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ORIGINAL ARTICLE

Chemical Composition, Characterization and Antioxidant Activity of Essential Oils from *Tinospora cordifolia (Willd.*) Stem.

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ABSTRACT
The present research reports hitherto unknown phytoconstituents and assessment of in vitro radical scavenging efficacy of essential oil extracted from fresh and green stem of Tinospora cordifolia (family: Menispermaceae). Hydrodistillation of essential oil was carried out by using Clevenger apparatus. The isolated essential oil was then analyzed by GC-MS analysis and found to contain 45 chemical constituents. Identification of 45 constituents comprising 96.8 % in stem essential oil was done by utilizing Wiley GC-MS and NIST libraries. It was further supported by comparing the stated retention indices with those empirically determined. The structural validation of 24 compounds were done through co-injection with standard chemicals. The identified phytoconstituents of the stem essential oil was found to contain phenols (29.3%), esters (23.4%), fatty acids (14.2%), alkanes (13.1%), terpenes (2.8%), alcohols (2.6%) and other categories of compounds (10.1%). The key constituents of the stem essential oil were identified as hydroquinone (29.3%), palmitic acid (11.3%), mono (2-ethyl hexyl) phthalate (11.1%), phenyl oxide (6.7%), and biphenyl (3.4%). The stem essential oil of Tinospora cordifolia demonstrated strong DPPH free radical inhibition activity, with an IC_{50} = 15±0.4µg/mL. They exhibited concentration -dependent reducing potential. Total phenolic content (TPC) of essential oil derived from stem is found to be 50 ± 0.6 milligram GAE/g fresh stem. This investigation proposes that stem essential oil derived from T. cordifolia could be serve as inherent source of antioxidants.
Keywords: *Tinospora cordifolia (wild), Gulvel, volatile oil, phenolic content, Gas Chromatography & Mass spectroscopy analysis, reducing potential capacity.*

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INTRODUCTION

T. cordifolia is a well-known medicinal plant found in India, China & Banladesh [2]. It is commonly available climbing shrub known as "Gulvel" and it belongs to the Menispermaceae family [1]. Root, leaves & stem of *T. cordifolia* have medicinal use. & they are used in ayurvedic medicine. The plant "Gulvel" is found to contain series of chemical compounds like alkaloids, phenolics, sesquiterpenoids aliphatic compounds, polysaccharides, steroids, lignans, glycosides, diterpenoids and lactones [3]. In Traditional medicine *T. cordifolia* is employed as "folk medicine". It is used to cure of various ailments like rheumatism, hepatobiliary disorders, peptic ulcer, urinary problems and infectious ailments (4, 5). It is also used for the treatment of diabetes mellitus [6, 7]. Anti-inflammatory & immunosuppressive activities of *T. cordifolia* were also reported (8). The stem extract of 'Gulvel' plant possess immunomodulatoy compound having antioxidant potential in cell-free systems [9]. Additionally, stem & root extract exhibit antimicrobial [10] as well as antibacterial activity respectively [11]. The ethanolic & aqueous extract of stem are known to prevent the cyclophosphamid-induced immunosuppression [12]. The water extract of stem demonstrates antipyretic, anti-inflammatory and analgesic properties [13]. Both aqueous & alcoholic extract of the *T. cordifolia* demonstrate anti-hyperglycemic effect [14]. The dichloromethane extract (DCM) of the plant exhibits antineoplastic properties [15]. Ethanolic extract obtained from the stem of the plant exhibits tissue-protective properties in Swiss albino mice exposed to irradiation from 60Co radiation [16]. Clinical studies have revealed its immunosuppressive effects in patients with obstructive jaundice [17, 18] as well as its antioxidant activity and ability to mitigate cyclophosphamide-

induced toxicity [19]. In recent years, there has been extensive research on free reactive radicals and reactive oxygen species (ROS). Free oxygen radicals are appearing in over 50 diseases, spanning conditions such as atherosclerosis, cancer, Alzheimer's, Parkinson's, chronic inflammation, stroke, arthritis & various degenerative ailments [20, 21]. Free radicals & ROS are associated with multiple disorders like inflammatory diseases, aging, and carcinogenesis, diabetes and neurodegenerative diseases [22]. ROS are usually reduced by natural as well as synthetic antioxidants. Available synthetic standard antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are recognized for their ability to scavenge ROS. Apart from their carcinogenic properties, these synthetic antioxidants are also associated with adverse effects on the liver [23, 24]. As a result, there is a growing interest in natural antioxidants [25-26]. Extract obtained from medicinal plants, vegetables and fruits which contain antioxidant components are frequently investigated for their antioxidant potential [27]. In recent times, essential oil extracted from medicinal plants have emerged as potential sources of natural and safer bioactive chemical components [28-34]. They have applications in various fields like Food industries, aromatherapy, Pharmaceutical, Agricultural etc [35, 36]. Numerous medicinal plants have been screened for essential oil composition with subsequent studies of their biological activities [37-46]. Thorough literature reviews suggest that essential oils containing robust antioxidant compounds may have the capacity to inhibit mutations induced by free radicals [47]. *Stephania hernandifolia* belonging to Menispermaceae family shows promising antioxidant and antidiabetic activities [48]. The fat-soluble components present in *T. crispa* exhibits remarkable potential for scavenging free radicals [49]. The *Cissampelos pareira (L.)* demonstrates notable free radical scavenging activity [50]. Considering pharmacological potential *T. cordifolia*, essential oil was extracted, and subsequent analysis of its phytoconstituents was conducted and then an assessment of its antioxidant properties was conducted.

MATERIAL AND METHODS

Collection of plant & authentication:

T. cordifolia (500 g) was collected from Mulashi, Maharashtra (Pune), India in January 2020. The plant sample was authenticated by Agharkar Research Institute (ARI), Pune, as per standard procedures. The material has been placed in the herbarium repository of ARI with identification number SB-102.

Chemicals:

Myristic acid, Palmitic acid, hydroquinone, 1-butanol, 1-pentanol, 1-hexanol, 1-octanol, biphenyl linalool and C10 to C14 alkanes were acquired from Hi-media. Butylated hydroxytoluene (BHT) was purchased from Loba Chemicals.
The Folin-Ciocalteu phenolic reagent was procured from Qualigens. Trichloroacetic acid (TCA) and DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical were obtained from Fluka, USA. All the solvents & chemicals utilized were of analytical grade.

Essential oil extraction Process:

The essential oil was isolated from the sticks of fresh stem (100g) of *Tinosporea cordifolia*. Clevenger hydrodistillation technique was adopted for extraction of essential oil. The process of hydrodistillation was carried out for about 4 to 5 hr. The experiment was conducted in duplicates, and average yield (%) of essential oil was recorded.

Chemical Analysis of the essential oil:

GC-MS analysis of the essential oil was carried out using a Varian CP-3800 instrument having DB-1 coloumn (30 m X 0.25 mm i.d., film thickness 0.25 µm) with ionization flame detector and Nitrogen was functioning as a carrier gas with steady flow velocity of 2.0 ml/min. Thermal conditions: 50^0C (2 min), 50^0C-200^0C at 2^0C /min, 200^0C (20 min.). The injector temperature was set to 220°C, while the detector temperature was adjusted to 300°C. Quantitative analysis data were generated from GC-FID area percentages. The relative quantities of components present in essential oil were represented as percentage. These percentages are obtained through peak area normalization, without employing correction factor. Gas chromatography & Mass spectroscopy examination of the essential oils were conducted using a Shimadzu QP-5000 spectrometer provided with a Supelcowax-10 column. Temperature profile: 50^0C (5 min), 50^0-200^0C at 2ºC/min, 200^0C for the period of 50 min. Injector temperature was maintained at 200°C, with an injection volume of 1.0 µl. Inlet pressure was maintained at 20 kPa. Helium was employed as the carrier gas, with a linear speed (u) of 46.8 cm/sec. Injection mode was split with a ratio of 1:29. The MS interface temperature was maintained at 230°C, operating in electron ionization (EI) mode with a detector voltage of 1.3 kV. Mass range was set from 45 to 450 u, with an ionization potential of 70 eV. Scanning rate was maintained at 1000 u/s with an interval of 0.50 s.

Component Detection & Identification:
The chemical components present in the extracted essential oil were interpreted through comparative analysis of their mass spectra with those available in the database of Wiley and NIST libraries. The analysis was supported by calculating their retention indices values using C10-C40 n-alkanes and comparing them with corresponding data published in the literature survey [51-53]. Further confirmation of this study was achieved through Co-GC-MS analysis of the available standards.

Assessment of Antioxidant Activity

a) **Evaluation of Antioxidant activity using DPPH assay:**

The free radical scavenging activity of the essential oil was investigated by using standardized methods. Test solutions were prepared by using essential oil and standard BHT in methanol to concentrations varies from 5 to 50 µg/mL. A solution of 0.1 mM, DPPH in methanol (1 mL) was added to each test solution (1 mL). The standard solution & test solutions were kept in dark for the period of 30 mins. After 30 min, absorbance of solutions was recorded at wavelength 515 nm. The inhibition rate (%) of DPPH antioxidant capacity was determined by using following formula:

$$\% \text{ Inhibition of DPPH} = \frac{[A_0-(A_t- A_b)]\ 00}{A_0} X\ 100$$

Where, A_t shows the absorbance readings of the test solutions/standard, A_0 represents absorbance readings of the control, A_b indicates the absorbance readings of the blank solution.

Antioxidant capacity of analyzed sample is expressed in terms of IC_{50} value and determined by employing graphical methods. The IC_{50} value denotes the concentration of antioxidant (essential oil) needed to inhibit 50 % of the DPPH radicals. Each experiment was performed in triplicate.

b) **Assessment of Reducing Capacity:**

The assay was conducted following the standard method [56]. Test solutions of different concentrations (100-1000 µg/mL) were added with 0.1% potassium ferricyanide (1 mL) and 1 mL of 0.2 M phosphate buffer (pH 6.6) as per described procedure. The prepared mixture underwent incubation at 50°C for 30 minutes. Aqueous 10% trichloroacetic acid (1 mL) was added to the mixture, followed by centrifugation at 3000 rpm for about 10 min. Supernatant solution was then added with 1% ferric chloride (2.5 mL), and all test solutions were subjected to absorbance measurements at 700 nm. BHT was employed as the reference in this study.

$$\% \text{ Inhibition} = \frac{(A_{Control} - A_{Test})\ X\ 100}{A_{Control}}$$

c) **Quantitative Estimation of Phenol content:**

The phenolic content of essential oil was assessed using standard protocol involving Folin-Ciocalteu reagent [55]. Test solutions of different concentration were prepared in methanol. Folin Ciocalteu reagent (1 mL, diluted to 1/10) was added to this test solution (1 mL). After 5 minutes, 10 mL solution of 7% sodium carbonate was mixed in prepared test solutions. The resulting solution was diluted up to 25 mL with distilled water. All prepared solutions were kept for incubation for about 90 minutes. The absorbance values of all test solutions were recorded at 750 nm. The experiment was performed at ambient temperature. The total phenolic content of the sample was expressed as milligrams of gallic acid equivalent (GAE) per gram of fresh weight of the sample. All the test samples were subjected for triplicate analysis.

Statistical Analysis**:** Results of antioxidant activity was submitted to statistical analysis & were performed on SPSS version 11. The results of the analysis were expressed as mean values ± standard from triplicate measurements.

RESULTS AND DISCUSSION

The extracted essential oil of *T. cordifolia* (0.17 g, 0.19%) was directly subjected for chromatographic examination (GC & GC-Ms). GC-MS evaluation of the essential oil extracted from *T.cordifolia* stem is illustrated in **Figure 1**. The mass spectra analysis was done using the resources of the Wiley as well as NIST libraries. Results of the study are presented in **Table 1**. The Gas chromatogram of stem essential oil indicates the presence of 45 chemical compounds, as shown in **Table I**. Characterization of 42 components, comprising 96.8% of total stem essential oil, was done through library data. These examinations were supported by comparing their linear retention indices values & determined by using standard formula [51-53]. Quantification was done using GC-FID analysis. Essential oil isolated from stem was found to contain phenols (29.3%), esters (23.4%), fatty acids (14.2%), alkanes (13.8%), terpenes (2.8 %), alcohols (2.6%) and other distinct group of compounds (10.1 %). The assignments were additionally validated through Co-GC-MS of 27 compounds.

Antioxidant Activity:

DPPH radical scavenging method is employed for the assessment of antioxidant capacity of substance. In DPPH assay, radical scavenging ability of essential oil was quantified in terms of its IC_{50} value. In general, a lower IC50 value indicates greater antioxidant activity in plant extracts. **Figure 2** depicts the plot correlating the percentage inhibition rate of DPPH free radicals by antioxidants with the concentration of test solutions. BHT was utilized as reference in this assay. The IC_{50} value of extracted essential oil was determined to be 15± 0.4 µg /mL. It is notable that observed antioxidant potential of essential oil is significantly exceeds that of BHT. The IC_{50} value for BHT is assessed to be 20 ± 0.1 µg/mL. Stem essential oil contains major chemical component hydroquinone (6.5%). The superior antioxidant potential of essential oil is due to presence of hydroquinone. Most of the Indian medicinal plant extracts shows reducing powers capacity. **Figure 3** displays the reducing power exhibited by essential oil and BHT. The hydrodistilled essential oil depicts greater reducing power capacity than previously reported leaf essential oil & found to be dose dependent up to the concentration 700 µg/mL and remains constant above this concentration. The presence of phenolic compounds accounts for the antioxidant properties found in medicinal plants [58]. Total phenolic content of essential oil was recorded as 50 ± 0.6 mg (GAE/g) fresh weight of the stem. The GC-MS spectra indicate the presence primary constituents, hydroquinone. Hydroquinone is known for its superior antioxidant properties. Medicinal plants give numerous antioxidants to mitigate oxidative imbalance induced by sunlight and oxygen, they can serve as a reservoir of novel compounds exhibiting antioxidant properties.

Since plants generate a large number of antioxidant agents to manage oxidative stress induced by oxygen & sunlight. They might serve as reservoir of novel compounds with antioxidant capabilities [59]. This research elucidates the phytochemical composition analysis and antioxidant potential of *T. cordifolia* essential oil. This study suggests that *T. cordifolia* could serve as a valuable of natural antioxidants, which hold significant therapeutic potential in mitigating aging processes and age-associated degenerative diseases caused by oxidative stress.

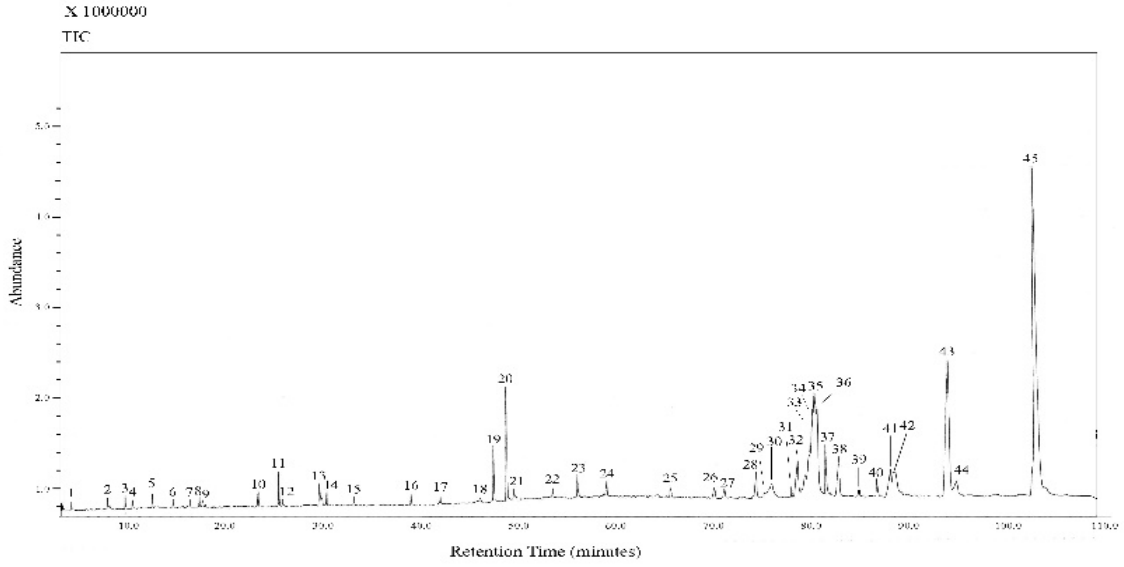

Fig. 1: Gas chromatogram obtained in GC/MS analysis of the essential oil of *T. cordifolia* stem.

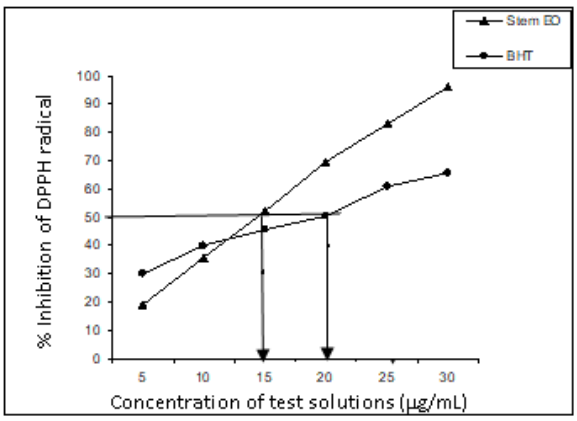

Fig. 2: Determination of IC_{50} value of *T. cordifolia* stem essential oil and BHT by DPPH radical scavenging assay .

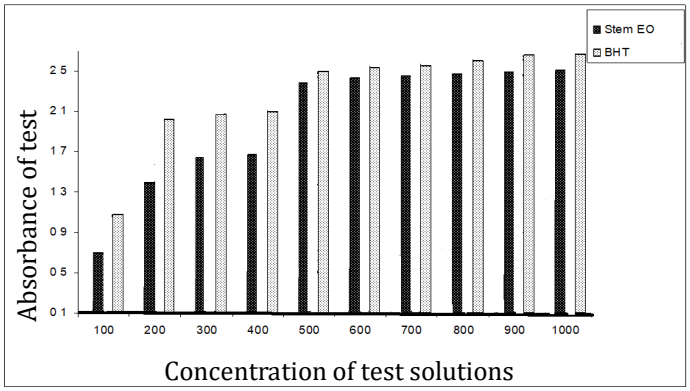

Fig. 3: Reducing power assay of *T.Cordifolia* stem essential oil & BHT

Table I: Chemical Composition of *T. cordifolia* stem essential oil determined by GC-MS analysis.

Sr. No.	Compound	Stem essential oil C[a] (%)	RI[b]	RI[c]	Method of Identification
1	1-butanol	t	1179	1175	MS, RI, CO-I
2	Dodecane	0.6 -	1201	1200	MS, RI, CO-I
3	1-Pentanol	0.6	1270	1255	MS, RI, CO-I
4	Tridecane	t	1300	1300	MS, RI, CO-I
5	Nonanal	t	1379	1390	MS, RI
6	2-Hexen-1-ol	t	1401	1414	MS, RI
7	Tetradecane	0.7	1407	1400	MS, RI, CO-I
8	Linalool oxide	t	1425	1443	MS, RI
9	Pentadecane	0.5	1507	1500	MS, RI, CO-I
10	Unidentified	t	1514	-	-
11	Linalool	2.8	1544	1549	MS, RI, CO-I
12	1-Octanol	t	1551	1551	MS, RI, CO-I
13	Phenyl acetaldehyde	0.6	1616	1613	MS, RI, CO-I
14	α-Humulene	t	1630	1661	MS, RI
15	Linalyl propionate	t	1680	1657	MS, RI
16	2,4 Decadienal	t	1762	1778	MS, RI
17	Geraniol	t	1840	1840	MS, RI, CO-I
18	Nonadecane	t	1920	1900	MS, RI, CO-I

19	Biphenyl	3.4	1946	1974	MS, R , CO-I
20	Phenyl oxide	6.7	1973	1991	MS, RI
21	Unidentified	0.5	1986	-	-
22	Elemol	0.5	2065	2079	MS, RI
23	2-phenoxy ethanol	0.9	2118	2103	MS, RI
24	Heneicosane	t	2124	2100	MS, RI, CO-I
25	Unidentified	0.9	2179	-	-
26	Triacosane	0.5	2310	2300	MS, RI, CO-I
27	Tetracosane	0.9	2411	2400	MS, RI, CO-I
28	Isobutyl phthlate	t	2517	2536	MS, RI
29	Pentacosane	1.6	2526	2500	MS, RI, CO-I
30	Lauric acid	0.6	2564	2523	MS, RI, CO-I
31	Phytol	0.6	2598	2614	MS, RI
32	Hexacosane	2.9	2614	2600	MS, RI, CO-I
33	Mono (2-ethyl hexyl) phthalate	11.0	2648	-	MS (t)
34	Bis (2-ethyl hexyl) phthalate	2.6	2665	-	MS (t)
35	Di-isooctyl phthlate	4.5	2668	-	MS (t)
36	Di -n- octyl phthlate	1.8	2671	-	MS(t)
37	Myristic acid	1.3	2691	2656	MS, RI, CO-I
38	Heptacosane	3.0	2725	2700	MS, RI, CO-I
39	Unidentified	0.5	2792	-	-
40	Pentadecanoic acid	1.0	2802	2791	MS, RI, CO-I
41	Octacosane	2.5	2813	2800	MS, RI, CO-I
42	11,14-Ecosadienoic acid methyl ester	3.5	2855	2847	MS, RI
43	Palmitic acid	11.3	2968	2946	MS, RI, CO-I
44	Triacontane	0.6	2990	3000	MS, RI, CO-I
45	Hydroquinone	29.3	3165	3165	MS, RI, CO-I
	Fatty acids Alcohols Phenols Terpenes Alkanes Esters Aldehydes Other identified Constituents	14.2 % 2.6 % 29.3 % 2.8 % 13.8 % 23.4 % 0.6 % 10.1 %			
	Total identified Constituents	96.8 %			

Table I. C[a]: Concentration of chemical constituents present in stem essential oil determined by GC-FID analysis RI[b] : Retention indices relative to C_{10} – C_{40} *n*- alkanes on Supelcowax -10 Column, RI[c] : Retention indices reported in the literature (51-53),.

Method of identification: MS – Comparison of the mass spectrum of the respective compounds from the Library, RI –Comparison of retention indices, Co-I – Co-injection with the authentic sample.

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