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PROTEIN PROFILING AND ISOLATION OF BIOACTIVE HYDROLYSATES WITH ANTIOXIDANT ACTIVITY FROM STEM CALLUS TISSUE OF *TINOSPORA CORDIFOLIA* (WILLD.) MIERS EXPOSED TO CYCLODEXTRIN

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Keywords:

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ABSTRACT: This study is aimed to evaluate the effects of different concentrations of cyclodextrin, an auxin stabilizing agent, on callus growth from the stem explants of *Tinospora cordifolia* and to assess the antioxidant properties of the bioactive hydrolysates of callus tissue using chymotrypsin, trypsin, pepsin, and papain. Cyclodextrin exerted a dose- and time-dependent effect on the callus growth. α-chymotrypsin hydrolysate showed the strongest 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging, while trypsin hydrolysate exhibited the highest 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) activity. Protein profiling of callus tissue revealed a few tissue-specific bands at 41, 30, 20, and 9 kDa with varying intensities. A growth medium containing sucrose has a specific impact on the expression of these polypeptides. Purification of 30 kDa polypeptide by Sephadex-G50 revealed distinct quantitative differences to different concentrations of cyclodextrin. This study suggested that *T.cordifolia* callus tissue protein hydrolysates exhibited antioxidant efficacy, leading to the development of a nutraceutical agent in promoting health.

INTRODUCTION: *Tinospora cordifolia* (willd.) Miers ex Hook. F. & Thoms. Belongs to the family Menispermaceae. In folk and tribal medicine, the whole plant, powdered root and stem bark, *decoction* of root and stem, juice of the root, leaves or stem of *T. cordifolia* is used to treat various ailments such as fever, jaundice, general debility, dyspepsia, cough, asthma, leucorrhoea, skin diseases, urinary diseases, gonorrhea, secondary syphilis, impotency, gout, viral hepatitis and eye disorders [26, 36, 16, 27]. The root and stem of *T. cordifolia* are prescribed in combination with other drugs as an antidote to snakebite and scorpion sting [16].

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T. cordifolia is extensively used in the Ayurvedic system of medicine for its tonic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-allergic, anti-stress, anti-oxidant, anti-pyretic, anti-inflammatory, anti-leprotic, anti-diabetic anti-malarial, and hepatoprotective properties [19, 8, 23-29]. It is also used to improve the immune system and the body's resistance against infections [25]. A variety of compounds have been isolated from aerial parts and roots of *T. cordifolia*. They belong to different classes such as alkaloids, diterpenoids, lactones, glycosides, steroids, sesquiterpenoid, phenols, aliphatic compounds, and polysaccharides.

Berberine, a benzyl tetra isoquinoline alkaloid, is pharmaceutically important and has been used in traditional Chinese medicine [34-35]. The therapeutic potential of berberine includes antileukaemia, antihepatoma, cardioprotective, anticancer, and AMP-activated protein kinase resulting in beneficial metabolic effects in diabetic and insulin-resistant states [9]. Unfortunately, these plant species

are now disappearing at an alarming rate due to rapid agricultural and urban development and indiscriminate collection. This plant is commercially exploited in pharmaceuticals due to its medicinal value. The tissue culture technique provides an ideal way for rapid mass propagation and conservation of these important multipurpose medicinal plants. Further, propagation of plant takes place mainly through stem cuttings; *In-vitro* culture method is considered the most suitable way to propagate *T. cordifolia*. *In-vitro* propagation increases the efficiency and scales up plant production [11]. The present study was therefore undertaken to establish an efficient protocol for *rapid in-vitro* propagation of this important medicinal plant as well as to study the effects of different concentrations of cyclodextrin (25 mg, 50 mg, 75 mg, and 100 mg), an auxin stabilizing agent on callus growth and protein profiling.

This study is also aimed to evaluate the antioxidant properties of the bioactive hydrolysates from callus tissue of *T. cordifolia* by hydrolyzing the proteins using several enzymes such as chymotrypsin, trypsin, pepsin, and papain. Their antioxidant efficacy was evaluated by measuring (1) 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging activity, (2) 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) radical scavenging capacity and (3) metal chelation and (4) superoxide anion radical scavenging activity.

MATERIALS AND METHODS:

Chemicals: Pepsin from porcine gastric mucosa (3000 U/mg of protein), Soybean trypsin inhibitor was obtained from Calbiochem, Merck. Porcine pancreatic trypsin (10,000 U/mg of protein), bovine pancreatic α-chymotrypsin (40 U/mg of protein), porcine pancreatin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azinobis (3-ethylbenzothiazoline-16-sulfonic acid) (ABTS), potassium persulfate and pyrogallol (HPLC grade) were procured from Sigma. Ferrozine and ferrous chloride were obtained from SRL.

Plant Material:

Source of Explants: Stems of *T. cordifolia* (0.5-1.5 cm long), which served as explants, were obtained from 1-2 months old healthy mother plant growing in the Greenhouse of Kalasalingam University.

Sterilization of Explants: The explants were first washed thoroughly in running tap water for about half an hour to remove soil and dust and then kept in double-distilled water for 3-4 h to facilitate the phenolics, a characteristic gummy substance of polysaccharide leaching out of explants. Later, the explants (1 cm each) were washed with liquid detergent for 5 min, followed by thorough washing under running tap water for 15 min. The explants were subsequently surface-sterilized with mercuric chloride (0.1%) for 5 min and then repeatedly washed with sterile distilled water under aseptic conditions in a laminar flow chamber.

Plant Tissue Culture Media: The surface-sterilized explants were then aseptically inoculated on sterile MS medium [18], comprising 3% sucrose as carbon source and 0.8% agar as a solidifying agent. The medium was also supplemented with cytokinin 2, 4-dichloro-phenoxyacetic acid (2, 4-D) (2.0 mg/L-1), and auxin Naphthalene acetic acid (NAA) (0.1 mg/L-1) and pH of the medium was adjusted to 5.8 before autoclaving at pressure 1.06 Kg/cm^{-3}. Besides, the callus induction from the explants of *T. cordifolia* was investigated on media containing different concentrations of cyclodextrin (25 mg, 50 mg, 75 mg, and 100 mg), an auxin stabilizing agent.

Culture Conditions: The cultures were incubated at 25 ± 2 °C under the cooldown, white, and fluorescent light of 2000-2500 lux intensity and relative humidity of about 55 ± 5%. The growth chamber was maintained with a 16/8 h photo and dark period, respectively. For each treatment, eight replicates were used and repeated at least thrice. The multiple shoot induction and elongation were periodical.

Extraction of Callus Tissue Proteins: Protein was extracted according to the method of [2] with minor modifications. The callus tissue was chopped into small pieces and air-dried to remove the residual moisture.

After drying, the callus tissue was ground into a fine dry powder, suspended in deionized water in the ratio of 1:5 (wt/vol), and stirred at 4 °C for 2 h. The suspension was centrifuged at 5000 x g for 20 min, and the supernatant obtained was subjected to salt precipitation by slowly dissolving ammonium

sulfate by stirring to 80% saturation. The suspension was kept at 4 °C overnight and then again centrifuged at 5000 x g for 20 min. The precipitated proteins were used for further experiments. Protein was quantified by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Preparation of Callus Stem Protein Hydrolysates: *Tinospora cordifolia* callus stem tissue proteins were hydrolyzed enzymatically using four selected proteases such as *α*-chymotrypsin, trypsin, pepsin, and papain.`

Papain Hydrolysates: Papain digestion was carried out following the method of [1]. For papain digestion, 5 mg of precipitated proteins from callus tissue was dissolved in 1 ml of 50 mM phosphate buffer (pH 6.8). Papain was activated in 20 ml of phosphate buffer (pH 6.8) containing 4 mg papain enzyme, 20 mM cysteine, and 50 mM EDTA. 50 mg of papain was dissolved in 250 µl of phosphate buffer and treated with a protein solution to yield an enzyme/substrate ratio of 1:100, which contained 1500 U papain per mg protein. The digestion mixture was incubated in an orbital shaker at 37 °C for 120 min and the reaction was arrested by boiling the reaction mixture for 6 min to irreversibly denature papain enzyme.

Pepsin Hydrolysates: Pepsin digestion was carried out following the method of Thomas, Aalbers, Bannon, Bartels, [28]. A suspension containing simulated gastric fluid (SGF), 10 mM HCl, 30 mM NaCl and pepsin ((2000 U/mg, 0.5 mg/ml) after adjusting the pH 2.2, was preheated to 37 °C for 10 min, before the addition of lyophilized powder obtained from callus stem tissue. This reaction mixture yielded 6000 U of pepsin/ mg of protein as a final digestion mix containing 10 mg of protein and 3.33 mg of pepsin (pepsin/ protein ratio of 1:3.33, w:w). Samples were incubated in a shaking incubator at 37 °C for 120 min, and the digestions were terminated by increasing the pH to 8 by adding 160 mM of sodium carbonate to irreversibly inactivate pepsin.

Trypsin and A-Chymotrypsin Hydrolysates: Trypsin and α-chymotrypsin enzymes were separately used for the hydrolysis of the proteins. The precipitated proteins from callus tissue were prepared in Tris-HCl buffer having pH 7.8 and 8.0, respectively, to yield a final concentration, 5 mg/ml. For α-chymotrypsin and trypsin digestion, solutions of enzymes were added to the protein solutions in Tris-HCl buffer with pH 7.8 and 8.0, respectively, to yield 8 U of bovine α-chymotrypsin and 2000 U of porcine trypsin per mg of protein (Thompson, Lawson, Barlow, & Goll, 2003). This yielded an enzyme/substrate ratio of 1:5 (w/w) for both trypsin and chymotrypsin (*i.e.*, 5 mg of protein incubated with 1 mg of trypsin and chymotrypsin, respectively). Digestions were carried out in a shaking incubator at 37 °C, and aliquots (100 µl) were taken out at 180 min for further analysis. Reactions were stopped by boiling at 100 °C for 6 min to irreversibly denature the α-chymotrypsin and trypsin in the digestion mixes.

Antioxidant Assays: The antioxidant properties of callus tissue protein hydrolysates were tested by quantifying four high-throughput radical scavenging assays, such as DPPH, ABTS·+, and superoxide radical scavenging assays and metal chelation.

DPPH Assay: The free radical scavenging activity of protein hydrolysates obtained from the callus grown in media supplemented with different concentrations of cyclodextrin was measured following the method of Brand-Williams, Cuvelier, & Berset, (1995). Briefly, 0.1 mM DPPH was dissolved in methanol to generate DPPH free radicals. Aliquots of protein digests (20 µl) obtained from the callus tissue grown in various concentrations of cyclodextrin (25. 50, 75, and 100 mg/ml), were added to 180 µl of DPPH solution. Methanol alone was used as a blank, while DPPH in methanol without the protein digests served as a positive control. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min, and the discolorization of the purple color was measured at 518 nm in a microplate reader. Measurements were carried out in triplicate. Lower absorbance of the reaction mixture indicated higher free radical activity. The radical scavenging activity of the samples was calculated as the percentage reduction of DPPH according to the following formula:

% Reduction = Blank OD – Sample OD / Blank OD x 100

The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical. Ascorbic acid was used as the standard. The experiment was done in triplicate, and the average is tabulated and graphically represented.

2, 2′- Azino - Bis (3 - Ethylbenzothiazoline – 6 - Sulfonic Acid) (ABTS•+) Radical Scavenging Assay: ABTS•+ scavenging activity of protein hydrolysates of callus grown in media supplemented with different concentrations of cyclodextrin was determined by ABTS radical cation decolorization assay [24]. The ABTS radical was generated by the oxidation of ABTS with potassium persulphate. An aqueous solution of ABTS (7 mM) was mixed with potassium persulphate (2.45 mM) and then incubated in the dark at room temperature for 12-16 h. The resulting dense-green colored solution was diluted with phosphate-buffered saline (PBS) (100 ml), pH 7.4 containing 81.0 ml Na_2HPO_4 (0.2 M) and 19.0 ml NaH_2PO_4 (0.2 M) until the absorbance at 734 nm was 0.70 ± 0.02. The solution was kept for 30 min in the dark before being used. For 10 µl of protein hydrolysates of callus grown in media supplemented with different concentrations of cyclodextrin (25 mg, 50 mg, 75 mg, and 100 mg) was added to 3.995 ml of diluted ABTS•+ solution and mixed well. The absorbance was measured after 30 min. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent inhibition of absorbance at 734 nm was calculated using the formula.

% Reduction = Blank OD – Sample OD / Blank OD x 100

Superoxide Radical Scavenging Activity: The superoxide radical (O_2•-) scavenging activity of protein hydrolysates of callus grown in media supplemented with different concentrations of cyclodextrin (0 mg, 25 mg, 50 mg, 75 mg, and 100 mg) was analyzed by the pyrogallol autoxidation method of [17]. The reaction mixture was prepared by mixing 100 µl of the sample in 50 mM phosphate buffer (pH 6.9) and 180 µl of 50 mM Tris-HCl buffer (pH 8.2) and incubated at 25 °C for 10 min. Superoxide radicals were generated by adding 10 µl Pyrogallol (prepared in 10 mM HCl). The absorbance of the final reaction mixture was measured at 320 nm up to 5 min.

Phosphate buffer (pH 6.9) alone was used as a blank. The percentage of scavenged superoxide anion was estimated from the following formula:

Superoxide scavenging activity = {[(ΔA/min) b – (ΔA/min)s] / (ΔA/minb} x 100

Determination of Ferrous Chelating Activity: The ability of protein hydrolysates of *T. cordifolia* callus grown in media supplemented with different concentrations of cyclodextrin to chelate the transition metal ion Fe2+ was determined according to the method of [33]. Samples (each 25 µl) were treated with 125 µl of 80 µM $FeCl_2$ (final concentration in the assay solution, 40 µM). After 3 min, the reaction was started by adding 100 µl of 0.5 mM ferrozine (final concentration of 0.2 mM). The mixture was incubated for 15 minutes. A blank was maintained without the sample. The absorbance values of blank (Ab) and sample (As) were measured at 562 nm using a spectro-photometer. The percentage chelating effect (%) was calculated using the following equation:

Metal chelating effect (%) = [(Ab – As) /Ab] x 100

Preparation of Protein and SDS-PAGE Gel Electrophoresis: Protein profiling was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis **(**SDS-PAGE) method. Total soluble proteins were extracted by grinding 500 mg of mature callus stem tissue (35 days old) grown in different concentrations of cyclodextrin separately. Protein extracts were prepared in Tris-HCl buffer (0.1 M, pH, 8.0) at 4 °C. The samples were sonicated (1 sec/ml, 5 times at setting 5) by keeping it in an icebox. Homogenates were then centrifuged at 12,000 rpm at 4 °C for 10 min. Protein concentration in the supernatant was determined by the method of Bradford (1976) using BSA as a standard.

Samples were denatured using 0.125 M Tris buffer (pH 6.8), containing 5% mercaptoethanol, 0.03% dithiothreitol, 40% glycerol and 2 % SDS. The denatured protein (20 µl) was incubated in a water bath at 100 °C for 3 min. Samples were cooled and loaded onto SDS-PAGE, which consisted of 12% separating gel and 5% stacking gel. After the gel was cast, a volume of each of 15 µl protein samples from callus tissue were then loaded onto gels separately.

A protein of known molecular weight marker standard (0.5 mg/ml) (Promega) was loaded in a separate lane adjacent to the sample wells. Bromophenol blue (5 µl) was used as a tracking dye. The gel was run in the refrigerator for proper cooling of the electrode buffer and plates. The gel was run at 50 mA current until the bromophenol blue reaches the bottom of the gel. After completion of the run, the gel was carefully removed from the plates and immersed in staining solution (0.5% Coomassie Brilliant Blue R-250) overnight. The gel was then transferred to a destaining solution (45% (v/v) methanol and 10% (v/v) acetic acid) for 12 h. After proper destaining, the gel was documented and photographed. The molecular weight of the protein bands was determined by comparing the protein bands of molecular weight marker standards.

Purification of Peptides: Of the four polypeptides, since 30 kDa polypeptide appeared prominent and more distinct, this peptide from control and those exposed to media supplemented with different doses of cyclodextrin was purified using diethyl aminoethyl (DEAE)-Sephadex (A-50) column chromatography. A DEAE-Sephadex (A-50) column (2X 30 cm) was prepared and equilibrated with buffer A as described by Atkin & Srivastava (1970). Proteins from the column were eluted by a combined stepwise and linear gradient elution procedure. An initial elution with 300 ml of buffer A was followed by two steps of linear salt gradient obtained first by mixing 300 ml of buffer A with 300 ml of buffer A containing 0.4M NaCl and then by mixing 200 ml of buffer A containing 0.4M NaCl with 200 ml of buffer A containing 1 M NaCl. Further elution was done with buffer A containing1 M NaCl (150) ml), with 0.5 N NaOH (175 ml), with 0.75 N NaOH (150 ml), and finally with 1 N NaOH (200 ml). All steps were performed at 2 C. Sixty-drop (5.2 ml) fractions were collected at a flow rate of 15 to 20 ml/hr. The final elution with 1 N NaOH was carried out at room temperature, and 50 ml fractions were collected. The A2SO Of each fraction was read against appropriate blanks.

Statistical Analysis: Experiments were carried out in triplicates. Data are presented as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was performed by SPSS 19.0 (SPSS, Chicago, IL, USA). The significance in differences was determined by Duncan's multiple range test ($p < 0.05$).

RESULTS:

Antioxidant Activity:

DPPH* Radical Scavenging Activity: DPPH scavenging activity registered an eight-fold but uniform increase in chymotrypsin hydrolysates obtained from callus exposed to 25, 50, and 75 mg of cyclodextrin. Such an increase in free radical scavenging activity was observed in chymotrypsin hydrolysates obtained from callus exposed to 100 mg of cyclodextrin (six-fold), but the effect is less than that of other doses of cyclodextrin. In trypsin hydrolysates, an increase in the percentage of DPPH radical scavenging effect was observed only in callus grown with 25 (26%) and 50 mg (28%) of cyclodextrin supplement.

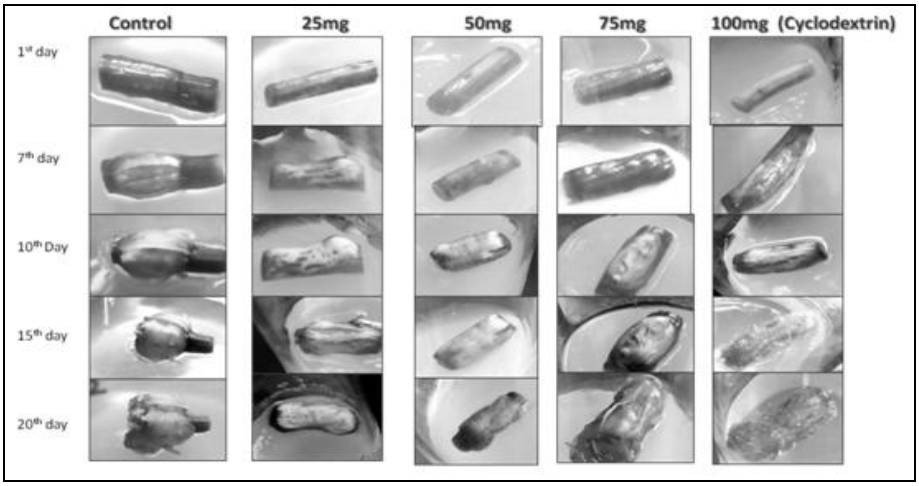

FIG. 1: DEVELOPMENT OF SHOOT EXPLANTS IN MS MEDIUM CONTAINING DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN

On the other hand, free radical scavenging activity was reduced (65%) in trypsin hydrolysates obtained from callus exposed to 100 mg of cyclodextrin. In pepsin hydrolysates, free radical scavenging activity decreased with increasing concentrations of cyclodextrin (5%, 12%, 18%, 28%, respectively). In papain digests, while 25 mg of cyclodextrin decreased the DPPH activity (26%), 50 and 75 mg doses of cyclodextrin increased the activity (66% and 25%, respectively) **Fig. 1.**

ABTS•+ Cation Radical Scavenging Activity: There was no appreciable change in ABTs activity in chymotrypsin hydrolysates obtained from callus exposed to 25, 50, 75, and 100 mg of cyclodextrin. However, trypsin hydrolysates of callus grown in media supplemented with 25, and 50 mg cyclodextrin registered an increase in ABTs activity (19%, 28%, 27%, and 22%).

Such a significant increase in ABTs activity was recorded in pepsin hydrolysates obtained from callus grown in media supplemented with 50 (23%) and 100 mg (29%) of cyclodextrin. On the contrary, a decrease in ABTs activity was recorded in papain hydrolysates obtained from callus exposed to only 25 mg of cyclodextrin **Fig. 2.**

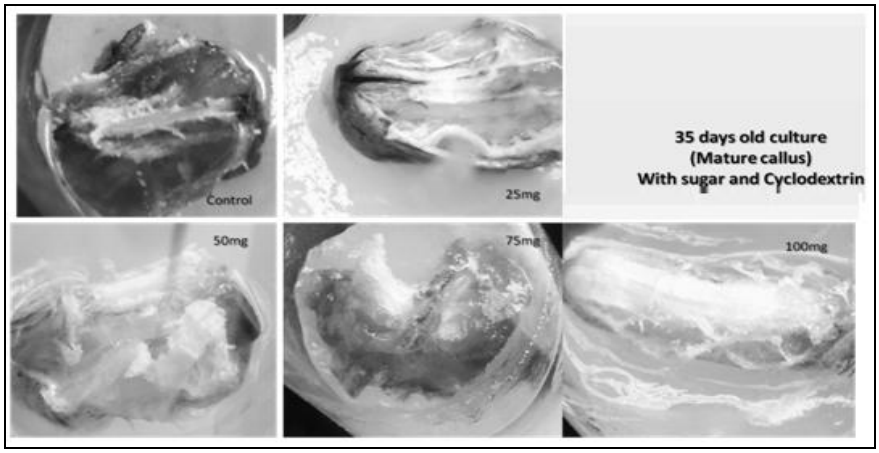

FIG. 2: CALLUS GROWTH IN MS MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN IN 35TH OLD CULTURE

Metal Chelating Activity: Metal chelation activity recorded a uniform decrease in chymotrypsin digests obtained from callus exposed to 25, 50, 75, and 100 mg of cyclodextrin (11%, 17%, 15%, 15%, respectively). Such a decrease in metal chelation activity was also observed in trypsin hydrolysates obtained from callus exposed to cyclodextrin, irrespective of the doses, with the effect being maximum in 50 mg cyclodextrin supplement. The same trend was noticed in pepsin digests also. In papain digests, metal chelation activity remained unaltered **Fig. 3.**

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity registered a four-fold decrease in hydrolysates obtained from callus grown in media supplemented with 50 mg of cyclodextrin **Fig. 6.**

Protein Expression in Stem Callus Tissue by SDS-PAGE: SDS-PAGE is the most widely used analytical method to resolve components of a protein mixture. In the present study, the total protein was estimated from mature cream-colored callus stem tissue.

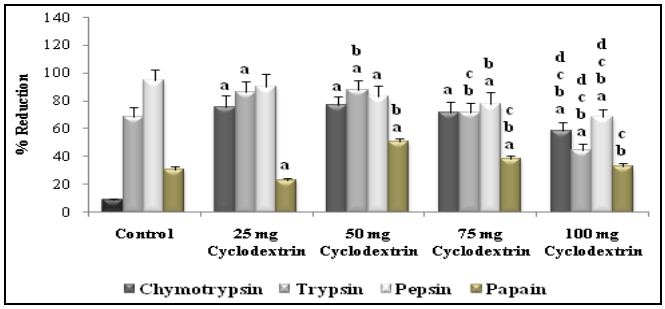

FIG. 3: DPPH• RADICAL SCAVENGING ACTIVITY IN DIFFERENT HYDROLYSATES OF CALLUS TISSUE OF *T. CORDIFOLIA* EXPOSED TO DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN. Values are expressed as mean ± SEM; **a** – Compared to respective protein hydrolysates of control; **b** – Compared to respective protein hydrolysates of callus grown in media supplemented with 25 mg cyclodextrin; **c**– Compared to respective protein hydrolysates of callus grown in media supplemented with 50 mg cyclodextrin; **d**– Compared to respective protein hydrolysates of callus grown in media supplemented with 75 mg cyclodextrin; Significant at $p < 0.005$.

Protein profiling of callus tissue by SDS-PAGE showed a few tissue-specific bands **Fig. 5**. Protein profiles showed variability in the number of bands, band pattern, and band intensity.

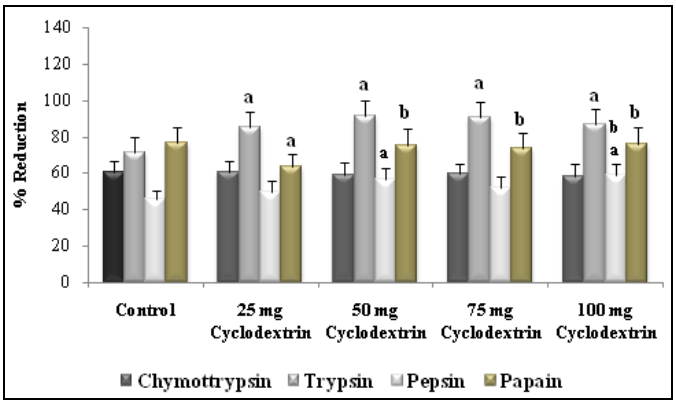

FIG. 4: ABTS RADICAL SCAVENGING ACTIVITY IN DIFFERENT HYDROLYSATES OF CALLUS TISSUE OF *T. CORDIFOLIA* EXPOSED TO DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN. Values are expressed as mean ± sem; **a** – compared to respective protein hydrolysates of control; **b** – compared to respective protein hydrolysates of callus grown in media supplemented with 25 mg cyclodextrin; **c**– compared to respective protein hydrolysates of callus grown in media supplemented with 50 mg cyclodextrin; d– compared to respective protein hydrolysates of callus grown in media supplemented with 75 mg cyclodextrin; significant at $p < 0.005$.

The callus tissue revealed 4 bands at 41, 30, 20, and 9 kDa. These four bands were feeble and weak in the negative control. However, all four bands were prominent in callus tissue exposed to media supplemented with 50, 75, and 100 mg cyclodextrin. From these observations, it is inferred that cyclodextrin at higher doses caused the expression of 41, 30, 20, and 9 kDa proteins.

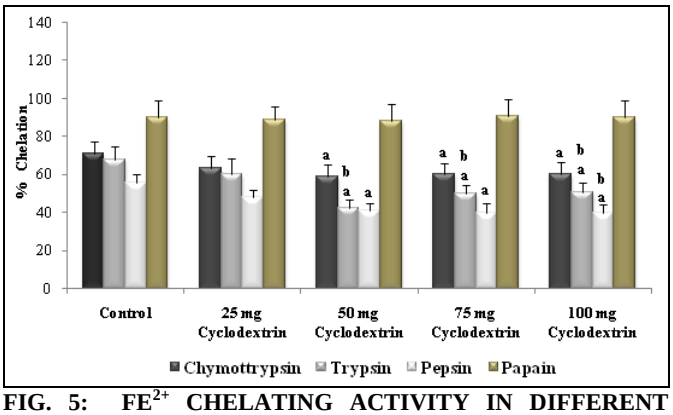

FIG. 5: FE^{2+} CHELATING ACTIVITY IN DIFFERENT HYDROLYSATES OF CALLUS TISSUE OF *T. CORDIFOLIA* EXPOSED TO DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN

These bands were confirmed by comparison with the reference sample. Secondly, there is a clear difference in the expression of 41 kDa polypeptide in the callus tissue exposed to sugar and different concentrations of cyclodextrin and those without sugar concentration. In the latter, the intensity of 41 kDa polypeptide expression was less as compared to the respective bands in the callus tissue grown in media supplemented with sugar and cyclodextrin **Fig. 6.**

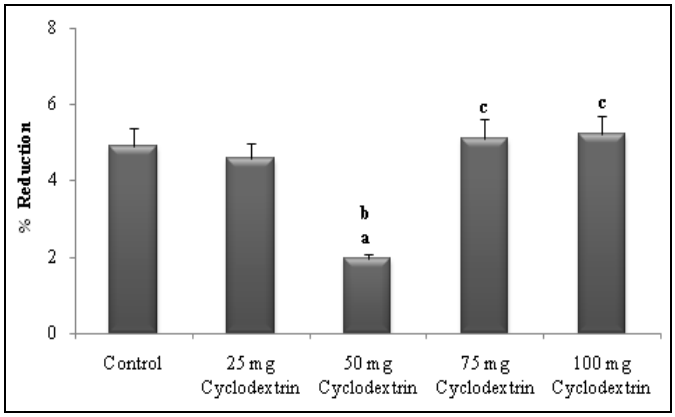

FIG. 6: SUPEROXIDE RADICAL SCAVENGING ACTIVITY IN DIFFERENT HYDROLYSATES OF CALLUS TISSUE OF *T. CORDIFOLIA* EXPOSED TO DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN. Values are expressed as mean ± SEM; a – Compared to respective protein hydrolysates of control; b – Compared to respective protein hydrolysates of callus grown in media supplemented with 25 mg cyclodextrin; c– Compared to respective protein hydrolysates of callus grown in media supplemented with 50 mg cyclodextrin; d– Compared to respective protein hydrolysates of callus grown in media supplemented with 75 mg cyclodextrin; Significant at $p < 0.005$.

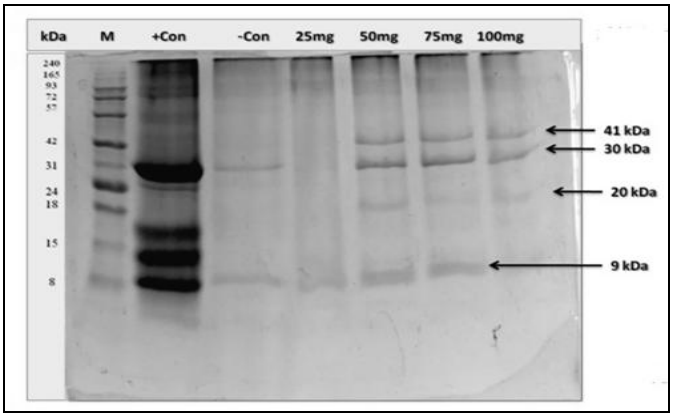

FIG. 7: PROTEIN EXPRESSION BY SDS-PAGE ANALYSIS IN 35 DAYS OLD MATURE CALLUS EXPOSED TO SUCROSE AND DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN. Fig. 7 shows protein analysis by sds-page in 35 days old stem callus tissue grown in ms medium supplemented with sucrose and different concentrations of cyclodextrin (25, 50, 75 and 100 mg). kda – kilo dalton; m - protein molecular weight marker marker; + con - positive control; – con - negative control.

Purification of Peptides: Of the four polypeptides, since 30 kDa polypeptide appeared prominent and more distinct, this peptide from control and those exposed to media supplemented with different doses of cyclodextrin was purified with a Sephadex G50 column. The typical DEAE-Sephadex elution profiles of the proteins from callus tissue presented in **Fig. 9** showed distinct quantitative differences in callus tissue peptides exposed to different concentrations of cyclodextrin. Peptide concentration increased greater in quantity in callus tissue exposed with increasing concentrations of cyclodextrin (25, 50, and 75 mg), while the same was not detectable in callus tissue supplemented with 100 mg of cyclodextrin.

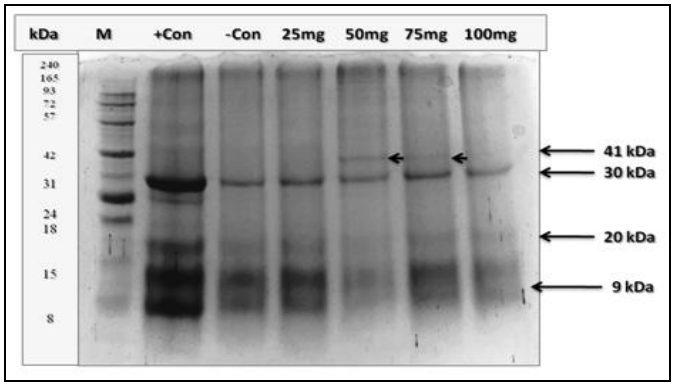

FIG. 8: PROTEIN EXPRESSION BY SDS-PAGE ANALYSIS IN 35 DAYS OLD MATURE CALLUS EXPOSED TO DIFFERENT CONCENTRATIONS OF CYCLODEXTRIN ALONE. Fig.8 Shows protein analysis by SDS-PAGE in 35 days old stem callus tissue grown in MS Medium supplemented with different concentrations of cyclodextrin (25, 50, 75 and 100 mg) Arrow mark indicate the expression of 41 kDa protein in callus tissue exposed to 50 and 75 mg concentrations of cyclodextrin). **kDa** – Kilo Dalton; **M** - Protein molecular weight marker marker; **+ Con** - Positive control; **– Con** - Negative control.

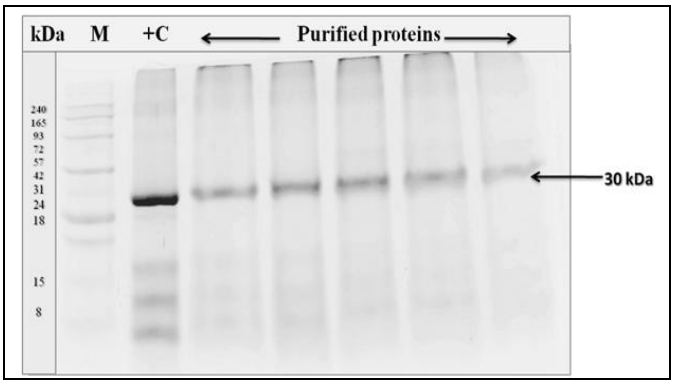

FIG. 9: PURIFICATION OF BIOOTIVE PEPTIDE 30 KDA PROTEIN BY SEPHADEX 50 COLUMN Fig. 9 shows t. cordifolia proteins from stem callus tissue exposed to different doses of cyclodextrin and the proteins were purified under sephadex 50 column kda – kilo dalton; m - protein molecular weight marker marker; + c - positive control.

DISCUSSION: The current study provided a protocol for large scale callus propagation of *T. cordifolia* stem explants. In the present study, callus growth was initiated on MS media supplemented with cytokinin 2, 4-D (2.0 mg/L-1), and auxin NAA (0.1 mg/L-1). This indicates that the MS basal medium fortified with the above concentrations of 2, 4-D, and NAA was optimal for callusing of stem explants. Also, the explants were exposed to different concentrations of cyclodextrin (25 mg, 50 mg, 75 mg, and 100 mg), an auxin stabilizing agent, and the effects of the same were investigated. Cyclodextrins are cyclic carbohydrates of six or more glucopyranose units linked by a-1,4 bonds.

Cyclodextrins have also attracted considerable attention as agents capable of inducing defense responses in plant cell cultures and therefore acting as true elicitors [6-15]. Cyclodextrin can promote auxin's stability and allows its slow release in favor of the plant cultured *in-vitro* [28]. In the present study, the addition of an auxin protective oligosaccharide, β-cyclodextrin, to MS medium exerted a dose- and time-dependent effect on callus growth.

Explants required an optimum concentration of growth regulators for their proliferation into an unorganized callus. The differentiation process is very complex in plant tissue and controlled by many factors, such as type and concentration of hormones, nutrient medium, and developmental stage of explant during *in-vitro* culture [10-13]. Differentiation was dependent on the synergistic effect of auxin along with cytokinins in the medium, which evoked good results for indirect shoot proliferation. Hydrolysis of proteins leads to the cleavage of peptide bonds, which makes proteins to peptides and amino acids. These mixtures of peptides and amino acids are called protein hydrolysates [14-20]. Peptides will be released by enzymatic digestion of proteins. Bioactive peptides (often 2–20 residues) encrypted in the parent or natural protein molecule are usually inactive within the sequence of the protein and their biological activities are based on their amino acid composition and sequence.

They are, however, released during gastrointestinal digestion or *in-vitro* protein hydrolysis with proteases and exhibit varying abilities to scavenge or quench DPPH, hydroxyl, peroxyl and superoxide radicals as well as chelate metal ions. Studies on the antioxidant potential of protein hydrolysates prepared from callus tissues are less common. In the present study, for the first time, bioactive peptides were prepared from the callus tissue of *T.cordifolia* by hydrolyzing the proteins using enzymes such as chymotrypsin, trypsin, pepsin, and papain. Their antioxidant efficacy was evaluated by determining (1) 1, 1-diphenyl- 2- picrylhydrazyl (DPPH·) radical scavenging activity, (2) 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) radical scavenging capacity, and (3) Fe2+ chelation.

DPPH assay was carried out to check the ability of antioxidant compounds to transfer hydrogen atom/ electron to neutralize free radicals of DPPH [5]. DPPH can readily undergo scavenging by an antioxidant and gets converted into 1, 1-diphenyl-2-picrylhydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant present in the sample. DPPH assay offers an accurate and convenient method for determining the antioxidant capacity due to the relatively short time required for analysis. In the present study, among the four hydrolysates, chymotrypsin hydrolysis stood out as the best for the production of protein hydrolysate exhibiting antioxidant activity. Chymotrypsin hydrolysates prepared from callus grown in media supplemented with cyclodextrin, irrespective of the doses, showed maximum DPPH radical scavenging activity about eight-fold. On the other hand, trypsin hydrolysates exhibited differential activity in free radical scavenging; while the trypsin hydrolysates obtained from callus exposed 25 and 50 mg doses of cyclodextrin showed an increase ib DPPH radical scavenging, hydrolysates obtained from callus exposed to a higher dose of cyclodextrin exposed callus showed a decrease in enzyme activity.

A similar decrease in free radical scavenging activity was observed in pepsin hydrolysates obtained from callus exposed with increasing concentrations of cyclodextrin, while papain hydrolysates from callus exposed to 50 and 75 mg doses of cyclodextrin exhibited an increase in free radical scavenging activity. An ABTS+ assay is an excellent tool to determine the antioxidant activity of hydrogen-donating antioxidants (scavenging aqueous phase radicals) and of chain breaking antioxidants (scavenging lipid peroxyl radicals). Trypsin-digested protein hydrolysates prepared from callus grown in media supplemented with all concentrations of cyclodextrin recorded a consistent enhancement in ABTS scavenging activity. Pepsin hydrolysates, except those prepared from callus exposed to 25 and 75 mg cyclodextrin showed a similar increasing trend in antioxidant activity. The increase in ABTS scavenging activity confirms the antioxidant activity of hydrolysates.

On the contrary, papain hydrolysates obtained from callus exposed to only 25 mg of cyclodextrin recorded a decrease in ABTs activity, while no appreciable change in ABTs activity was noticed in chymotrypsin hydrolysates obtained from callus exposed to all doses. These findings indicate that ABTs activity depends on the enzyme used for hydrolysis and the dose of cyclodextrin exposed. Iron is an extremely reactive metal and will catalyze oxidative changes in lipid, protein, and other cellular components. Since, Fe^{2+} has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2}+ concentration in Fenton reactions affords protection against oxidative damage. In the present study, except papain, the other three hydrolysates obtained from callus exposed to 50, 75, and 100 mg doses of cyclodextrin, however, showed lower iron-chelating activity as compared to their respective controls, suggesting the specific effect of cyclo-dextrin. Similarly, the four-fold decrease in superoxide radical scavenging activity observed in the hydrolysates from callus tissue grown in media supplemented with 50 mg of cyclodextrin also implies the specific effect of cyclodextrin. Superoxide anion, which is the primary ROS produced in the body by a single electron reduction of molecular oxygen, is short-lived, very reactive, and toxic and can react with nitric oxide (NO) to form peroxynitrite (OONO-) [21].

Uncontrolled production of free radicals (superoxide, hydroxyl, singlet oxygen, peroxyl) by ROS during cellular metabolism/oxidation leads to oxidative stress, which consequently causes damages to cellular biomolecules such as proteins,

lipids, carbohydrates, enzymes, and DNA [7]. To protect the cells and organ system of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system that functions interactively to neutralize free radicals [29]. Thus, antioxidants are capably stabilizing or deactivating free radicals before they attack cells [22]. Antioxidants are substances that can prevent or retard oxidation of lipids, proteins, and DNA and protect the compounds or tissues from damage caused by oxygen or free radicals. The number of endogenous antioxidants present under normal physiological conditions may be insufficient to neutralize free radicals generated. Therefore, it is obvious to enrich our diet with antioxidants to protect against harmful diseases.

In the present study, electrophoretic profiling of protein was done by SDS-PAGE, the most widely used analytical method to resolve components of a protein mixture. Protein profiling of callus tissue is important to understand the changes which occur during growth. Protein profiling of callus tissue by SDS-PAGE showed a few tissue-specific bands at 41, 30, 20, and 9 kDa **Fig. 7** and **8**. These polypeptides showed variability based on the presence or absence and intensities of protein bands with banding patterns.

Protein banding patterns also exhibited a considerable range of variability about their mobilities and intensities. These four bands were feeble and weak in the negative control. However, all four bands were prominent in callus tissue exposed to media supplemented with 50, 75, and 100 mg cyclodextrin. From these observations, it is inferred that cyclodextrin at higher doses caused the expression of 41, 30, 20, and 9 kDa proteins. These bands were confirmed by comparison with the reference sample. Further, this suggested that the differentiation process was dependent on the concentration of cyclodextrin. These polypeptides could be potentially used as a marker to decipher the differentiation pathway and selection of organogenic potential callus or tissue.

Secondly, it was observed that the presence of sugar concentration in the MS media has a specific impact on the expression of these polypeptides in the callus tissue. In particular, 41 kDa polypeptide expressions was feeble and less distinct in callus tissue exposed to different concentrations of cyclodextrin without sugar concentration, as compared to the respective bands in the callus tissue grown in media supplemented with sugar and cyclodextrin. Of the four polypeptides, purification of 30 kDa polypeptide by DEAE-Sephadex G50 revealed distinct quantitative differences in response to different concentrations of cyclodextrin. Peptide concentration increased appreciably in callus tissue exposed with increasing concentrations of cyclodextrin (25, 50, and 75 mg), while the same was not detectable in callus tissue supplemented with 100 mg of cyclodextrin.

CONCLUSION: The overall results obtained in the present study demonstrate the interesting antioxidant properties of chymotrypsin and trypsin hydrolysates. In particular, these hydrolysates. Showed the most promising free radical scavenging effects evaluated by DPPH and ABTS tests and a good antioxidant activity through different mechanisms of action. This finding also agrees with the proposition that chymotrypsin and trypsin are relatively more efficient in the production of hydrolysates with promising radical scavenging effects, as compared with plant protease, papain. SDS-PAGE studies revealed that there was a synthesis of four callus stem-specific polypeptides (41, 30, 20, and 9 kDa). Expression of these shoot-specific polypeptides could be used as potential markers to characterize the differentiation pathway during *in-vitro* propagation. In conclusion, the above findings inferred that the callus tissue of *T. cordifolia* may be propagated not only for food consumption but also as a source of healthy compounds for the development of dietary supplements and to protect against oxidative stress.

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