

1 **Effect of growth regulators on direct shoot formation from leaf explant and antioxidant**
2 **activity of leaf and *in vitro* plants of *Cadaba fruticosa* (Linn.) Druce - an endemic**
3 **medicinal plant**

4 **Abstract**

5 The present study aimed to develop an efficient simple and reproducible *in vitro*
6 propagation technique for an multipurpose endangered medicinal plant *Cadaba fruticosa*(L.)
7 Druce. Multiple shoot formation was observed from the surface sterilized leaf segments of
8 mature plant through direct regeneration on Murashige and Skoog (1962) medium containing
9 13.32 µM benzyl amino purine (BAP), 1.16 µM kinetin (KIN) and 1.35 µM α naphthalene
10 acetic acid (NAA). The *in vitro* regenerated shoots were rooted in MS medium supplemented
11 with NAA (2.68 µM) with 95% response. The plantlets were successfully hardened in the
12 decomposed coir waste and compost combination with 85% survival rate. Antioxidant
13 activity of the leaf and *in vitro* plants through DPPH and FRAP assay confirmed the
14 similarity.

15 **Keywords:***Cadaba fruticosa*, Benzyl amino purine, *in vitro* regeneration, naphthalene acetic
16 acid, DPPH and FRAP.

17 **1. INTRODUCTION**

18 Medicinal plants play an important role in human life to control diseases since time
19 immemorial. The World Health Organization has estimated that up to 80% of people still rely
20 on herbal remedies for their health care [1]. Pharmaceutical industries collect plant materials
21 from wild strands resulting in indiscriminate large-scale exploitation of this natural resources.
22 Moreover no significant endeavor is being made for the cultivation and replenishment of the
23 wild stock. Therefore, now there is an urgency to conserve wild populations for future uses
24 and at the same time, produce enough planting materials by adopting improved and efficient
25 propagation approaches including mass cultivation of these species [2].

26
27 Though India has rich biodiversity and one among the twelve mega diversity centre, the
28 growing demand is putting a heavy strain on the existing resources causing a number of
29 species to be either threatened or endangered category. Global concern about the loss of
30 valuable genetic resources. Within past decade several conservation strategies were
31 developed [3,4] mainly in the terms of *in situ* and *ex situ* conservation.

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Advances in biotechnology, especially *in vitro* culture techniques and molecular biology, provide some important tools for conservation and management of plant genetic resources. Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. Cultivation of medicinal plants is also difficult due to lack of standardized agronomic practices for the most species and unavailable of source plant materials. So *in vitro* regeneration is an efficient means of *ex situ* conservation of plant diversity [5,6] because with this technology many endangered species can be quickly propagated and preserved from a minimum of plant material and with little impact on wild population. Moreover this technique has the unique advantage of propagation of the desired taxon, independent of season, reproductive barriers, germination hurdles and so on [7]. In fact *in vitro* propagation and cryopreservation of medicinal plants help us to conserve biodiversity.

Cadaba fruticosa (L.) Druce or the Indian Cadaba is a shrub, it is a member of the Capparaceae family and commonly known as ‘vizhuthi’ in Tamil and ‘Capper bush’ in English. The shrub is widely distributed in the Indian subcontinent and is commonly used in Siddha medicine in the northern districts of Tamilnadu. A rare plant species *Cadaba fruticosa* has been locally used to treat by the indigenous people since time immemorial and needs immediate attention for conservation. Some of these are believed to promote positive health and maintain organic resistance against infection by re-establishing body equilibrium and conditioning the body tissues. The folk use of plants in medicinal value is an important part of the health care system [8]. The leaf juice is used internally to treat diarrhoea, dysentery and general weakness and also used as an anti-allergic, antidote, antiscorbutic, hypoglycemic and anti-helminthic herbal drug [9,10]. Further leaf extracts possesses antimicrobial activity [11] and used in traditional medicine to treat syphilis and gonorrhea [12], anti-pyretic activity [13], anti-diabetic activity [10]. Literatures survey reveals that there is only one report on tissue culture of *Cadaba fruticosa* through nodal culture [14]. In spite of the high demand and pharmacological importance, there are not any conventional methods known for propagation of *C. fruticosa*. Due to in effective pollination the seed setting is very poor [15]. For the first time we report an efficient, simple and reproducible micropropagation protocol from leaf explant of *Cadaba fruticosa* (L.) Druce.

2. MATERIALS AND METHODS

2. 1. Plant collection and authentication

66 The plants of *Cadaba fruticosa* were collected along with areal part like node and leaves
67 from the foothills of Western Ghats, Coimbatore, Tamilnadu, identified by Botanical Survey
68 of India. Plant identification reference number is BSI/SRC/5/23/2015/Tech/931(Fig 1).

69 2. 2. Plant sterilization and media preparation

70 Leaf parts were collected and washed with Tween 20 (5% v/v) for 5 minutes and then
71 surface sterilized with 70% alcohol for 30 seconds followed by mercuric chloride (0.12 %
72 w/v) solution for 3 minutes. The segments were washed thoroughly with sterile distilled
73 water before cutting into appropriate size explants.

74 The culture medium [16] was fortified with 30g/l sucrose, solidified with 0.8% (w/v) agar.
75 The pH of the medium was adjusted to 5.8 before autoclaving. All the cultures were
76 incubated in sterile culture room at 25±2 °C with 16/8 h photoperiod under white florescent
77 light (60µE²/S irradiance) and with 60 -70 % relative humidity. All the cultures were sub-
78 cultured on the fresh medium after 15 to 20 days. For culture establishment, MS medium
79 were supplemented with various cytokinins, viz., Benzyl amino purine (BAP), alone or with
80 Kinetin (KIN), Thidiazuron (TDZ) and auxin like naphthalene acidic acid (NAA) in
81 combination. *In vitro* raised shoots were excised and transferred to the same medium for
82 rooting.

83 Micropropagated shoots were transferred to MS medium with different concentrations of
84 NAA, IAA and IBA along with activated charcoal (2 g/l). The highest percentage of rooting
85 was observed in MS medium containing NAA (2.68 µM) and activated charcoal (2 g/l). The
86 rooting percentage, (95%) and mean number of roots per shoot (4.55 ± 0.71) and mean root
87 length (5.5 ± 1.28) was recorded on that medium (Table 2).

89 2. 3. Acclimatization

90 The rooted shoots were washed to remove the adhering gel, and planted in net pot, containing
91 various potting material (Fig 2). After 10 days, plantlets were transferred to pots containing
92 organic manure, red soil and forest humus (1:1:1). The survival rate of regenerated plants
93 were recorded one month after transfer to pots. Each experiment was repeated at least three
94 times with 10 replicates for each treatment. Data were analyzed and recorded.

95 2. 4. Extract preparation

A 100 g of *C. fruticosaleaf* and *in vitro* plants were subjected to cold extraction using successive method like low polar solvent to high polar solvent such as petroleum ether, chloroform, ethyl acetate, ethanol and methanol (Each 500 ml). Each plant extracts collected and evaporated in room temperature and finally stored for further study.

2. 5. Antioxidant activity

2. 5. 1. DPPH radical scavenging activity [17]

DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The color change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 517 nm.

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Various concentrations of ethanol extract of the sample (4.0 ml) were mixed with 1.0 ml of methanol solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM. The mixture were shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. Ascorbic acid was used as control. The percentage of inhibition in DPPH radical scavenging activity was calculated as follows;

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

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2. 4. 2. FRAP assay [18]

The total antioxidant potential of sample was determined using ferric reducing ability of plasma FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe II-tripyridyltriazine compound from colorless oxidized Fe III form by the action of electron donating antioxidants.

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The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl₃.6H₂O and 0.3 M acetate buffer (pH 3.6) was prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. Then, 900 µl FRAP reagent was mixed with 90 µl water and 30 µl test sample/methanol/ethanol/standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 593 nm. An intense blue colored complex were formed when ferric tripyridyltriazine (Fe³⁺-TPTZ) complex were reduced to ferrous (Fe²⁺) form. The absorption at 540 nm was recorded. The calibration was plotted with absorbance at 593 nm vs concentration of ferrous sulphate in the

range 0.1 mM both aqueous and ethanol solutions. The concentrations of FeSO₄ were in turn plotted against concentration of standard antioxidants L-ascorbic acid.

3. RESULTS

3.1. Shoot formation

In our experiments, we used young tender green leaf explants obtained from wild plant for efficient shoot regeneration. After ten days the explant began to expand and start producing shoot primordia without callus formation. After 8-9 weeks of culture significant differences were observed among the treatments for percentage of explants forming shoots. Multiple shoot formation was achieved using different concentration and combination of BAP, TDZ, KIN and NAA for leaf explant. The maximum shoot sprouting frequency is 79.2 % was observed on MS basal medium augmented with 13.32 μ M BAP, 1.16 μ M KIN and 1.35 μ M NAA (Table 1; Fig 1). The highest number of shoots per explants in initiation (15.98 $\pm$ 0.13) and in subculture (36.01 $\pm$ 0.14) with a mean shoot length (6.98 $\pm$ 0.34) was obtained in MS medium with BAP (13.32 μ M), KIN (1.16 μ M) and NAA (1.35 μ M) (Table 1 and Fig 1).

Table 1. Effect of BAP, TDZ, NAA and KIN on initiation and multiple shoot induction from leaf explants of *Cadaba fruticosa* cultured on MS medium

S.NO	BAP μ M	TDZ μ M	KIN μ M	μ M NAA	Shoot sprouting frequency %	Shoot No/explant	Shoot No/explants subculture	Shoot length (cm)
1	2.22	-	1.16	1.135	30.17 $\pm$ 0.19	5.04 $\pm$ 0.10	18.96 $\pm$ 0.13	6.05 $\pm$ 0.13
2	4.44	-	1.16	1.135	45.84 $\pm$ 0.25	12.93 $\pm$ 0.10	19.01 $\pm$ 0.10	6.41 $\pm$ 0.10
3	6.66	-	1.16	1.135	43.17 $\pm$ 0.16	9.98 $\pm$ 0.12	22.01 $\pm$ 0.14	6.25 $\pm$ 0.07
4	8.88	-	1.16	1.35	38.92 $\pm$ 0.16	10.03 $\pm$ 0.12	25.05 $\pm$ 0.13	6.28 $\pm$ 0.06
5	11.10	-	1.16	1.35	51.94 $\pm$ 0.11	11.96 $\pm$ 0.13	28.01 $\pm$ 0.11	6.45 $\pm$ 0.09
6	13.32	-	1.16	1.35	68.92 $\pm$ 0.10	15.98 $\pm$ 0.13	36.01 $\pm$ 0.14	6.98 $\pm$ 0.14
1	2.22	-	1.16	-	31.95 $\pm$ 0.12	08.01 $\pm$ 0.11	20.00 $\pm$ 0.15	5.01 $\pm$ 0.14
2	4.44	-	1.16	-	35.16 $\pm$ 0.12	10.10 $\pm$ 0.21	21.05 $\pm$ 0.13	5.03 $\pm$ 0.08
3	6.66	-	1.16	-	58.11 $\pm$ 0.10	11.01 $\pm$ 0.11	19.06 $\pm$ 0.14	4.06 $\pm$ 0.09
4	8.88	-	1.16	-	23.16 $\pm$ 0.11	12.03 $\pm$ 0.17	21.01 $\pm$ 0.14	4.36 $\pm$ 0.08
5	11.10	-	1.16	-	47.09 $\pm$ 0.09	13.78 $\pm$ 0.21	15.98 $\pm$ 0.11	5.18 $\pm$ 0.09
6	13.32	-	1.16	-	36.07 $\pm$ 0.12	14.03 $\pm$ 0.12	29.91 $\pm$ 0.20	4.54 $\pm$ 0.06
1	2.22	1.135	-	-	49.06 $\pm$ 0.14	08.05 $\pm$ 0.13	18.01 $\pm$ 0.11	7.47 $\pm$ 0.12
2	4.44	1.135	-	-	35.08 $\pm$ 0.11	10.12 $\pm$ 0.14	17.05 $\pm$ 0.13	6.47 $\pm$ 0.08
3	6.66	1.135	-	-	42.12 $\pm$ 0.14	11.90 $\pm$ 0.19	20.68 $\pm$ 0.19	6.28 $\pm$ 0.09

4	8.88	1.35	-	-	35.17 ± 0.13	12.13 ± 0.16	21.95 ± 0.11	5.38 ± 0.08
5	11.10	1.35	-	-	28.29 ± 0.13	12.43 ± 0.12	24.98 ± 0.09	5.17 ± 0.07
6	13.32	1.35	-	-	33.18 ± 0.12	13.13 ± 0.14	26.96 ± 0.12	4.85 ± 0.06
Basal medium	-	-	-	-	-	-	-	-

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Figure 1. *In vitro* propagation of *Cadaba fruticosa*

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147 **Figure 1. *In vitro* propagation of *Cadaba fruticosa* - A: Habit with flower. B, C& D:**

148 **Direct shoot initiation from leaf explants on MS medium with BAP (13.32 μM), KIN**

(1.16 μ M) and NAA (1.35 μ M). E&F: Different stages of shoot development on the same medium. G, H& I: Multiplication of shoots after subculture on the same medium.

3. 2. Root formation

In vitro developed shoots obtained from leaf explants were excised and inoculated on MS medium supplemented with different concentration of NAA, IAA and IBA for root induction. The root formation was observed after 10-15 days of inoculation maximum root induction response of 95% was obtained on MS medium supplemented with 2.68 μ M/l NAA and well developed plantlets were observed after 22-24 days (Table 2). The other auxins IAA and IBA also induced roots but not effective like NAA.

S. No	NAA (μ M)	IAA (μ M)	IBA (μ M)	Response %	Mean number of root / shoot	Mean root length (cm)
1.	2.68	-	-	95	4.55 $\pm$ 0.71	5.5 $\pm$ 1.28
2 .	5.36	-	-	80	3.20 $\pm$ 0.58	5.0 $\pm$ 1.39
3 .	8.04	-	-	40	1.70 $\pm$ 0.32	3.0 $\pm$ 0.85
4 .	10.72	-	-	28	1.20 $\pm$ 0.31	2.0 $\pm$ 0.47
5 .	13.40	-	-	19	1.15 $\pm$ 0.28	2.0 $\pm$ 0.56
6..	-	2.86	-	55	3.38 $\pm$ 1.12	4.2 $\pm$ 0.32
7.	-	5.72	-	48	3.19 $\pm$ 0.32	3.5 $\pm$ 2.17
8.	-	8.58	-	41	2.95 $\pm$ 2.14	3.2 $\pm$ 4.29
9.	-	11.44	-	38	2.01 $\pm$ 0.31	2.8 $\pm$ 5.01
10.	-	14.30	-	27	1.63 $\pm$ 3.31	2.3 $\pm$ 0.96
11.	-	-	2.46	53	3.51 $\pm$ 0.41	4.1 $\pm$ 1.56
12.	-	-	4.92	46	3.10 $\pm$ 2.11	3.8 $\pm$ 3.21
13.	-	-	7.38	43	2.37 $\pm$ 0.34	3.5 $\pm$ 1.42
14.	-	-	9.84	39	1.72 $\pm$ 4.21	2.7 $\pm$ 3.16
15.	-	-	12.30	32	1.36 $\pm$ 0.18	1.6 $\pm$ 1.83
16.	Basal medium	-	-	-	-	-

Table 2. Effect of auxins on root formation of *Cadaba fruticosa* in MS medium

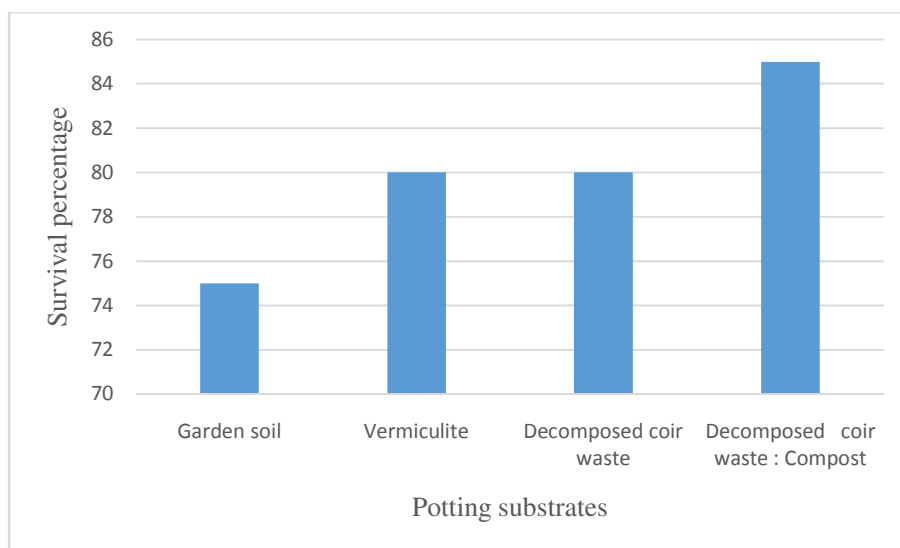
Values are mean $\pm$ SEM of three samples

3. 3. Hardening

The regenerated plantlets of *Cadaba fruticosa* were successfully hardened in net pots containing different potting media. Among the different potting media tried, the percentage of plant survival rate was high (85%) in decomposed coir waste and compost combination (Fig 2).

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Figure2. Acclimatization of *Cadaba fruticosa*



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168 3. 4. Antioxidant

169 The antioxidant activity of *C. fruticosa* *in vivo* leaf and *in vitro* plant ethanol and methanol
 170 extracts were assessed by DPPH and FRAP. Both the extracts had significant scavenging
 171 activity with increasing concentration of extracts from 1.0 mg/ml to 5.0 mg/ml. The data
 172 observed from different concentration of the extracts through DPPH are shown in the table 4.
 173 Highest scavenging activity *in vivo* leaf and *in vitro* plant ethanol and methanol extracts was
 174 58.22 ± 0.23 , 55.34 ± 0.38 , 53.15 ± 0.60 and 52.60 ± 0.27 respectively against the control
 175 (63.55 ± 0.40). The IC_{50} value of the extracts was 4.25 ± 1.27 , 4.13 ± 0.28 , 4.77 ± 2.01 and
 176 4.88 ± 1.16 respectively against the control ascorbic acid ($3.98.99 \pm 0.23$) (Fig 3). The DPPH
 177 scavenging activity was almost equal with *in vitro* plant extracts.

178 **Table 4. Antioxidant activity (DPPH) of *in vivo* leaf and *in vitro* plant ethanol and**
 179 **methanol extracts of *C. fruticosa***

S. No.	Samples	% inhibition				
		1(mg/ml)	2 (mg/ml)	3 (mg/ml)	4 (mg/ml)	5 (mg/ml)
1.	<i>In vivo</i> ethanol extract	11.31 ± 0.75	23.42 ± 0.70	35.16 ± 0.60	47.20 ± 0.30	58.22 ± 0.23
2.	<i>In vitro</i> ethanol extract	10.45 ± 0.48	22.27 ± 0.50	33.36 ± 0.78	45.39 ± 0.27	55.34 ± 0.38
3.	<i>In vivo</i> methanol extract	9.21 ± 0.15	19.40 ± 0.22	27.40 ± 0.50	39.20 ± 0.33	53.15 ± 0.60

4.	<i>In vitro</i> methanol extract	9.32±0.76	20.23±0.83	31.23±0.59	42.38±0.28	52.60±0.27
5.	Standard (Ascorbic acid)	18.20±0.44	27.10±0.32	38.22±0.42	50.12±0.30	63.55±0.40

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The experiment was conducted in triplicates (n=3)

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Ferric reducing power of the *C. fruticosa* extracts are presented in the table 5. Decrease in the

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absorbance was found with increasing concentration of the extract as showed in the table

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5. The absorbance is 0.85±0.20, 0.81±0.39, 0.79±0.30 and 0.77±0.49 at the concentration

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2.5 mg/ml, whereas the standard ascorbic acid absorbance is 0.89±0.30 respectively in *in*

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vivo leaf and *in vitro* plant ethanol and methanol extracts. The absorbance of both the plant

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samples are almost equal.

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Table 5. Antioxidant activity (FRAP) of *in vivo* leaf and *in vitro* plant ethanol and

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methanol extracts of *C. fruticosa*

S. No.	Samples	Absorbance at 593 nm				
		0.5 (mg/ml)	1 (mg/ml)	1.5 (mg/ml)	2 (mg/ml)	2.5 (mg/ml)
1	<i>In vivo</i> ethanol extract	0.38±0.20	0.52±0.33	0.60±0.20	0.72±0.30	0.85±0.20
	<i>In vitro</i> ethanol extract	0.35±0.30	0.45±0.28	0.58±0.40	0.69±0.50	0.81±0.39
2	<i>In vivo</i> methanol extract	0.30±0.40	0.46±0.10	0.55±0.22	0.67±0.22	0.79±0.30
	<i>In vitro</i> methanol extract	0.29±0.30	0.42±0.30	0.53±0.30	0.65±0.38	0.77±0.49
3	Standard (Vitamin C)	0.40±0.10	0.55±0.20	0.64±0.15	0.76±0.55	0.89±0.30

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The experiment was conducted in triplicates (n=3).

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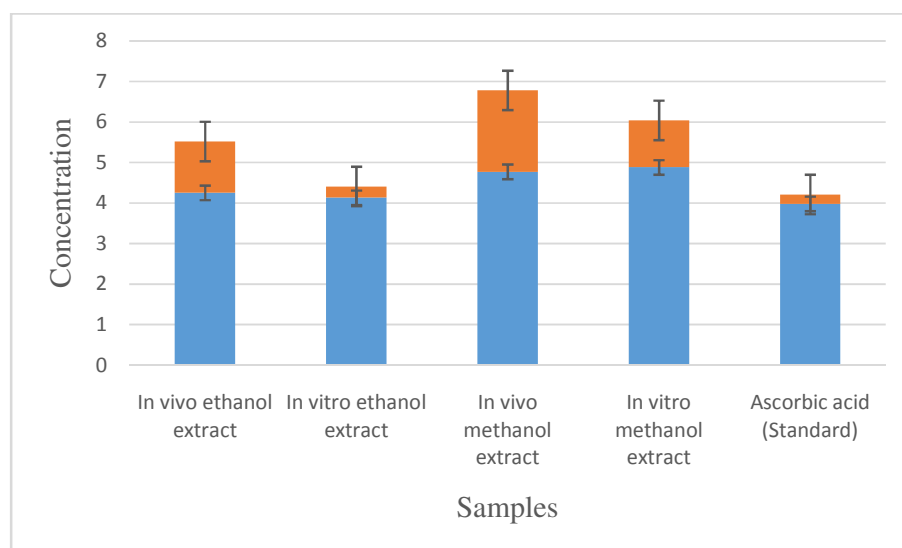
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Figure 3. Inhibition concentration value (IC₅₀ value) of antioxidant assay (DPPH) of *in vivo* leaf and *in vitro* plant ethanol and methanol extracts of *C. fruticosa*



4. DISCUSSION

Plant tissue culture can produce the plantlets with similar valuable natural metabolites like the wild plants. The secondary metabolites obtained from tissue culture plants could be a substitute for the natural metabolites and this will reduce the pressure on the collection of wild plants. In the present study also we compared the antioxidant activity of both natural and tissue cultured plants. The standardization of medium for regeneration of plantlets is a major steps in plant tissue culture. Here, we defined such a system for *C. fruticosa* using different growth regulators, for achieving maximum regeneration from leaf explant without callus formation. The type, concentration and combinations of plant growth regulators also have a strong effect on plantlets induction and multiplication of many medicinal plants [19,20,21,22,23].

This study has presented 68.92% regeneration at BAP (13.32 μ M), KIN (1.16 μ M) and NAA (1.35 μ M), because there is a combination relationship among the BAP, KIN and NAA. The result of shoot sprouting frequency (68.92%) and mean number of shoots per explant (15.98) in initiation and in subculture (36.01) with 6.98 cm shoot length showed that this is the best protocol for *C. fruticosa* regeneration. Because to our knowledge there is no report on direct shoot formation of this plant from leaf explant.

The natural plant metabolites have protective, disease preventive or act as a defense mechanism for plants. The role of antioxidant is to prevent damage to the cell against

chemical reaction involving free radicles[24].The synthetic antioxidant used in food is having carcinogenic potential [25]. This will leads to find out and replace the synthetic food additives with natural plant antioxidant [26]. The DPPH is a model system, which is wildly used to test the plant extract for its scavenging activity [27]. The DPPH radical scavenging activity of the extracts showed concentration depended scavenging activity. The present study results were confirmed by the earlier studies on methanolic leaf extracts of *C. fruticosa*[28]. The extracts of *in vivo* leaf and *in vitro* plants inhibition activity was similar. Antioxidant potential of medicinal plants is measured by reducing power assay [29]. In our study *in vivo* leaf, *in vitro* plant ethanol and methanol extracts at various concentrations showed dose-dependent reducing power. The results agreed with the earlier report on *C. fruticosa* methanolic leaf extract [28] and *C. farinose* ethyl acetate and aqueous fraction[30].

The DPPH and FRAP assay results in this study shows that the *in vivo* leaf and *in vitro* plant ethanol and methanol extracts of *C. fruticosa* are a source of natural antioxidant. Due to the similar activity of the *in vivo* leaf and *in vitro* plant, instead of collecting the wild plant, micro propagated plants can be used for the pharmaceutical purpose. By using the tissue culture plant the conservation of *C. fruticosa* is possible.

5. CONCLUSION

An efficient simple and reproducible micropropagation system of *C. fruticosa* was reported for the first time from leaf explants. The best regeneration was achieved in an appropriate medium MS+ BAP (13.32 μ M), KIN (1.16 μ M), NAA (1.35 μ M). Micropropagated shoots were rooted in medium MS+ NAA (2.26 μ M) and with good survival rate (95%). The present protocol, could be of great value for commercial purpose, conservation and extraction of bio active compounds.The result of antioxidant activity recorded that the ethanol and methanol extracts of both *in vivo* leaf and *in vitro* plant confirmed the similarity. This study provide a basic for isolation and evaluation of bioactive compounds and their efficiency from *in vitro* plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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