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Original Article

IN VITRO CALLUS INDUCTION AND ANTIOXIDANT POTENTIAL OF DECALEPIS HAMILTONII (WIGHT AND ARN)

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ABSTRACT

Objective: To develop *in vitro* callus induction in nodal segments of *Decalepis hamiltonii* and investigate the antioxidant potential of the callus.

Methods: In this study, nodal segments of *Decalepis* were cultured on Murashige and Skoog (MS) medium supplimented with various concentration of 2,4-D for callus induction and later subcultured on various concentration of NAA and BAP. The antioxidant activity of tuberous root and callus were investigated along with the quantification of phenolics and flavonoid contents. The ability of scavenging activity of methanolic extract using DPPH, FRAP assays and total antioxidant activity using Phosphomolybdenum assay were carried out .

Results: Callus was induced on MS medium supplemented with various concentration and combination of auxins and cytokinins. Callus formation was best on media suplemented with 2mg / L 2,4-D . However the callus formation was slow on different combinations of NAA and BAP. The total phenolic content in tuberous root and callus was 100.7 ± 13.24 µg (GAE) / mg and 18.86 ± 0.1485 µg (GAE) / mg respectively. The total flavonoids were 180.5±63.36 µg (QE) / mg and 130.6 ± 3.571 µg (QE) / mg of extract respectively. The experiments further revealed that *Decalepis* callus had FRAP values of 17.25 ± 0.04738 µmol of AAEs/mg extract and for root 110.4±7.397 µmol of AAEs / mg. The total antioxidant capacity of root extracts showed higher degree (194.6+41.74 µg AAE /mg extract) of antioxidant capacity than the callus extract (119.3+10.55 µg AAE / mg extract). The IC_{50} values of the DPPH radical scavenging property of callus and root extract were in the range of 148±4.39 µg/ml and 20±1.54 µg/ml respectively.

Conclusion: This work has shown that *D.hamiltoni* callus extracts contained substantial amount of phenolic compounds including flavonoids exhibiting potent antioxidant activity by effectively scavenging free radicals. If appropirate stratagies are developed for enhancing the antioxidant molecules, possibilites of making plant cell factories using callus or suspension cultures can be established, which could preserve the endangered population of *Decalepis hamiltonii* from extinction.

Keywords: *Decalepis*, Callus, Antioxidant, FRAP, Phenolics.

INTRODUCTION

The demand for plant-based contemporary and alternative medicines, particularly foods rich in antioxidants are assoicated with promoting health and lowering risk of various age-related diseases [1]. Nearly 80% of the world's human population relies mainly on traditional medicines for their health care [2]. Many medicinal plants have become source of new lead molecules in theraupeutics [3]. Plants with history of traditional use such as the ethnomedicinal species of *Decalepis* are of particular interest as herbal medicines and a source for novel bioactive compounds [4].

Decalepis comprises five species of twining vines and erect shrubs, four of which are endemic to rocky hillsides and open areas of the Eastern and Western Ghats of India; the exception, *Decalepis khasiana*, has been documented in the Meghalaya state of eastern India and in neighboring Bangladesh, Laos, Myanmar, and the Guangxi, Guizhjou, and Yunnan provinces of southern China. Two species, *Decalepis hamiltonii* and *D.khasiana*, are distributed fairly widely, while the others are narrow endemics, each restricted to a different mountain range of the southern western ghats of India [5]. *Decalepis hamiltonii Wight & Arn*. commonly called as swallow root, is a monogeneric medicinal shrub belonging to the family *Apocynaceae* [6] known for its antioxidant property. The young roots contain about 92% fleshy matter and 8% woody core. The roots of this plant are highly aromatic and contain metabolites like aldehydes, alcohols, ketones, sterols and triterpenes, of which 2-hydroxy-4-methoxybenzaldehyde is the principle component [7,8]. This compound is an isomer of vanillin, due to which it offers aroma to the roots and hence justifies its use in pickles [9]. The root extract exhibits antibacterial, antifungal, anti-inflammatory, antipyretic, chemoprotective, hepatoprotective and most importantly, antioxidant properties [10]. When consumed, it cools the system, gives good appetite and also acts as a blood purifier [11]. The root extract also acts as neuroprotectant [12] and attenuates the age-related decline in cognitive ability, in addition to ameliorative effect on the memory of the offspring in *Drosophila* [13]. The methanolic root extract also alleviates hepatotoxicity in rats [14] which is attributed to its antioxidant activity. The root extacts have antitumour effect [15] and inhibits tumour progression and metastasis [16].

Antioxidant compounds when added to food products, especially to lipids and lipid-containing food, are belived to increase the shelf life by retarding the process of lipid peroxidation, which deteriorates food products during processing and storage. *Decalepis* roots have been extensively used as flavouring agent for various culinary preparations and health drink nannari sharabat [17]. Increased levels of antioxidant activity have been exihibited with high levels of total phenolics and flavonoids [18] in the leaves of *Decalepis hamiltonii*. Many compounds having antioxidant potential have been isolated from roots including Decalepin [19], ellagic acid [20], 2-(hydroxymethyl)-3-methoxybenzaldehyde [21], Decalpoline [22], 4-hydroxyisophthalic acid (4-HIPA) [23] among others**.** *Decalepis hamiltoni* contains a cocktail of antioxidant biomolecules and has been considered as edible plant source for neutraceuticals and pharmacaeutical applications.

The demand for the *Decalepis* roots and its unregulated harvesting has lead to near extinction of the species. Plant tissue culture techniques offer powerful tools for mass multiplication of many plant species and provide alternative ways for extraction of pharmaceutically important bioactive components under controlled conditions [24]. In the present investigation, induction of callus from *Decalepis hamiltonii* nodal segments, biochemical charecterisation and the antioxidant potential of the callus is reported, which could be used as an alternative source to *Decalepis* roots.

MATERIALS AND METHODS

Culture conditions for callus induction

Decalepis hamiltonii is an endangered plant and hence not found in all parts of Karnataka. The strong belt for the growth of *Decalepis* is in the Western ghats of India. The mother plant of *Decalepis* was procured from Dhanavantri vana, a nursery situated in the Bangalore University Campus. Healthy nodal segments were used as explants for *in vitro* callus induction. The explants were washed under tap water for 30 minutes to remove surface adhered soil particles. The explants were transferred to laminar air flow and were primarily surface sterilized with 0.2% mercuric chloride for a minute followed with several washes with sterile double distilled water. The explants were later surface sterilized with 2% sodium hypochlorite for 5 minutes followed by thorough wash in sterile double distilled water. The surface sterilized explants were blotted on sterile filter papers to remove excess of water. The explants were inoculated on MS basal medium [25] supplemented with various concentration and combination of auxins and cytokinins (Table-1) and maintained at 28 °C under white fluorescent light for 16 h / 8h light/dark cycle.

Preparation of plant extracts

About 25gms of 30 day old subcultured matured green friable callus inoculated on MS + 2, 4-D 2mg/L) was collected, and powdered in liquid nitrogen and immediately suspended in 50ml of methanol and kept at room temperature for 24 hrs with periodic shaking. After 24 hrs the solution was passed through whatman filter paper, the methanolic extract was concentrated till dry .The dried methanolic extract was dissolved in minimum amount of methanol and stored at 4 ^{0} C until further use. The roots of *Decalepis hamiltonii* were collected from local market in Bangalore and extract was prepared as described above. All the chemicals used were of analytical grade and procured from Himedia, Bombay.

Estimation of total flavonoids

The total flavonoid content in the methanolic extract of callus tissue was determined by Aluminum chloride method [26] with slight modifications. Briefly 0.5 ml of callus extract was mixed with 2.5 ml of 95% ethanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water was added to the mixture to bring the final volume up to 9 ml . After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with spectrophotometer (Elico SA 165). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank (r^2 = 0.9945). The final absorbance of each sample was compared with a standard curve plotted from Quercetin. The total flavonoid content was expressed in micrograms of Quercetin equivalents (QEE) per mg extract.

Estimation of Total Phenolic Content (TPC)

The total phenolic content in the methanolic callus extract was determined using Folin-Ciocalteau method [27]. 0.5 ml of callus / root extract (1 mg/ml) was mixed with .2 ml of 2N Folin-Ciocalteau's reagent and allowed to stand at room temperature for 5 min. Then 2 ml of sodium carbonate (Na_2CO_3, 7.0 %, w/v) was added and the mixture was made upto 5ml with double distilled water .The reaction was allowed to stand for another 90 min and kept in the dark with intermittent shaking. The absorbance of the blue color that developed was measured at 725 nm using spectrophotometer (Elico SA 165). The experiment was carried out in triplicates. Gallic acid was used for constructing the standard curve (5 to 50 µg / ml; r^2= 0.999) and the total phenolic compounds concentration in callus / root extract was expressed as micrograms of gallic acid equivalent per mg (µg GAE/mg) of extract.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability

Radical scavenging activities of *Decalepis* were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay [28] . The extract (20µl) was added to 1 ml of 50µM DPPH solution in methanol. The extracts tested ranged from 0–250µg/ml .The mixtures were mixed well and incubated in the dark for 20 min. The reduction of DPPH absorption was measured at 515 nm. Ascorbic acid was used as the positive control. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following equation:

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

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

Ferric reducing antioxidant power (FRAP) Assay

The antioxidant activity based on the ferric reducing ability of *D.hamiltonii* callus / root extracts was estimated based on the assay [29] with some modifications. A working reagent was prepared fresh by mixing 10 ml of 300 mM acetate buffer with 1ml of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM of hydrochloric acid (HCl) and 1 ml of 20 mM $FeCl_3.6H2O$. The freshly prepared FRAP reagent was pre-warmed at 37°C after which a blank reading was taken at 595 nm. Subsequently, 30 µl of sample / standard, 90 µl of water was added to 900 µl of the FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent and again at 30 min after the start of the reaction. The change in absorbance in the 30 min reaction was calculated by comparison to the absorbance changes of ascorbic acid against a standard curve tested in parallel. Results were expressed as micromoles of ascorbic acid equivalents (AAEs) per milligram of extract (µmol of AAEs/mg). All experiments were carried out in triplicate.

Phosphomolybdenum assay (Total antioxidant capacity)

Total antioxidant activity of the extract was evaluated by the phosphomolybdate method [30] using ascorbic acid as a standard. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank. Methanol (0.1 mL) in the place of extract is used as the blank. Ascorbic acid equivalents were calculated using standard graph of AA. The experiment was conducted in triplicates and values were expressed as equivalent of ascorbic acid per mg of extract.

Statistical Analysis

All the experiments were carried out in triplicates (n=3) and the results were expressed as mean ± standard deviation (SD). Statistical tests as well as mean and SD calculations were performed using Graph Pad Prism v 5.

Table 1: Effect of plant growth regulators on callus induction from nodal explants of *D.hamiltonii*

Growth regulator	Concentration	Nature of callus	%Response ± SD
2, 4 -D	0.4mg/l	Yellow, nodular	35 ± 1.47
	1 mg/l	Creamish yellow	35 ± 0.89
	2 mg/l	Greenish friable	55 ± 2.31
NAA + BAP	2mg/l + 0.5mg/l	Creamish yellow	20 ±3.47
	1mg/l + 0.5mg/l	Friable	15±2.47
	0.5mg/l + 1mg/l	Friable green callus	16 ±1.07

RESULTS

In vitro callus induction from nodal explant of *Decalepis hamiltonii*

The induction of callus was observed in nodal explants of *Decalepis hamiltoni* incubated on MS media supplemented with various PGRs as shown in table 1. In nodal explants from young plants, callusing was robust, without any shoot regeneration. Callus formation failed to occur from nodal region of relatively old plants. Callus initiation and growth were best on MS media containing 2 mg / L 2,4-D. However, no striking difference was observed with higher auxin concentrations (5, 7.5 and 10 mg / L 2,4-D) except for the slow growth of callusing. The response for callusing was very slow when nodal explants innoculated on MS media with different combinations of NAA and BAP. The nodal explant after 65 days showed creamish nodular callusing on 2, 4-D (figure-1A). The callus after 65 days was subcultured and grown on MS medium supplemented either with 2mg / L 2,4-D alone or 2mg / L NAA and 0.5mg / L BAP. The subcultured calli proliferated robustly within 26-28 days to give rise to light green friable calli irrespective of the above hormone combinations without any regeneration. Though addition of BAP (1mg/L) showed initial green regenerating calli, later failed to regenerate further to form shoots. The callus tends to become brown due to leaching of phenolics as it was maintained for more than 50-60 days. Due to this, callus was subcultured on MS medium supplemented with 2mg / L 2, 4-D for every 30 days to maintain the proliferating state of callus. Further, there were no observable differences in callus growth or viablity between cultures maintained in dark or in light conditions. `

Total Phenolics: The content of phenolic compounds (µg / mg) in methanolic extract of roots and callus were determined using regression equation of callibration curve (r^2= 0.999) and expressed as gallic acid equivalents (GAE) was found to be 100.7±13.24 µg (GAE) / mg in methanolic root extract and 18.86±0.1485 µg (GAE) / mg in callus extracted in methanol.These phenolic compounds are likely to contribute to the radical scavenging activity of callus and root extract.

Total Flavonoids : Flavonoids, the most common polyphenolic compounds have antioxidant activity and are ubiquitously found in plants.The content of flavonoid compounds (µg/mg) in methanolic extract of roots and callus was determined using the regression equation of callibration curve (r^2 = 0.9945) and expressed as Quercetin Equivalent (QE). The total flavonoids in methanolic extract of roots and callus was determined spectrophotometrically as 180.5±63.36 µg (QE) / mg and 130.6±3.571 µg (QE) / mg of extract respectively.

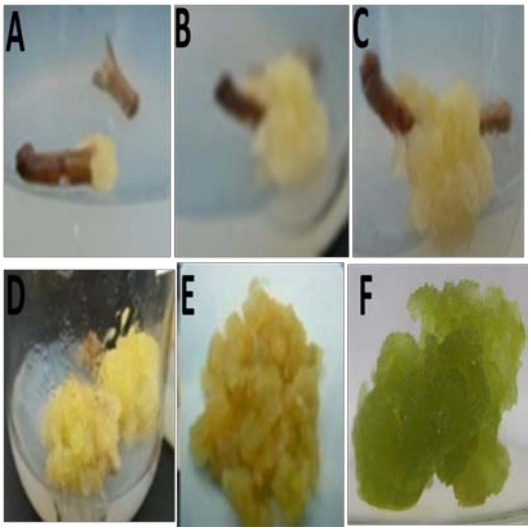

Fig. 1: *In-vitro* callusing from nodal explant of *Decalepis hamiltonii.*A: Callus initiation from the nodal explant grown on MS mediun supplemented with 0.4 mg/L 2, 4-D. B: Callus formation in media containing 1mg / L 2, 4-D. C: Callus from MS medium supplemented 2 mg / L 2, 4-D. D: *In vitro* callus subcultured grown from nodal explant with MS medium supplemented with NAA + BAP (2mg/L + 0.5mg/L). E: *In vitro* callus subcultured grown from nodal explant with MS medium supplemented with 1mg/L NAA and 0.5mg/L BAP. F. *In vitro* callus subcultured grown from nodal explant with MS medium supplemented with NAA + BAP (0.5mg/L + 1mg/L).

Table 2: Quantitative estimation of phytochemcials and antioxidant activities of root and callus extracts of *D.hamiltonii*

	Phosphomolybednum assay µgAAE / mg extract	FRAP assay µg AAE /mg extract	Phenolics µg GAE /mg extract	Flavonoids µg QEE / mg extract	DPPH assay IC_{50} µg/ml
Methanol Extract of roots	194.6 ± 14.74	110.4 ± 7.397	100.7 ± 13.24	180.5 ± 13.36	20 ± 1.54
Callus extract	119.3 ± 10.55	17.25 ± 0.04738	18.86 ± 0.1485	130.6 ± 3.571	148 ± 4.39

Ferric reducing antioxidant power (FRAP) Assay

The FRAP assay measures the antioxidant effect of the substance in the reaction medium as reducing ability. Antioxidant potential of the callus / root extract of *Decalepis* was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. The antioxidant capacities of methanol extract of callus and that of root varied significantly (Table 2) .The callus extract had 17.25 ± 0.04738 µmol of AAEs / mg and for root 110.4 ± 7.397 µmol of AAEs / mg.

Phosphomolybdenum assay

The phosphomolybednum quantitative method was utilised to evaluate the total antioxidant capacity of the callus extract. The root and callus extract exhibited different degrees of activity as shown in table 2. The results indicate that under the same conditions of extraction both callus and root extracts of swallow root exhibit high antioxidant activity. However, the differences in the degree of Mo reduction between two types of extracts were observed. Results indicated that root extracts showing higher degree (194.6+41.74 µg AAE /mg extract) of antioxidant capacity than the callus extract (119.3+10.55 µg AAE / mg extract).

DPPH Radical Scavenging activity:

The DPPH radical scavenging property of the compounds extracted in methanol from *D.hamiltonii* root and callus is shown in (Table 2). The values of IC_{50} of callus and root extract were in the range of 148±4.39 µg/ml and 20±1.54 µg/ml respectively. The scavenging activities of these sample were significant The IC_{50} values of callus and root extract were relatively high compared to positive control of ascobic acid (IC_{50} 3.19±0.23 µg/ml) implying the low potent in free radical scavenging activity . However, the root extract showed relatively high anitoxidant activity than callus.

DISCUSSION

Antioxidants are considered to be important nutraceauticals on account of its immense health benefits [31]. When plant materials become scarce, biotechnological methods based on *in vitro* culture of tissue and cell suspension cultures are considered as alternate sources. *Decalepis* an endangered plant is considered to be having huge cocktail of antioxidant biomolecules. In the present investigation, an *invitro* method has been developed for the callus production using MS media .The media was suplemented with the

auxin 2,4-D alone at different concentrations (Table 2) was able to induce callus from nodal explant at maximum concentration of 2mg/L .The callus was friable without any regeneration. Similar observations were made [32], where leaf sections had produced callus in response to 2,4-D and BA. The callus was also produced when nodal explants were grown on NAA and BAP but comparatively showed slow growth. The slow growth of callus was enhanced to its robustness when it was subcultured on media contianing either 2,4-D or NAA +BAP combination without any regeneration. The resultant callus was utilised for examining the antioxidant activity.

Plant phenolics acts as one of the important group of compounds having primary antioxidant activity. In the present study, the phenolic amount in methanolic extract of roots was 100.7±13.24 µg (GAE) / mg and 18.86±0.1485 µg (GAE) / mg in callus, exihibiting more in extracts of root. This low amount of phenolics in callus can be attributed to the proliferating callus of 30 days. The low amount of phenolics is enough to function as potential antioxidant molecules, as many other non phenolic molecules also contribute to the antioxidant activity. It is also postulated that a network of antioxidants with different chemical properties present in low amounts could work synergisticaly protecting cells from oxidative damage [33]. The flavonoids content in calli was 130.6±3.571 µg Quercetin equivalents/mg extract as compared to 180.5±13.36 µg QE / mg in tuberous roots. The combined effect of phenolics and flavonoids would result in comparitively higher antioxidant activity in *Decalepis* callus.

The free radical scavenging activities of callus extract and tuberous root extract along with the standard ascorbic acid was determined by the DPPH and results are shown in table (2) .The decrease in absorbance of the DPPH radical caused by antioxidant was due to scavenging activity which was noticeable as colour change from purple to yellow. A lower IC_{50} for tuberous root (20±1.54 µg/ml) comapred to the callus IC_{50} (148±4.39 µg/ml) indicates significantly lower amount of scavenging activity of callus compared to root extract and ascorbic acid (IC_{50} 3.19±0.23 µg/ml). Similar decreased DPPH radical scavenging activity has been reported in *Biophytum sensitivum* callus extract [34]. Salinity induced elevated level of antioxidant capacity has been reported [35] in the callus cultures of *Salvadora persica.* The increased antioxidant activity in callus would also depend on effect of different plant growth regulators with concommitant increase in phenolics in the callus [36]. The ferric reducing ability of plasma (FRAP) assay, which is non-specific, measures the ability of antioxidant compounds to reduce complex (Fe(III)-TPTZ) to (Fe(II)-TPTZ). The Fe(II)-TPTZ complex gives a blue color with an absobance maximum at 593nm.This assay is used for analysis of single antioxidant and total antioxidant power of plant extracts [37] .The results of FRAP assay are presented in table (2). In this assay, the highest activities were noticed in tuberous root extracts which showed 6.4 fold more compared to callus. The presence of high concentration of phenolics and flavonoids in root extract may explain the high FRAP activities owing to its high antioxidant property. Finally, Phosphomolybdenum assay, which is quantitative method to evaluate the antioxidant capacity, was used to determine the total antioxidant capacity of callus.The extracts exhibited different degrees of activity as shown in Table 2. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity[38]. Results indicated that the root extract showed 194.6 ±14.74 µg (AAE) / mg of extract compared to callus (119.3±10.55 µg (AAE) / mg indicating 1.6 fold less effective total antioxidant activity.

CONCLUSION

This work has shown that *D.hamiltoni* callus extracts contained substantial amount of phenolic compounds including flavonoids exhibiting potent antioxidant activity by effectively scavenging various free radicals. The antioxidant activity in callus might be due to the bioactive compounds present in them which could be similar to the one found in roots. However, it also warrants extensive studies on chemcial profiles and mechanistic action of antioxidant activity of each of these molecules in callus. Even though the extracts of undifferentiated callus proved to be less active in its antioxidant activity compared to root extact, it would be interesting to see whether the biomolecules and their antioxidant activity can be enhanced by elicitation either by different hormone regimen or elicitors for callus production or cell culture, so that the callus / cell culture can be effectively used as an alternate source for production of antioxidant molecules. If appropriate strategies are developed for enhancing the antioxidant molecules, possibilites of making plant cell factories for enhanced production of active principles with higher antioxidant property can be established, which would preserve the already endangered population of *Decalepis hamiltonii* from extinction.

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