

Characterization and bioactivity of β -ecdysterone from cell cultures of *Trianthema decandra* L.

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In the present study, cell cultures from the nodal explants of *Trianthema decandra* L. were established on Murashige and Skoog's (MS) medium supplemented with different concentrations (0.5, 1, 1.5, and 2 mg/L) of indole 3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn). Induction of callus was promising in MS medium supplemented with 1.5 mg/L 2,4-D after 25 days of inoculation. Their total phenolics and flavonoids levels were estimated and compared with those of *in vivo*. Antimicrobial (agar well diffusion method) and antioxidant (DPPH and FRAP methods) activities were studied. Further, six-weeks-old callus grown on MS medium with 2,4-D (1.5 mg/L) were harvested, dried and extracted using absolute methanol. Methanolic extracts of callus showed maximum inhibition against *B. subtilis*, *P. aeruginosa*, and *A. flavus* (IZ 14.33 \pm 0.32 mm, 15.66 \pm 0.32 mm and 13.33 \pm 0.65 mm, respectively) as compared to *in vivo* plant extract. Similarly, in the case of DPPH activity, methanolic extract of the callus demonstrated more antioxidant potential than *in vivo*. At 0.8 mg/mL concentration of callus extract showed inhibition of 85.02% as compared to 83.38% of plants grown *in vivo*. Likewise, in the case of the FRAP method, there is no significant difference in antioxidant potential (303 \pm 3.37 ascorbic acid equivalent/ mg in callus; AAE/mg) as compared to *in vivo* plant (310 \pm 10.01 AAE/mg). A new compound β -ecdysterone (20-hydroxyecdysone), was isolated and identified from the callus of this plant, which was also tested for its antimicrobial and antioxidant activity. Isolated pure compound β -ecdysterone were tested against selected strains of bacteria and fungi and prove effective *E. aerogenes* and *K. pneumoniae* with Minimum inhibitory concentration of 250 μ g/mL concentration (IZ 11.00 \pm 0.00 mm and 12.00 \pm 0.00). compound does not impressive for antifungal profile.

Keywords: Antimicrobial, Antioxidant, Phytochemical analysis, *Trianthema decandra*

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Introduction

Trianthema decandra L. is a short struggling herb and is used in migraine, orchitis, headache, hepatitis, fever, toothache, inflammation of testicles, asthma, suppression of menses, fertility, strengthening of muscles, and aspirant¹⁻³. Leaves are used for jaundice and skin diseases, and juice is dropped in the nostrils to relieve one-sided headache^{4,6}.

So far, roots of this plant species have been investigated for antioxidant, hepatoprotective, cancer chemopreventive and antibacterial activities⁷⁻⁹. Phytochemically, from *T. portulacastrum* trianthenol, flavonoids, leptorumol, 5-hydroxy-2-methoxy benzaldehyde, 3-acetyl aleuritic acid and *p*-methoxy benzoic acid¹⁰⁻¹¹ have been isolated. Several medicinal herbs, which contain ecdysteroids, have been used for several centuries in various herbal traditions. Up to 2001, about 6% of the plant species

have been found to contain 1–2% (w/w) ecdysteroids (on a dry plant weight basis). A review of the literature suggests that ecdysterone may have several therapeutically active properties viz. anabolic¹², immunostimulant¹³, stimulation of carbohydrate metabolism¹⁴, normalisation of lipid biosynthesis and renal function¹⁵, hepatoprotection¹⁶, antioxidant, antiarrhythmic, cardiovascular protection¹⁷, antifertility¹⁸, and antibacterial¹⁹.

In the present study, we report the successful establishment of cell cultures of *T. decandra* and evaluation of its chemical constituents and bioefficacies, viz. antimicrobial and antioxidant activity and compared with *in vivo*. Ecdysterone has several biological properties and it is used in gym supplement to gain muscle power. To make it cost effective and safer alternate to chemical derivatives plant tissue culture experiment have been conducted and the isolated product have been tested against market purchased synthetic ecdysterone.

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Materials and Methods

Collection of plant material

Plants of *T. decandra* were collected from the fields of the University itself in July 2021. The botanical identity was confirmed by the herbarium, Botany Department, University of Rajasthan, Jaipur, India. A voucher specimen (No. 110) was deposited at the herbarium and another in the laboratory for further reference.

Callus regeneration

Young sterilized nodal explants were inoculated on Murashige and Skoog's medium²⁰ with 3% (w/v) sucrose, 0.8% (w/v) agar (HiMedia, India) and singly supplemented with specific concentrations (0.5, 1, 1.5, and 2 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D), indole 3-acetic acid (IAA) and kinetin (kn). After adding the growth regulators, the pH of the medium was adjusted to 5.8±0.2 and autoclaved (121°C) at 15 psi for 20 min. Inoculated media were maintained in the culture room at 25±2°C at fluorescent light of 300-400 lux for 16 hours of photoperiod. The callus was regularly maintained by repeated sub-culturing (6–8 weeks).

Preparation of callus extracts

Six-week-old callus grown on MS medium with 2,4-D (1.5 mg/L) was used for the study. Accurately, 50 g of callus and *in vivo* plants were collected, shade dried (at 60°C), powdered and extracted in Soxhlet apparatus with 100 mL of methanol (3 times x 8 h). Each extract was filtered through Whatman No. 1 filter paper, dried in a vacuum, weighed and stored at 4°C for further studies.

Isolation and identification of compound

Thin layer chromatography (TLC) of both the extracts (*in vivo* and *in vitro* extracts) along with related markers were carried out on a silica G plate (0.4-0.5 mm) using ethyl acetate: methanol (8.7:1.3) as solvent system and sprayed with vanillin-sulfuric acid reagent. Spots coinciding with the reference marker (β -ecdysterone) from the extract were scrapped from unsprayed plates, eluted with methanol, filtered, evaporated to dryness, reconstituted and crystallised. The melting point of the isolated compound was determined in capillary tubes (Toshniwal melting point apparatus) and subjected to IR and NMR spectral studies.

Using the colourimetric method, the quantification of ecdysterone was carried out²¹. Standard β -

ecdysterone (1 - 5 mg/mL; purchased from Wuhan Senwayer Century Chemical Co. Ltd, China) dissolved in 5 mL of methanol, followed by the addition of 5 mL of 1% (w/v) vanillin reagent + 1 mL of 25% sulfuric acid, kept at 60°C for 60 min, allowed to cool and the optical density (OD) was read at 518 nm (Pharmaspec UV-Vis spectrophotometer by Shimadzu). The results were expressed as mg of β -ecdysterone/g dw callus. All determinations were carried out in triplicates and statistically analysed (Mean±S.E.).

Total phenolics contents

Total phenolic contents were determined using the Folin-Ciocalteu method²². A standard calibration curve of gallic acid (10-500 mg/mL) was prepared, and OD was measured at 750 nm. A similar procedure was repeated with 1 g of extract, and total phenolics in the extract were expressed in mg of gallic acid equivalents (mg GAE/g dw of extract). All determinations were carried out in triplicate and statistically analysed.

Total flavonoids contents

Total flavonoids were estimated by AlCl₃ spectrophotometric method²³. A standard quercetin solution (10-100 mg/mL) was prepared. The total flavonoids were expressed as mg of quercetin equivalents (mg QE/g dw) and statistically analysed.

Antioxidant activity

DPPH method

Oxidation of DPPH radical was determined for estimation of antioxidant potentials²⁴. Different concentrations of extract (0.8, 0.6, 0.4, 0.2, 0.1 mg/mL) were prepared in methanol and mixed with 2.5 mL of DPPH (2 mg/10 mL methanol). After 30 min of incubation, OD was measured at 517 nm using a UV-Vis spectrophotometer (Varian type Cary PCB 150 Water Peltier System). Data was recorded in triplicate and processed using EXCEL. The concentrations that caused a 50% reduction in absorbance (IC₅₀) were evaluated. Per cent inhibition of DPPH was calculated by the following equation:

$$\% \text{ Inhibition} = 1 - (\text{OD}_{\text{Sample}} / \text{OD}_{\text{Control}}) \times 100$$

where OD_{Sample} is the absorbance of the test sample, and OD_{Control} is the absorbance of the test control.

Ferric ion reducing antioxidant potentials method

The total reducing power of extracts was determined according to the FRAP (Ferric ion

reducing antioxidant potentials) method²⁵. A specific concentration of the standard (ascorbic acid) and extract (62.5-1000 µg/mL) was prepared in 1.0 mL absolute ethanol separately, mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 % (w/v)). After incubation for 20 min at 50°C, 2.5 mL of 10 % (w/v) trichloroacetic acid, 2.5 mL of distilled water and 0.5 mL of 1.0 % (w/v) ferric chloride (0.5 mL, 1%; chromogenic reagent) were added in the sequence, and absorbance was measured at 700 nm. A standard calibration curve of ascorbic acid was prepared using 10-500 mg/l concentrations, and total antioxidant potentials were calculated in mg of ascorbic acid equivalents (mg AAE/g of extract). All determinations were carried out in triplicates and statistically analysed.

Antimicrobial activity

For antibacterial screening, pure cultures of test bacteria, *Bacillus subtilis* (MTCC 441), *Enterobacter aerogenes* (MTCC 111), *Escherichia coli* (MTCC 443), *K. pneumonia* (MTCC 412), *Pseudomonas aeruginosa* (MTCC 741), *Raoultella planticola* (MTCC 530) and *Staphylococcus aureus* (MTCC 740) obtained from the IMTECH, Chandigarh, India and for antifungal screening *Aspergillus flavus* (ATCC 16870), *A. niger* (ATCC 322), *Candida albicans* (ATCC 4718), *Penicillium chrysogenum* (ATCC 5476) and *Tricophyton rubrum* (ATCC 2327) obtained from IARI, New Delhi, India were used. The antimicrobial assay was performed by agar well diffusion method²⁶. Inoculums were prepared by suspending bacteria in nutrient broth and fungus in Sobouraud dextrose broth (SDB) medium overnight to attain a cell count of 10^6 - 10^7 CFU/mL concentration. Bacterial and fungal suspensions were inoculated in Müller-Hinton agar and Sobouraud's Dextrose agar poured plates, respectively, along with the test extracts. These plates were incubated at 37°C

for bacteria and 25°C in the case of fungi for appropriate time under aerobic conditions. The diameter of the inhibition zone around each well was measured and recorded by an inhibition zone recorder (HiMedia, India).

Disc diffusion method²⁷ was used for the determination of MIC of isolated compound. Serial dilutions of the extracts ranging from 2000 µg to 62.5 µg were prepared and administered in previously inoculated plates. Both experiments were carried out in triplicates and statistically analysed. Gentamycin (10 mcg/disc) and ketoconazole (10 mcg/disc) were used as positive controls (antibacterial for antifungal activity, respectively).

Results and Discussion

Tissue culture studies

Trianthema decandra L. is a short perineal herb species and only grow in rainy season, so to extract active ingredient in continuous supply, tissue culture experiments were carried out. To Out of all the concentrations of growth hormones used, the growth of the callus was appreciable in the MS medium supplemented with 2,4-D (1.5 mg/L). The callus was green and friable in nature in MS supplemented with Kn. In contrast, it is brown and compact in MS supplemented with IAA and 2,4-D (Fig. 1). In general, the callus was rhizogenic in nature (Table 1). The presence of β-ecdysterone (20-hydroxyecdysone) (Fig. 2) in the six-weeks-old callus was established based on m.p. (241–242°C), IR spectra (ν , cm⁻¹: 3420, 1723, 1650, 2968, 1381, 1446, 1289, 1144, 1056, 879) and ¹H NMR spectral data (δ , ppm; κ_{cc} , Hz; $W/2$, Hz): 3.87 (m, 22.3, 2-Ha), 4.02 (bs, 9, 3-He), 2.43 (dd, 13.1, 4.2, 5-H), 5.86 (d, 2.5, 7-H), 2.89 (bt, 22.0, 9-Ha), 2.34 (bt, 8.8, 17-H), 3.45 (m, 22-H), 0.86 (s, 18-Me), 0.98 (s, 19-Me), 1.21 (s, 21-Me), 1.24 (s, 26-Me), 1.25 (s, 27-Me).

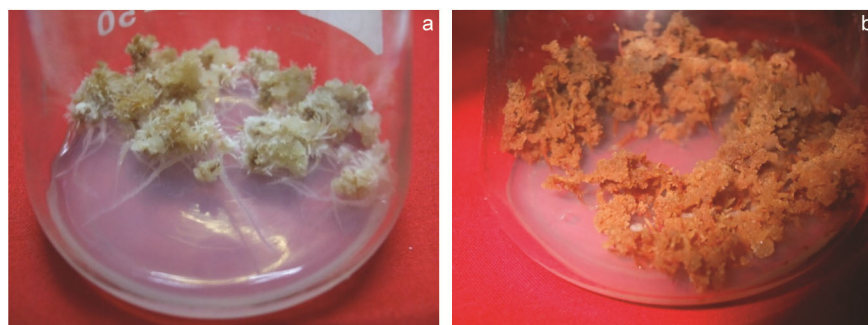


Fig. 1 — a) Four-week-old callus in MS supplemented with Kn; and b) 2,4-D and IAA.

On quantification, the maximum yield of β -ecdysterone was found in a six-weeks-old callus grown on MS supplemented with Kn (2.8 mg/g dw of callus) followed by 2,4-D (2.4 mg/g dw) and IAA (2.1 mg/g dw; Table 2).

Total Phenolic and flavonoid contents

Total phenolics were higher in the whole plant (64.66 ± 3.21 mg GAE/g dw), and total flavonoids were higher in callus extract (31.5 ± 3.24 mg QE/g dw). In the case of antioxidant activity, callus

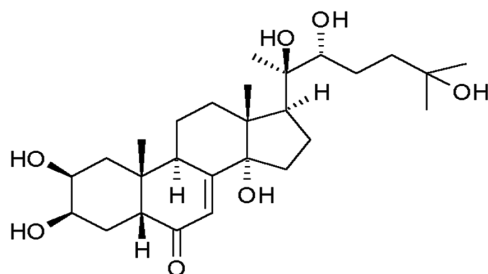


Fig. 2 — Structure of 20-hydroxyecdysterone.

demonstrated more antioxidant potential than that of *in vivo* plants. In DPPH activity at 0.8 mg/mL concentration IC_{50} value was found to be 0.065 in the callus as compared to *in vivo* plant (0.075; standard quercetin - 0.06; Table 3), whereas in FRAP method, there is no significant difference in antioxidant potential in callus as compared to *in vivo* plant (303 ± 3.37 mg AAE/g dw in callus with reference to 310 ± 10.01 mg AAE/g dw in whole plant; Table 4).

Antioxidant activity

β -Ecdysterone demonstrated moderate antioxidant activity with 75.03% inhibition at 0.8 mg/mL concentration with an IC_{50} value of 0.27 mg/mL. In the FRAP method, 1000 μ g/mL concentration demonstrated 33.33 ± 0.88 AAE /mg antioxidant potential.

Antimicrobial activity

The results of the antimicrobial activity of callus and the whole plant of *T. decandra* against human pathogenic bacteria are presented in Table 5. From the results, it is evident that in the

Table 1 — Callus regeneration from nodal explants in *T. decandra*

MS medium supplemented with	Concentration (mg/l)	% Response	Callus growth	Texture	Colour	Response
2,4-D	0.5	30 ± 8.4	+	CM	BN	Rhizogenic
	1	45 ± 7.9	++	''	''	''
	1.5	80 ± 19.8	++++	''	''	''
	2	60 ± 1.32	+	''	''	''
IAA	0.5	30 ± 8.7	+	CM	BN	Rhizogenic
	1	33 ± 3.9	+	''	BN	''
	1.5	40 ± 8.33	+	''	BN-GN	''
	2	20 ± 6.34	+	''	BN-GN	''
Kinetin	0.5	20 ± 9.01	+	FR	GN	Rhizogenic
	1	20 ± 8.4	+	''	''	''
	1.5	45 ± 11.04	++	''	''	''
	2	60 ± 2.11	++	''	''	''

Evaluation after 8 week of culture initiation. +, Low; ++, Moderate; +++, High; +++, Intense. FR., Friable; CM, Compact; GY, Grey; BN, Brown

Table 2 — Effect of auxins and cytokinin on growth of *T. decandra* callus and ecdysterone production

MS Medium supplemented with (1.5 mg/l)	Growth Index (6 weeks age)	% Ecdysterone in extract
2, 4-D	3.00 ± 1.23	0.24
IAA	2.27 ± 2.11	0.21
Kinetin	2.08 ± 0.89	0.28

Growth index = Final wt. of callus - Initial wt. of callus/Initial wt. of callus.

All experiments were performed in 5 multiples.

Table 3 — Total phenolics, total flavonoids and antioxidant activity by DPPH method.

Nature of extracts	Total phenolics (mg GAE/g dw) ^a	Total flavonoids (mg QE/g dw) ^b	% Inhibition (mg/mL) ^d					
			IC_{50}	0.1	0.2	0.4	0.6	0.8
Callus	73.75 ± 0.72	28.33 ± 3.22	0.065	67.66 ± 0.11	70.55 ± 2.43	73.26 ± 1.11	81.72 ± 3.13	83.38 ± 2.01
<i>In vivo</i>	99.76 ± 1.60	31.50 ± 3.24	0.075	76.02 ± 6.13	78.76 ± 0.19	81.85 ± 2.18	85.66 ± 0.12	85.02 ± 0.19
β -Ecdysterone	-	-	0.2	44.02 ± 3.22	44.07 ± 1.13	58.94 ± 8.01	62.95 ± 1.13	75.03 ± 1.34
Quercetin	-	-	0.06	64.42 ± 3.24	80.58 ± 5.12	93.38 ± 9.32	93.82 ± 4.16	94.71 ± 3.14
Ascorbic acid	-	-	-	-	-	-	-	-

^aGAE/g = Gallic acid equivalents/g of extract; ^bQE/g = Quercetin equivalents/g of extract;

^d% Inhibition = $1 - (\text{Absorbance of the sample} / \text{Absorbance of the control}) \times 100$.

Table 4 — Antioxidant activity by FRAP method

Nature of extracts	Antioxidant activity in AAE/g dw ^c (concentration in µg/mL)				
	62.5	125	250	500	1000
Callus	220.78±0.00	273.56±3.34	276.06±3.37	286.67±3.37	310.71±10.01
<i>In vivo</i>	250.65±3.37	270.09±0.00	260.32±0.00	266.19±3.37	303.07±3.37
β-Ecdysterone	23.03±0.00	25.64±0.66	24.07±0.00	25.37±0.32	33.39±0.88
Quercetin	-	-	-	-	-
Ascorbic acid	62.5±0.00	250±0.00	250±0.00	500±0.0	1000±0.00

^cAAE/g = Ascorbic acid equivalent/ mg extract;

Table 5 — Antimicrobial activity of *T. decandra* callus and *in vivo* plant extracts

Microorganisms	Collection No.	Antimicrobial activity		
		^a Callus	^a <i>In vivo</i> plant	
A. Bacteria				
<i>B. subtilis</i>	MTCC 441	^b IZ	14.33±0.32	10.00±0.00
		^c AI	0.65	0.45
<i>E. aerogenes</i>	MTCC 111	IZ	15.66±0.32	10.33±0.32
		AI	0.78	0.73
<i>E. coli</i>	MTCC 443	IZ	12.66±0.65	12.33±0.32
		AI	0.66	0.64
<i>K. pneumoniae</i>	MTCC 109	IZ	11.66±0.66	9.66±0.66
		AI	0.58	0.48
<i>P. aeruginosa</i>	MTCC 741	IZ	15.66±0.32	11.33±0.32
		AI	0.78	0.56
<i>R. planticola</i>	MTCC 530	IZ	-	12.00±0.57
		AI	-	0.54
<i>S. aureus</i>	MTCC 740	IZ	16.00±0.00	14.00±0.00
		AI	0.76	0.66
B. Fungi				
<i>A. flavus</i>	ATCC 16870	IZ	13.33±0.65	12.33±0.32
		AI	0.48	0.45
<i>A. niger</i>	ATCC 322	IZ	12.00±0.00	10.66±0.66
		AI	0.44	0.39
<i>C. albicans</i>	ATCC 4718	IZ	9.33±0.66	-
		AI	0.44	-
<i>P. chrysogenum</i>	ATCC 5476	IZ	12.00±0.57	10.66±0.65
		AI	0.57	0.50
<i>T. rubrum</i>	ATCC 2327	IZ	10.66±0.65	11.66±0.32
		AI	0.36	0.40

^aTest samples: 4 mg/well.

^bIZ = Inhibition zone (in mm) including the diameter of the well (6 mm);

^cAI = Activity index= Inhibition zone of the sample/Inhibition zone of the standard.

Gram-Positive group, the methanolic extract of callus better inhibition activity was recorded against *B. subtilis* (IZ 14.33±0.32 mm) as compared to *in vivo* plant extract. However, in Gram-negative bacteria, appreciable activity was demonstrated against *P. aeruginosa* (15.66±0.32 mm) compared to *in vivo* plants (IZ 11.33±0.32 mm). Maximum antifungal inhibitory activity was demonstrated by the callus extract against *A. flavus* (IZ 13.33±0.65 mm) as compared to *in vivo* plant extract (12.33±0.32 mm).

It is noteworthy that β-ecdysterone demonstrated moderate activity against the selected microbes. No activity was found against *R. planticola*, whereas it showed appreciable inhibitory against *E. aerogenes*

and *K. pneumoniae* (MIC 250 µg/mL in both cases, Table 6).

Ecdysteroids were reported with potent antimicrobial activity in several antibiotic resistant microorganism such as *Staphylococcus aureus*, *Escherichia coli*, *Serratia* sp., *Klebsiella pneumoniae*, and *Candida albicans*²⁸⁻³¹. Several plant extract with different forms of Ecdysteroids inhibit the growth of bacteria and fungal species, causes in unknown but it seems they produce reactive oxygen species (as act as neuroprotective and anti-inflammatory agents) inhibits the growth and proliferation on pathogens. Ecdysteroids considered as natural antioxidant as they prevent and delay both collagenase-related

Table 6 — Antimicrobial activity of the isolated compound on selected microbe

β-Ecdysterone (µg/mL)	Zone of microbial suppression (in mm)					
	<i>E. aerogenes</i>	<i>K. pneumoniae</i>	<i>T. rubrum</i>	<i>A. flavus</i>	<i>A. niger</i>	<i>P. chrysogenum</i>
1000	11.00±0.00	12.00±0.00	11.66±0.6	9.00±0.00	12.66±0.66	12.00±0.00
500	10.00±0.00	10.66±0.32	-	-	10.66±0.66	11.00±0.57
250	9.33±0.33	9.33±0.33	-	-	-	-
125	-	-	-	-	-	-
MIC	250	250	1000	1000	500	500

MIC = Minimum inhibitory concentration in µg/mL

skin damages and oxidative stress. They release thromboxane A₂ (TXA₂), endothelin (ET), malondialdehyde (MDA), and cyclooxygenase-2 (COX-2) as well as promote the activities of endothelial nitric oxide synthase (eNOS) and superoxide dismutase (SOD)^{32,33}.

As the plant extract as well as active ingredient β-ecdysterone both exhibited potent antimicrobial and antioxidant properties and establishment of tissue culture will help in continues supply of active ingredient through the year in equal quantity and same quality.

Conclusion

In this study, callus cultures of *T. decandra* retain the potential to biosynthesise β-ecdysterone and other bioactive substances having antibiotic and antioxidant activities. Furthermore, maximum growth of callus was recorded in 2,4-D supplemented MS medium while the maximum yield of ecdysterone was in MS medium with kinetin. β-Ecdysterone also demonstrated appreciable antimicrobial and antioxidant activities. The presence of a biologically important phytoecdysteroid, β-ecdysterone, in the callus also raises its importance as a natural remedy for common diseases. It thus justifies the use of this plant in traditional medicine.

Conflict of interest

The authors declare no conflict of interest.

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