

Original Article

A COMPARATIVE STUDY OF THE PHYTOCHEMICALS, ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF METHANOLIC EXTRACTS OF *TRICHOSANTHES CUCUMERINA* (L.) VAR. *CUCUMERINA* UNDER *IN VITRO* CULTURE AND NATURAL CONDITIONS

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ABSTRACT

Objective: To compare the phytochemicals, antioxidative capacity and antibacterial profile of methanolic extracts of callus and naturally propagated plant species-*Trichosanthes cucumerina* (L.) var. *cucumerina* and to optimize an ideal protocol for *in vitro* callus and shoot induction.

Methods: The sterilized seeds of *Trichosanthes cucumerina* (L.) var. *cucumerina* were inoculated in half Murashige and Skoog (MS) basal medium devoid of growth hormones to raise aseptic seedlings. Explants from aseptic seedlings used for callus induction in MS medium fortified with varying combinations of N⁶-Benzyl amino purine (BAP), 1-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D). For *in vitro* shoot induction, MS medium supplemented with different concentrations of 2,4-D, BAP and Kinetin-either alone or in combinations were employed. The callus harvested on 21st and 45th days were analyzed for a comparison of the influence of age of callus on the quantity of secondary metabolites. For a comparison with the naturally grown plant, all experiments were carried out with extracts from callus and wild plants. The antioxidant capacity of methanolic extracts was evaluated by 2,2-Diphenyl-1 Picryl Hydrazyl (DPPH) free radical scavenging assay and Ferric Reducing Antioxidant Power (FRAP) analysis. The antibacterial activity of were screened by the agar diffusion method using pathogenic bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and documented through measurement of the diameter of growth inhibition zone (IZ).

Results: The results on *in vitro* culture indicated that MS medium with BAP (0.5 mg/l) and 2,4-D (1 mg/l) was ideal for callus induction. For shoot induction, supplementation of MS medium with BAP-0.5 mg/l, 2,4-D-1.0 mg/l and Kinetin-0.5 mg/l was found to be most favourable. Direct root induction from the callus was found to occur in medium fortified with BAP-0.5 mg/l, 2,4, D-0.5 mg/l and NAA-1.0 mg/l. The phytoconstituents quantified were alkaloids, flavonoids, tannins, phenols and terpenoids and their levels were higher in wild plant in comparison to callus. Naturally grown plant possesses higher free radical scavenging ability and ferric reducing power than callus. Results of antibacterial activity indicated that the Gram-positive strain (*Staphylococcus aureus*) was more sensitive than the Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The highest antibacterial activity recorded for naturally propagated plant extract against *S. aureus* (IZ = 13 mm) and was quite comparable with standard antibiotic cephaloxim (IZ = 20 mm) at 100µg concentration.

Conclusion: Results concluded that this overexploited medicinal plant with lesser seed longevity could be successfully propagated by *in vitro* methods. The phytoconstituents with antioxidative and antibacterial potential were more abundant in naturally propagated plants than undifferentiated callus tissue. The extracts are potent antibacterial agents.

Keywords: *Trichosanthes cucumerina*, *In vitro* culture, Antioxidant, Antibacterial, DPPH, FRAP

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INTRODUCTION

The importance of herbal medicines in the treatment of acute and chronic ailments and their relevance as a safe and stable device is being highlighted in the present scenario [1] *Trichosanthes cucumerina* (L.) var. *cucumerina*, selected for the present study has a prominent place in alternative systems of medicine like Ayurveda and Siddha due to its various pharmacological activities like antidiabetic, hepatoprotective, cytotoxic, anti-inflammatory and larvicidal effects [2, 3].

All parts of this medicinal herb are extremely bitter and therefore the fruits are not consumed as a vegetable. Owing to bitterness, this species is commonly called "bitter snake gourd" This species is used exclusively as a medicine and is cultivated on a large scale by the tribals and stakeholders in eco-development committee (EDCs) under the Department of Forest and Wildlife in Kerala. Annual production of this plant by the EDCs of Kanthalloor and Marayoor (Idukki District) amounts to the range of 35-45 tonnes, for meeting the demand of the dried plant material by "Oushadhi" and "Kottakkal arya vaidya pharmacy" for the preparation of Ayurvedic formulations. Traditional literature like Charak Samhita mentions the protective role of the edible species *Trichosanthes* on important body organs like liver, spleen etc, many of which are now scientifically proven. Clinical investigation on peptic ulcer with the polyherbal formulation, where species of *Trichosanthes* is an integral part, has shown promising

results. The genus is characterized by the abundance of an array of secondary metabolites like flavonoids, carotenoids, phenolics which render the plant pharmacologically and therapeutically active [4].

Generally, these secondary metabolites impart antioxidant property to medicinal plants and render them as natural agents to protect our body from the oxidative damage of biomolecules. Though free radicals can be effectively scavenged by synthetic antioxidant agents, natural antioxidants can always be considered as a better option in view of their limited side effects and higher safety [5, 6].

The increasing demand of this medicinal plant in large quantities for preparation of herbal medicines as well as the occasional failure of seed propagation due to lower seed longevity necessitates its mass multiplication. The present investigation was carried out to characterize the level of phytoconstituents, antioxidative potential and antimicrobial profile in samples from cultivated plants and under *in vitro* conditions. Attempts were carried out to establish an ideal protocol for callus and shoot induction to explore the possibility of mass propagation.

MATERIALS AND METHODS

Chemicals and reagents: source

MS medium, Nutrient agar, Nutrient broth, N⁶-Benzyl amino purine (BAP), 1-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy

acetic acid (2,4-D), Kinetin, DPPH (2,2-Diphenyl-1 Picryl Hydrazyl)-HiMedia Laboratories Pvt. Ltd. Mumbai.

Mercuric chloride, acetic acid, trichloroacetic acid, hydrochloric acid, sulfuric acid, ferric chloride, sodium carbonate, potassium ferricyanide, potassium ferrocyanide-Sisco research Laboratory Pvt. Ltd. Mumbai.

Methanol, chloroform, Folin's reagent, magnesium turnings, aluminium chloride, gallic acid, tannic acid, ascorbic acid-sd fine chemicals Ltd. Mumbai.

Sodium nitrite, sodium hydroxide, sodium hydrogen phosphate (mono and dibasic)-Qualigens fine chemicals, Mumbai

In vitro culture-establishment of aseptic seedlings as explant source

The seeds were washed in running tap water for few min and then washed repeatedly in double distilled water. Under aseptic conditions, the seed coats were removed and the dehusked seeds surface sterilized with 70% ethanol for one min and then rinsed 3-5 times in sterile double distilled water followed by a seven min treatment with 0.1% (w/v) mercuric chloride and rinsed 5 times in sterile double distilled water. The sterilized seeds were then inoculated in ½ MS basal medium. About 6-10 seeds were cultured per bottle and five replicates were taken for each combination of growth regulators in 50 ml of the medium for raising *in vitro* sterile seedlings as explant source.

Callus induction

Different explants including cotyledon, epicotyl and hypocotyl were excised from the *in vitro* raised seedlings (fig. 1A-B) on 14th day and inoculated in MS media supplemented with plant growth regulators in order to study the *in vitro* response and callus formation. Segments of each type of explants were inoculated in MS medium supplemented with different concentrations and combinations of BAP (0.5 mg/l), NAA (1 mg/l), 2,4 D (0.5-2 mg/l) for callus induction. Six different combinations of the three growth regulators were compared for their frequency of callus formation. After inoculation, the culture bottles were sealed properly and placed in the culture room maintained at 25±2 °C with 2500 lux of light intensity provided by cool, white fluorescent tubes.

Subculture of callus and nodal explants for shoot induction

Callus formed were sub-cultured after 20-25 d of inoculation. Sub-culturing was carried out in ½ strength MS medium supplemented with different concentrations and combinations of BAP (0.5 mg/l) 2,4 D (0.5 mg/l) and kinetin (0.5 mg/l). Direct shoot development was also documented for the nodal explants dissected out from aseptically grown seedlings using the same media compositions.

Preparation of extracts for phytochemical analysis

The callus tissues were harvested as two sets of samples-21 d old calli and 45 d old calli to carry out a comparative evaluation of maturity level of callus on quantitative variation in secondary metabolites. 20 g of callus tissue was dried at 40 °C in an oven until constant weight, powdered and 5 g of the dry powdered callus was extracted with 20 ml 90% methanol. The plants grown in the experimental garden of the college were harvested during flowering phase, washed, shade dried until constant weight and powdered. About 25 gm of dried powder was extracted in 100 ml 90 % methanol. These samples, suspended in methanol were placed in a water bath at 45 °C for 10 h and then placed in a gyratory shaker at 120 rpm for 48 h. Each type of extract was filtered using Whatman No.1 filter paper, dried to attain constant weight and the yield and quality of extract were recorded. A stock solution (mg/ml) of extract from the sample was prepared using methanol. These extracts were analyzed quantitatively for the presence of various phytochemicals. Phytochemical examinations were carried out for the extracts of callus and naturally propagated *Trichosanthes cucumerina* (L.) var. *cucumerina* as per the standard methods.

Quantitative analysis of phytochemicals

Determination of alkaloids-For estimation of alkaloids, 5 g of each sample was blended with 200 ml of 20% acetic acid and kept for 40

h. This mixture was filtered and the volume was reduced to one quarter using a water bath. To this sample, concentrated ammonium hydroxide was added dropwise until the precipitation was complete. The solution was allowed to settle and the precipitate was collected by filtration and weighed [7].

The percentage of total alkaloid content was calculated as:

$$\text{Percentage of total alkaloids (\%)} = \frac{\text{weight of residue}}{\text{weight of sample taken}} \times 100$$

Determination of total phenolics

Phenolic content was determined by mixing 3.9 ml of distilled water and 0.5 ml of Folin's reagent with 0.1 ml of the extract. The solution was incubated at room temperature for 3 min and then added 2 ml of 20% (w/v) sodium carbonate solution to the mixture. The solution was kept in boiling water bath for 1 min, cooled and the absorbance recorded at 650 nm. Gallic acid standard was used to express the total phenol content [8].

Determination of total flavonoid

The content of total flavonoids was measured spectrophotometrically by using the aluminium chloride colourimetric assay [9]. A total of 1 ml of plant extracts were diluted with 200 µl of distilled water followed by the addition of 150 µl of sodium nitrite (5%) solution. This mixture was incubated for 5 min and then 150 µl of aluminium chloride (10%) solution was added and allowed to stand for 6 min. Then 2 ml of sodium hydroxide (4%) solution was added and made up to 5 ml with distilled water. The mixture was shaken well and left for 15 min at room temperature. The absorbance was measured at 510 nm.

Determination of total tannin

a) Reagents

Coloring agent: Dissolve 1.6221 g of ferric chloride (0.1M), 0.9 ml of hydrochloric acid (0.1N) and 263.4 mg of potassium ferrocyanide (0.008M) in 100 ml of water.

Working standard solution: A stock solution of 100 µg/ml tannic acid was prepared and used as a standard.

b) Procedure: The powdered samples, 1 g each were boiled with 100 ml of water for 30 min, cooled and filtered through a Whatman no.1 filter paper and made up to 100 ml with distilled water. Added 0.5 ml of coloring agent the blue colour developed was measured at 760 nm against reagent blank after 30 min at room temperature. Added colouring reagent to the standard (tannic acid at concentration ranges-20-100 µg/ml) and calculated the amount of tannic acid equivalent. The tannin content of the samples is expressed as mg of tannic acid equivalent (TE)/g of the dried sample [10].

Determination of total terpenoid

To estimate total terpenoid content in the plant extract, 1 g of each powder was blended in 10 ml methanol and 15 ml water. The mixture was shaken well and centrifuged at 10000 rpm for 10 min. The filtrate was taken. To 1 ml of extracts, 2 ml of chloroform followed by 3 ml of concentrated sulphuric acid was added. Formation reddish brown colour indicated the presence of terpenoids. Terpenoids estimated by reading the absorbance at 538 nm against blank of 95% methanol.

In vitro antioxidant assay

(a) DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The antioxidant activity of the extracts was estimated using the DPPH radical scavenging protocol [11]. DPPH solution (0.004% w/v) was prepared in methanol. A stock solution of DPPH solution-0.004% (w/v) prepared in 95 % methanol. Methanolic extract and standard ascorbic acid were prepared in the concentration of mg/ml. From stock (methanolic extract and ascorbic acid) solution 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of this solution were taken in five test tubes respectively. With the same solvent made the final volume of

each test tube up to 1 ml whose concentration was then 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml respectively. To each sample added 2 ml of freshly prepared DPPH solution. The reaction mixture was incubated in the dark for 30 min and the optical density was recorded at 517 nm against the blank. For the control, 2 ml of DPPH solution in methanol was mixed with 1 ml of methanol and the optical density of the solution was recorded after 30 min. The assay was carried out in triplicates. The decrease in optical density of DPPH on the addition of test samples in relation to the control was used to calculate the antioxidant activity, as percentage inhibition of DPPH radical. The capability of scavenging DPPH radical was calculated using the following equation.

$$\text{DPPH scavenged (\%)} = \frac{(A'_{\text{control}} - A'_{\text{test}})}{A_{\text{control}}} \times 100$$

Where A 'control' is the absorbance of the control reaction and A 'test' is the absorbance of the sample of the extracts.

(b) Ferric reducing antioxidant power (FRAP assay)

Methanolic extracts of callus and plant were compared for their Ferric reducing power [12], using ascorbic acid as standard.

Test samples (1 ml) in different concentrations (100–500 µg/ml) were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated in a water bath at 50 °C for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. The mixtures were then centrifuged for 10 min at room temperature. The supernatant obtained (1 ml) was added with 1 ml of deionized water and 200 µl of 0.1% FeCl₃. The blank was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by distilled water. The absorbance of the reaction mixture was measured at 700 nm. The reducing power was expressed as an increase in absorbance after blank subtraction.

Antibacterial activity

Microbial strains

The microorganisms used for the evaluation of antibacterial activity were two Gram-negative strains—*Escherichia coli* and *Pseudomonas aeruginosa* and one Gram-positive strain—*Staphylococcus aureus*. All the organisms used in this study were clinical isolates obtained from Department of Microbiology, Medical College, Thiruvananthapuram.

Culture medium and inoculation

The stock cultures of microorganisms used in this study were maintained in nutrient agar slants at 4 °C. Inoculum was prepared by suspending a loopful of bacterial culture from stock slant into 25 ml of sterilized nutrient broth (prepared by dissolving 5 g Nutrient broth—readymade Himedia in 200 ml distilled water), maintained in a gyratory shaker at 120 rpm for 48 h to get uniform growth of bacteria.

Medium for culture plate

Nutrient agar (11.2g) was dissolved in 400 ml distilled water, as per the instruction provided by the suppliers (Himedia) and heated

gently to dissolve the powder completely. The sterilized nutrient agar medium was cooled to 45–50 °C and 30 ml was poured into each culture plate. The plates were examined after 24 h for any contamination before carrying out the antibacterial analysis of the extracts.

Preparation of antibiotic (positive control)

The positive control employed for comparison of the antibacterial potential of the extracts was the standard cefotaxim. A stock solution (mg/ml) of the standard was prepared in saline solution and 0.1 ml aliquot (100 µg/ml) was used to test bacterial sensitivity.

Antibacterial analysis

Determination of antibacterial potential of the extracts was carried out using agar well diffusion method [13]. The solidified nutrient agar in the Petri plates was inoculated by dispensing 0.2 ml of the inoculum using a sterilized pipetting device and spread evenly with sterile cotton swabs. Four wells were prepared in each plate with the help of a sterile cork-borer of 5 mm diameter. Each extract was diluted to obtain the concentration 1 mg/ml. Each diluted extract (0.1 ml; concentration-100 µg/ml) was introduced in each well. Wells introduced with 0.1 ml of methanol and 0.1 ml of cephotaxim (100 µg) served as negative and positive controls respectively. All the plates containing loaded wells were incubated for 24 h at 37 °C. After the incubation period zone of inhibition for each concentration of extract and antibiotic cefotaxime were measured by calculating the difference between the diameter of cork-borer and diameter of clear zone [14]. The same method of experiment was carried out in the triplicates and the mean values were reported for the final consideration.

RESULTS

In vitro culture for callus and shoot induction

In the present study, several different combinations of auxins were tested for initiation of friable and vigorous growing callus from hypocotyl, epicotyl and cotyledonary explants. Various auxin concentrations used in this study produced different types of callus morphology and callus induction percentages. When cultured on a medium with varying concentrations of BAP alone, the explants produced no callus. In contrast, in MS medium supplemented with auxin, cultured explants were swollen after five days of inoculation and callus emerged at the cut edges within 10 d of culture (fig. 1C–F). MS with 1 mg/l 2,4-D in combination with 0.5 mg/l BAP was the most effective medium for friable callus induction from cotyledons and the highest frequency recorded was 83.33% (table 1). Substitutions with other auxins (NAA) or increasing or decreasing the concentration of 2,4-D resulted in a reduced percentage of callusing (table 1).

The calli were white, nodular with roots at 0.5 mg/l 2,4-D (fig. 2A) and relatively brownish with a dry friable appearance at 2 mg/l 2,4-D (fig. 2B). However, when MS in combination with higher or lower concentrations of NAA was used, the callus morphology and callus induction percentages differed but induction occurred with moderate frequency—42.85 to 75 % (table 1). The study concluded that the best result on callus induction could be recorded for cotyledon explants cultured on MS medium with BAP (0.5 mg/l) and 2,4-D (1 mg/l).

Table 1: Percentage of callus induction in different growth hormone concentration

S. No.	Growth hormone conc (mg/l)			Percentage of callus induction
	BAP	2,4-D	NAA	
1	0.5	0.5	-	25.28
2	0.5	1	-	83.33
3	0.5	1.5	-	50.27
4	0.5	2	-	55.55
5	0.5	1	1	71.42
6	0.5	1.5	1	75.08
7	0.5	2	1	42.85

(N⁶-Benzyl amino purine (BAP), 1-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D)

One of the objectives of this study was to investigate the effect of growth regulators in shoot induction from the callus and nodal explants of *Trichosanthes cucumerina* (L.) var. *cucumerina*. Direct root formation (fig. 2A and 2B) from callus was detected in MS medium containing BAP-0.5 mg/l, 2,4-D-0.5 mg/l and NAA-1 mg/l. As depicted in fig. 2C and 2D, direct shoot initiation was monitored from the callus in MS medium with hormone combination (BAP-0.5 mg/l, 2,4-D-1.5 mg/l) along with kinetin (0.5 mg/l). Nodal

explant of *T. cucumerina* showed direct shoot induction (fig. 2E and 2F) with hormone supplementation in the range (BAP-0.25 mg/l and NAA-0.5 mg/l). The differential responses indicated by various explants in MS media with the best combinations of hormones are summarized in table 2. The results suggested that *in vitro* shoot differentiation and plant regeneration can be effectively carried out for mass propagation of this valuable medicinal plant.

Table 2: The explant response to different growth hormone combinations

<i>In vitro</i> response	Hormone combination (mg/l)
Maximum callus induction	BAP-0.5, 2,4-D-1
Direct root induction from the callus	BAP-0.5, 2,4-D-0.5, NAA-1
Direct shoot induction from the callus	BAP-0.5, 2,4-D-1.0, Kinetin-0.5
Direct shoot induction from nodal explant	BAP-0.25, NAA-0.5

(N⁶-Benzyl amino purine (BAP), 1-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D))

Quantitative phytochemical analysis

The results of quantitative estimation of various phytoconstituents in callus and *in vivo* plant samples are illustrated in table 3. Alkaloids and tannins were absent in callus extract but were substantially high

in the naturally grown plant. Both callus and the wild grown plant contained flavonoids, phenolics and terpenoids.

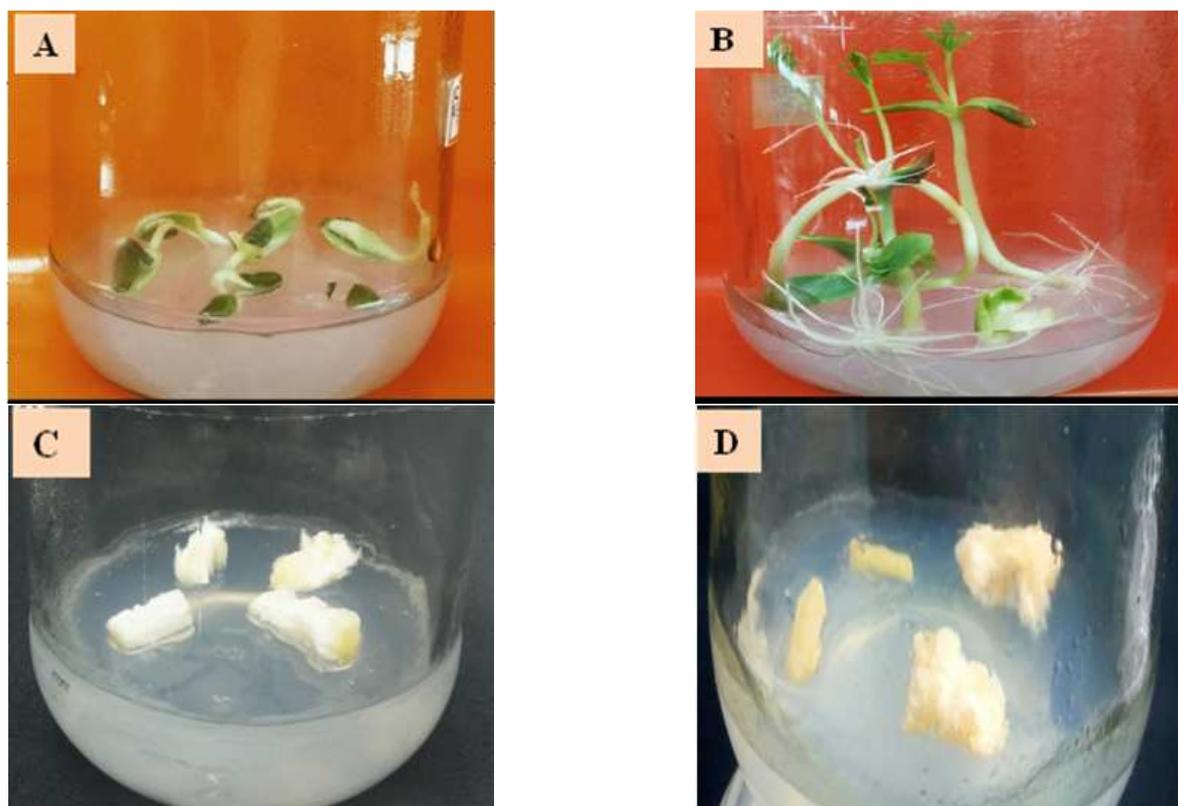
In general, all phytoconstituents were present in higher levels in the extract of *in vivo* plant sample.

Table 3: Quantitative analysis of phytochemicals in methanolic extracts of *Trichosanthes cucumerina* (L) var. *cucumerina*

Source of extract	Alkaloids (mg/g)	Flavonoids (mg/g)	Phenols (mg/g)	Tannins (mg/g)	Terpenoids (mg/g)
Callus (21 d)	0	0.052	8.09	0	0.051
Callus (45 d)	0	0.306	9.07	0	0.177
Wild plant	9.75	1.52	13.04	2.54	1.08

The effects of age of callus were also investigated to determine the impact of cellular ageing on the level of phytochemicals under *in vitro* conditions. A Comparative study between fresh friable callus (21 d

old) and aged callus (45 d old) induced in MS medium supplemented with BAP (0.5 mg/l and 2, 4-D (1 mg/l) revealed that the level of flavonoids, phenolics and terpenoids increase with callus ageing.



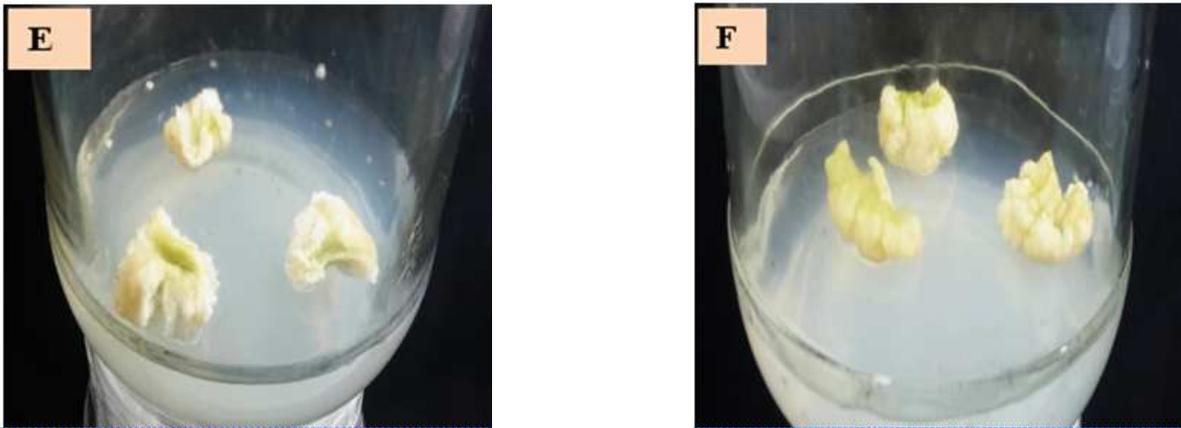


Fig. 1: *In vitro* seed germination and callus induction, A-Germination B-aseptic seedlings C-hypocotyl explants, D-epicotyl explant E-cotyledon explant F-Leaf explants

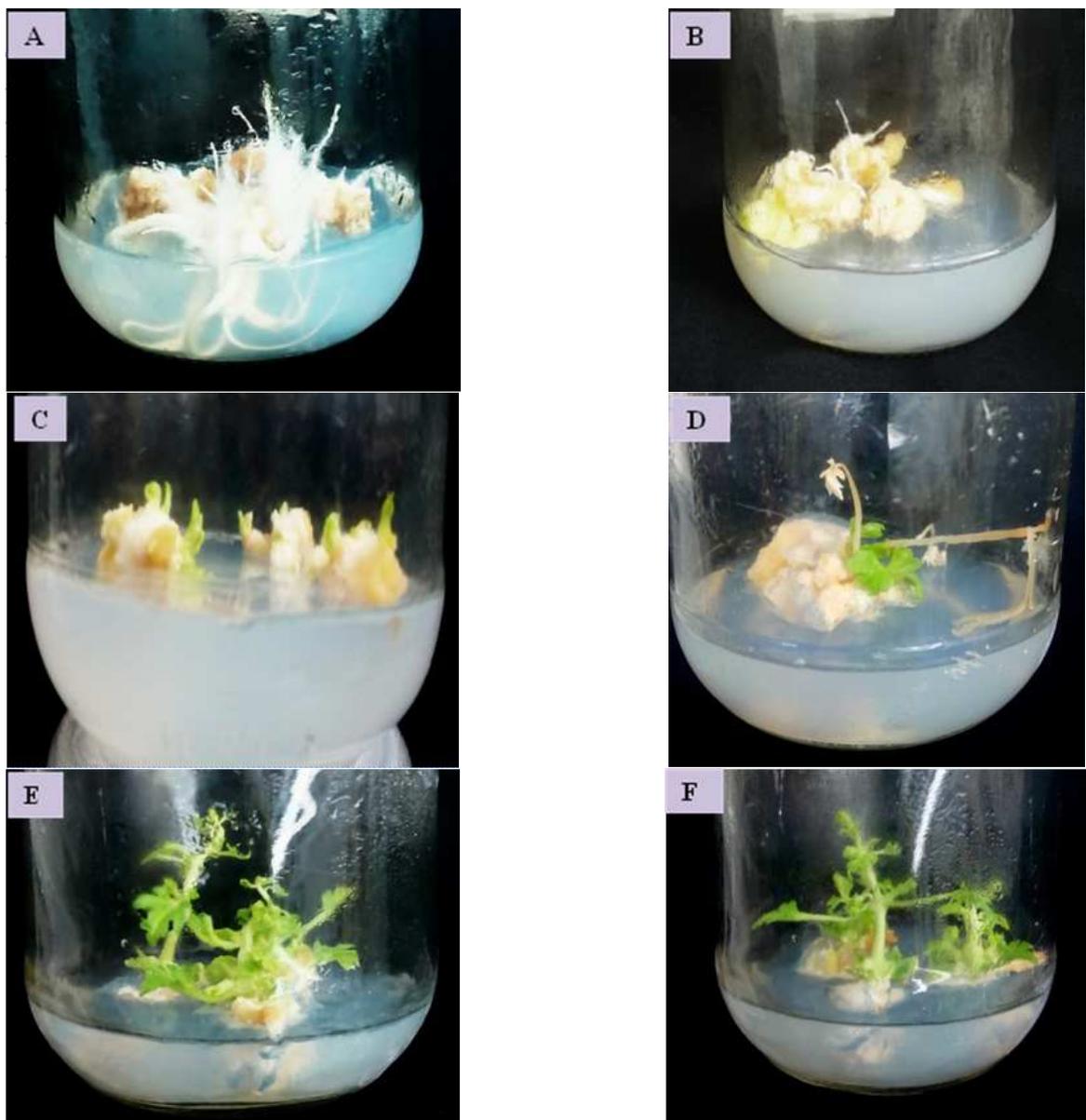


Fig. 2: Direct root induction and shoot induction, A-Root induction from hypocotyl explant: B-root induction from cotyledonary explants, (MS with BAP-0.5 mg/l; 2,4,D-0.5 mg/l; NAA-1 mg/l), CandD-Stages of shoot initiation from callus (MS with BAP-0.5 mg/l; 2,4,D-1.5 mg/l; Kinetin-0. mg/l), EandF-Shoot initiation and callusing from nodal explant (MS with BAP-0.25 mg/l; NAA-0.5 mg/l)

Antioxidant activity**(a) DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging assay**

The results of free radical scavenging ability of a methanolic extract of the plant material and callus at different concentration are illustrated in the table 4. It is expressed as the percentage of inhibition of free radicals by antioxidants present in the extract. The

results on DPPH scavenging activity suggested that there is an increase in the percentage of radical scavenging activity with an increase in the concentration of the extracts.

Among the extracts, the highest scavenging activity at 500 µg/ml extract concentration was recorded (91.85 %) for extract of *in vivo* plant sample. For callus extract, the maximum scavenging activity recorded was 63.48%.

Table 4: DPPH radical scavenging potential of the extracts

Conc (µg/ml)	Standard (Ascorbic acid)	IC ₅₀ * (standard)	Wild plant (in vivo)	IC ₅₀ ** (in vivo)	Callus	IC ₅₀ * (callus)
100	28.97±0.17	207.90 µg	20.85±0.05	393.82 µg	14.61±0.07	355.23 µg
200	48.10±0.21		34.86±0.18		22.98±0.16	
300	71.94±0.06		49.83±0.24		38.91±0.07	
400	87.76±0.18		73.35±0.06		56.30±0.22	
500	98.44±0.08		91.85±0.12		63.48±0.15	

*Each value represented as mean±SD (n = 3)**-IC₅₀-Half maximal inhibitory concentration

Ferric reducing antioxidant power (FRAP) assay

In the FRAP assay, a linear increase in reducing power was observed over the concentration range 100–500 µg/ml. A comparison of the ferric reducing power in relation to standard-ascorbic acid is summarized table 5. A linear progression in reducing ability over a concentration range of 100–500 µg/ml was recorded in FRAP assay.

Extracts from naturally grown *T. cucumerina* indicated ferric reducing power either greater than or at comparable levels with the standard ascorbic acid. The reducing capacity of callus was lesser than the wild plant. In conclusion, the present study demonstrated that extracts from tissues of both *in vitro* (callus) and *in vivo* (wild plant) samples of *Trichosanthes* possesses strong antioxidant activity.

Table 5: Ferric reducing power of extracts–influence of extract concentration

Conc (µg/ml)	Absorbance (700 nm)		
	Callus	Wild plant	Standard (ascorbic acid)
100	0.127±0.065	0.004±0.033	0.089±0.017
200	0.223±0.042	0.121±0.009	0.167±0.004
300	0.257±0.008	0.235±0.014	0.253±0.014
400	0.271±0.015	0.311±0.008	0.321±0.012
500	0.393±0.026	0.456±0.033	0.484±0.024

*Each value represented as mean±SD (n = 3)

Antibacterial activity

The methanolic extract of *Trichosanthes cucumerina* (L.) var. *cucumerina* showed moderate antibacterial activity against all the microorganisms tested (table 6). The methanolic extract of the wild

plant showed appreciable activity against *Staphylococcus aureus* (inhibition zone-13 mm/100 µg/ml) and *Escherichia coli* (11 mm/100µg/ml). The bacterial sensitivity to the plant extract is quite comparable with that of the standard antibiotic cephotoxim (20 mm/100µg/ml).

Table 6: Antibacterial activity of *Trichosanthes cucumerina* (L) var. *cucumerina* against different strains of bacteria

Type of extract	Diameter of Inhibition Zone (mm)		
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Callus	5.03±0.05	4.8±0.28	4.00±0.00
Wild plant	13.00±1.00	4.00±0.06	11.16±0.28
Positive control	20.00±1.00	11.00±0.05	13.00±0.00
Negative control	3.00±0.05	2.00±0.00	2.03±0.05

(Extract-100 µg/ml; Period of incubation-24 h; Positive control-Cefotaxim-100 µg/ml (Antibiotic) Negative control-Methanol; Well diameter-5 mm. Each value represented as mean, n=3)

DISCUSSION

The responses of *in vitro* culture analysis indicated that 2,4-D was the best auxin in combination with BAP as regards callus induction. In earlier studies, 2,4-D was reported to be an effective growth hormone that produced rapid proliferating friable callus in *Curcuma amada* [15], *Gymnema sylvestris* [16] and *Brassica napus* [17] during *in vitro* cultures.

Relatively lesser level of phytochemicals in callus can be interpreted as follows. Callus being an undifferentiated meristematic tissue

accumulates only minor quantity of plant secondary metabolites. However, in general, the majority of callus and suspension cultures produce lesser quantities of secondary metabolites. This is mainly due to the lack of fully differentiated cells in the cultures.

In the present study, no alkaloids and tannins were detected in callus samples but these were found in samples from cultivated plants. The absence of alkaloids and tannins in callus tissues could be explained as due to lack of cellular differentiation and organogenesis in undifferentiated tissue like calli. Accumulation and storage of secondary metabolites such as alkaloids and tannins

might be associated with organogenesis. Differentiated plant parts such as leaves, stem and roots normally serve as effective storage centres of these phytoconstituents. The observations of the present study agree with an earlier report on comparative phytochemical yield in wild and *in vitro* derived tissues of *Harpagophyllum procumbens* [18].

The correlation between age of culture and accumulation of natural product may possibly be due to the reason that secondary metabolites accumulated in the later stage when the growth of callus slow down. Enhancement in yield of secondary metabolites with the age of the callus has been reported for the accumulation of cryptotanshinone in *Salvia miltiorrhiza* [19]. In *Morinda citrifolia* also chemical constituents accumulate in the later stage of growth cycle when growth slows down [20].

The phenolics and flavonoids are characterized by their potential to act as antioxidants. Flavonoids are oxidized by radicals resulting in a more stable, less reactive radical. It appears that the flavonoids stabilize the reactive oxygen species (ROS) by reacting with the reactive compound of the radical [21]. In general, a positive correlation exists between antioxidation capacities and total polyphenol and flavonoid contents. Earlier reports on the antioxidant potential of *Ulva prolifera* [22] and *Garcinia* species [23] confirmed this correlation. In the present study also, a strong positive correlation was documented between total phenolic and flavonoid content and antioxidant capacity. The results suggested that wild grown plant with comparatively higher concentrations of secondary metabolites could exhibit maximum DPPH scavenging ability than the callus. The current observations were in agreement with the reports of Bouba *et al.* [24], where a positive correlation between total phenolics and flavonoids and antioxidant profile in extracts of Cameroonian spices was reported. Similar observations have also been reported by Chai and Wong [25].

The present study indicated that all extracts of *Trichosanthes cucumerina* (L.) var. *cucumerina* were potent against the Gram-positive strain (*Staphylococcus aureus*) than the Gram-negative bacteria (*Scherichia coli* and *seudomonas aeruginosa*). These results are similar and consistent with reports on related plant extracts which reported inhibition zone sizes varying from 7 mm for *Pseudomonas aeruginosa* to 33 mm for *Staphylococcus aureus* [26-31]. It could be explained on basis of the inherent properties that are related to the composition of cell wall architecture and cell surface permeability to the extracts tested. The antibacterial activity of these extracts could be correlated as due to the presence of secondary metabolites such as flavonoids, phenolic compounds, terpenoids, tannin and alkaloids that adversely affect the growth and metabolism of microbes. The comparatively poor antibacterial profile indicated by the callus extract which contains no alkaloids and tannin supports this correlation.

CONCLUSION

A comparative evaluation of phytochemicals in extracts from naturally propagated and *in vitro* derived plant parts suggested that *Trichosanthes cucumerina* (L.) var. *cucumerina* is a rich source of biologically active phytoconstituents. All samples compared exhibited moderate to high level of antioxidant and antibacterial potential and substantiated its medicinal uses. Results of *in vitro* culture indicated that this highly valued medicinal species with poor seed longevity could be successfully regenerated in artificial media and this could be suggested as a remedial measure to counteract the occasional failure in natural seed germination.

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AUTHORS CONTRIBUTION

The first author (Sudha Bai R): Conception and design of the project, data interpretation and manuscript preparation.

The second author (Remakanthan A): Planning and layout of experiments on *in vitro* culture.

Third author (Hareesh Kumar H): Conducted experiments on *in vitro* culture, antibacterial study and data acquisition.

Fourth author (Aryakrishna U K): Conducted experiments on phytochemical evaluation, antioxidant assay and data acquisition.

CONFLICT OF INTERESTS

Declare none

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