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**Supplementary Data**
**Phytochemical, antioxidant, antimicrobial, and protein binding qualities of hydro-ethanolic extract of *Tinospora cordifolia***

## S1. Qualitative analysis of phytochemicals

Small branches and stem bark extracts (petroleum ether, acetone and methanol) of TC were analyzed for the presence of various phytochemicals using the respective chemical tests as follows.

### S1.1. Test for glycosides

0.5 mL extract was taken in a test tube, 0.2 mL of 10 % ferric chloride solution and (50 %) glacial acetic acid added. Few drops of concentrated sulphuric acid were added. A blue color production shows the presence of glycosides.

### S1.2. Tests for terpenoids

Extract was mixed with chloroform and a few drops of conc. $H_2SO_4$ were added, shaken well and allowed to stand for some time. Red color appeared at the lower layer indicated the presence of steroids and formation of yellow colored layer indicated the presence of terpenoids.

### S1.3. Test for proteins

An aliquot of 2 mL of extract was treated with one drop of 2% copper sulphate solution. To this, 1 mL of ethanol (90%) was added, followed by excess of potassium hydroxide pellets. Pink color in ethanol layer indicated the presence of proteins.

### S1.4. Test for amino acids

Two drops of ninhydrine (5%) were added to 1 mL of extract. A characteristic purple color indicated the presence of amino acids.

### S1.5. Test for alkaloids

Two millilitre of 1 % HCl was mixed with 0.1 gm of crude extract and heated slightly. After cooling Wagner's reagent and Mayer's reagent were added to it. The presence of buff colored precipitate indicated the presence of alkaloids.

### S1.6. Test for carbohydrates

Benedict's reagents was mixed with the 1 mL of crude extract and slightly boiled, appearance of reddish brown precipitate indicated the presence of the carbohydrates.

### S1.7. Test for flavonoids

The appearance of pink scarlet color when 1 mL of crude extract was mixed with few drops of concentrated HCl and Mg pellets indicated the presence of flavonoids.

### S1.8. Test for phenols

2 mL of 2 % ferric chloride was mixed with the 1 mL of crude extract and the presence of blue-green or black coloration indicated the presence of phenols.

**S1.9. Test for saponins**

1 mL of crude extract was mixed well in water and shaken; the appearance of foam indicated the preliminary evidence for the presence of saponins.

**S1.10. Test for steroid**

2 mL of extract was mixed with 2 mL $H_2SO_4$ and slowly added to 2 mL of acetic anhydride. The color change from violet to green or blue indicated the presence of steroids.

**S111. Test for tannins**

1 mL of extract mixed in distilled water and filtered. Few drops of ferric chloride solution were added to the filtrate. The green or blue-green precipitate indicated the presence of tannins.

**S2. Antioxidant assays**

***S2.1. DPPH radical scavenging assay***

100 µL of 0.01 mM solutions of different extracts and ascorbic acid were added to 2.0 mL of 0.01 mM solution of DPPH (prepared in ethanol) in 5.0 mL amber color volumetric flasks. After 5 minutes, the absorbance at 517 nm was recorded. Pure DPPH solution was used as a control. The radical scavenging ability was calculated according to the formula: Radical scavenging activity % = $(A_0-A_T/A_0) \times 100$. Where, $A_0$= absorbance of pure DPPH solution, and $A_T$ = absorbance of (DPPH + 100 µL extract or ascorbic acid).

**S2.2. Metal chelating activity**

200 µL of the extract or control was incubated for 10 min together with 100 µL of $FeSO_4$ (2 mM) and 400 µL ferrozine (5 mM). After 5 minutes 2 mL methanol was added and incubated at 37°C for 15 min. The absorbance was measured at 560 nm. The % chelating activity was calculated by the following formula: Metal chelating ability (%) = $[(A_0-A_1)/A_0] \times 100$ Where $A_0$ = absorbance of the control; $A_1$ = absorbance of the sample/ standard. EDTA was taken as a positive control.

**S2.3. Ferric reducing ability of plasma (FRAP) assay**

The antioxidant capacity of extracts were estimated according to the procedure given below. FRAP reagent (900 µL), prepared freshly and incubated at 37°C, was mixed with 90 ìL of distilled water and 30 µL of test sample (for the reagent blank). The test samples and reagent blank were incubated at 37°C for 30 minutes in a water bath. The FRAP reagent contained 2.5 mL of 20 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. At the end of incubation period the absorbance readings were recorded immediately at 593 nm using a spectrophotometer. Percentage inhibition of FRAP activity was calculated as: % Inhibition = $((A_0-A_1)/A_0) \times 100$; Where $A_0$ is the absorbance of the control (Fe(II)) and $A_1$ is the absorbance of the sample.

**S2.4. Ferric reducing power**

100 µL of extract or control was added to 2.5 mL of potassium ferricyanide (1% prepared in phosphate buffer pH 6.6) and incubated at 50°C for 20 min. After incubation, 2.5 mL of 10 % trichloroacetic acid was added to the reaction mixture and centrifuged at 1000 r/min for 8 min. About 5 mL of supernatant was extracted and made up to 10 mL with deionized water. To this solution, 0.5 mL of 0.01% ferric chloride was added and absorbance was measured at 700 nm. Ascorbic acid was used as a standard.

***S2.5. Scavenging activity of superoxide anion***

This activity was carried out by mixing the 100 µL of extract(s) or control to 1 mL of nitroblue

tetrazolium (NBT) solution (156 ìM prepared in Tris – HCl buffer), 1 ml of nicotinamide adenine dinucleotide (NADH) solution (468 ìM prepared in Tris – HCl buffer), 700 µL Tris – HCl buffer (pH 8.0) and 0.1 mL of phenazine methosulphate (PMS) solution (60 µM prepared in Tris – HCl buffer), and incubated at 25°C for 5 min. After 5 min the absorbance was read at 560 nm. Ascorbic acid was used as a positive control. Percentage inhibition of scavenging activity of superoxide anion was calculated as: % Inhibition = $((A_0-A_1)/A_0)\times100$; Where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample.

**S2.6. Nitric oxide scavenging activity**

Nitric oxide was generated from sodium nitroprusside and its quantity was determined using Griess reagent. 100 µL of extract(s) and control were mixed with 1 mL of sodium nitroprusside solution (10 mM; prepared in phosphate buffer pH 7.2), and incubated at 37°C for 120 min. 1 mL sulphanilic acid (1 % , prepared in 2 % $H_3PO_4$) was added and incubated at 37°C for 5 min. 0.5 mL of naphthylethylenediamine dihydrochloride (0.1 %) was added and incubated at 37°C for 20 min. The absorbance was measured at 540 nm. The percentage of nitric oxide scavenging by the following formula: Nitric oxide scavenging (%) = $[(A_0-A_1)/A_0] \times100$ Where $A_0$ = absorbance of the control; $A_1$ = absorbance of the sample/ standard. Ascorbic acid was used as the standard.

**S3. Total phenolic content**

Total phenolic content of the extract was analyzed using Folin's Ciocalteau reagent. Briefly, 1 mL of sample of varying concentrations was incubated in 5 mL of Folin-Ciocalteu reagent and 4 mL of 1 mol/L $Na_2CO_3$. After 15 min of incubation, absorbance was measured at 765 nm by spectrophotometer. Gallic acid dissolved in ethanol was used as standard. The total phenol content was reported in terms of mg of gallic acid equivalents/g of dry eight (GAEs/ g DW). All the tests were performed in triplicates.

**S4. Total flavonoids content**

The total flavonoids content was estimated by taking 0.3 mL of 5 % (w/v) $NaNO_2$ to every 4 mL of sample of varying concentrations. After 5 min of incubation, 0.3 mL of 10 % (w/ v) $AlCl_3$ was added. After 6 min, 2 mL of 1 mol/L NaOH was added. The total volume was made up to 10 mL with distilled water. After vortex shaking for 1 min, the absorbance was noted at 510 nm. The total flavonoids content was reported in terms of mg of quercetin equivalents/g of dry weight of drug (QRE/ g DW). All the tests were performed in triplicates.

**S4. Protein (BSA) binding assay**

Aspirin (control) (of concentrations 50 µL, 100 µL, 150 µL, 200 µL and 250 µL) was added to 2 mL of BSA solution (5 mM) (prepared in phosphate buffer of pH 7.2). Final volume was make up to 5 mL. Absorbance was noticed at 280 nm. An increase in the concentrations resulted in an increase in UV light absorption and shifting of BSA band from 280 to 272 nm that can be related to complex formation. The binding constants of the complexes are determined by using UV-vis spectroscopic method. The double reciprocal plot of $1/(A-A_0)$ versus 1/(ligand concentration) is found linear and the binding constant (k) can be estimated from the ratio of the intercept to the slope. Same was repeated with various extract of DP. All the tests were performed in triplicates.

**S6. Anti-inflammatory activity**

Inhibition of protein denaturation was evaluated by the method given below. 2 mL of 1 % bovine serum albumin (BSA) was added to 200 ìL of plant extract. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetyl salicylic acid was taken as a

positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using: % Inhibition = 100"(($A_1$-$A_2$)/$A_0$)×100); Where $A_1$ is the absorbance of the sample, $A_2$ is the absorbance of the product control and $A_0$ is the absorbance of the positive control. All the tests were performed in triplicates.

**S7. Antimicrobial activities**

Antimicrobial activities of all the extracts were performed on three fungal and four bacterial strains. The fungal strains **(***Aspergillus parasiticus* NCIM 696, *Candida albicans* MTCC 183, and *Aspergillus niger* NCIM 501) and bacterial strains (*Streptococcus mutans* MTCC 497 (Gram +ve), *Salmonella enteric* MTCC 164 (Gram -ve), *Staphylococcus aureus* MTCC 7443 (Gram +ve) and *Pseudomonas aeruginosa* MTCC 4673 (Gram -ve)) were purchased from the Institute of Microbial Technology, Chandigarh and National Chemical Laboratory with unique code number. Antimicrobial activities of various extract of small branches and stem bark of DP was analyzed against three bacterial and three fungal strains as protocol given below.

**S7.1. Antibacterial activities:**

*In vitro* antibacterial studies were carried out by agar disc diffusion method against test organisms. Nutrient broth (NB) plates were swabbed with 24 hrs old broth culture (100 ml) of test bacteria. Sterile paper discs (5 mm) were put into each petriplate. Different concentrations of various extracts (250, 500, 1000 mg/L) were added into the discs by dipping individual disc into solution containing test tubes. In our experiment, solvent(s) acted as negative control and chloramphenicol as positive control. The plates were incubated at 37$^{o}$C for 24 hrs. After appropriate incubation, diameter of zone of inhibition of each disc was measured. All the tests were performed in triplicates.

**S7.2. Antifungal activities**

*In vitro* antifungal studies were carried out by potato dextrose agar medium disc diffusion method against test organisms. All fungi were cultured in potato dextrose agar medium. For this purpose, potato dextrose agar medium was poured in the sterilized petri dishes and allowed to solidify, where dishes were inoculated with a spore suspension of ($10^6$ spores/mL of medium). Test crude extracts to a final concentration of 250, 500 and 1000 mg/L and soaked in filter paper discs, which were placed on the already seeded plates and incubated at 37$^{o}$C for 96 hrs. After 96 hrs, inhibition zone appeared around the discs in each plate was measured. In this experiment solvent acted as negative control and ketoconazole as positive control. All the tests were performed in triplicates.

**S8. Quantitative analysis of phytochemicals present in the hydro-alcoholic extract of *T. cordifolia***

| Mass | m/z [2] | Name of compound |
|---|---|---|
| 102 | 75 , 57, 45 | N-Methoxy-N-methylacetamide |
| 112 | 112, 94, 68, 44 | 4-Imidazolecarboxylic acid |
| 141 | 138, 124, 105 | 4-Hydroxy-3,6-dimethylpyran-2-one |
| 143 | 131, 102 | Methyl 3-hydroxyisoxazole-5-carboxylate |
| 178 | 177, 144, 141, 113, 102 | 5-Hydroxy-1,4-naphthalenedione |
| 183 | 102, 73, 56, 43 | D-Mannitol |
| 219 | 127, 141, 113, 98, 27 | Diethyl bis(hydroxymethyl)malonate |
| 227 | 193, 186, 152, 131 | 7,8-Dihydroxy-6-methoxychromen-2-one |
| 256 | 253, 124, 75, 63 | Pinocembrin |
| 264 | 220, 192, 166, 102 | 2-(2-(5-Nitro-2-furyl)vinyl)quinoxaline |
| 294 | 273, 236, 227, 178 | 2-(4,5-Dihydroxy-2-methylphenyl)-6-hydroxy-4-methoxybenzoic acid |
| 301 | 257, 228, 198, 76 | Quercetin |
| 315 | 181, 152, 136, 83 | (3,4-Dimethoxyphenethylaminomethyl) methyl-malonic acid |
| 418 | 399, 381, 303, 189, 135 | β-Sitosterol |
| 342 | 167, 145, 131 | Magnoflorine |
| 376 | 272, 232, 141, 123, 112 | Syringin |
| 396 | 363, 184, 155, 103 | Tinocordioside or Heptacosanol |
| 451 | 248, 207, 203, 133, 119 | Betulinic acid |
| 465 | 301, 167, 144, 131 | 20-Hydroxyecdysone |
| 624 | 451, 301, 183, 143 | Cordifolide A |
| 656 | 356, 198, 152, 91 | p-Octylphenyl 2-chloro-4-(p-heptylbenzoyloxy) benzoate |
| 678 | 566, 356, 157, 141 | 5-Cholestene-3beta,4beta-diol bis(p-chlorobenzoate) |
| 736 | 225, 124, 87, 53 | Trihexadecyl borate |
| 792 | 390, 363, 303, 299, 152 | Hexopyranoside, 1-[7-[[4-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]oxy]-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2,4b,8,8-tetramethyl-2-phenanthrenyl]-2-hydroxyethyl |

**S9.** Direct mass quantitative analysis of hydro-alcoholic extract of *T. cordifolia*