Research Article

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ANTIOXIDANT ACTIVITY AND RP-HPLC ANALYSIS OF DIOSGENIN FROM THE CALLUS OF *TRIBULUS TERRESTRIS* LINN.

Yogendra Kumar Gautam^{1*} and Vimala Y²

¹Research Scholar, Department of Botany, CCS University, Meerut, UP, India

²Professor, Department of Botany, CCS University, Meerut, UP, India

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*Corresponding author

Dr. Yogendra Kumar Gautam, Dept. of Botany, CCS University Meerut 250 004 UP, India

E-mail: yogendra.gautam79@gmail.com

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ABSTRACT

The aim of present study was to develop TLC method for determining the contribution of individual compound to overall antioxidant activity and effect of varying concentrations of NaCl on diosgenin content in stem explant derived-callus of *Tribulus terrestris*. A modified TLC method in combination with DPPH assay was employed for antioxidant activity and RP-HPLC method for estimation of diosgenin content by using mobile phase consisting of acetonitrile: water, in the ratio of 30:70 v/v at flow rate of 1 mL/min with 203 nm wavelength in PDA detector at an ambient temperature of $22 \pm 2^\circ\text{C}$. Statistical analysis was carried out using one way ANOVA followed by post hoc test in Microsoft excel-2010. It was clear from TLC investigation that antioxidant activity in *Tribulus terrestris* was due to the presence of a compound of R_f value (0.58) rather than gallic or ascorbic acid. Further, TLC was developed for testing sapogenin-like activity by using a different mobile phase and spraying reagent. All the loaded samples showed the visible spots with similar R_f value (0.94) as of standards (β -sitosterol and Diosgenin). During RP-HPLC profiling, diosgenin was detected in acetonitrile: water (30:70) as mobile phase and the peak of standard as well as of samples was at the retention time of 3.1 ± 0.1 minutes at 203 nm wavelength. Diosgenin content decreased as concentration of NaCl increased and it was maximum at 50 mM NaCl treated callus as compared to explant used for callus development.

Keywords: Callus, DPPH (1, 1-diphenyl-2-picrylhydrazyl), HPLC (High performance liquid chromatography), NaCl, TLC (Thin layer chromatography) and *Tribulus terrestris*.

INTRODUCTION

Tribulus terrestris has the long history of use in Ayurveda and Unani system of India. The decoction of leaves is useful as a gargle from mouth trouble, painful gums and reduced inflammation. It is also used in traditional Indian medicine in the therapy of a variety of health conditions affecting liver, kidney, and cardiovascular and immune system. The fruits yield protodioscin, the terrestrosins A-E, desgalactotigonin and furostanol glycosides with β -sitosterol and stigmasterol. The flowers also contain steroidal sapogenins based on diosgenin, hecogenin and ruscogenin^{1,2}. The antioxidant potential of ethanolic extract of the fruits of *Tribulus terrestris* for hydroxyl radical and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical scavenging activity³ and for inhibition of lipid peroxidation has been assayed *in vitro*⁴. The extracts showed considerable inhibition of lipid peroxidation. Preliminary detection of sterols in the methanol extract of different parts of some selected plants including *Tribulus terrestris* showed positive response for Burchard's or Salkowski test⁵. In the present study, a simple, rapid, cost-effective and precise TLC and HPLC method has been developed for quantification of antioxidant activity of diosgenin in stem explant-derived callus raised on MS medium⁶ supplemented with NAA (3.0 mg/L) and BA (1.0 mg/L).

MATERIAL AND METHODS

For *in vitro* studies, a well-defined protocol⁷ was followed for callus induction with a modification at surface sterilization step. Plant parts were wiped with 70 % ethanol and then washed with Tween-20, agitated on a rotator shaker at 100 rpm for 30 minutes and then washed

thoroughly with double distilled water to reduce frequency of contamination due to presence of characteristics silky hairs on the surface of plant. Followed by standard surface sterilization methods, explants were inoculated on sterile MS medium containing 3.0 mg/L NAA and 1.0 mg/L BA for callus development and subculture on same medium with similar PGRs along with different concentration of NaCl (50 -150 mM) for 4 and 8-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 60°C and then finely powdered. The weighed powder was extracted three times at room temperature with 80 % methanol, using ultra sonicator (Citizen # CD-4820) for 30 minutes. After centrifugation and fractionation of extracts with equal volume of ethyl acetate, filtration was done by using Whatman filter paper No.1, kept in a vacuum desiccator for complete removal of solvent. The resulting extracts were dissolved in absolute HPLC grade methanol for TLC and HPLC analysis. A modified TLC method⁸ in combination with DPPH assay was employed for antioxidant activity and for sapogenins⁹. Absorption spectra of all the extracts were recorded against methanol using a Shimadzu UV-1800 spectrophotometer attached to a PC with UV Probe software (Shimadzu, Kyoto, Japan). The RP-HPLC analysis was performed on UFLC prominence system (Shimadzu, Kyoto, Japan) consisting of LC-6AD binary pump system, 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 C18

column (250 × 4.6 mm, 5 µm particle size, Phenomenex, Luna, USA) by using mobile phase consisting of acetonitrile: water, in the ratio of 30:70 v/v at flow rate of 1 mL/min at 203 nm at an ambient temperature of 22 ± 2°C. 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 done to detect the significant differences ($p < 0.01$) of three separate replicates.

RESULTS AND DISCUSSION

Out of three types of explants viz. leaflet, stem and fruit; stem proved to be the best in terms of callus formation with higher frequency and low contamination. Maximum number of explants showed callusing in minimum number of days when raised on MS medium containing NAA (3.0 mg/L) with BA (1.0 mg/L). TLC analysis was carried out using mixtures of toluene: ethyl acetate: formic acid: methanol (6: 2: 1: 0.5) as mobile phase. After spraying 0.05 % DPPH, antioxidant potential could be noted. Plant parts showed more spots than the callus did, and the standards showed only one spot each for ascorbic acid and gallic acid. It was clear from TLC investigation that antioxidant activity in *Tribulus terrestris* was due to the presence of a compound of R_f value (0.58) rather than gallic or ascorbic acid (Figure 1a). Furthermore, to confirm sapogenin-like activity, TLC was developed using mixtures of Chloroform: Glacial acetic acid: Methanol: Water (60: 32: 12: 8) as mobile phase. After spraying with anisaldehyde reagent (prepared by mixing 0.5 mL anisaldehyde, 10 mL glacial acetic acid, 85 mL methanol and 5 mL concentrated sulfuric acid), the plate was heated at 95°C for 10 minutes. All the loaded samples showed the visible spots with similar R_f value (0.94) as of standards (β -sitosterol and Diosgenin). However, there

were some additional spots, observed in samples of plant parts as well as of callus (Figure 1b). UV-VIS spectrum analysis resulted in detection of diosgenin in ethyl acetate fraction of all plant parts and callus extract of *Tribulus terrestris* with similar $\lambda_{\max}$ as was observed with standard solution of diosgenin 203 nm $\lambda_{\max}$ (Figure 2a, b). During RP-HPLC profiling, using acetonitrile: water (30:70) as mobile phase at ambient temperature in binary mode at 203 nm wavelength, diosgenin was detected by PDA detector. The peak of standard as well as of samples was detected at the retention time of 3.1 ± 0.1 minutes (Figure 3a, b). Diosgenin decreased as concentration of salt increased in 4-week old calli and it was maximum at 50 mM salt treated callus as compared to explant used for callus development. Same result was noted after 8-weeks of growth, again it was higher in calli treated with 50 mM salt (compared to control) which decreased at 100 mM salt and further increased at 150 mM salt concentration (Figure 4). Ageing resulted in significant accumulation of higher amount of diosgenin as compared to 4-week old calli; there was 2-fold gain ($p < 0.01$). In the present investigation, TLC results showed the R_f value of spots of sample extracts to be same as that of standards (β -sitosterol and diosgenin). Diosgenin in the callus of *Costus speciosus* (Zingiberaceae) was also analyzed by performing TLC analysis¹⁰. With UV-VIS spectrophotometric analysis, the peaks of samples and standard with same $\lambda_{\max}$ were recorded which advocate the presence of diosgenin. HPLC results further verified the same. Similar approach was used for analysis of major phenolic compounds in extracts of traditional Chinese prescription¹¹ and characterization and isolation of sapogenin in *Moringa oleifera* pods¹². The TLC results from all extracts showed total absence of the spots of ascorbic acid and gallic acid. The HPLC results further confirmed the absence of peak. High antioxidant activity was spectrophotometrically confirmed in samples showing high diosgenin content.

Fig-1: (A) TLC for antioxidant activity through DPPH assay (B) TLC for sapogenin activity (L- Leaf, S- Stem, F- Fruit, C- Control, AA- Ascorbic Acid, GA- Gallic Acid, T1-Callus on PGR +50mMNaCl, T2- PGR+100mM, T3- PGR+150mM, B- β -sitosterol and D- Diosgenin)

Fig-2: Absorption spectrum of (A) Standard (diosgenin), (B) Sample

<Chromatogram>

Fig-3: HPLC chromatograph of (A) Diosgenin (Standard), (B) Sample

CONCLUSION

The existing protocol for determination of the antioxidant potential of plant extracts is based on broad spectrum spectrophotometric assay. However, this approach does not allow for determining the contribution of individual compounds to overall antioxidant activity. Our modified TLC method including DPPH assay proves to be a more reliable and applicable method for analysis of individual compounds for antioxidant potential in *Tribulus terrestris* and has been carried out for the first time with optimization for the diosgenin, not laid down earlier in protocols for selected plant nor reported in the literature within accessible limits. This has been further standardized and confirmed by absorption spectrum and HPLC against standards. In conclusion, our method proves to be a sound, accurate and rapid method for identification and quantification of diosgenin possessing antioxidant activity. The method can be equally used for pharmaceutical formulations that contain diosgenin.

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