TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF *TINOSPORA CORDIFOLIA* (ROOT): A UNANI HERBAL NEUTRACEUTICAL WITH PROMISING THERAPEUTIC ACTIVITY

Nazish Siddiqui[1*], Tarannum Khanam[2] and Moinuddin[3]
[1]Associate Professor, Department of Ilmul Advia, (Unani Pharmacology and Pharmaceutical Sciences), Faculty of Unani Medicine, Aligarh Muslim University, Aligarh, U. P., India;
[2]Medical Officer, Bareilly, UP, India;
[3]Assistant professor, Department of Ilmul Advia, Hakim Rais unani medical college and hospital, Moradabad road, Sambhal UP, India).
[*]E-mail: nazish_sadat@rediffmail.com

Abstract
Antioxidants are the neutraceuticals which delays the oxidation of biomolecules and prevents the damage due to free radicals or oxidative stress. In natural antioxidants chemical constituents like alkaloids,phenols, flavonoids etc work synergistically to protect human body and arrests progression of diseases with no or lesser side effects. Recently scientist are interested in searching naturally occurring antioxidants, Tinospora cordifolia roots used as carminative, blood purifier, anti-arthritic, general tonic etc. in unani medicine was selected for the present study. Total phenolics and antioxidant activity of various extracts were determined spectrophotometrically.
The total phenolic content in aqueous, ethanolic and hydroalcoholic extracts was estimated quantitatively using Folin Ciocalteu reagent and in-vitro antioxidant activity was determined by DPPH radical scavenging, spectrophotometrically using Gallic acid and Ascorbic acid as standard respectively.
The total phenolics in aqueous, ethanolic and hydroalcoholic extracts was found to be 13.09, 41.50 and 14.0 mg/g Gallic acid Equivalent(GAE), respectively. Ethanolic extract showed good antioxidant activity with low IC50 value 67.47± 0.034 ($p<0.01$) as compared to aqueous and hydroalcoholic extracts. Antioxidant activity of various extracts of Tinospora cordifolia was found to be in the following order Ethanolic >Aqueous > Hydroalcoholic.
The present study provides scientific support to Unani description of the test drug Giloe (Tinospora cordifolia) like Mohallil (Resolvent/ anti-inflammatory), Muqawwi-e-Aaza (general tonic) etc. and now itproves that it could be used as next generation potent therapeutic agent and to cure many diseases related to free radicals like arthritis, neurodegenerative disorders, cancer, cardiovascular diseases etc.

Keywords: Antioxidants; Free radical; Unani medicine; Giloe; Oxidative stress

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1. INTRODUCTION

Antioxidants are the substances which inhibits the oxidation of biomolecules like carbohydrates, proteins, DNA and lipids etc. at low concentration [1]. The antioxidant prevents the damage caused by free radicals [2]. When the mitochondria in the body uses oxygen for the oxidation of these biomolecules free radicals are generated in the process [3].
A free radical is an uncharged chemical species contains unpaired electrons and can exist independently. Free radicals undergo chain reactions generating new free radicals at each step with very short half- life and excessive reactivity [4]. They cause damage to biomolecules, cells, and tissues [5]. It quickly pairs up with an electron to attain the stability. This oxidizes the surrounding molecule and in turn will lead to oxidation of another neighboring molecule, and a chain reaction is generated to release more and more free radicals, thus damaging the cellular components in large number. When the accumulation of free radicals cannot be destroyed in the body they produce a condition known as oxidative stress [6]. It is a result of an imbalance between free radicals generated(generally reactive oxygen species) and antioxidant defense mechanism [6] and lead to different abnormal physiological and anatomical conditions leading to cardiovascular

diseases, autoimmune diseases, asthma, arthritis, carcinogenesis, neuro-degenerative diseases, diabetes, and ageing etc [7-8], [5-6].
In the human body we have various mechanisms to cope with oxidative stress mainly by the production of antioxidants [6]. Antioxidants are of two types, when generated in the body naturally (*in-situ*) they are called endogenous antioxidants and if they are supplied externally through food or as suppliments are called exogenous antioxidants. Antioxidants present in the body are essential for the body as they maintain various functions of the cells and keep the body healthy.
But under the oxidative stress the body becomes deficient of antioxidants generated in the body and cellular functions are not maintained. Thus externally supplied antioxidants are needed which can be absorbed in the body and scavange free radicals [3].
Unani medicine is an age-old traditional system of medicine which utilizes herbal, mineral and animal products in the management of diseases. Plants contain potential natural antioxidants which possess anti-aging properties also and are highly appreciated foe these properties [9].
Phytochemicals are the chemical compounds produced in the plants during usual metabolic processess like coumarins, alkaloids, carbohydrates, phenolic compounds, flavonoids, terpenes, and terpenoids, glycosides, tannins etc [10]. Many of them possess antioxidant activity and are capable of reducing the risk of a number of diseases [11]. These chemical substances create specific physiological actions on the human body. Antioxidant mechanism of polyphenolic compounds is based on their ability to donate hydrogen. When they donate hydrogen they form a radical which is stabilized by resonance and as a result chain reactions of free radicals is arrested and do not proceed further. Plants specially with phenolics have been found to possess antibacterial, anti-inflammatory, anti-viral anti-allergic, anti-thrombotic anti-mutagenic, vasodilatory, anti-ageing and antineoplastic activities, which may be due to their antioxidant potential [12]. Numerous studies have shown that phenolic compounds are effective hydrogen donors and are powerful antioxidants [9] which can scavenge various radicals such as lipid peroxyl, hydroxyl radicals and superoxide radicals [12-16].
It has been observed that under oxidative stress, the Reactive Oxygen Species (ROS) like hydroxyl (OH**.**) , peroxyl and superoxide ($O_2^{\cdot}$) radicals are being produced. The ROS plays a cruicial role in the pathological conditions of various diseases like cancer, atherosclerosis, neurodegenerative disorders, inflammation and cardiovascular diseases. Scientific reports suggests that antioxidants decrease the chances of serious diseases including cardiovascular diseases and cancer. Antioxidants supplements or natural food rich in antioxidants may be helpful in reducing oxidative damage by reactive oxygen and free radicals[10]. Therefore, keeping in mind the role played by antioxidants the search for naturally occurring herbal antioxidants has been increased that may be used to prevent the damage caused by free radicals in the human body and also to inhibit the progression of many chronic diseases.
The most commonly used synthetic antioxidants have been found to show various disadvantages like liver diseases and liver cancer in experimental animals [17]. Natural antioxidants such as ascorbic acid and Vitamin E (α- tocopherol) are considered safe and are used widely for therapeutic purposes. Therefore, the development of effective and safe antioxidants of natural sources is treasured to replace the synthetic antioxidants [9].
For the present work *Tinospora cordifolia* (Giloe in unani medicine) was selected on three bases: firstly it was found to contain phenols as chemical constituents known to possess antioxidant activity [18-19], secondly from the literature we came to know that Giloe is used as antipyretic, expectorant, anti-emetic, aphrodisiac, carminative, blood purifier, anti-arthritic, diuretic, antiperiodic, digestive, febrifuge, general tonic and stomachic etc. It is indicated in fever, cough, jaundice, nausea, vomiting, flatulence, decreased libido, diabetes, leprosy, gonorrhea, palpitation, periodic fever,

urinary affections, general debility, chronic diseases of diarrhea and dysentery [20].
Thirdly, Unani descriptions were correlated to the characters of antioxidant activity and used as a basis for selection [21]. Giloe showed antioxidant activity in animals [22] and their Unani actions like Mohallil (anti-inflammatory/Resolvent), Muqawwi-e-Aaza (General tonic) etc also supports their antioxidant property [23-28].
Besides this National Medicinal Plant Board (NMPB), New Delhi by the Government of India
[29] has classified *T. cordifolia* as one of the highly prioritized plant of importance. So keeping in view these facts, Giloe (*T.cordifolia)* root was selected for the total phenolic content estimation by Folin Ciocalteau reagent in various extracts and antioxidant activity was also determined by *in-vitro* chemical method using DPPH as free radical scavenger. Standard taken for the study was Ascorbic acid. The experiment was done thrice and results were given as mean ± SEM. The IC50 values were compared by one way ANOVA test and p value < 0.05 will be considered statistically significant.

2. MATERIALS AND METHODS

Collection and Authentication

The test drug Giloe (*Tinosopra cordifolia*) root was collected fresh from herbal garden of Department of Ilmul advia, Ajmal Khan tibbiya College, AMU, Aligarh, U.P., India. Giloe root was used as drug and it is a member of Menispermaceae family. The drug was properly identified according to the botanical & unani literature & then confirmed in pharmacognosy section of Department of Ilmul Advia, A.K.T.C and in Botany department of A.M.U, Aligarh.
After identification a herbarium sample of the test drug was prepared & submitted to *mawalid- salasa* museum of the Department of Ilmul Advia, A.K.T.C, A.M.U, Aligarh for further reference (Giloe, Voucher No. SC-0213/17). After this the drug was properly cleaned, dried and powder was made for the present work.
The present study was done in the Medicinal Chemistry Laboratory, Department of Ilmul advia, Faculty of Unani Medicine, AMU, Aligarh, with an objective of screening the antioxidant activity of the test drug Giloe (*Tinospora cordifolia).*
The study consists of two parts:

(I) Quantitative determination of Total phenolic content in the test drugs with spectrophotometer.

(II) Antioxidant activity determination by DPPH radical scavenger.

Preparation of crude extract
The coarse powdered drug was subjected to soxhlet extraction method using various solvents successively for 6 hours, then the extracts were recovered by drying at 40-50°C under reduced pressure and was kept under refrigerator till further use. The ethanolic (alcoholic) and aqueous extracts were used for the proposed study.
Besides this further 50% hydro alcoholic solution (50:50, ethanol and distilled water) was also prepared using reflux method of extraction. After 6 hours of extraction the solution was filtered, concentrated by removing solvent at reduced pressure and the crude extract was obtained, which was used for the present study.

Chemicals

2, 2 – Diphenyl – 1 - picrylhydrazyl (DPPH); Ascorbic acid; Folin Ciocalteau reagent; Gallic acid, Methanol and Sodium carbonate, all reagents used were of analytical grade.

Determination of Total Phenolic Content by Spectrophotometer

Total phenolics in aqueous, ethanolic and 50% hydroalcoholic extract of test drug *T. Cordifolia* were estimated by Folin Ciocalteau reagent, using the Singleton and Rossi (1965)[30] method. Calibration curve (fig-1) was drawn by taking 1 ml from 50, 100, 200, 300, 400 and 500 µg/ml of gallic acid solutions and mixing them with 5.0 ml of Folin's reagent (ten times diluted) and 4.0 ml solution of Na2CO3 (75gm/litre). The absorbance was observed at

765 nm after 30 minutes. One ml of aqueous, ethanolic and hydroalcoholic extract (1000µg/ml) was separately mixed in the same manner with same reagents as above in making of Calibration Curve, then absorbance was recorded after one hour for the estimation of phenolic compounds in test drug extracts separately. Methanol was used as the blank. To attain the reproducibility and accuracy of the results three observations were recorded for each and every solution. Results are summarized in Table- 1, Fig. 2 and total phenolic content was expressed in mg/g Gallic Acid Equivalent (GAE).

In- vitro Antioxidant study by DPPH free radical scavenging activity

The measurement of antioxidant capacity or free radical scavenging ability of substances by using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical is a widely used simple, rapid and not very costly method.

Here in this paper the free radical scavenging property of aqueous, ethanolic and 50% hydroalcoholic extracts of giloe was assessed in terms of radical scavenging / hydrogen donating ability by the use of DPPH stable radical, by the method of Chang *et al.*, 2001[31]. After the addition of the test drugs the absorption by the solution of DPPH decreases and was measured at 517 nm, that forms the bases of this assay. Ascorbic acid (1000µg/ml) was taken as the standard.

Principle

DPPH is a stable (in powder form) free radical having dark purple color, which becomes yellow when scavenged. The DPPH assay uses this property to show free radical scavenging activity. The reaction of the DPPH and test drug is based on the capability of stable 2, 2-diphenyl-1- picrylhydrazyl free radical to react with phenolics or polyphenols as H-donors [32].

The scavenging property of antioxidant RH with DPPH can be represented by the following reaction:

$$DPPH + RH \rightarrow H\text{-}DPPH + R \quad [1]$$

The absorbance decreases when antioxidant reduces DPPH to DPPH-H, as a result of this reaction the color changes. The degree of discoloration is an indication of the antioxidant potential of the extracts (mainly phenolic compounds) in terms of hydrogen donating capacity [31].

Preparation of Working Solutions:

DPPH stock (0.1mM) solution

Freshly made 0.1 mM DPPH solution in methanol was used for the study to ensure the dissolution and stability of DPPH, then the flask was protected from the light by keeping in dark
and stored in refrigerator.

Ascorbic acid stock (1000µg/ml) solution

The stock solution of ascorbic acid (1000µg/ ml) was prepared using methanol as solvent. Then different solutions of concentration from 10 to 1000µg/ ml were made by using stock solution.

Test drug solutions

The aqueous, ethanolic and 50% hydro alcoholic extracts were dissolved in methanol to obtain 1000µg/ml stock solution. Then from the stock solution 10 to 1000µg/ml solutions were prepared by dilution method and were used for the study.

Working Procedure

1.0 ml of 0.1mM solution of DPPH solution was added to 2.0 ml of test extract solution in methanol at different concentration (10-1000µg/ml) and mixed thoroughly. The test tubes were covered with aluminium foil to protect from light and kept in dark condition for 30 minutes. After 30 minutes the absorbance of the mixture was read at 517 nm using methanol as blank. The decrease in absorbance of test drug mixture shows increase in free radical scavenging activity. Ascorbic acid was used as standard. Experiment was done in triplicate. The difference in absorbance of the control and the test was calculated and expressed as percent radical scavenging activity (Table: 2) (Fig. 3-6).

$$\%\ Radical\ scavenging\ Activity = \frac{A\ control - A\ sample}{A\ control} \quad (2)$$

$$A\ control = Absorbance\ of\ Solvent + DPPH \quad (3)$$

$$A\ sample = Absorbance\ of\ plant\ extract + DPPH \quad (4)$$

All the absorbance values were taken as the mean ±S.E.M of three consecutive readings.
IC50 value is the concentration of the extract of test drug required to inhibit 50% of DPPH free radical and was estimated by drawing a graph between radical scavenging activity and the concentration of extracts.

Statistical analysis:

The experiments were done in triplicate and results are given as mean ± SEM. The IC50 values will be compared by one way ANOVA test and p value <0.05 will be considered statistically significant.

3. RESULTS AND DISCUSSION

Total Phenolic content

Plants have varying levels of total phenolic content. First the presence of phenols in aqueous, ethanolic, and hydroalcoholic extract of *T. cordifolia* was checked by phytochemical screening qualitatively. All the three extracts of the test drug giloe were found to contain phenols .Then the total phenolics in all the test extracts were estimated by Foiln Ciocalteu reagent using the method given by Singleton and Rossi (1965)[30] with the help of spectrophotometer in mg/g Gallic acid equivalent(GAE) by drawing a calibration curve of gallic acid. The standard curve equation was found to be y=0.0044 x + 0.7824, where R^2 =0.9962 (figure1). The total phenolic content in aqueous, ethanolic, and hydroalcoholic extracts were determined as 13.09, 41.5 and 14.0 mg/g Gallic acid equivalent, respectively (Table 1).

Table 1: Estimation of Total Phenolic Content (in mg/g Gallic Acid Equivalent)

S. No.	Drug Name	Aqueous extract	Alcoholic extract	Hydroalcoholic extract
1	Giloe	13.09	41.5	14

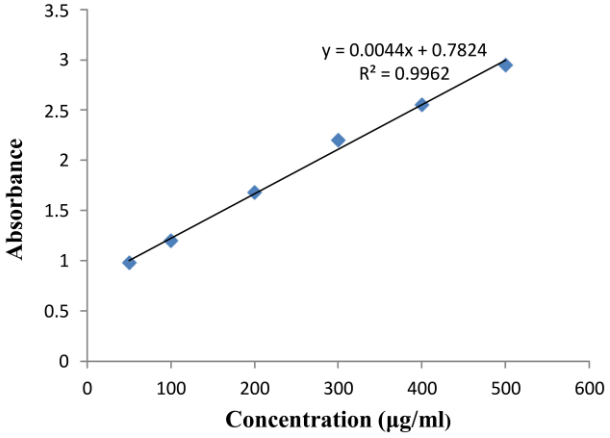

Fig. 1: Standard curve of gallic acid

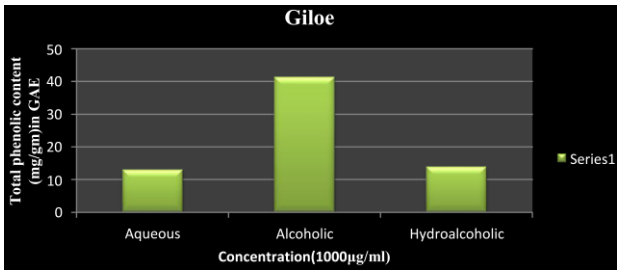

Fig. 2: Total phenolic content of Giloe (*Tinospora cordifolia).*

Antioxidant study

The antioxidant activity of the alcoholic (ethanolic), hydroalcoholic (1:1) and aqueous extract of the test drug was determined by DPPH radical scavenging method and compared with the Ascorbic acid as standard (Figure 3-6).

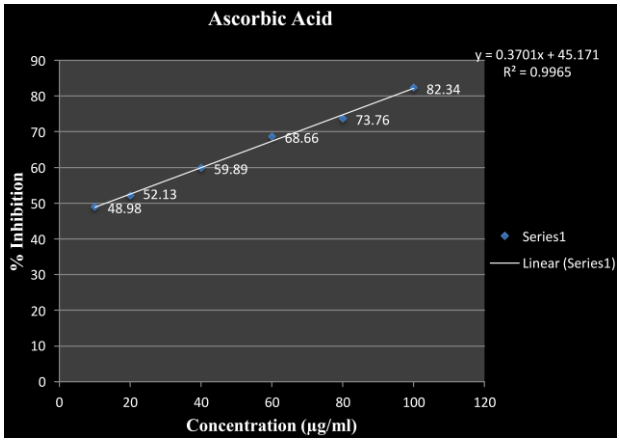

Fig. 3: Standard curve of Ascorbic acid

Experiment was repeated three times to get the mean value. The overall % inhibition, respective IC50 values and R^2value from regression analysis is represented in Table- 1. One way ANOVA test was used for analysis of results, p value <0.05 will be considered significant statistically. The % inhibition of free radical activity by different concentration of ethanolic extract of Giloe was found to be 25.94, 35.67, 48.1, 55.96 and 65.67 % at 20, 40, 60, 80,100 µg/ml concentration. The standard curve equation was determined to be y=0.4988x + 16.343 (R^2=0.9957) and IC50 of alcoholic extract was determined to be 67.47±0.034 (p<0.01) µg/ml.

The free radical % inhibition by Hydroalcoholic extract of Giloe was found to be 12.34, 16.45, 24.76, 33.45, 41.8 and 50.01 % at 10, 20, 40, 60, 80, 100 µg/ml concentration respectively. The standard curve equation was found to be y=0.4202x+8.0908 (R^2=0.9999) with IC50 value 99.73±0.080 (p<0.01) µg/ml was determined for hydroalcoholic extract.

The % of inhibition of free radical activity by different concentration of Aqueous extract of Giloe was found to be 23.52, 29.09, 36.11, 44.39, 50.03 % at concentration of 20, 40, 60, 80, 100 µg/ml with equation y=0.3416x+16.132, where R^2=0.9957 and IC50 of alcoholic extract was determined to be 99.14±0.030 (p<0.01) µg/ml (Table-2) (Fig. 3-5).

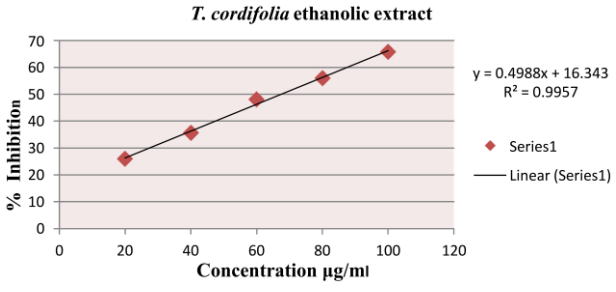

Fig. 4: DPPH radical scavenging assay of *T. cordifolia* (Giloe) ethanolic extract

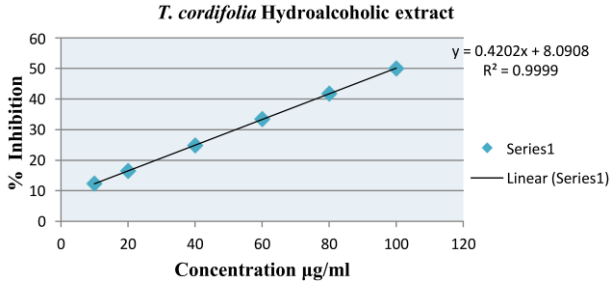

Fig. 5: DPPH radical scavenging assay of Giloe Hydroalcoholic extract

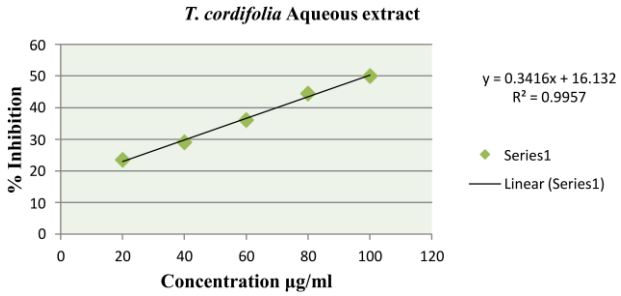

Fig. 6: DPPH radical scavenging assay of Giloe Aqueous extract

The scavenging activity in all three extracts was found to increase with increased concentration of test drug. The result was found to be significant, among them alcoholic (ethanolic) extract showed good antioxidant activity with low IC50 value 67.47± 0.034 (p<0.01) as compared to aqueous and hydroalcoholic extracts. For ascorbic acid IC50 value was 13.04 µg/ml and R^2=0.9965, which was the standard compound (Results are summarized in table- 2) .

The various extracts of *Tinospora cordifolia* showed antioxidant activity in the following Order:

Ethanolic >Aqueous > Hydroalcoholic

Table 2: Antioxidant Activity of Giloe (*Tinospora cordifolia*)

S. No.	Extract	Equation	R^2 Value	IC_{50} (µg/ml)
1.	Alcoholic	y=0.4988x + 16.343	0.9957	67.47±0.034**
2.	Hydroalcoholic	y=0.4202x + 8.0908	0.9999	99.73±0.080**
3.	Aqueous	y= 0.3416x +16.132	0.9957	99.14±0.030**
4	Ascorbic acid	y=0.370x+45.17	0.996	13.04

Discussion

Plants especially rich in phenolic compounds have been demonstrated to possess numerous biological properties and wide pharmacological potential. Anilkumar *et al.* (2010) [33] have studied the in vitro antioxidant activity of stem of *Tinospora cordifolia*. Methanolic extract of *T. cordifolia* stem has been reported to possess antioxidant activity by increasing the erythrocyte membrane lipid peroxidase and catalase activity. The water extract of *T.cordifolia* roots has already shown the antioxidant effect in alloxan induced diabetic rats [22].

Here, free radical scavenging activity of *T. cordifolia* roots exhibited excellent antioxidant activity in ethanol, water and 50% hydroalcoholic extracts, which confirmed its potential as natural neutraceutical with antioxidant property besides various reported therapeutic properties. This experimentally proved information can benefit the health by reducing oxidative stress generated by free radicals in the body, which will be helpful in the development of new drug and standardization of drug.

It was observed that all three extracts, the aqueous, alcoholic and hydoalcoholic extract of Giloe (*Tinospora cordifolia*) possess significant antioxidant activity. The alcoholic extract possess good antioxidant activity while other two extracts showed average antioxidant activity.

4. CONCLUSION

The present study provides scientific support to Unani description of the test drug Giloe (*Tinospora cordifolia*) like Mohallil (Resolvent/ anti-inflammatory), Muqawwi-e-Aaza (general tonic) etc and it also supports the fact that the drugs which contain phenolic compounds possess antioxidant activity and now it has been proved that it could be used as next generation potent therapeutic agent and also to cure many diseases related to free radicals like arthritis, neurodegenerative disorders, cancer, cardiovascular diseases etc.

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