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ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL EVALUATION OF TRIBULUS TERRESTRIS FRUIT

Pallavi Duse*, Tanvi Pingale*, Vipul Duse and Dr. Arti Jagtap

Assistant Professor, VIVA Institute of Pharmacy, Virar (E.), Maharashtra.

***Corresponding Author: Pallavi Duse**

Assistant Professor, VIVA Institute of Pharmacy, Virar (E.), Maharashtra.

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ABSTRACT

Reactive oxygen species (ROS) are thought to underline the process of ageing and the pathogenicity of various diseases, such as cardiovascular diseases. The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs. The purpose of our study is to investigate the antioxidant activity and phytochemical evaluation of *Tribulus terrestris* fruit. (Tanvi Pingale and Kedar Prabhavalkar, 2016). In the present study, DPPH assay was used to estimate the antioxidant activity of ATT and it showed IC 50 at 400µg/ml. It suggests that ATT has good free radical scavenging activity, as lower the IC 50 value higher is the free radical scavenging activity, another method used for the estimation of ATT antioxidant potential was total reducing capacity. This method is based on the conversion of ferric (Fe 3+) – ferricyanide complex to ferrous (Fe 2+) form by ATT. Higher the total reducing capacity greater is the antioxidant activity of plant extract. (Yerra Rajeshwar et. al 2005). ATT has shown good total reducing capacity.

KEYWORDS: *Tribulus terrestris*, DPPH, HPTLC fingerprint, *Zygophillaceae*, ferricyanide.

INTRODUCTION

Traditional herbal remedies are found to have better therapeutic effects and lesser side effects as compared to modern medicines (Saied Kianbakht and Fereshteh Jahaniani, 2003). Plants reported to have antioxidant property and proved to be protective against DXR induced cardiotoxicity are: *Terminalia arjuna* (Gurvinder Singh, Anu T. Singh et.al 2008), *Nigella sativa* (Mahmoudn. Nagi and Mahmoudn et. al 2000), *Silybum marinum* (milk thistle) (Nagla A. El-Shitany et. al 2008), *Zingiber officinale* (ginger) (T A Ajith et. al 2008). Another traditional herb which is well known for its medicinal uses in Ayurveda is *Tribulus terrestris* (TT) (*Zygophillaceae*). TT is commonly known as "Gokshura" in Sanskrit. Literature revealed the beneficial effects of TT on cardiovascular system.

Body produces free radicals, Reactive Oxygen Species (ROS) like hydroxyl, hydrogen peroxide etc. DPPH which is a stable free radical provides the information about preliminary antioxidant activity. These studies indicate that ATT has certain antioxidant compounds which can effectively scavenge free radicals like ROS under in vitro conditions.

Thus the present study was carried out to evaluate antioxidant and phytochemical potential of aqueous extract of TT (ATT). In this article the antioxidant activity and phytochemical evaluation of ATT was evaluated using DPPH free radical scavenging activity and total reducing capacity and various Phytochemical screening tests respectively. Also the Phytochemical investigation of ATT such as HPTLC and various chemical tests evaluated in this article.

MATERIAL AND METHODS

Plant Material

Fruits of *Tribulus terrestris* were collected and taxonomically identified and authenticated by Dr. Aashish Phadke M.D. (Ayur), an Ayurvedic consultant Mumbai. The sample specimen was preserved in our laboratory for future reference.

Plant Processing

The Fruits of *Tribulus terrestris* were dried under shade; then pulverized by a mechanical grinder, passed through a 40- mesh sieve, and stored in a well closed container for future use.

Preparation of Extracts (Muneer Al-Ali et.al, 2003)

250 gm of the dried powder of the fruits of TT was extracted for 4-5 hours with 1 litre of distilled water at 80 ^{0} c with occasional shaking. The extract then filtered and solvent was evaporated at room temperature to get the dry crude extract. The extract was stored in refrigerator for further use.

$$\text{Percentage yield} = \frac{\text{Weight of the extract}}{\text{Weight of the dried root powder}} \times 100$$

PHYTOCHEMICAL SCREENING

Characterization of the extract

Aqueous extract of *Tribulus terrestris* was characterized using following parameters:

i. Evaluation of physicochemical parameters.
ii. General phytochemical evaluation.
iii. HPTLC analysis.

i. Evaluation of Physicochemical parameters

ATT was subjected to the study of organoleptic properties like colour, odour, taste, and consistency.

ii. General phytochemical screening (Khandelwal, 2004)

The following phytochemical tests were performed.

ATT was dissolved in distilled water so as to give a concentration of 10% w/v solution which was further subjected to the following tests as a 'test solution'.

Table 1: Phytochemical Tests.

Name of the Phytochemicals	Name of the Tests
Tests for Carbohydrates	▪ Molish's test ▪ Fehlings test
Tests for Proteins	▪ Biuret's test ▪ Millon's reagent test
Test for Essential oil	--
Test for steroids	▪ Salkowski test ▪ Libermann-burchard test
Test for tannins/phenolic compounds	▪ Ferric chloride ▪ Pottasium permagnate
Test for Flavonoides	▪ Lead acetate ▪ Shinoda Test
Test for saponin glycosides	▪ Foam test
Tests for alkaloids	▪ Dragendorff's test ▪ Mayer's test ▪ Wagner's test

iii. HPTLC fingerprint of Aqueous extract of *Tribulus terrestris* (ATT) (H. Wagner et. al 2nd Ed.)

ATT (2mg/ml) was subjected to HPTLC analysis for fingerprinting. The samples were spotted on silica gel F_{254} pre coated aluminium TLC plates. The plate was developed by ascending technique in a chamber saturated with solvent system Chloroform: Glacial acetic acid: methanol: water (64: 32: 12: 4 % v/v) until the solvent moved through a distance of 8 cm. The appropriate wavelength at which ATT showed a separate peak with UV absorbance was selected. Peak was observed, photo documented and its respective Rf value was noted. Plate was sprayed with Vanillin sulphuric acid reagent to allow the chromatogram to be seen in visible light.

Table 2: Optimized Chromatographic condition.

Applicator	CAMAG LINOMAT V
Scanner	CAMAG TLC Scanner 3, win CATS (version 1.2.2)
Syringe	Hamilton syringe 100 µl
Development mode	CAMAG twin through chamber
Plate material	Silica gel 60 F254 precoated TLC plates
Developing solvent	Chloroform: Glacial acetic acid: methanol: water (64: 32: 12: 4 % v/v)
Chamber saturation time	15 min
Developing time	20 min

IN VITRO* ASSESSMENT OF ANTIOXIDANT PROPERTIES OF AQUEOUS EXTRACT OF *TRIBULUS TERRESTRIS

Clinical Significance

Many drugs are responsible for generation of reactive oxygen species (ROS) such as H_2O_2, OH free radicals etc. which induces the oxidative stress more specifically in heart because; heart has low levels of antioxidant enzymes such as Superoxide dismutase, glutathione and catalase as compared to other organs such as kidney, liver etc. Potential antioxidant therapy should, therefore, include either natural free radical scavenging enzyme or agents, which are capable of augmenting these oxidative mechanisms. (Roohollah Babaei Kelishomi et. al 2008).

A. Free radical scavenging by DPPH activity (Blosis M.S 1958)

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical and is commonly used in antioxidant assays. The molecule of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as

a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to deep violet colour, characterized by an absorption band in ethanol solution at about 517 nm. When a solution of DPPH is mixed with a substance that can donate electron or hydrogen atom this electron becomes paired off, the absorption vanishes and the resulting decolourisation is stochiometeric with respect to the number of electrons taken up.

Procedure

The following solutions were pipetted in tubes labelled as follows:-

Table 3: Preparation of solutions.

Solution	Blank	DPPH	Test
Test agent	-	-	1.5 ml
DPPH solution	-	1.5 ml	1.5 ml
Methanol	3 ml	1.5 ml	-

After addition of DPPH solution and the test agent in amber coloured test tubes, the mixture was kept aside for 20 min in dark conditions to allow the reaction to take place. After 20 min, the absorbance was measured at 517 nm. The absorbance of DPPH solution A $_{(Control)}$ was measured against methanol blank. The violet colour developed was proportional to the free radical generation. Measurements were performed in triplicates. Different concentrations of test agent were prepared from the stock (1mg/ml) to find the "effective concentration" or EC_{50} (otherwise called as IC_{50} value).This was defined as the concentration of substance that causes 50% loss of DPPH activity (colour). Calculation: Free radical scavenging activity was calculated on the basis of % inhibition.

$$\% \text{ inhibition} = \frac{(A_{control} - A_{test})}{A_{control}} \times 100$$

B. Total reducing capacity of extract (Yerra Rajeshwar et. al 2005)

Principle

The antioxidant activity has been reported to be concomitant with the development of reducing power. In this assay, the yellow colour of the test solution changes to various shades of green, blue depending on the reductive agent content. The presence of antioxidant substances in the sample causes the reduction of the Fe^{3+} / ferricyanide complex to ferrous form. Therefore Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700nm (Ilhami et al 2006).

Procedure

Different concentrations of ATT (100-1000ug/ml) in 1ml of distilled water were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2ml of 1% Potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50^0C for 20 min. An aliquot of 2ml of TCA (10%) was added to the mixture & centrifuged. The 2.5ml from upper layer of the solution was mixed with o.5 ml of 1% Ferric chloride & mixture was allowed to stand for 10 min. The absorbance was measured at 700nm against blank reagent in absence of extract.

Measurements were performed in triplicates. Increased absorbance of the reaction mixture indicates increase in reducing capacity.

RESULTS AND DISCUSSION

Preparation and Characterization of Aqueous Extract Of Tribulus Terrestris

A. Yield of extract

The yield of extract was 21.48 %w/w.

B. Characterization of the extract

i. Physicochemical Parameters

Table 4: Physiochemical properties of ATT.

Organoleptic Property	Activity
Colour	Dark brown
Odour	sweet
Taste	sweet
Consistency	Semisolid

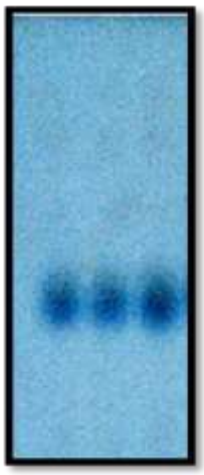
ii. General phytochemical screening of the aqueous extract of *Tribulus terrestris* ATT

Table 5: Qualitative phytochemical evaluation of EIR

TESTS	Results
Test For Carbohydrates	
Molisch's test	Positive
Fehling's test	Positive
Test For Proteins	
Millon's test	Positive
Biuret test	Positive
Test For Essential oil	Negative
Test for Steroids	
Salkowski test	Positive
Libermann-burchard test	Positive
Test for Tannins/Phenolic compounds	
Potassium permanganate	Positive
Ferric chloride	Positive
Test For Saponins	Positive
Test for Flavonoides	
Lead acetate test	Positive
Shinoda test	Positive
Test For Alkaloids	
Dragendorff's test	Positive
Mayer's test	Positive
Wagner's test	Positive

iii. HPTLC fingerprint of Aqueous extract of *Tribulus terrestris* (ATT)

At 254 nm proper separation of peak was seen in ATT with the mobile system used for separation. HPTLC fingerprinting of ATT revealed the presence of saponins as major constituent, which was confirmed by post derivatisation of HPTLC plate with vanillin-sulphuric acid reagent showing blue colour spots corresponding to saponins.



"Fig.1": HPTLC of ATT (2mg/ml) Rf: 0.3.

***IN VITRO* ASSESSMENT OF ANTIOXIDANT POTENTIAL**

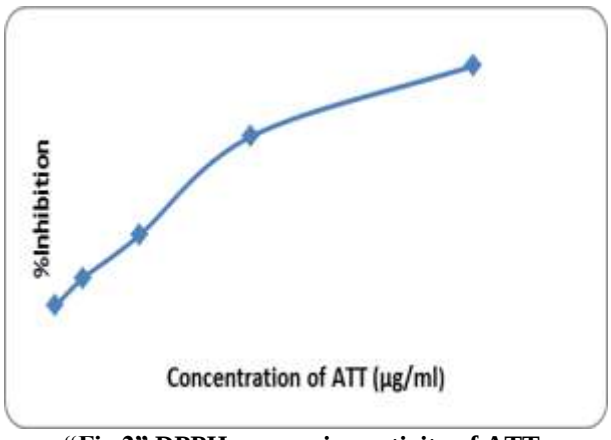
1) Free radical scavenging by DPPH activity

- The molecule of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is characterized by the presence of a stable free radical. The absorbance of this solution in the absence of extract was taken as 100%.
- The different conc of ATT (50-800 µg/ml) caused scavenging of free radical in a dose dependent manner.
- **The IC_{50} value of ATT for inhibition of free radicals was found to be 400µg/ml**

Table 6: DPPH scavenging activity of ATT

Conc. of ATT (µg/ml)	%Inhibition
50	17.97 ± 0.126
100	23.35 ± 0.259
200	31.56 ± 0.282
400	50.65 ± 0.211
800	64.37 ± 0.198

(Results are expressed as mean ± S.D, n=3)



"Fig.2" DPPH scavenging activity of ATT.

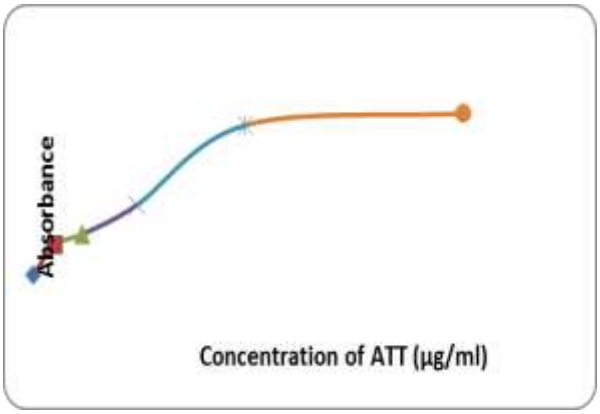
2) Total reducing capacity of extract:-

- The capacity of ATT to reduce ferric-ferricyanide complex to the ferrous-ferricyanide complex of prussion blue was determined by recording the absorbance at 700nm after incubation.
- The reducing power of ATT was found to be dose dependent as shown in above graph.
- Higher absorbance of reaction mixture indicates greater reducing power. The presence of reductant such as antioxidant substance in the sample (ATT) causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Increase in absorbance was seen with increase in the conc of ATT.

Table 7: Reducing capacity of ATT on Fe^{3+} /ferricyanide complex.

Conc. of ATT (µg/ml)	Absorbance at 700 nm
10	0.0152 ± 0.0012
50	0.0196 ± 0.0029
100	0.0211 ± 0.0035
200	0.0253 ± 0.0033
400	0.0369 ± 0.003
800	0.0386 ± 0.0031

(Results are expressed as mean ± S.D, n=3)



"Fig. 3": Reducing capacity of various conc of ATT on Fe3+ /ferricyanide complex.

CONCLUSION

Preliminary Phytochemical estimation of ATT shown presence of saponins, flavonoids, alkaloids, lignanamides and cinnamic acid amides. These phytochemicals are known to have antioxidant potential.

The ATT was evaluated for *in vitro* antioxidant activity using DPPH method. The IC_{50} value of ATT for inhibition of free radicals was found to be 400µg/ml. The low IC_{50} value indicates higher free radical scavenging activity. Thus it indicates that ATT possess good antioxidant properties. ATT was also evaluated for its total reducing capacity. A direct correlation between concentration and reducing power of ATT has been observed. The reducing properties are generally associated with the presence of reductant, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. This reductant nature of ATT further supports its antioxidant properties.

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CONFLICT OF INTEREST: None.

ABBREVIATION
Reactive oxygen species (ROS), *Tribulus terrestris* (TT), aqueous extract of TT (ATT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

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