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IN VITRO ESTIMATION OF POTENTIAL ANTIOXIDANT COMPOUNDS IN *TINOSPORA CORDIFOLIA* BY CHROMATOGRAPHY

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ABSTRACT

Tinospora cordifolia (Willd.) Miers belonging to famiy Menispermaceae is an important medicinal plant. It is used as a rejuvenator and to improve the immunity and body resistance against a wide range of pathogenic attacks. In the present study, we have analysed the antioxidant activity of leaf explant-derived callus raised on WPM supplemented with NAA (2.0mg/L) and BA (1.0mg/L) in *Tinospora cordifolia* by qualitative/quantitative chromatographic techniques. Methanolic extracts of callus and plant parts showed the presence of ascorbic acid and gallic acid on TLC analysis. These extracts were estimated for gallic acid and ascorbic acid through HPLC using PDA detector. Higher ascorbic acid content as well as gallic acid was estimated in callus treated with 100mM NaCl after 4- weeks of growth.

KEYWORDS: *Tinospora cordifolia*, TLC, HPLC, Ascorbic Acid, Gallic Acid and NaCl

INTRODUCTION

Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae (Anonymous, 2003). It is distributed throughout tropical Indian subcontinent and China, ascending to an altitude of 300 m. The chemical constituents reported from this shrub belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. The literature survey records a variety of medicinal properties such as anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, heap to protective, immune modulatory and anti-neoplastic (Singh *et al*., 2003). Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. Due to the complex nature and inherent variability of the chemical constituents of the plant-based drugs, it is difficult to establish quality control parameters and modern analytical techniques like HPLC and HPTLC are expected to help in circumventing this problem (Patel *et al*., 2010). Several analytical methods have been reported for the determination of antioxidant activity based on DPPH assay, HPLC and TLC but there are very few analytical for estimation of antioxidant activity by TLC and HPLC in *Tinospora cordifolia*. Advances in the area of plant tissue culture for the production of secondary metabolites have made it possible to increase the yield of a wide variety of substances with pharmaceutical value such as alkaloids, terpenoids, steroids, phenolics and flavonoids[4]. In the present study, we have analysed the antioxidant activity of *Tinospora cordifolia* leaf explant-derived callus raised on WPM (Lloyd and McCrown, 1980) supplemented with NAA (2.0mg/L) and BA (1.0mg/L) by qualitative/quantitative chromatographic techniques.

MATERIAL AND METHODS

Dissected young leaves of ca 45 ± 2 cm^2 were put under running tap water for the removal of soil and other superficial contamination and then allowed to stay for 3 to 4 h in double distilled water to facilitate leaching out of phenolics and characteristic gummy substance. The surface was wiped with 70% ethanol followed by thorough washing

with Tween-20 under running water. These washed explants were transferred into a container containing 5.0 % sodium hypochlorite (v/v) for 5-8 min., surface sterilized with 0.1%$HgCl_2$(w/v) for 5-8 min followed by rinsing 4-5 times with sterile DDH_2O (double distilled water) under laminar hood. After trimming the edges, explants were prepared upto 1.0 cm in size and transferred to a sterile petri-dish containing 0.1% ascorbic acid (w/v). The explants were inoculated on sterile WPM medium containing 2.0 mg/L NAA and 1.0 mg/L BA for callus development and subculture on liquid medium with similar PGRs along with different concentration of NaCl (50-150mM). The cell suspensions were incubated on a gyratory shaker at 100 rpm and 25 ± 2 ^{0}C temperature for 4-weeks and used for TLC and HPLC analysis after extraction of desired metabolites.

Sample Preparation

The explant and calli were washed, dried in hot air oven at 40^{0}C and then finely powdered. The weighed powder was extracted three times at room temperature with 80% methanol, assisted by ultra sonicator (Citizen CD-4820) for 30 min. After centrifugation extracts were filtered using Whatman filter paper No.1, kept in a vaccum desiccator for complete removal of solvent. The resulting extracts were dissolved in absolute HPLC grade methanol for TLC and HPLC analysis.

A modified TLC method (Jasprica *et al*., 2007) in combination with DPPH assay was employed for antioxidant activity by using silica gel 60 F_{254} pre-coated plate (Merck) as stationary phase and mixture of ethyl acetate: formic acid: water (82:9:9)as mobile phase; 20µL of diluted crude extract and standard markers were loaded. After development the plate was sprayed with 0.05% DPPH w/v (in methanol) solution. Pale yellow spots were observed after 12 h of incubation in dark with pink background under light exposure.

The HPLC analysis was performed on UFLC prominence system (SHIMADZU, Kyoto, Japan) consisting of LC-6AD binary isocratic pump, a manual injector with a 20 µL fixed loop and a SPD-20A Prominence UV- visible diode array detector (DAD) and CTO-20A column oven. The separation was performed on a Phenomenex C^{18} column (250 × 4.6 mm, 5 µm particle size, Phenomenex, Luna, USA), mobile phase consisting of 8% acetonitrile: water acidified with 0.1% phosphoric acid v/v at flow rate of 0.8 mL/min with UV detection at 245 nm for ascorbic acid and methanol: water (80: 20) at flow rate of 1 mL/min with UV detection at 280 nm for Gallic acid at an ambient temperature of 22 ± 2 ºC. Chromatographic data was recorded and processed using Shimadzu LC solution software.

STATISTICAL ANALYSIS

One-way ANOVA followed by post hoc test ascertaining significant difference using the Microsoft Excel-2010 was performed to detect the significant differences ($p<0.01$) of three separate replicates.

RESULTS AND DISCUSSIONS

Methanolic extracts of explants and callus (control and treated) were loaded on TLC for antioxidant activity along with standard ascorbic acid and gallic acid. Pale yellow spots with purple background, after spraying with 0.05% (w/v) DPPH solution were analyzed for R_f value, which was recorded to be similar to standard's R_f value. The presence of gallic acid in all samples was detected in the form of spots coherent with standard Gallic acid'sR_f value (0.97). Similarly ascorbic acid was also detected in the form of spot with similar R_f value (0.30 or 0.38) as of standard ascorbic acid (Table 1 and Figure 1).

Ascorbic acid was detected in almost all the samples from plant parts as well as from callus (Control and Treated)

by HPLC (Figure 2, A-G). Peak of ascorbic acid was detected at 245 nm wavelength using PDA detector, solvent A (8% acetonitrile): solvent B (0.1%v/v phosphoric acid in water) as mobile phase at ambient temperature in binary mode with a RT (Retention Time) of 2.93 ± 0.1min in standard as well as in samples. In control callus, ascorbic acid increased significantly ($p<0.01$) upto 11% and in callus treated with 100mM NaCl upto 24%, as compared to leaf which was used as explant for callus development. Ascorbic acid content in stem and root was lower than in leaf. Also in salt treated calli at the concentration of 50mM and 150mM NaCl, ascorbic acid content was lower than in leaf explant (Figure 2 (A-C) and Figure 3).

Gallic acid was detected in methanol extracts of plant parts and calli treated with salt in *Tinospora cordifolia* (Figure 2, A-E). The RT (2.4 min) of peak in all samples was similar to RT of standard Gallic acid detected at 286 nm wavelength using PDA detector and methanol: water (80: 20) in binary mode. A significant increase of Gallic acid was detected in calli treated with 100mM NaCl as compared to leaf explant ($p<0.01$). Maximum fall was recorded in root (Figure 2 (D-F) and Figure 4).

CONCLUSIONS

The developed TLC and HPLC method was precise, specific, accurate and robust for the determination of antioxidant compounds in methanolic extracts of *Tinospora cordifolia* plant parts as well as callus. Our TLC method in combination with DPPH assay proves to be a reliable and applicable method for analysis of antioxidant capacity in selected plant. This has been standardized and carried out for the first time with optimization for the selected plants of the present investigation with authentic markers successfully. Statistical analysis proves that NaCl at 100mM concentration enhanced significant amount of ascorbic acid and gallic acid in 4-week old callus raised on WPM supplemented with 2.0 mg/L NAA and 1.0mg/L BA.

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APPENDICES

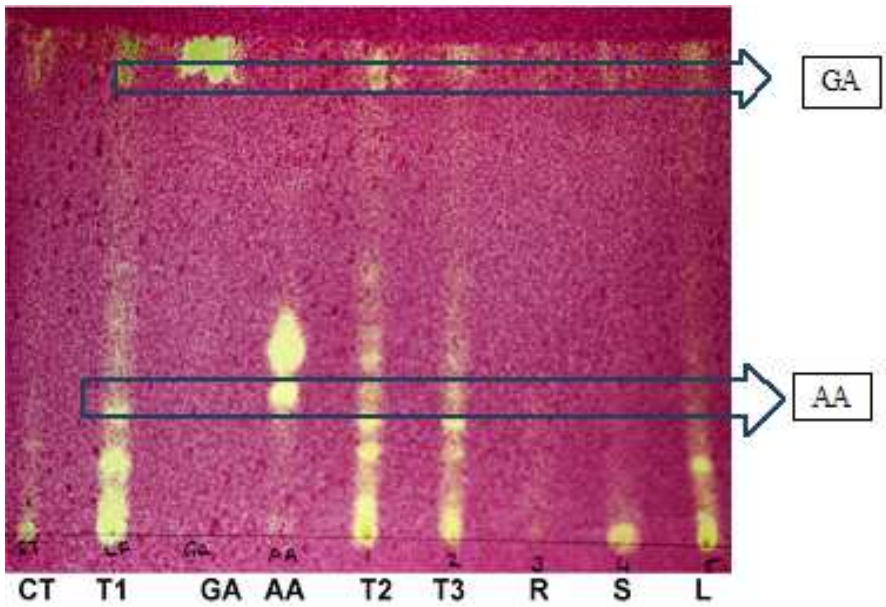
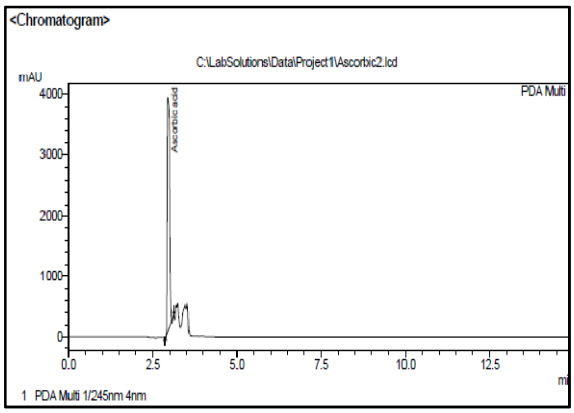


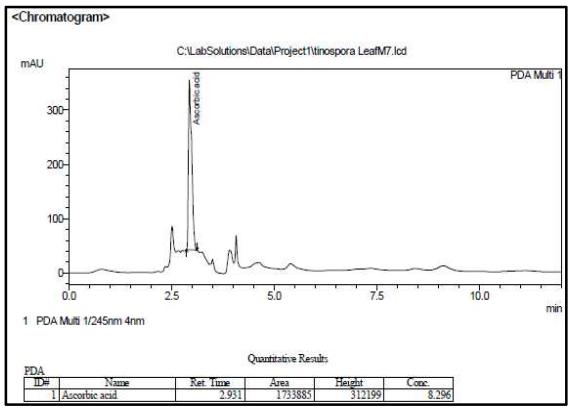
Figure 1: TLC Plate Showing Antioxidant Potentiality in *Tinospora cordifolia*, after Spraying with 0.05% DPPH by Using a Mixture of Ethyl Acetate: Formic Acid: Water (82: 9: 9) as Mobile Phase (CT-Control Callus, GA- Gallic Acid, AA- Ascorbic Acid, T1-Callus on PGR +50mM, T2- PGR+100mM, T3- PGR+150mM NaCl, R-Root, S-Stem and L-Leaf)

Table 1: R_f Value of Visible Band after TLC Separation of Methanolic Extracts of Plant Parts and Callus in *Tinospora cordifolia* by Spraying DPPH Solution

S.No.	Extract	Spots	R_f Values
1	Leaf	2	0.24, 0.97
2	Stem	1	0.97
3	Root	1	0.97
4	Control callus	2	0.24, 0.97
5	Callus+50mM	3	0.21, 0.28, 0.97
6	Callus+100mM	5	0.21, 0.28, 0.30, 0.38, 0.97
7	Callus+150mM	5	0.21, 0.28, 0.30, 0.38, 0.97
8	Gallic Acid	1	0.97
9	Ascorbic Acid	2	0.30, 0.38



(A)



(B)

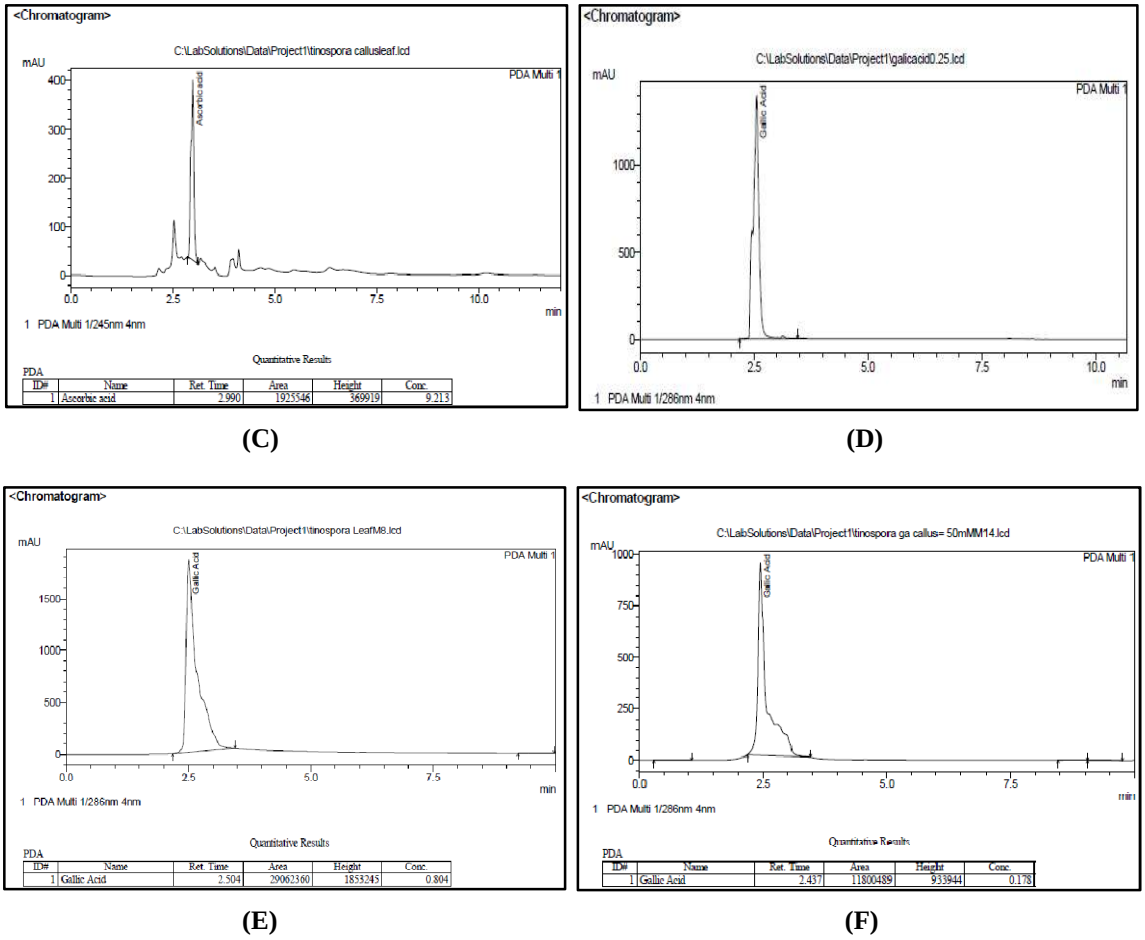


Figure 2: HPLC Chromatogram of Ascorbic Acid and Gallic Acid of Methanolic Extracts of Explant and Callus of *Tinospora cordifolia* Raised on WPM Containing 2.0mg/L NAA and 1.0 mg/L BA along with Salt. (A-Standard Ascorbic Acid, B-Leaf, C-Control-Callus, D-Standard Gallic Acid, E-Leaf and F-Callus on 50mM NaCl)

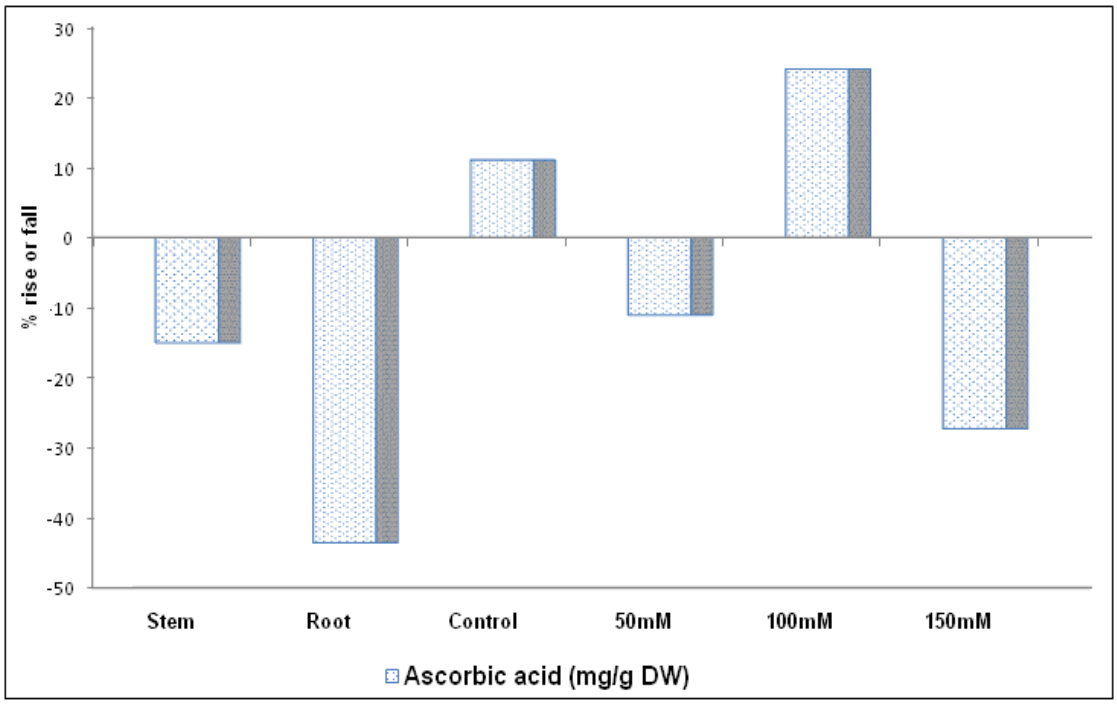


Figure 3: % Rise or Fall in Ascorbic Acid Content in Stem, Root and Calli as Compared to Leaf Explant in *Tinospora cordifolia* by HPLC

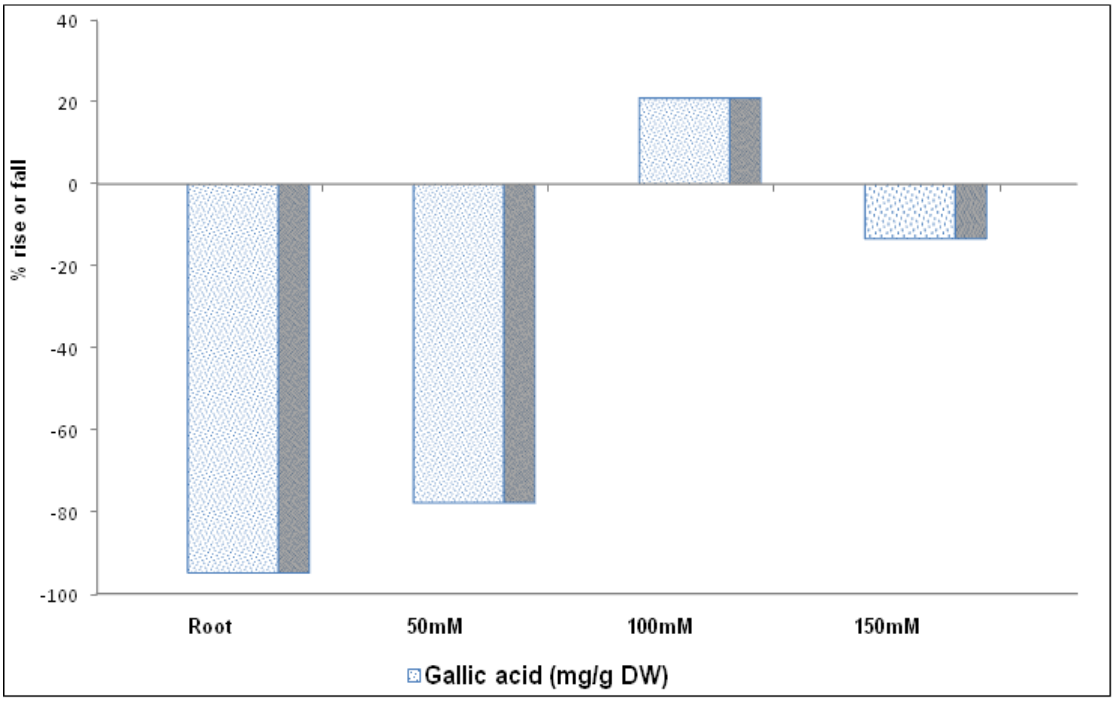


Figure 4: % Rise or Fall in Gallic Acid Content in Root and Calli as Compared to Leaf Explant in *Tinospora cordifolia* by HPLC