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In vitro Propagation and Evaluation of Antioxidant Properties of the Medicinal Plant Species, *Hypochaeris radicata* L. (Asteraceae) Distributed in Nilgiris, the Western Ghats.

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Abstract

In the recent years phytomedicines are employed for curing many diseases as alternative source of synthetic drugs due to many reasons; affordability, low price, no side effects and ecofriendly. Higher demand and hence over exploitation have depleted the plant sources severely. There is an urgent need to conserve the natural stalk by developing conservation strategies. *Hypochaeris radicata,* one such species needed in large quantity is severely exploited for its medicinal uses in high hills of Nilgiris, the Western Ghats, India. Further, no major scientific reports are available with respect to its bioactive compounds and therapeutic properties. To address this lacuna, the study was carried out to develop protocol for *in vitro* propagation, to know the phytochemicals by preliminary investigation and quantification and to evaluate the antioxidant properties. The results of the study report that higher number of shoots were obtained from cotyledonary leaf explants in the MS medium fortified with BAP at 2mg/L (100%) followed by BAP and GA_3 at 2mg/L and 0.5mg/L (95.63%) respectively. Rooting percentage (98.48%) was better which subcultured onto the MS medium contained NAA at 1mg/L. Rooted plantlets were hardened (garden soil: sand: vermicompost=1:1:1) and established in pots with 75.22% survival rate. The determination of phytochemical constituents by qualitative and quantitative analysis revealed the presence of rich variety of secondary metabolites. The *in vitro* antioxidant activities of *in vitro* derived methanolic leaf extract of *H. radicata* proved this species as potential source for pharmaceutical drug preparations.

Keywords: *Hypochaeris radicata, in vitro* regeneration, phytochemical analysis and antioxidant properties.

Abbreviations: BAP-6-Benzyladenine, NAA-Napthalene acetic acid, IAA-Indole acetic acid, IBA-Indole Butyric acid, Kn-Kinetin, GA_3-Gibberllic acid, MS-Murashige and Skoog medium.

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INTRODUCTION

Hypochaeris radicata an edible, perennial herb belongs to the family Asteraceae, is commonly called as 'hairy cat's-ear'. It is native to South Africa and distributed in forest margins of Nilgiris, the Western Ghats, Tamil Nadu above 2000m msl. It possesses several medicinal properties such as antiinflammatory, anticancer, antioxidant[1], antibacterial[2], antifungal[3] and antidiuretic. It is being prescribed for the treatment of jaundice, rheumatism, dyspepsia, constipation, hypoglycemia and kidney related problems in traditional medicinal practices of Tamil Nadu, India[4]. This species is used for medicinal purpose in Meghalaya[5] and also used as food for ruminants in British Columbia[6]. Further, *H. radicata* is reported to have many bioactive compounds[7]. The leaves are usually blanched, steamed and cooked like any other leafy vegetable. Salads are made with the leaves, raw as well as boiled or steamed. The flowers can be battered and made into fritters. A number of dishes are flavored with the petals of the flowers, which are also added to sautéed dishes. In Greece and Crete, leaves of this plant are eaten raw, along with other similar herbs such as pachies and agrioradika. Alcoholic beverages such as wine are flavored with the petals of these flowers. The roots of this herb are ground and roasted and used as a substitute for coffee[8].

Unhampered large-scale exploitation for its medicinal uses has been lead to the marked diminution of wild stock of *H. radicata.* Further, its propagation through conventional means has been unreliable due to poor seed germination, low seed set and viability[9]. Therefore, there is an immediate need to generate this plant through appropriate propagation techniques. In such a scenario, *in vitro* culture methods strongly provide an alternative mode of plant propagation for material supply as well as conservation of germplasm for posterity. In addition, to large scale application, evaluation of phytochemical constituents and antioxidant properties of *in vitro* produced leaf explants are also necessary to know the reliability of clones to be produced by employing tissue culture technology. Hence, in the current situation, studies on *in vitro* propagation and determination of phytochemical constituents by preliminary qualitative analysis and quantification of antioxidant phytochemicals and to evaluate the antioxidant potential to corroborate the medicinal properties of *in vitro* leaf of *H. radicata.*

MATERIALS AND METHODS

Collection of the plant materials:

For contamination problem (the presence of numerous hairs present on the surface of the leaf) the seeds were taken for the source to produce the plant via *in vitro* condition. The seeds of *Hypochaeris radicata* were collected from the Kattabettu, Nilgiris, the Western Ghats, Tamil Nadu, India during the month of February 2013 when the seeds were available. The authenticity of the plant was confirmed in Botanical Survey of India, Southern Regional Circle, Coimbatore by referring the deposited specimen. The voucher number of the specimen is BSI/SRC/5/23/2010-11/Tech.153.

Sterilization of seeds:

The seeds were washed with running tap water for 15minutes followed by 3-4 rinses in double distilled water. These seeds were sterilized with 0.1% (w/v) $HgCl_2$ for 5 minutes followed by 4-5 rinses with sterilized double distilled water.

Preparation of the MS medium:

Seeds were inoculated on MS media[10] containing 30% sucrose solidified with 8% agar (Tissue culture grade, Hi-Media, India). The pH of the medium was adjusted to 5.6-5.8 with 0.1 N NaOH or 0.1 N HCl prior to the addition of agar. The medium was autoclaved at 121°C and 15lbf/Inch^2 pressure for 15 minutes.

Culture conditions:

The cultures were maintained under white fluorescent light having 2000 lux light intensity. The incubation temperature was 25±2°C with 16 hours light and 8 hours dark period in every 24 hours cycle.

Seed germination and multiple shoot induction:

The surface sterilized seeds were inoculated in the test tubes containing MS medium without any addition of growth regulators. After the germination of seeds the cotyledonary leaf portion was removed and cultured on a MS medium containing various growth hormones. Thirty explants were used for each culture and the experiments were repeated thrice. The per cent explants responding for multiple shoot induction, time taken for this induction, total number of shoots per explant, morphogenetic response and length of the shoots were recorded after 2 weeks of culture.

Rooting and green house condition:

The regenerated multiple shoots of 2-3cm were excised from the culture and transferred to MS medium supplemented with different concentrations of IAA, IBA and NAA for root initiation. After two weeks, the percentage of shoot forming roots, roots per shoot and root length were measured. Rooted shoots were thoroughly washed to remove the adhering gel and dipped in 0.1% bavistin, to remove the fungal contamination. The plantlets were transplanted to pots filled with mixture of different proportions of red soil, sand, garden soil, vermicompost, decomposed coir waste and perlite. After acclimatization, the plantlets were transferred to the garden and the rate of survivability percentage was calculated.

Preparation of *in vitro* leaf extract:

Fresh leaves were collected from *in vitro* produced plantlets and shade dried then grounded into a coarse powder. 10g of powder was extracted with methanol (150mL) using SOXTRON™, SOX-2, Tulin equipments, Chennai. The solvent present in the extract was reduced under room temperature and this crude *in vitro* leaf extract was used for the analysis of phytochemicals and evaluation of antioxidant properties.

Preliminary qualitative phytochemical analysis:

Preliminary qualitative phytochemical analysis was carried out to identify the presence or absence of secondary metabolites such as alkaloids (Dragendorff's test), cardiac glycosides (Keller-Killiani test), flavonoids, glycosides, phenols, resins, saponins (Frothing test), tannins, terpenoids and triterpenoids according to standard phytochemical methods as described by Trease and Evans[11], Sofowora[12] and Harbone[13,14].

Quantification of antioxidant phytochemical compounds:

Antioxidant compounds present in the *in vitro* methanolic leaf extract of *H. radicata* was determined by using standard quantitative methods. The total phenolics and tannin contents were determined by using a standard curve gallic acid equivalents (GAE) mg/100g extract as per the method of Siddhuraju and Becker[15]. The total flavonoids content was estimated and expressed as rutin equivalents (RE) mg/100g extract according to the method described by Zhishen *et al.* [16].

Determination of *in vitro* antioxidant activities:

Reducing power activity:

The reducing capability of the extract was determined according to the method suggested by Yildirim *et al.* [17]. The *in vitro* derived leaf extracts (50-250µg/mL) were mixed with 1mL of 0.2M phosphate buffer, pH 6.6 and 1mL of 1% potassium ferric cyanide and the mixtures were incubated at 50°C for 20 minutes. The reaction was terminated by adding 1mL of 10% trichloroacetic acid (w/v) and the mixture was centrifuged at 3000rpm for 10minutes. After the completion of process, 2mL of the upper layer was mixed with 2mL of distilled water and 0.5mL of 1% ferric chloride (w/v). Absorbance was read at 700nm. Rutin, quercetin, BHA, BHT, AT, TX and AA were served as reference standards. The reductive capability of the extracts was noted by increased absorbance values.

DPPH• radical scavenging activity:

The DPPH• radical scavenging activity was measured according to the procedure described by Blois[18]. Briefly, 0.1mM DPPH• solution was prepared by using methanol in dark. The extract (50-250µg/mL) were mixed with 500µL of DPPH• solution. This solution containing mixture was incubated at 27°C for 30 minutes and the absorbance was measured at 517nm. Results were compared with the activity of rutin, quercetin, BHA, BHT and AT. The DPPH• radical scavenging activity of the extract was calculated by using the following formula:

Inhibition (%) = (A_0-A_1) X $100/A_0$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts. The IC_{50} (the microgram of extract to scavenge 50% of the radicals) value was calculated using linear regression analysis. Lower IC_{50} value indicates greater antioxidant activity.

Trolox equivalent antioxidant capacity assay:

Antioxidant activity was performed by using $ABTS^{\bullet+}$ method proposed by Siddhuraju and Manian[19]. $ABTS^{\bullet+}$ radical cation was generated by a reaction of 7mM $ABTS^{\bullet+}$ and 2.45mM potassium persulphate and the mixture was incubated for 12-16h at room temperature in dark. Prior to assay, the solution was diluted in ethanol (about 1:89v/v) and equilibrated to obtain an absorbance of 0.700±0.01 at 734nm. The generated $ABTS^{\bullet+}$ solution (2mL) was mixed with 20µL of sample extracts or trolox standards (10-15µM). After 30 min of incubation, the absorbance was read at 734nm. Trolox was used a reference material.

Statistical analysis:

The data obtained were assessed for the analysis of variance (ANOVA) and means of different experiments were compared by using Duncan's Multiple Range Test ($p<0.05$)[20].

RESULTS

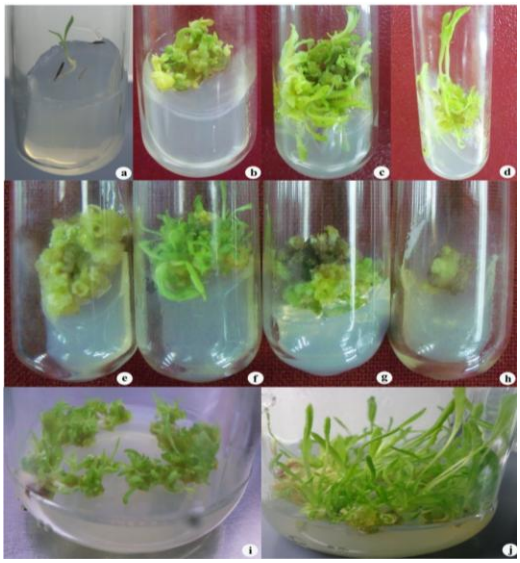

Figure 1: Micropropagation of *Hypochaeris radicata.* a) Seed germination on MS medium without any growth regulators. b) Induction of multiple shoot in MS medium supplemented with BAP at 2mg/L. c) After 2 weeks of culture. d) Induction of multiple shoot in MS medium with BAP+GA_3 (2+0.5mg/L). e-h) Multiple shoot induction on different concentrations of BAP (1, 2, 3, 4 mg/L) respectively. i) Similar cluster of multiple shoots formed when cultured on BAP at 2mg/L. j) After 4 weeks of culture.

Growth regulators (mg/L)				Days required	% of response*	No. of shoots/ explants*	Morphogenetic response	Average length of the shoot (cm)*
BAP	GA_3	Kn	IAA					
0.5	0.0	0.0	0.0	25	45.54±0.55^{c}	-	C	2.32±0.06^{a}
1.0	0.0	0.0	0.0	19	54.06±0.03^{d}	05±0.04^{b}	C+MS	5.42±0.32^{b}
1.5	0.0	0.0	0.0	17	64.53±0.11^{e}	07±0.19^{c}	C+MS	8.66±0.64^{cd}
2.0	0.0	0.0	0.0	14	100.00±0.10^{i}	20±0.02^{f}	MS	4.79±0.04^{ab}
2.5	0.0	0.0	0.0	16	87.05±0.21^{g}	09±0.73^{d}	C+MS	3.22±0.12^{a}
3.0	0.0	0.0	0.0	19	33.95±0.22^{b}	04±0.32^{a}	C+MS	-
3.5	0.0	0.0	0.0	25	20.32±0.02^{ab}	-	C	-
4.0	0.0	0.0	0.0	30	13.06±0.20^{a}	-	C	-
0.5	0.5	0.0	0.0	26	15.05±0.64^{a}	-	C	-
1.0	0.5	0.0	0.0	23	34.65±0.22^{b}	-	C	7.93±0.54^{c}
1.5	0.5	0.0	0.0	21	44.12±0.75^{c}	04±0.80^{a}	C+MS	9.54±0.03^{d}
2.0	0.5	0.0	0.0	18	95.63±0.20^{h}	12±0.11^{e}	MS	8.77±0.33^{cd}
2.5	0.5	0.0	0.0	23	81.10±0.66^{fg}	06±0.21^{bc}	C+MS	-
3.0	0.5	0.0	0.0	27	40.00±0.49^{bc}	-	C	-
0.5	0.0	0.5	0.0	25	56.90±0.09^{d}	-	C	-
1.0	0.0	0.5	0.0	19	78.33±0.01^{f}	-	C	2.00±0.09^{a}
1.5	0.0	0.5	0.0	18	83.88±0.19^{fg}	09±0.42^{e}	C+MS	4.30±0.25^{ab}
2.0	0.0	0.5	0.0	16	92.21±0.05^{gh}	-	MS	-
2.5	0.0	0.5	0.0	20	87.00±0.01^{g}	-	C	-
3.0	0.0	0.5	0.0	28	69.01±0.12^{ef}	-	C	-
0.5	0.0	0.0	0.5	31	42.02±0.08^{bc}	-	C	-
1.0	0.0	0.0	0.5	28	56.00±0.21^{d}	-	C	-
1.5	0.0	0.0	0.5	24	78.01±0.29^{f}	-	C	1.04±0.52^{a}
2.0	0.0	0.0	0.5	18	90.04±0.33^{gh}	03±0.15^{a}	C+MS	3.74±0.45^{a}
2.5	0.0	0.0	0.5	20	88.05±0.19^{g}	13±0.18^{e}	MS	2.32±0.06^{a}
3.0	0.0	0.0	0.5	23	65.01±0.20^{e}	-	C	5.42±0.32^{b}

Table 1: Effect of different concentrations of growth regulators on multiple shoot induction from *in vitro* cotyledonary leaf explants of the species, *Hypochaeris radicata.*

*Mean of three replicates of 30 explants ± standard deviation.

Values with the column followed by the same superscript are not significantly different as determined by ANOVA at P<0.05.

C-Callus, MS-Multiple Shoot.

Growth regulators (mg/L)			Shoots rooted (%)*	No. of roots/shoot*	Root length (cm)*
IAA	IBA	NAA			
0.0	0.3	0.0	16.54±0.76^{a}	1±0.09^{a}	0.51±0.02^{a}
0.0	0.5	0.0	25.72±0.83^{b}	2±0.02^{b}	2.90±0.03^{b}
0.0	1.0	0.0	41.66±0.13^{d}	3±0.34^{c}	5.21±0.43^{e}
0.0	0.0	0.3	10.43±0.55^{a}	1±0.02^{a}	2.04±0.05^{b}
0.0	0.0	0.5	32.32±0.95^{c}	3±0.01^{c}	4.21±0.44^{d}
0.0	0.0	1.0	98.48±0.32^{g}	9±0.67^{e}	8.97±0.10^{f}
0.3	0.0	0.0	-	-	-
0.5	0.0	0.0	65.10±0.63^{e}	3±0.75^{c}	2.03±0.34^{b}
1.0	0.0	0.0	89.88±0.64^{f}	4±0.40^{d}	3.63±0.32^{c}

Table 2: Effect of different concentrations of growth regulators on root initiation, root number and root length from *in vitro* shoots of the species, *Hypochaeris radicata.*

*Mean of three replicates of 30 explants ± standard deviation.

Values with the column followed by the same superscript are not significantly different as determined by ANOVA at P<0.05.

Hardening medium composition (v/v)	No. of plantlets under hardening	No. of plantlets survived	Survivability (%)
Garden soil: sand: vermicompost (1:1:1)	30	24±0.09^{d}	75.22±0.32^{e}
Red soil: sand: vermicompost (1:1:1)	30	18±0.12^{c}	65.04±0.11^{d}
Vermicompost: soil (1:1:1)	30	13±0.10^{b}	48.00±0.25^{b}
Red soil: sand (1:1:1)	30	11±0.20^{a}	43.03±0.22^{a}
Decomposed coir waste: perlite: compost (1:1:1)	30	17±0.22^{c}	60.12±0.43^{c}

Table 3: Effect of various composition of hardening medium on survivability of plantlets of *Hypochaeris radicata.*

*Mean of three replicates of 30 explants ± standard deviation.

Values with the column followed by the same superscript are not significantly different as determined by ANOVA at P<0.05.

Alkaloids	Cardiac glycosides	Flavonoids	Glycosides	Phenols	Resins	Saponins	Steroids	Tannins	Terpenoids	Triterpenoids
+++	-	+++	+++	-	-	-	+++	+++	+++	-

Table 4: preliminary phytochemical analysis of *in vitro* methanolic leaf extract of *Hypochaeris radicata.*
(+++) – Present, (-) – Absent.

Sample	Percentage yield (w/w)	Total phenolics*	Total flavonoids^	Tannins*
In vitro leaf	20	4.45±0.24[a]	3.48±0.05[a]	0.97±0.22[a]

Table 5: Percentage yield, total phenolics, total flavonoids and tannins content in the methanolic leaf extract of *Hypochaeris radicata.*
(*)-(mg GAE/100g extract), (^)-(mg RE/100g extract). GAE - Gallic Acid Equivalent, RE - Rutin Equivalent.
Values were performed in triplicates and represented as mean ± SD.
Mean values followed by different superscript in a column are significantly different (p<0.05).

Sample concentration (µg/mL)	*In vitro* leaf	Sample concentration (µg/mL)	RU	QE	BHA	BHT	AT	TX	AA
50	0.471±0.02[a]	20	0.238±0.003[a]	0.359±0.012[a]	0.236±0.010[b]	0.224±0.001[a]	0.147±0.006[a]	0.210±0.011[a]	0.0257±0.010[a]
100	0.655±0.32[b]	40	0.350±0.013[c]	0.632±0.023[c]	0.396±0.017[c]	0.368±0.009[b]	0.152±0.001[b]	0.308±0.016[b]	0.341±0.016[b]
150	0.687±0.54[c]	60	0.408±0.013[c]	0.718±0.019[b]	0.496±0.028[d]	0.478±0.013[c]	0.155±0.002[c]	0.389±0.012[c]	0.469±0.028[c]
200	0.764±0.21[d]	80	0.476±0.006[b]	0.833±0.044[e]	0.593±0.008[a]	0.517±0.017[d]	0.158±0.002[d]	0.453±0.011[d]	0.578±0.015[d]
250	0.832±0.11[e]	100	0.557±0.014[c]	0.973±0.029[d]	0.644±0.011[b]	0.584±0.012[c]	0.164±0.002[e]	0.549±0.024[e]	0.673±0.014[e]

Table 6: Reducing capability of methanolic leaf extract of Hypochaeris radicata.
RU-Rutin, QE-Quercetin, BHA-Butylated hydroxylanisole and BHT-Butylated hydroxyltoluene, AT-Alfa Tocopherol, TX-Trolox, AA-Ascorbic acid.
Values were performed in triplicates and represented as mean ± SD.
Mean values followed by different superscript in a column are significantly different (p<0.05).

Plant part	Sample concentration (µg/mL)					IC_{50}
	50	100	150	200	250	
In vitro leaf	20±0.20[a]	38±0.48[b]	44±0.32[c]	61.33±0.27[d]	80±0.35[e]	159±0.12[e]
Standards	10	20	30	40	50	IC_{50}
RU	40±0.20[a]	46±0.20[b]	51±0.10[c]	52±0.10[c]	60±0.20[d]	35.71±0.03[b]
QE	48±0.08[a]	60±0.07[b]	69±0.05[c]	79±0.05[c]	89±0.07[d]	24.21±0.12[a]
BHA	37±0.40[a]	50±0.12[b]	67±0.14[c]	76±0.30[d]	90±0.30[e]	25.18±0.02[a]
BHT	17±0.13[a]	24±0.08[b]	33±0.60[c]	42±0.09[d]	54±0.19[e]	45.70±0.24[c]
AT	02±0.30[a]	11±0.30[b]	15±0.15[c]	22±0.50[d]	34±0.30[e]	84.17±0.52[d]

Table 7: DPPH• assay for methanolic leaf extract of Hypochaeris radicata.
RU-Rutin, QE-Quercetin, BHA-Butylated hydroxylanisole and BHT-Butylated hydroxyltoluene, AT-Alfa Tocopheral.
Values were performed in triplicates and represented as mean ± SD.
Mean values followed by different superscript in a column are significantly different (p<0.05).

Induction of multiple shoot:

Fig. 1(a) shows the germination of seed on MS medium devoid of any growth regulators. The *in vitro* cotyledonary leaf explants showed good response to different concentrations of growth regulators. However, no shoot induction was observed on MS medium without any growth regulators even after 6 weeks of culture. The highest 100% response was observed on MS medium containing BAP (2mg/L) alone [Fig. 1(b and c)]. The higher number of 20 shoots per explant was recorded on the medium with the same amount of BAP mentioned above (Table 1). But the greater shoot length (9.54cm) was observed on MS medium fortified with BAP and GA_3 (2+0.5mg/L respectively) and percentage of response was also higher in this prescribed medium (95.63%) (Fig. 1d.). Moreover, medium supplemented with BAP (2mg/L) and Kn (0.5mg/L) and BAP (2mg/L) and IAA (0.5mg/L) showed the production of substantial amount of multiple shoots (92.21% and 90.05%). The production of higher number of 200 shoots from single cotyledonary leaf explant can be achieved with ten subcultures at every 4 week interval.

Root induction:

For the induction of the roots, shoots (2-3cm) were excised and cultured on to the MS medium with different combinations of IAA, IBA and NAA at 3 different concentrations such as 0.3, 0.5, 1.0mg/L.

However, the percentage of shoots forming roots and the number of roots per shoot and length of roots are significantly varied depending on concentration of auxin (Table 2). The higher percentage of rooting (98.48%) with highest number of roots per shoot (9.33) and the highest root length (8.97cm) were observed on MS medium supplemented with 1mg/L NAA [Fig. 2 (a and b)] followed by 1 and 0.5 mg/L IAA (89.88 and 65.10% respectively).

Figure 2: Micropropagation of *Hypochaeris radicata.*
a) Root induction on MS medium containing NAA at 1mg/L.
b) After 4 weeks of culture.
c) *H. radicata* plant was established in soil.

Acclimatization:

Survived rate of plantlets was determined to be higher (75.22%) in the hardening medium composed by garden soil: sand: vermicompost (1:1:1) by volume (Table 3) followed by 65.04% in red soil, sand and vermicompost in the ratio of 1:1:1 by volume.

Preliminary qualitative phytochemical analysis:

Preliminary qualitative phytochemical screening of the *in vitro* methanolic leaf extract of *H. radicata* showed the presence of alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids (Table 4) with high degree of precipitation (+++). However, cardiac glycosides, phenols, resins, saponins and triterpenoids were marked to be absent in this extract.

Quantification of antioxidant compounds:

The calculated percentage yield of *in vitro* methanolic leaf extract of *H. radicata* was 20% (Table 5). The total phenolics and tannin content of extract was determined to be 4.45 and 0.97 mg GAE/100g extract. The total flavonoids content of the extract was 3.48mg RE/100g extract (Table 5).

Determination of *in vitro* antioxidant activities:

Reducing power activity:

Table 6 shows the reducing power of *in vitro* methanolic leaf extract of *H. radicata.* Their reductive ability was increased exponentially with the increase in concentration from 50µg/mL (0.47±0.02 OD at 700nm) to 250µg/mL (0.832±0.10 OD at 700nm).

DPPH· radical scavenging activity:

The DPPH· radical scavenging activity of *in vitro* methanolic leaf extract of *H. radicata* along with the best known natural and synthetic antioxidant standards, *viz.,* rutin, quercetin, BHA, BHT and AT are presented in Table 7. The antioxidant activity was increases with increase in concentration from 50µg/mL (20.02%) to 250µg/mL (80.06%). The IC_{50} value of the extract (159.30µg/mL) was comparable to that of the standard antioxidants.

Trolox equivalent antioxidant capacity assay:

In the evaluation of total antioxidant capacity by $ABTS^{\bullet+}$ method, the *in vitro* methanolic leaf extract was able to quench $ABTS^{\bullet+}$ radicals more effectively (2767.5 µmol Trolox equivalent/g extract).

DISCUSSION

In vitro propagation has been found to be extremely difficult in the members of the family, Asteraceae an account of several constraints such as presence of inhibitors during seed germination, non availability of the elite germplasm and poor germination due to short term viability[21-23]. However, some of the articles have proved *in vitro* regeneration of some Asteraceae members[24-28]. But no work has been made so far on the medicinal plant, *Hypochaeris radicata.*

In the present investigation, *in vitro* regeneration has been achieved successfully by employing cotyledonary leaf explants. When compared to other growth regulators, 100% of the response was obtained only in BAP at 2mg/L. In addition greater number of shoots was also noted in the same medium. It is of common fact that cytokinin is the major growth hormone involved in shoot formation in many plant species[29]. The cytokinin can activate and regulate the expression of genes associated with cell division and differentiation in shoot formation in many plants[30-32]. However, the highest shoot length was achieved in the MS medium fortified with BAP and GA_3 (2+0.5mg/L). Bipasha *et al.*[33] reported that addition of GA_3 in the BAP fortified MS medium evoked shoot elongation of other Asteraceae member, *Wedelia chinensis.* Among the three auxins tested, NAA at 1mg/L was found to be a more suitable hormone for root induction. The root induction was gradually increased with increasing concentration of auxin. However, the lower concentration of NAA less than 1mg/L induced callus and not root formation and no rooting was observed on auxin omitted medium. From the results, NAA is known to be the best root inducing hormone for *H. radicata.* It indicates that the auxin is most essential for rooting attribute. Similar findings have also been reported by Shamima *et al.*[34]. The *in vitro* produced plantlets were transferred to hardening medium which gave 75.22% survival rate. The acclimatized plants showed normal

growth and were morphologically similar to the mother plants.

Preliminary qualitative phytochemical analysis of *in vitro* methanolic leaf extract of *H. radicata* revealed the presence of alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids. These phytochemicals in plants are reported to have various protective and therapeutic properties[35,36]. Thus, the leaf extract of the study species containing phytochemical compounds may serve as a potential source of bioactive compounds for the treatment of various diseases. The quantitative analysis of the extract gives the positive results for total phenolics, tannins and flavonoids. Phenolic compounds are considered to be the most important antioxidants of plant materials[37,38] and so they are known to be the primary antioxidants or free radical terminator[39]. Several studies demonstrated a significant correlation between the phenolic content and the antioxidant activity of the plant extracts[40]. Tannins possess physiological astringent properties which hasten the wound healing activity. They are usually found in large quantities in the bark of trees where they act as a barrier for micro-organisms and protect the tree and they have been reported to have antioxidant[41], antihyperglycemic[42] and insecticidal activity[43]. Flavonoids, the most diverse and widespread group of natural compounds are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity, antidiabetic and antihypertensive[44-46].

The *in vitro* radical scavenging activity results of the study clearly indicates that the *in vitro* methanolic leaf extract of *H. radicata* was potentially active. DPPH• is a stable free radical. It is based on measurement of the scavenging ability of antioxidants towards the stable radical DPPH•. Because of its extra electron, the DPPH• radical gives a strong absorption band at 517nm under visible spectroscopy (a deep purple colour), which vanishes in the presence of a free radical scavenger. DPPH• is usually employed as a reagent to evaluate the free radical scavenging activity of antioxidants. The results of DPPH• scavenging assay indicate that the plant possess high antioxidant activity. It may be due to the presence of phytochemical compounds in plant extracts that were capable of donating hydrogen ions to a free radical scavenger. At higher concentration (250μg/mL) the extract has significantly increased the DPPH• radical scavenging activity. Similar studies were already made by Manickam *et al.*[47].

The reducing power of the *in vitro* methanolic leaf extract of *H. radicata* also showed that its potential antioxidant activity[48]. The presence of reductones could contribute in breaking the free radical chain by donating a hydrogen atom. These reductants (i.e antioxidants) in the extracts apparently reduces the Fe^{3+}/Ferricyanide complex to its Fe^{2+} forms which can be monitored by measuring the formation of Perl's Purssian blue at 700nm. The reducing power increased with the increase in the extract concentration. Hence, the study presumed the extract of *H. radicata* may have high amount of reductones contributing its antioxidant property.

$ABTS^{\bullet+}$ is a blue chromophore produced by the reaction between $ABTS^{\bullet+}$ and potassium persulphate[49]. It has been widely used to measure the antioxidant capacity of natural extracts[50]. In the present investigation, the extract of *H. radicata* leaf showed high TEAC and it neutralizes the radical ion more effectively and also it have high amount of hydrogen donating compounds.

In the present study, an efficient regeneration through cotyledonary leaf explant was achieved by *in vitro* technique. Subculture of the separated individual shoots on the same medium could induce twice the number of multiple shoots without any formation of callus. This *in vitro* propagation is used to supplement the natural stock of plants in wild population. The present protocol will facilitate the propagation and conservation of *H. radicata* in an efficient manner. The results of *in vitro* radical scavenging potential of *H. radicata* were comparable with those of standard compounds. These data further support the view that the extract of this plant are the promising sources of natural antioxidants and could be seen as potential sources of useful drug as well.

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