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COMPARATIVE ANALYSIS OF HPTLC, SECONDARY METABOLITES AND ANTIOXIDANT ACTIVITIES OF *TINOSPORA CORDIFOLIA* STEM POWDERS

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Keywords:

Tinospora cordifolia, HPTLC, TLC, secondary metabolites, DPPH, total antioxidants

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ABSTRACT: Aim of this study was to develop a HPTLC fingerprint profiles of *Tinospora cordifolia* stems powdered by different procedures viz; Sample 1 (fresh aqueous powder), 2 (freeze drying powder), 3 (aqueous freeze drying powder) and Sample 4 (dry powder). The study also includes qualitative, quantitative secondary metabolites and the antioxidant potential of all the samples. Result of various physiochemical and phytochemical screening shows a variation among all powdered stems. HPTLC profile revealed a similarity between the samples 1, 2 and 3 while sample 4 shows much lesser no. of constituents. TLC profile of all the samples followed a similar trend like HPTLC fingerprinting. Besides this, secondary metabolite profiles *viz;* total phenolics, flavonoids, proanthocyanidin and anthocyanidin content of these samples reveals that maximum phenolics and flavanoids content in sample 1 (51.5 µg GAE/mg Wt) and 3 (14.0 µg QE/mg Wt). However, sample 4 was greatly enriched with maximum proanthocyanidin (27.7 µg CE/mg Wt) and anthocyanidin (175 µg CE/mg Wt) contents. Antioxidant potential of these powders was evaluated by total antioxidant, reducing power and DPPH assay which was maximum in sample 4 (170.8, 27.7 µg ascorbic acid/ mg Wt and 61.08 % respectively). A positive correlation between the sum of polyphenolic content and total antioxidant activity were also checked. Sample 4 shows a maximum amount of polyphenols (213.31 µg /mg Wt) and total antioxidants (170.8 µg ascorbic acid/mg Wt) than others. The presence of high levels of polyphenolic contents could be a possible reason behind the higher amount of total antioxidants.

INTRODUCTION: Medicinal plants have been used as a natural source of medicine since ancient times [1-2]. Traditional knowledge of these plants has significantly contributed to the modern pharmaceutical industries [3]. In recent advancement of this many modern medicines have been developed by exploring the traditional knowledge [4].

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In last few years, demand of natural medicines around the world has increased and which promotes the well organized and systemic research to develop newer pharmaceutical drugs from medicinal plants [5]. Therefore, in view of this importance many traditionally known medicinal plants have been documented in recent years which possesses various medicinal properties *viz*, anticancer activity, antidiabetic activity, hepatoprotective activity, antioxidant activity, antimicrobial activity, larvicidal activity, anti-inflammatory activity and haemolytic activity [6].

Tinospora cordifolia (Menispermaceae) is an herbaceous vine which is indigenous to the tropical

areas of India, Myanmar and Sri Lanka [7]. It is also known as 'Giloy' in Hindi and 'Guduchi' in Sanskrit [8]. The stem of *T. cordifolia* is succulent with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey, deeply left rosette like lenticels [9]. The large numbers of compounds have been isolated from the aerial parts and roots of *T. cordifolia* which includes berberin, tinosporaside, tinosporin, tinocordifolioside, cordifolioside A, cordifolioside B, isocolumbin, magnoflorine [10]. It shows the presence of terpenoids, alkaloids, lignan, carbohydrates, bitters, steroids and glycosides.

One of the most important constituent present in stem of *T. cordifolia* is berberin, an isoqunoline alkaloid which is yellow in colour and shows various pharmacological actions which enhances the therapeutic efficacy of this plant [11]. *T. cordifolia* a medicinal herb used in the Indian system of medicine due to their health benefits. In modern medicine it is used for the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia [10]. It is more recently used as immunomodulatory, antioxidant, antineoplastic, anti-stress, antihyperglycemic, antidiabetic agents. However, a systemic study to support the traditional applications of *T. cordifolia* could increase the uses of this plant in drug discovery [1].

Therefore in the present study, powdered extract of *T. cordifolia* stems by four different methods *viz;* fresh aqueous extraction, freeze drying powder, aqueous freeze drying powder and dry powder. These were investigated for their physiochemical, phytochemical, thin layer chromatography (TLC) profile and high performance thin layer chromatography (HPTLC) fingerprints. Beside this, these four powdered extracts were also studied for their secondary metabolites compositions *viz;* total phenolics, flavonoids, proanthocyanidin and anthocyanidin contents. In addition this, these four samples also were screened for their antioxidant potential by various *in vitro* methods.

MATERIALS AND METHODS

Plant Materials:

Fresh stems of *T. cordifolia* were collected in the month of January 2016 from Patanjali Research Foundation, Haridwar (Uttarakahnd, India). The plant stems was authenticated by Acharya Balkrishna, Chancellor, University of Patanjali, Haridwar. These stems were powdered by using different systems and named as Sample 1 (fresh aqueous extract), Sample 2 (freeze drying powder), Sample 3 (aqueous freeze drying powder), and Sample 4 (dry powder).

Chemicals:

Quercetin and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemical Co. (USA). Sodium phosphate (NaH_2PO_4) and Sodium carbonate (Na_2CO_3) was purchased from Hi media Laboratories Pvt. Ltd. (Mumbai, India). Ferrous chloride ($FeCl_2$), Methanol, Potassium ferri cyanide ($K_3Fe(CN)_6$), Trichloroacetic acid, Ascorbic acid, Gallic acid, Folin-Ciocalteau reagent, Ethanol, Ammonium molybdate ($(NH_4)_2MoO_4$) and Aluminium chloride ($AlCl_3$) were purchased from SRL Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Physiochemical Studies:

Physiochemical parameters like bitter, pH, water solubility, methanol solubility, total ash, acid insoluble ash and water soluble ash extractive values were determined as per Indian Pharmacopoeia.

Phytochemical screenings:

Preliminary phytochemical screening was carried out on *T. cordifolia* stems four powders revealed the presence of a wide range of phytoconstituents including alkaloids, flavonoids, fixed oil, phenols and tannin, carbohydrates, glycosides and sterols.

Thin layer chromatographic separation of four powdered extracts of *T. cordifolia* stems:

Total fractions powdered extracts of four powdered extracts of *T. cordifolia* stems were obtained by preparative TLC (Thin layer chromatography) [12]. Samples were loaded on TLC plate in the form a streak with the help of micropipette and dried with dryer. The TLC plates were developed in two solvent systems separately consists of Chloroform: Methanol (17:1 *v/v*) and Toluene: Ethyl acetate: Formic acid (5:4:1 *v/v*) respectively at room temperature. The plates were removed, dried by

evaporation of solvent then sprayed with anisaldehyde. Different bands were visualized at light 366 nm were observed and their Rf values were calculated.

Fingerprint analysis of powdered extract of four *T. cordifolia* stems by HPTLC:

HPTLC fingerprint analysis was carried out on four powdered extracts of *T. cordifolia* stems with solvent system i.e. Chloroform: Methanol: Ethyl acetate (9.5:0.5:0.1 *v/v*) using CAMAG HPTLC system consisting of linomat v spotting and scanner 3. The chromatogram obtained was studied under 254 nm, 366 nm.

Methodology:

100 mg of powder from each powdered sample was weighed and dissolved in 5ml of methanol. The sample was subjected for sonication for 20 minutes and further be dissolved in 5ml. Samples are filtered and used for fingerprint analysis. Precoated aluminium sheet (10x10cm, Merck, Darmstadt, Germany) with silica gel 60 F254 of thickness 0.2 mm were used on sample which were applied in the form of band with the help of Linomat 3 applicator attached to HPTLC system which was programmed through winCATS, the software which were installed with the apparatus. 1ml of each of the sample was applied in the form of band of 3mm and chromatogram was developed in CAMAG twin through TLC chamber using solvent system Chloroform: Methanol: Ethyl acetate (9.5:0.5: 0.1 *v/v*) using by using anis- aldehyde sulfuric acid as a detecting agent. The developed Chromatograms were then scanned using CAMAG TLC Scanner 3 at 254 nm and 366 nm using slit dimension 4x0.30m[12].

Determination of Secondary Metabolites:

Secondary metabolites *viz;* total phenolics, flavonoids, proanthocynidins and anthocyanidin were measured as per the previously published procedures. For determination of total phenolics content Folin-Ciocalteu method was used [13]. To the 0.5 ml of methanol extracts (concentration 1 mg/ml) of each sample 0.5 ml of the Folin-Ciocalteu reagent was added and content were mixed gently. After 2 min, 0.5 ml of sodium carbonate (100 mg/ml) was added by mixing gently and allowed to stand for 2 h. The optical density of the blue developed was measured at 765 nm. Total phenolic contents were expressed as Gallic acid equivalent (GAE) µg /mg weight (Wt.).

Flavonoids content was measured as per the method of Ganjewala et al. (2013) [14]. Aliquots (500 μl) of each extract (concentration 1 mg/ml) were added to a test tube containing 1.25 ml of distilled water. To this, 75 μl of 5% sodium nitrate solution was added and left for 5 min. Then, 150 μl of 10% ammonium chloride was added. After 6 min, 500 μl of 1 M sodium hydroxide was added. The content was diluted with 275 μl of distilled water. Absorbance of the solution was measured at 510 nm. Total flavonoids content was expressed as Quercetin equivalent (QE) µg /mg Wt.

Proanthocyanidin content was measured according to the previously published report[15]. 0.5 ml of extract (concentration 1 mg/ml) solution was mixed with 3 ml of 4% vanillin methanol solution and 1.5 ml of hydrochloric acid. The content was mixed well and left at room temperature for 15 min. The absorbance of the solution was measured at 500 nm. Total proanthocyanidin content was expressed as Catechin equivalent µg (CE) /mg Wt.

For determination of anthocyanidin content, 0.5 ml of extract (concentration 1 mg/ml) solution was incubated over night in 150 µ1 L of methanol acidified with 1 % HCl (*v/v*). Then added 100 µL of distilled water and anthocyanins were separated from chlorophylls with 250 µL of chloroform. The absorbance of the aqueous phase containing total anthocyanidin was recorded at 530 and 657 nm. Total anthocyanidin content were also expressed as milligrams of catechin equivalent (CE) µg /mg Wt of the tissue (13).

Antioxidant activity:

Estimation of total antioxidant activity:

0.5 milliliter extract (concentration 1 mg/ml) from each sample was mixed with 3.3 ml of the reaction mixture which contain 3.3 ml of concentrated H_2SO4, 0.335 g of sodium phosphate monobasic and 0.495 g of ammonium molybdate which was dissolved in 96.67 ml of water. The mixture was kept in water bath for one hour at 95º C. The solution containing 3 milliliter of reaction mixture and 1 milliliter of distilled water was then used as

blank and the absorbance was measured using UV-Visible spectrophotometer at 695 nm. Experiment was performed in triplicates [16].

DPPH radical scavenging activity:

The DPPH Radical scavenging activity of powdered extract of *T. cordifolia* stems was determined by using standard protocols reported earlier. Two milliliter of each extract (concentration 1 mg/ml) was mixed with one milliliter of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20ºC for 40 min. Absorbance was measured at 517 nm using a UV-Visible spectrophotometer with methanol as blank. Each experiment was performed in triplicates [17].

The percentage of DPPH radical scavenging was calculated according to the following formula:

% DPPH radical scavenging = [(Ac-At)/Ac] 100

Where
AC is the absorbance of the control
At is the absorbance of test

Reducing power assay:

The reducing power of four powdered extracts of *T. codifolia* stems were determined by ferric ion reducing power assay. One milliliter volume of each extract (concentration 1 mg/ml) was mixed with phosphate buffer of pH 6.6 and 2.5 ml of potassium ferric cyanide was added. The mixture was incubated at 50ºC for 20 min. A volume of 2.5 milliliter of trichloroacetic acid was added to the mixture and was centrifuged at 3000 rpm for 10 min in a cooling centrifuge. Then 2.5 milliliter of the supernatant was mixed with equal volume of distilled water and 0.5 ml of ferric chloride was added to the solution. Absorbance was measured at 700 nm using a UV-Visible spectrophotometer. Higher absorbance indicated greater reductive power. Each experiment was performed in triplicates[18].

RESULTS:

Physio-chemical Analysis: Physio-chemical parameters like bitter, pH, water solubility, methanol solubility, total ash, acid insoluble ash, water soluble ash extractive value were shown in **Table 1**.

TABLE 1: PHYSIOCHEMICAL PARAMETERS (%W/W) OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
Bitter	3.3%	3.3%	4.3%	1.7%
pH	7.0	6.5	6.8	6.4
Water Solubility	76%	48%	92%	64%
Methanol Solubility	24%	8%	44%	16%
Total Ash	8%	5%	6%	8%
Acid insoluble ash	5%	3.25%	2.9%	3.2%
Water soluble ash	5.6%	3.70%	3.2%	3.5%

Preliminary Phytochemical Screening:

Preliminary phytochemical screening was carried out on the powdered extract of stems of four different *T. cordifolia* samples revealed the presence of a wide range of phytoconstituents including alkaloids, flavonoids, tannins, protein, fixed oil, glycosides and carbohydrates as shown in **Table 2**.

TABLE 2: QUALITATIVE ANALYSIS OF SECONDARY METABOLITES OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
Alkaloids	-	-	-	-
Flavonoids	+	-	-	-
Protein	-	-	-	-
Fixed Oil	+	+	+	+
Phenol and tannin	-	-	-	-
Glycosides	+	+	+	+
Sterols	-	-	-	-

TLC Analysis of Powdered Extract of Four *T. Cordifolia* Stems:

Stems of *T. cordifolia* plants collected from PRF, Haridwar were analyzed by developing the TLC plates in two solvent systems Chloroform: Methanol (17:1 *v/v*) and Toluene: Ethyl acetate: Formic acid (5:4:1 *v/v*). To visualize the bands TLC plates were sprayed with anisaldehyde-sulphuric acid Vanillin and sample 1, 2 and 3 show maximum numbers of bands with similar Rf values in both solvent system. However, sample 4 shows lesser bands with differing Rf values than other extracts as shown in **Table 3** and **Table 4**. The spot of Rf 0.58 was found to be Tinosporaside, one of the major constituents of the plant.

TABLE 3: TLC ANALYSIS OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS

No.	Samples	Solvent system	No. of bands	Rf
1	Sample 1	(Chloroform: Methanol :: 17:1)	17	0.06,0.10,0.16,0.22,0.29,0.34,0.41, 0.49,0.53, 0.57,0.64,0.72, 0.76,0.81,0.84,0.88,0.92
2	Sample 2			
3	Sample 3			
4	Sample 4		14	0.06,0.085,0.121, 0.28,0.39,0.43, 0.46, 0.50, 0.54, 0.46,0.50,0.54,0.58,0.60,0.65, 0.78,0.82

TABLE 4: TLC ANALYSIS OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS

No.	Samples	Solvent system	No. of bands	Rf
1	Sample 1	(Toluene: Ethyl Acetate: Formic Acid::5:4:1)	20	0.06,0.10,0.14,0.24,0.28,0.32,0.37,0.42,0.46, 0.50,0.53,0.54,0.58,0.65,0.71,0.78,0.82,0.85, 0.91, 0.93
2	Sample 2			
3	Sample 3			
4	Sample 4		17	0.085,0.14,0.19,0.25,0.29,0.32,0.36,0.41,0.47, 0.52,0.56,0.58,0.63,0.78, 0.84,0.86,0.90

Fingerprint Analysis of Powdered Extracts of Four *T. Cordifolia* Stems at 254nm and 366 nm:

HPTLC fingerprint analysis was carried out on powdered extracts of four *T. cordifolia* stems with solvent system i.e. Chloroform: Methanol: Ethyl acetate (9.5: 0.5: 0.1 *v/v*) using CAMAG HPTLC system. The Rf value of different compounds at 254 nm is shown in **Table 5**. After derivatization with anisaldehyde the Rf value of different compounds at 366 nm is shown in **Table 6** and **Fig.1-4**.

TABLE 5: FINGER PRINT ANALYSIS OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS AT 254 NM

No.	Samples	Solvent system	No. of spots	Rf (area % > 5.0)
1	Sample 1	(Chloroform: Methanol: Ethyl acetate::9.5:0.5:0.1)	12	0.06,0.09,0.32,0.49,0.63,0.73
2	Sample 2		13	0.08,0.18,0.43,0.64,0.72
3	Sample 3		10	0.08, 0.18,0.70
4	Sample 4		10	0.04,0.10,0.32,0.70

TABLE 6: FINGER PRINT ANALYSIS OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS AT 366 NM

No.	Samples	Solvent system	No. of spots	Rf (area % > 5.0)
1	Sample 1	(Chloroform: Methanol: Ethyl acetate::9.5:0.5:0.1)	11	0.07,0.12,0.18,0.26,0.34,0.38,0.44,0.63
2	Sample 2		16	0.08,0.12,0.18,0.26,0.31,0.36,0.43,0.49,0.67
3	Sample 3		13	0.10,0.17,0.34,0.41,0.48,0.53,0.62,0.67
4	Sample 4		09	0.08,0.16,0.37,0.44,0.53,0.72

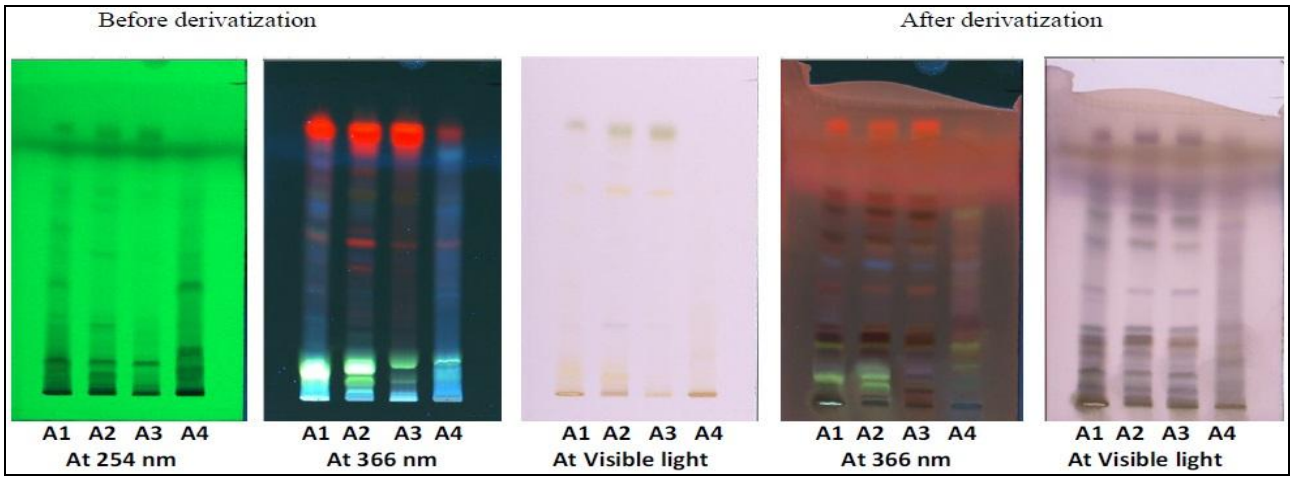

FIG.1: IMAGE OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS [SAMPLE 1 (A1), 2 (A2), 3 (A3) AND 4 (A4)] AT 254 AND 366 NM (BEFORE AND AFTER DERIVATIZATION)

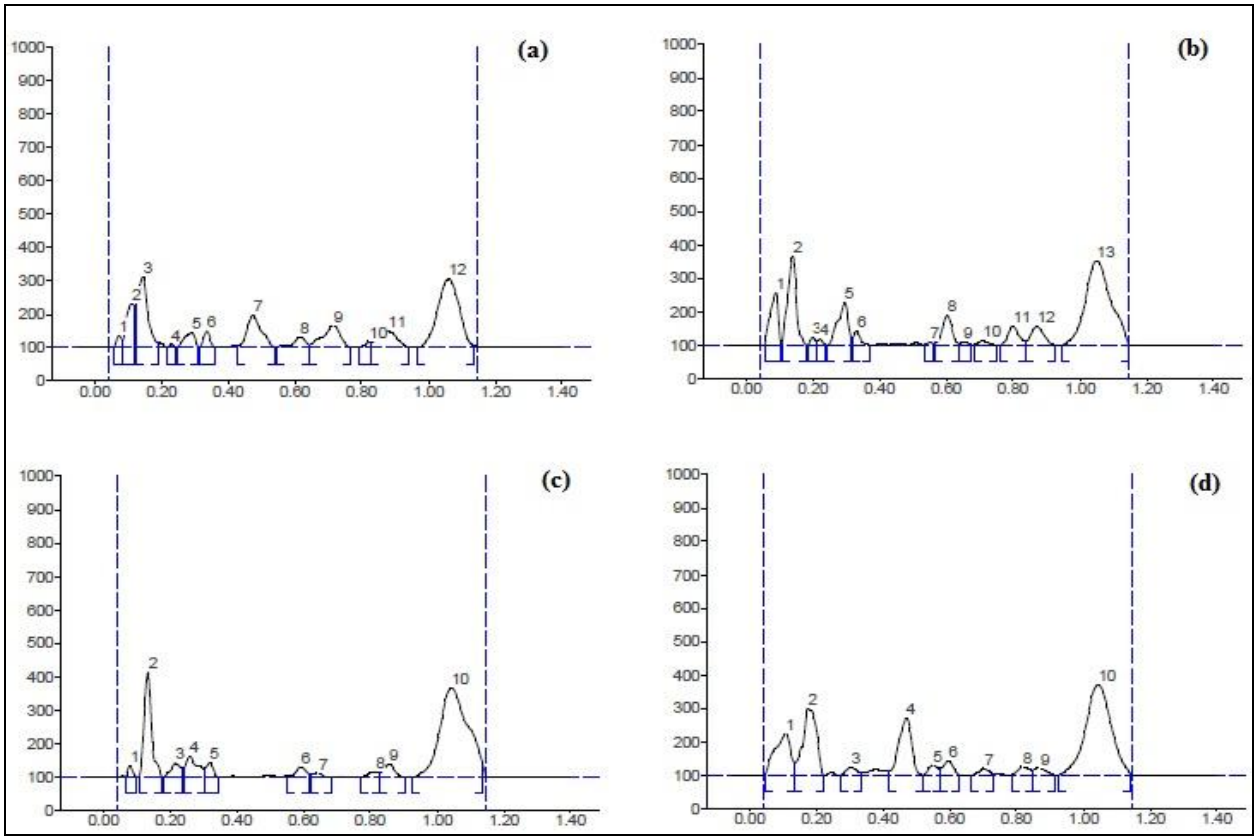

FIG.2: FINGERPRINT ANALYSIS OF FOUR POWDERED EXTRACT OF *T. CORDIFOLIA* STEMS [SAMPLE 1(A), 2(B), 3(C) AND 4 (D)] AT 254 NM

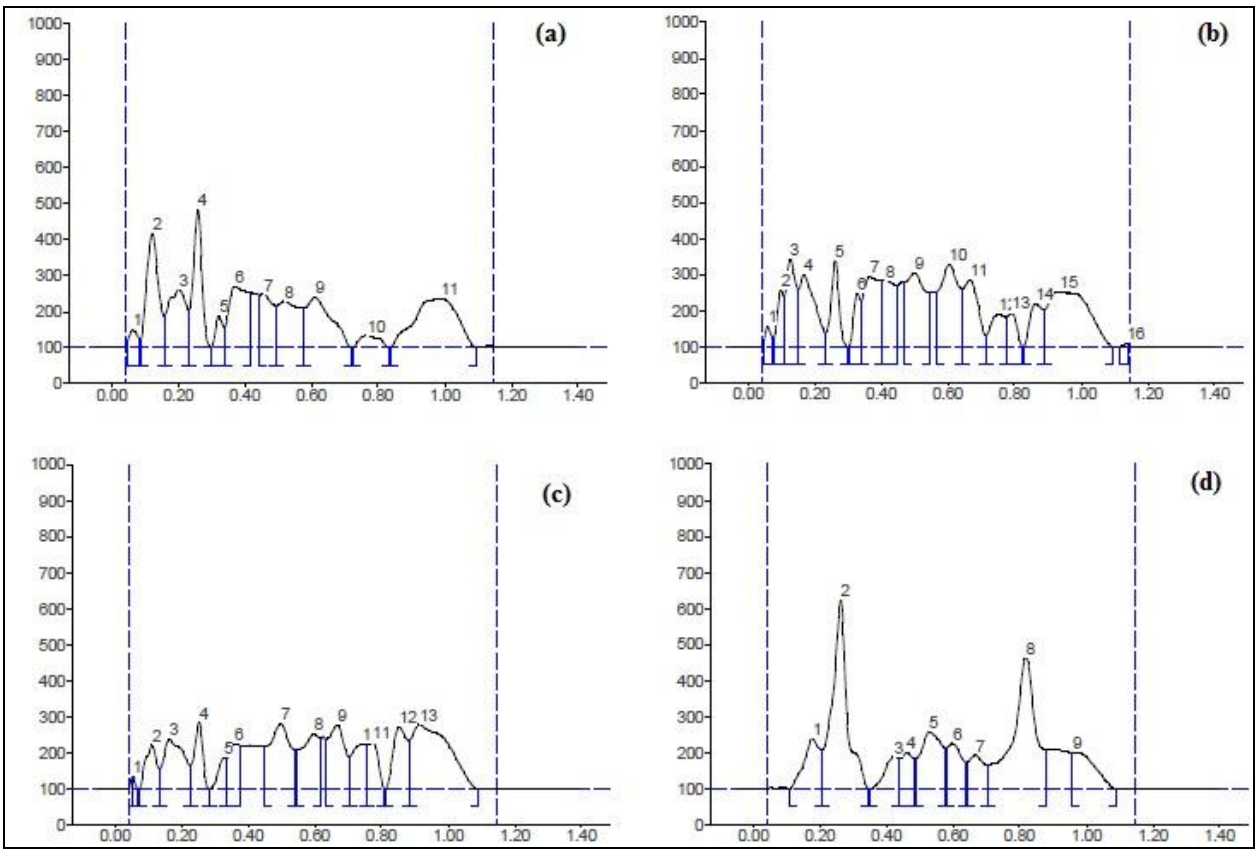

FIG.3: FINGERPRINT ANALYSIS OF FOUR POWDERED EXTRACT OF *T. CORDIFOLIA* STEMS [SAMPLE 1(A), 2(B), 3(C) AND 4 (D)] AT 366 NM (AFTER DERIVATIZATION)

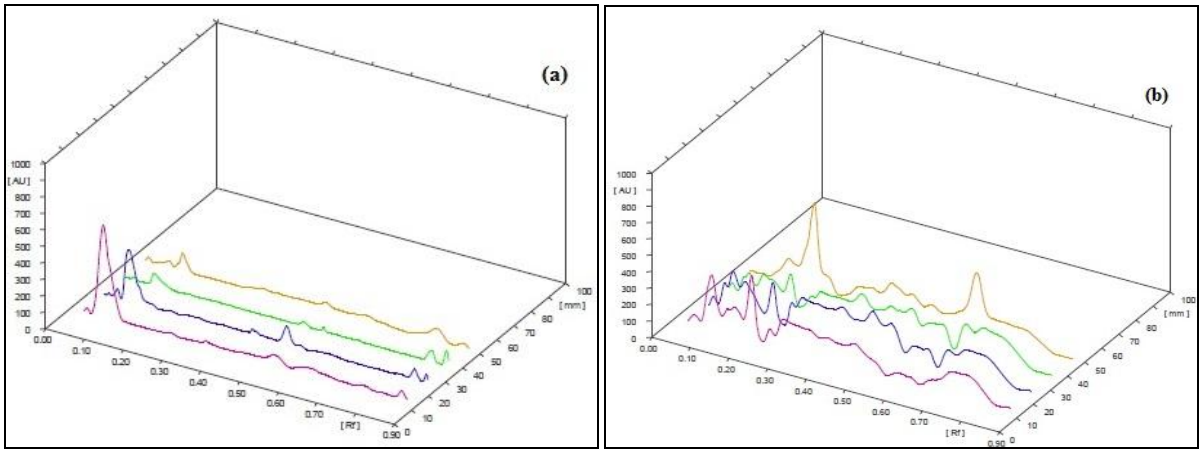

FIG.4: HPTLC CHROMATOGRAPHIC PROFILE (3D) OF FOUR POWDERED EXTRACT OF *T. CORDIFOLIA* STEMS AT (A) 254 NM AND (B) 366 NM

Determination of Secondary Metabolites and Antioxidant Activities:

Total phenolics, flavonoids, proanthocyanidin and anthocyanidin contents in different powdered extracts are shown in **Table 7**. In these powders, total phenolics content were higher in sample 3 (51.5 µg GAE /mg Wt) while flavonoids and proanthocynaidins content were more or less similar in all the four samples of *T. cordifolia* stems. However, anthocynaidins contents were observed much higher in sample 4 with the value of 175 mg CE/mg Wt than other samples quantity (71, 141 and 93 µg CE / mg Wt respectively).

Similarly, antioxidant potential of all the *T. cordifolia* samples was tested by using standard protocols. Antioxidant potential was determined by using total antioxidant activity, reducing power activity and DPPH radical scavenging activity. It shows sample 4 is better antioxidants in respect to other samples. It shows total antioxidant activity and reducing power activity as 170.8 and 27.7 ascorbic acid equivalence µg while scavenges DPPH up to 61.08 %. However, reducing power and DPPH radial scavenging activity is almost analogous in all the samples as shown in **Table 8**.

These all four differently processed stem powders were also checked for a correlation between total secondary metabolites and total antioxidant activity. A positive correlation was recorded in all the samples and sample 4 is enriched with maximum amount of total secondary metabolites (243.31 µg Std./mg Wt) as well as a highest total antioxidants (170.8 ascorbic acid equivalence µg) than all other samples of *T. cordifolia* stems as shown in **Table 9**.

TABLE 7: QUANTITATIVE ANALYSIS OF SECONDARY METABOLITES OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS (CONCENTRATION 1mg/ml)

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
Phenolics (GAE equivalence µg)	29.7±3.2	17.25±2.4	51.5±4.6	29.25±2.0
Flavonoids (Qurecetin equivalence µg)	11.81±1.2	14.0±1.23	12.27±1.3	11.36±1.3
Proanthocyanidin (Catechin equivalence µg)	24.7±2.31	20.3±2.19	20.4±1.0	27.7±0.98
Anthocyanidin (Catechin equivalence µg)	71.0±7.90	141.0±14.32	93.0±12.83	175.0±20.1

TABLE 8: ANTIOXIDANT PROFILE OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS (CONCENTRATION 1mg/ml)

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
Total secondary metabolites (µg Standards/ mg Wt)	137.21±2.45	192.55±16.09	177.17±12.34	243.31±23.34
Total antioxidant activity (ascorbic acid equivalence µg)	28.27±1.98	157.96±10.76	142.8±13.56	170.8±14.59

TABLE 9: TOTAL SECONDARY METABOLITES VS TOTAL ANTIOXIDANT ACTIVITY OF FOUR POWDERED EXTRACTS OF *T. CORDIFOLIA* STEMS (CONCENTRATION 1mg/ml)

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
Total antioxidant activity (ascorbic acid equivalence µg)	28.27±1.34	157.96±9.2	142.8±9.54	170.8±17.6
Reducing power activity (ascorbic acid equivalence µg)	24.7±1.0	20.3±0.98	20.4±1.0	27.7±2.11
DPPH radial scavenging activity (%)	59.21±3.54	58.0±3.89	55.90±4.9	61.08±6.12

DISCUSSION: In the current work attempts were made to generate fingerprint of differently processed *T. cordifolia* stems powder by useing of HPTLC which creates interest in prospective researcher from herbal science[19]. The developed HPTLC method and its outcome can be used as a quality control tool for rapid authentication of wide variety of herbal samples and or also for herbs which were differently processed. Previously also HPTLC-fingerprinting applications were demonstrated in quality control and assessment of herbal drugs [20]. The described HPTLC result above in current work also marked nearly a similar number of constituents with their respective percentages of differently processed *T. cordifolia* stem powders. These results are also consistent with the previously published reports [10].

Total secondary metabolites (quantitative) and different antioxidant assay shows a variations among all samples. Here, sample 4 is found to be a potent antioxidant which contains maximum amount of secondary metabolites which reveals a highest antioxidant activity is correlated with a maximum amount of total phenolics/ flavonoids/ proanthcyanidin/anthocyanidin contents. Thus, secondary metabolite compounds are the major contributor to the antioxidant activity of these samples. These results are consistent with the previously correlations evidence on some plants like *Luffa acutangula* (L.) Roxb [21] and others [22].

CONCLUSION: Differently processed *T. cordifolia* stems powders viz; 1, 2, 3 and 4 were tested for their physiochemical parameters, qualitative secondary metabolites, TLC profile, HPTLC profile before (at 254 nm) and after derivatization (366 nm). These giloy samples also were tested for quantitative secondary metabolites *viz;* total phenolics, flavonoids, proanthocyanidin, anthocyanidin content as well as for their antioxidant potentials (total antioxidant, reducing power and DPPH radial scavenging activity). Results shows the presence of different phyto-constituents in all the samples while HPTLC profile of different samples depicts a similarity between sample 1, 2 and 3 in reference to their constituents and area %. However, sample 4 (freeze drying powder) is different among all the provided samples in its HPTLC and TLC profile. During secondary metabolites (quantitative) and antioxidant assay shows a major and minor variations among all the samples. However, sample 4 is found to be a potent antioxidant which contains a higher amount of secondary metabolites.

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CONFLICT OF INTERESTS: The authors declare that there is no conflict of interests regarding the publication of this paper.

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