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Effect of Growth Regulators on Direct Shoot Formation from Leaf Explant and Antioxidant Activity of *in situ* and *in vitro* Plants of *Cadaba fruticosa* - An Endemic Medicinal Plant

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present study aimed to develop an efficient simple and reproducible *in vitro* propagation technique for an multipurpose endangered medicinal plant *Cadaba fruticosa* (L.) Druce. Multiple shoot formation was observed from the surface sterilized leaf segments of mature plant through direct regeneration on Murashige and Skoog medium containing 13.32 µM benzyl amino purine (BAP), 1.16 µM kinetin (KIN) and 1.35 µM α naphthalene acetic acid (NAA). The *in vitro* regenerated shoots were rooted in MS medium supplemented with NAA (2.68 µM) with 95% response. The plantlets were successfully hardened in the decomposed coir waste and compost

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combination with 85% survival rate. Antioxidant activity of the *in situ* leaf and *in vitro* plants through DPPH and FRAP assay confirmed the similarity.

Keywords: Cadaba fruticosa; benzyl amino purine; in vitro regeneration; naphthalene acetic acid; kinetin; DPPH and FRAP.

1. INTRODUCTION

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

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

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 or small tree, 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

The plants of *Cadaba fruticosa* were collected along with areal part like node and leaves from the foothills of Western Ghats, Coimbatore throughout the year. Tamilnadu, identified by Botanical Survey of India.

Plant identification reference number is BSI/SRC/5/23/2015/Tech/931(Fig. 1A).

2.2 Plant Sterilization and Media Preparation

Leaf parts were collected and washed tap water with Tween 20 (5% v/v) for 5 minutes and then surface sterilized with 70% alcohol for 30 seconds followed by mercuric chloride (0.12 % w/v) solution for 3 minutes. The segments were washed thoroughly with sterile distilled water before cutting into 1 cm length appropriate size explants.

The culture medium [16] was fortified with 30g/l sucrose, solidified with 0.8% (w/v) agar (Hi-Media, Mumbai). The pH of the medium was adjusted to 5.8 before autoclaving. All the cultures were incubated in sterile culture room at 25±2 ℃ with 16/8 h photoperiod under white florescent light (60μE^2/S irradiance) and with 60 - 70 % relative humidity.

2.3 Induction of Multiple Shoots

For culture establishment, MS medium were supplemented with various cytokinins, viz., Benzyl amino purine (BAP 2.22 -13.32 µM), alone or with Kinetin (KIN 1.16 µM), Thidiazuron (TDZ 1.135 µM) and auxin like naphthalene acidic acid (NAA 1.135 µM) in combination. All the cultures were sub-cultured on the fresh medium after 15 to 20 days (All the chemicals used in this experiment was obtained from Hi-Media, Mumbai).

2.4 Induction of Root

Micropropagated shoots were transferred to MS medium with different concentrations of NAA, IAA and IBA along with activated charcoal (2 g/l) for rooting. All the cultures were incubated under the same conditions as during plantlets regeneration. After a month, the percentage of plantlets inducing root, mean number of roots per shoot, mean root length were evaluate (Table 2).

2.5 Acclimatization

The rooted shoots with 5.5 cm length were washed to remove the adhering gel, and 20 planted in net pot, containing various potting material (Table 3). After 10 days, 20 plantlets were transferred to pots containing organic manure, red soil and forest humus (1:1:1). The survival rate of regenerated plants were recorded one month after transfer to pots. Each experiment was repeated at least three times with 10 replicates for each treatment. Data were analyzed and recorded.

2.6 Extract Preparation

A 100 g of *C. fruticosa* leaf and fifty days old *in vitro* plantlets 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.

2.7 Antioxidant Activity

2.7.1 2,2-Diphenyl-1-PicrylhyDrazyl (DPPH) radical scavenging activity

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 [17].

Various concentrations (1 mg/ml to 5 mg/ml**)** of ethanol and methanol extracts of the samples (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;

% Inhibition = A_0- A_1/A_0 X 100 (A_1 – sample value and A_0 – control value)

2.7.2 Ferric Reducing Ability of Plasma (FRAP) assay

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 [18].

The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM$FeCl_3.6H_2O$ 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℃. 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℃ for 30 minutes and the absorbance was recorded at 593 nm. An intense blue colored complex were formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex were reduced to ferrous (Fe^{2+}) 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 $FeSO_4$ 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 68.92 % was observed on MS basal medium augmented with 13.32 µM BAP, 1.16 µM KIN and 1.35 µM NAA (Table 1; Fig 1A, B, C). The highest number of shoots per explants in initiation (15.98±0.13) and in subculture (36.01±0.14) with a mean shoot length (6.98±0.34) was obtained in MS medium with BAP (13.32 µM), KIN (1.16 µM) and NAA (1.35 µM) (Table 1 and Fig 1D,E,F,G,H,I).

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 µM/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.

Fig. 1. *In vitro* propagation of *Cadaba fruticosa* - A: Habit with flower. B, C& D: Direct shoot initiation from leaf explants on MS medium with BAP (13.32 µM), KIN (1.16 µM) and NAA (1.35 µM). E&F: Different stages of shoot development on the same medium. G, H& I: Multiplication of shoots after subculture on the same medium

3.3 Acclimatization

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 (Table 3).

3.4 Antioxidant

The antioxidant activity of *C. fruticosa in situ* leaf and *in vitro* plant ethanol and methanol extracts were assessed by DPPH and FRAP. Both the extracts had significant scavenging activity with increasing concentration of extracts from 1.0 mg/ml to 5.0 mg/ml. The data observed from different concentration of the extracts through DPPH are shown in the Fig. 2. Highest scavenging activity *in situ* leaf and *in vitro* plant ethanol and methanol extracts was 58.22±0.23, 55.34±0.38, 53.15±0.60 and 52.60±0.27

respectively against the control (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 4.88±1.16 respectively against the control ascorbic acid (3.98.99±0.23) (Fig 4). The DPPH scavenging activity was almost equal with *in vitro* plant extracts.

Ferric reducing power of the *C. fruticosa* extracts are presented in the Fig. 3. Decrease in the absorbance was found with increasing concentration of the extract as showed in the Fig. 3.The absorbance is 0.85±0.20, 0.81±0.39, 0.79±0.30 and 0.77±0.49 at the concentration 2.5 mg/ml, whereas the standard ascorbic acid absorbance is 0.89±0.30 respectively in *in situ* leaf and *in vitro* plant ethanol and methanol extracts. The absorbance of both the plant samples are almost equal.

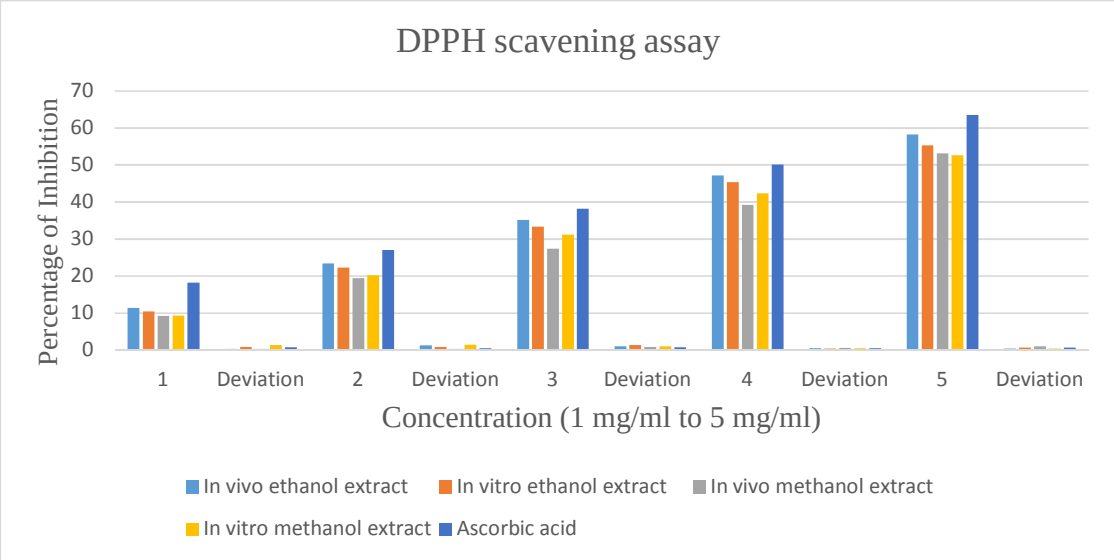

Fig. 2. Antioxidant activity (DPPH) of *in situ* leaf and *in vitro* plant ethanol and methanol extracts of *C. fruticosa*

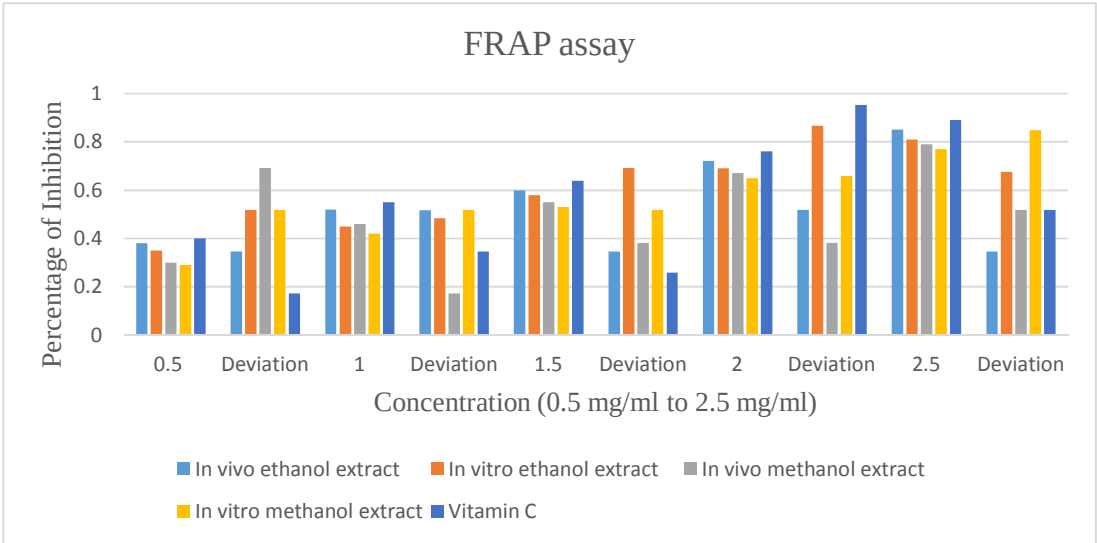

Fig. 3. Antioxidant activity (FRAP) of *in situ* leaf and *in vitro* plant ethanol and methanol extracts of *C. fruticosa*

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

BAP µM	TDZ µM	KIN µM	µM NAA	Mean of Shoot sprouting	Deviation	Mean of Shoot No/explant	Deviation	Mean of Shoot No/explants subculture	Deviation	Mean of Shoot length (cm)	Deviation
2.22	-	1.16	1.135	30.17	0.109	5.04	0.057	18.96	0.075	6.05	0.075
4.44	-	1.16	1.135	45.84	0.114	12.93	0.057	19.01	0.057	6.41	0.057
6.66	-	1.16	1.135	43.17	0.192	9.98	0.069	22.01	0.080	6.25	0.040
8.88	-	1.16	1.135	38.92	0.092	10.03	0.069	25.05	0.075	6.28	0.034
11.10	-	1.16	1.135	51.94	0.063	11.96	0.075	28.01	0.063	6.45	0.051
13.32	-	1.16	1.135	68.92	0.057	15.98	0.075	36.01	0.080	6.98	0.080
2.22	-	1.16	-	31.95	0.069	08.01	0.063	20.00	0.086	5.01	0.080
4.44	-	1.16	-	35.16	0.069	10.10	0.212	21.05	0.075	5.03	0.046
6.66	-	1.16	-	58.11	0.057	11.01	0.063	19.06	0.080	4.06	0.051
8.88	-	1.16	-	23.16	0.063	12.03	0.981	21.01	0.080	4.36	0.046
11.10	-	1.16	-	47.09	0.051	13.78	0.212	15.98	0.063	5.18	0.051
13.32	-	1.16	-	36.07	0.069	14.03	0.069	29.91	0.115	4.54	0.034
2.22	1.135	-	-	49.06	0.080	08.05	0.075	18.01	0.063	7.47	0.069
4.44	1.135	-	-	35.08	0.063	10.12	0.080	17.05	0.075	6.47	0.046
6.66	1.135	-	-	42.12	0.080	11.90	0.109	20.68	0.109	6.28	0.051
8.88	1.35	-	-	35.17	0.075	12.13	0.192	21.95	0.063	5.38	0.046
11.10	1.35	-	-	28.29	0.075	12.43	0.069	24.98	0.051	5.17	0.040
13.32	1.35	-	-	33.18	0.069	13.13	0.080	26.96	0.069	4.85	0.034
Basal medium		-	-	-	-	-	-	-	-	-	-

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

NAA (µM)	IAA (µM)	IBA (µM)	Response %	Mean number of root / shoot	Deviation	Mean root length (cm)	Deviation
2.68	-	-	95	4.55	0.409	5.5	0.739
5.36	-	-	80	3.20	0.334	5.0	0.802
8.04	-	-	40	1.70	0.184	3.0	0.490
10.72	-	-	28	1.20	0.178	2.0	0.271
13.40	-	-	19	1.15	0.616	2.0	0.323
-	2.86	-	55	3.38	0.646	4.2	0.184
-	5.72	-	48	3.19	0.184	3.5	1.252
-	8.58	-	41	2.95	1.235	3.2	2.467
-	11.44	-	38	2.01	0.178	2.8	2.892
-	14.30	-	27	1.63	1.911	2.3	0.554
-	-	2.46	53	3.51	0.236	4.1	0.900
-	-	4.92	46	3.10	1.218	3.8	1.853
-	-	7.38	43	2.37	0.196	3.5	0.819
-	-	9.84	39	1.72	2.430	2.7	1.824
-	-	12.30	32	1.36	0.103	1.6	0.750
Basal medium	-	-	-	-	-	-	-

Table 3. Acclimatization of *Cadaba fruticosa*

Soil combinations	Number of the plant hardening	Deviation	Number of the plant survive	Deviation	Percentage of plant survive	Deviation
Garden soil	20	0.132	15	0.063	75	0.121
Vermiculite	20	0.109	16	0.138	80	0.028
Decomposed coir waste	20	0.623	16	0.051	80	0.103
Decomposed coir waste : Compost	20	0.150	17	0.093	85	0.051

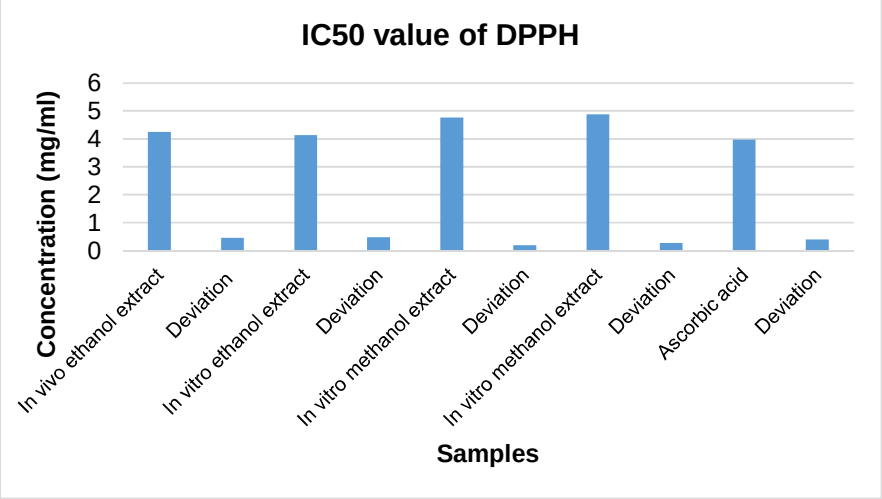

Fig. 4. Inhibition concentration value (IC_{50} value) of antioxidant assay (DPPH) of *in situ* leaf and *in vitro* plant ethanol and methanol extracts of *C. fruticosa*

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 µM), KIN (1.16 µM) and NAA (1.35 µM), 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 situ* 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 situ* 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 situ* 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 situ* 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 µM), KIN (1.16 µM), NAA (1.35 µM). Micropropagated shoots were rooted in medium MS+ NAA (2.28 µM) 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 situ* 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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