00^{bc}	Friable green callus
	0.5+3	20	18.00±1.00^{a}	Creamish green callus
	0.5+4	20	17.00±1.00^{a}	Friable green callus
NAA+BAP	0.5+0.5	20	18.00±1.00^{ab}	Friable green callus
	0.5+1	20	19.33±0.58^{c}	Greenish callus
	0.5+2	20	19.00±1.00^{bc}	Friable green callus
	0.5+3	20	15.00±1.00^{a}	Creamish green callus
	0.5+4	20	14.00±1.00^{a}	Creamish green callus
	1+1	20	19.00±1.00^{a}	Creamish green callus
	1+2	20	18.00±1.00^{a}	Creamish green callus
	2+1	20	16.00±1.00^{a}	Creamish green callus

Values represent the Mean ± SD in triplicates. Means with the different letters in columns indicate significant differences at 5% level.

Table.3: Effect of combination of cytokinins on callus induction from leaf explants of *D. arayalpathra*

Growth hormones	Concentration mg/L	Number of explants	Mean ± SD	Nature of callus
BAP+KIN	0.5+1	20	00.00±0.00	----
	0.5+2	20	00.00±0.00	----
	0.5+3	20	17.00±1.00^{b}	Friable green callus
	0.5+4	20	18.00±1.00^{b}	Creamish green callus
KIN+BAP	0.5+1	20	13.00±1.00^{a}	Creamish green callus
	0.5+2	20	12.00±1.00^{a}	Creamish callus
	0.5+3	20	15.00±1.00^{b}	Creamish green callus
	0.5+4	20	14.00±1.00^{b}	Creamish green callus

Values represent the Mean ± SD in triplicates. Means with the different letters in columns indicate significant differences at 5% level.

Table 4: Total phenolic and DPPH activity in leaf and callus extracts

Methanolic extracts	Total Phenolics mg /g GAE	DPPH IC_{50} µg/ml
Leaf	18.12±0.38	30.25±0.2814
Callus	5.88±0.70	69.38±0.6972

Values represent the Mean ± SD in triplicates.

Shoot regeneration and root induction

The presence of cytokinin along with auxin is necessary for shoot regeneration [24]. In the present study, shoot regeneration was observed in combination of cytokinins and auxins as shown in **Fig. 1** (**g-i**). The maximum shoots were observed in 1 mg/L of BAP + 0.2 mg/L of NAA and 0.5 mg/L of IBA from nodal and shoot tip explants respectively. Similar results were reported in *D. arayalpathra* where in MS media fortified with cytokinins in combination of NAA/IAA/IBA was best for shoot organogenic response [15]. The shoots were then transferred to half strength MS media supplemented with auxins. The maximum root induction was noticed in media fortified with 0.2 mg/L of NAA and 0.5 mg/L of IBA (**Fig.1 j**). Our results indicate that lower concentrations of NAA and IBA induced maximum number of roots. Samydurai *et al.* reported maximum root induction in *D. hamiltonii* cultured on half strength MS media supplemented with lower concentration of NAA and IBA [22]. The well-developed rooted plants were hardened under greenhouse conditions and around 70% survival rate was recorded (**Fig. 1 k & l**).

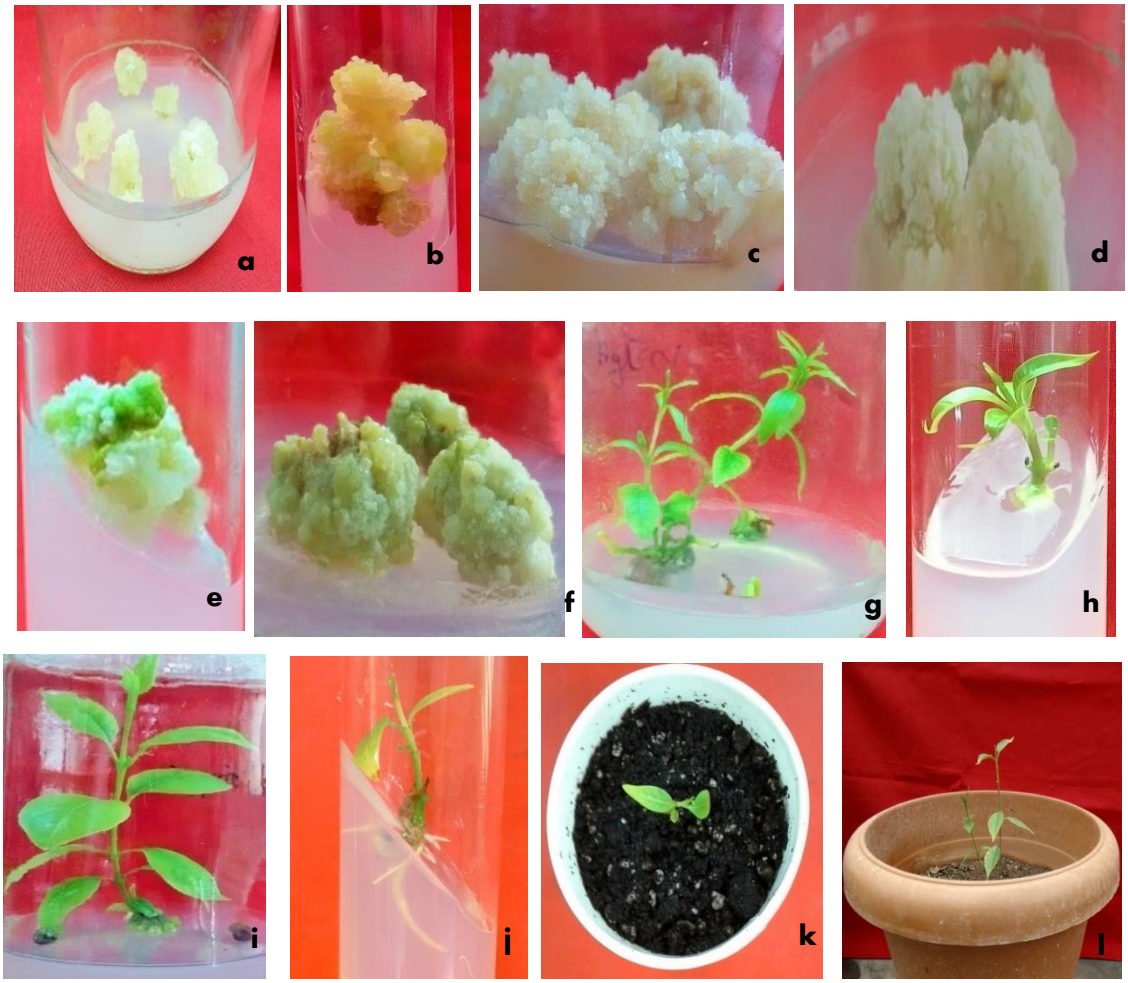
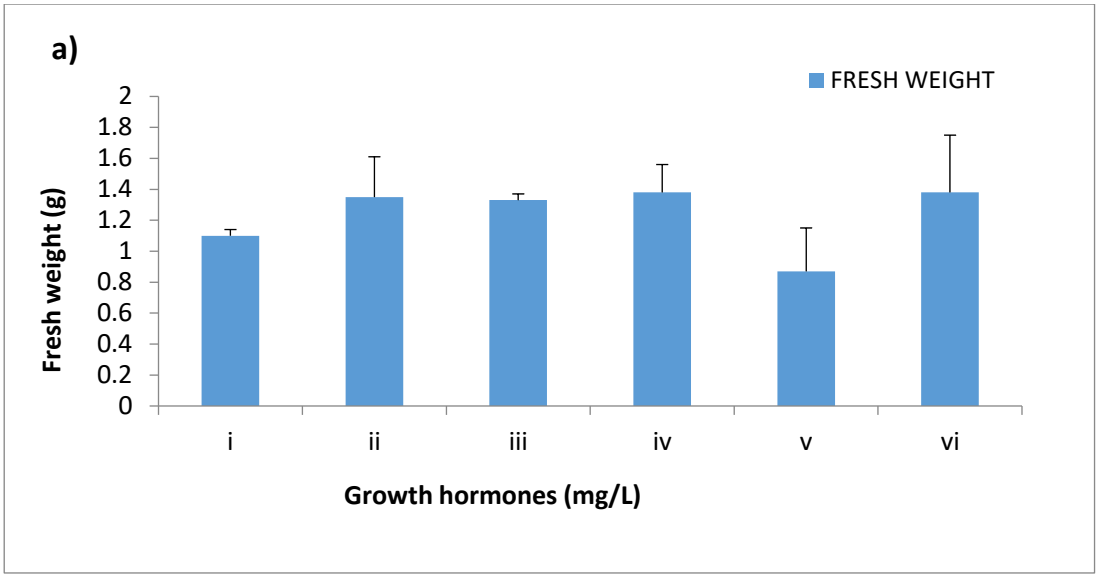


Fig.1: a-f – Induction of callus from leaf explant: **a**) MS +BAP (0.5 mg/L) + 2, 4-D (1 mg/L), **b**) MS + BAP (1 mg/L), **c**) MS + 2, 4-D (2 mg/L), **d**) MS + BAP (2 mg/L) + 2, 4-D (1 mg/L), **e**) MS + NAA (0.5 mg/L) + BAP (1 mg/L), **f**) MS + BAP (1 mg/L) +NAA (0.5 mg/L), **g** - Direct regeneration from Shoot tip MS + BAP (2 mg/L) + NAA (0.5 mg/L), **h & i** - Direct regeneration from nodal explant; **h**) MS + BAP (0.5 mg/L) + NAA (0.5 mg/L) & **i**) MS + BAP (1 mg/L) + NAA (0.5 mg/L), **j**) MS + NAA (0.2 mg/L), **k & l**- Hardening and acclimatization.



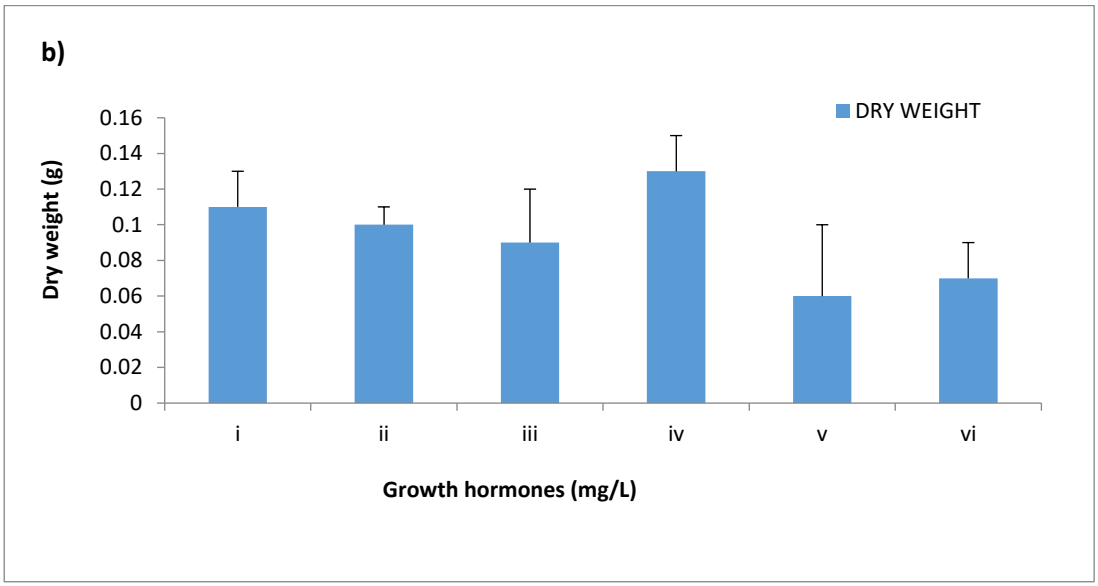


Fig. 2: Effect of plant growth regulators on a) Fresh weight and b) dry weight of callus biomass induced from leaf explants. i) MS + 2, 4-D (2 mg/L) ii) MS + BAP (1 mg/L) +NAA (1 mg/L) iii) MS + BAP (1 mg/L) + NAA (2 mg/L) iv) MS + BAP (2 mg/L) + NAA (1 mg/L) v) MS + BAP (1 mg/L) + 2, 4-D (2 mg/L) vi) MS + BAP (2 mg/L) + 2, 4-D (1 mg/L).

Values represent the Mean ± SD in triplicates. Means with the different letters in columns indicate significant differences at 5% level.

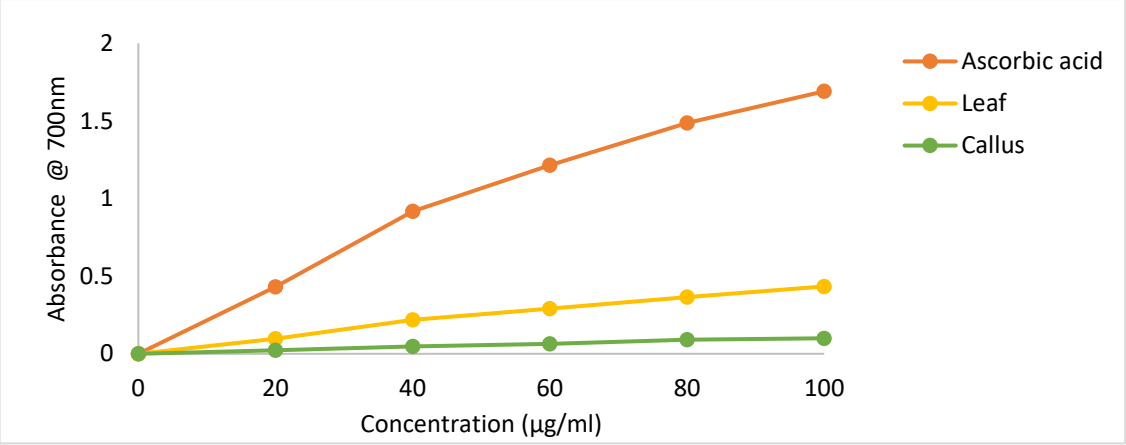


Fig. 3: Reducing power assay from leaf and callus extracts of *D. arayalpathra.*

Callus biomass

Figure **2a** & **b** represents the callus biomass derived from leaf explants. The fresh and dry weight callus was recorded maximum in combination of 2 mg/L of BAP + 1 mg/L of NAA by 20% and 15% over 2, 4-D. The biomass was higher in combinations of auxins and cytokinins due to the compact greenish callus. Our results are in concordance with the studies of Bakar *et al*. and Gopi and Vatsala where higher callus biomass was observed in media supplemented with different auxins and cytokinins [25, 26].

Total phenolic contents

Phenolic compounds are the major class of plant metabolites with diverse functions such as antioxidant, anticancer and antimicrobial activities etc. The total phenolic content is estimated using the FC reagent and calculated from regression equation of calibration curve ($y=0.058x + 0.0531$, $R^2= 0.9978$). In the present study, total phenolic content in methanolic leaf extract and callus were found to be 18.12±0.38 and 08.24±0.64 mg/g GAE respectively **Table 4**. The higher total phenolic content in the leaf is may be due to the biosynthesis of polyphenols, which is accelerated by the light exposure. Studies by Rehman *et al*. also reported lower phenolic content in the callus compared to plant extracts in *Caralluma tuberculata* [27].

DPPH radical scavenging assay

The DPPH is a stable free radical, has been widely used to evaluate the radical scavenging ability of antioxidants. The DPPH radical scavenging activity of methanolic extracts of leaf and callus is represented in **Table 4**. The DPPH activity showed dose dependent relationship with leaf extract exhibited higher radical scavenging activity compared to callus with an IC_{50} value of 30.25 µg/ml. The standard antioxidant BHT showed potent radical activity with an IC_{50} value of 8.20 µg/ml. The results are in agreement with the studies of Umesh where in the root extracts of *D. hamiltonii* showed higher DPPH activity over callus [28].

Reducing power

To examine the reducing power assay of the extracts, the reduction of Fe^{3+} to Fe^{2+} was investigated. The reducing power ability of methanolic extracts of leaf and the callus showed dose dependent relationship as represented in the **Fig. 3.** The leaf extract exhibited higher level of reducing power than leaf derived callus. Higher reducing ability in the leaf extract may be due to the presence of higher phenolic content which can act as free radicals' scavenger by donating an electron or hydrogen.

CONCLUSION

The present study highlights the effective micropropagation protocol for rapid callus induction and large-scale propagation of *D. arayalpathra* an important endangered medicinal plant. Callus induction was most effective on medium supplemented with lower concentration of auxins and cytokinins. The developed protocol for callus induction may be used for secondary metabolite production they're by reducing the over exploitation of plants from the natural habitats.

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***Corresponding Author:**
Raveesha H R
Email: hrraveesh74@gmail.com