Preliminary phytochemical evaluation and *In Vitro* antidiabetic acitivity of Ethanolic leaf extract of *Tribulus terrestris* Linn.

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Abstract

The phytochemical screening *Tribulus terrestris* leaves showed that tannin, saponin, flavonoids, steroids, terpenoids, anthraquinone, polyphenol, glycoside and coumarins were present while triterpenoids and alkaloids were absent. The spectral analysis of leaves showed that the extract contains phenolic and flavonoid compounds. *In vitro* antidiabetic activity of aqueous and ethanolic leaves extracts of *T. terrestris* was evaluated by α-amylase and α-glucosidase enzyme inhibitory assay. The extracts exhibited significant inhibitions of α-amylase and α-glucosidase enzyme in dose-dependent manner. It is concluded that from the present study that *T. terrestris* leaves contain a rich source of phytochemicals. The present findings indicate that ethanolic leaves extracts of *T. terrestris* have *in vitro* antidiabetic activity.

Key words: Acarbose, α-glucosidase, α-amylase; *In Vitro* Antidiabetic Activity

INTRODUCTION

Diabetes is a metabolic disorder of carbohydrate, fat and protein, affecting a large number of populations in the world (Pareek *et al*., 2009). *Diabetes Mellitus* is not a single disorder but it is a group of metabolic disorders characterized by chronic hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. Increased thirst, increased urinary output, ketonemia and ketonuria are the common symptoms of *Diabetes Mellitus*, which occur due to the abnormalities in carbohydrate, fat, and protein metabolism. When ketones body is present in the blood or urine, it is called ketoacidosis, hence proper treatment should be taken immediately, else it can lead to other diabetic complications (Craig *et al*., 2009). *Diabetes Mellitus* has caused significant morbidity and mortality due to microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications (Thevenod, 2008). Diabetes is mainly attributed to the rapid rise in unhealthy life style, urbanization and aging.

Hyperglycemia which is the main symptom of *Diabetes Mellitus* generates reactive oxygen species (ROS) which cause lipid peroxidation and membrane damage. ROS plays an important role in the development of secondary complications in *Diabetes Mellitus* such as cataract, neuropathy and nephropathy. Antioxidants protect-cells from oxidation by inhibiting the peroxidation chain reaction and thus they play an important role in the diabetes. Plants contain natural antioxidants such as tannins, flavonoids, vitamin C and E which can preserve-cell function and prevent diabetes induced ROS formation. Polyphenols, which are classified into many groups such as flavonoids, tannins and stilbenes, have been known as health-beneficial properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, anti-inflammatory action and antidiabetogenic potentiality (Patel *et al*., 2011). Aldose reductase as a key enzyme, that catalyze the reduction of glucose to sorbitol and is associated in the chronic complications of diabetes such as peripheral neuropathy and retinopathy. Use of aldose reductase inhibitors and.-glucosidase inhibitors has been reported for the treatment of diabetic complications (Jung *et al*., 2011).

Considering the fact that diabetes is regarded as a chronic metabolic disease, numerous antidiabetic therapies with conventional drugs are often not a single-dose program as most drugs require frequent injections, sometimes for the entire life of the diabetic patient. However, many of these conventional drugs have been reported for their inefficiency with prominent adverse side effects (Atanasov *et al*., 2015). These limitations have largely prompted the exploration of management strategies involving the use of medicinal plants reported to be cost- effective antidiabetic agents

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with fewer reported side effects (Deutschlander, 2010) . However, the majority of these traditional plants have not been scientifically validated for their efficacy in the treatment of diabetes. Therefore, determination of the efficacy is very important as these plants may play a significant role in the management of *Diabetes Mellitus*.

Tribulus terrestris Linn. (family Zygophyllaceae), commonly known as *Gokshur* or *Gokharu* or puncture vine, has been used for a long time in both the Indian and Chinese systems of medicine for treatment of various kinds of diseases. Its various parts contain a variety of chemical constituents which are medicinally important, such as flavonoids, flavonol glycosides, steroidal saponins, and alkaloids. It has diuretic, aphrodisiac, antiurolithic, immunomodulatory, antidiabetic, absorption enhancing, hypolipidemic, cardiotonic, central nervous system and hepatoprotective, anti-inflammatory, analgesic, antispasmodic, anticancer, antibacterial, anthelmintic, larvicidal, and anticariogenic activities. For the last few decades or so, extensive research work has been done to prove its biological activities and the pharmacology of its extracts. It is distributed along a wide geographic perimeter. It is found all over India up to 11,000 ft in Kashmir, Ceylon, and all warm regions of both hemispheres. It is a common weed of the pasture lands, road sides, and other waste places, chiefly in hot, dry, and sandy regions including West Rajasthan and Gujarat in India (Kokate *et al*., 2007). In this study, its antidiabetic activity is evaluated.

MATERIALS AND METHODS

Collection of Plant Materials

The leaves of *Tribulus terrestris* leaves were collected in December 2019 from Okkanadu Keeliyur, Thanjavur District, Tamil Nadu, India. The *Tribulus terrestris* leaves were washed several times with distilled water to remove the traces of impurities from the leaves. Leaves were spread out in a plain paper and shade dried at room temperature for about 10 days and made a fine powder using grinder mixture. The powdered materials were used for further studies.

Preparation for Extract

One gram of the leaves powder of *Tribulus terrestris* were transferred into conical flask (250ml) containing 50ml of ethanol and shaken well for 30 minutes by free hand. After 24 hrs, the extracts were filtered using Whatman filter paper No.1 and filtrate was used for further analysis.

PHYTOCHEMICAL SCREENING

Chemical tests were carried out on the extract using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973 and 1984).

Test for Tannins: About 1ml of sample was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration that indicated the presence of Tannins.

Test for Saponin: About 2 ml of sample was boiled in 20 ml of distilled water in a water bath and filtered. Ten ml of the filtrate is mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing is mixed with 3 drops of olive oil and shaken vigorously and then observed for the formation of emulsion, that indicated the presence of Saponin.

Test for Flavonoids: Five ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2S0_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

Test for Steriods: Two ml of acetic anhydride was added to 1ml of extract of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples that indicated the presence of steroids.

Test for Terpenoids (Salkowski test): 5 ml of extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration formed of the interface was taken to show the presence of terpenoids.

Test for triterpenoids: One ml of the extract was added with 1 ml of chloroform and 1 ml of acetic anhydride on addition of 2 ml of concentrated H_2SO_4, formation of reddish violet colour indicated the presence of triterpenoids.

Test for alkaloids: Mayer's test: To a few (one) ml of the extract, a drop of Mayer's reagent was added by the side of the test tube. A creamy or white precipitate indicated the presence of alkaloids.

Test for anthraquinones: Five ml of the extract solution was hydrolysed with diluted concentrated H_2SO_4 extracted with benzene. One ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

Test for Polyphenols: Ethanol (4 ml) was added to the extract (1ml) and the resulting solution was transferred to a test tube and warmed in a water bath (15 minutes). Three drops of freshly prepared ferric cyanide solution were added to the extract solution. Formation of a blue green colour indicated the presence of polyphenols.

Test for Cardiac glycosides (Keller-Killani test): Five

ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated H_2SO_4. A brown ring of the interface indicated a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Table 1. Qualitative analysis of Phytochemicals in ethanolic leaf extract of *Tribulus*

S. No	Phytochemicals	Ethanolic leaf extract of *Tribulus terrestris*
1	Tannin	++
2	Saponin	++
3	Flavonoids	++
4	Steroids	+
5	Terpenoids	+
6	Triterpenoids	-
7	Alkaloids	-
8	Antroquinone	++
9	Polyphenol	++
10	Glycoside	++
11	Coumarins	++

(+) Presence, (++) High concentration and (–) Absence

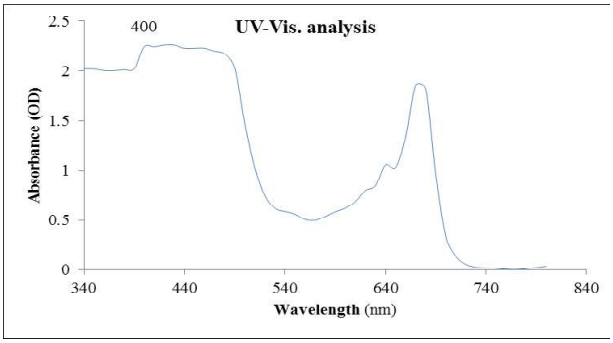

Fig. 1. UV-Visible analysis ethanolic leaf extract of *Tribulus terrestris*

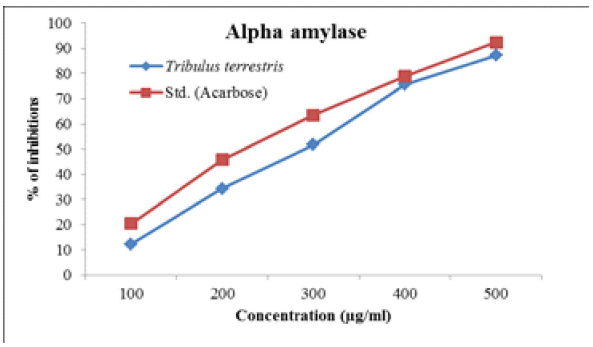

Fig. 2. *In vitro* anti-diabetic activity (Alpha amylase) of ethanolic leaf extract of *Tribulus terrestris*

Test for coumarins: Two ml of extract was treated with 3 ml of 10% NaOH. A yellow colouration observed, indicated the presence of coumarins.

UV-VISIBLE SPECTRAL ANALYSIS

The extract was examined under visible UV-Visible spectrum. The sample was dissolved in same solvent. The extracts were scanned in the wavelength ranging from 340-800 nm using Systronic Spectrophotometer. These solutions were scanned in turn at intervals of 10 nm and the characteristic peaks were detected. The peak value of the UV-Visible spectrum was recorded.

IN VITRO ANTI-DIABETIC ACTIVITY

In vitro α-amylase inhibition study

α-amylase (0.5 mg/ml) was mixed with the sample at various concentrations (100-500 µg/ml) to which 1% of starch solution and 100 µl of 0.2 mm of phosphate buffer (pH-6.9) were added. The reaction was allowed to be carried out at 37°C for 5 min and terminated by addition of 2 ml of 3, 5-dinitrosalicylic acid reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. α-amylase activity was determined by measuring color intensity at 540 nm in spectrophotometer.

The results were expressed as % inhibition using the formula:

% inhibitory activity = (Ac-As)/Ac ×100

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

In vitro α-glucosidase inhibition study

The inhibitory activity was determined by incubating 1 ml of starch solution (2% w/v maltose) with 0.2 M tris buffer (pH 8) and various concentration of sample (100-500µg/ml). The reaction mixture was incubated at 37°C for 10 min. The reaction was initiated by adding 1 ml of α-glucosidase enzyme (1 U/ml) to it and incubation at 35°C for 40 min. Then the reaction was terminated by the addition of 2 ml of 6 N HCl. The intensity of the color was measured at 540 nm in spectrophotometer.

The results were expressed as % inhibition using the formula:

% inhibitory activity = (Ac-As)/Ac ×100

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

STATISTICAL ANALYSIS

Tests were carried out in triplicate for 3 separate experiments. The result was graphically determined

Table 2. *In vitro* ant-diabetic activity (Alpha amylase) of ethanolic leaf extract of *Tribulus terrestris*

Concentration (µg/ml)	% of inhibitions				
	100(µg/ml)	200(µg/ml)	300(µg/ml)	400(µg/ml)	500(µg/ml)
Tribulus terrestris leaf extract	12.31±0.86	34.24±2.39	51.80±3.62	75.63±5.29	87.30±6.11
Std.(Acarbose)	20.49±1.43	45.78±3.20	63.49±4.44	78.83±5.51	92.46±6.47

Table 3. *In vitro* ant-diabetic activity (Alpha glucosidase) of ethanolic leaf extract of *Tribulus terrestris*

Concentration (µg/ml)	% of inhibitions				
	100(µg/ml)	200(µg/ml)	300(µg/ml)	400(µg/ml)	500(µg/ml)
***Tribulus terrestris* leaf extract**	18.19±1.27	36.25±2.53	55.66±3.89	68.46±4.79	86.88±6.08
Std. (Acarbose)	21.32±1.49	49.64±3.47	68.74±4.81	76.43±5.35	91.65±6.41

by a linear regression method using Ms-Windows based graph pad InStat (version 3) software. Results were expressed as graphically and mean ± standard deviation.

RESULTS AND DISCUSSION

Phytochemical analysis of *Tribulus terrestris*

Phytochemicals are chemical compounds produced by plants, generally to help them thrive or thwart competitors, predators, or pathogens. Some phytochemicals have been used as poisons and others as traditional medicines. As a term, phytochemicals are generally used to describe plant compounds that are under research with unestablished effects on health and are not scientifically defined as essential nutrients. Regulatory agencies governing food labeling in Europe and the United States have provided guidance for industry limiting or preventing health claims about phytochemicals on food product or nutrition labels. In the present study the Phytochemicals were screened from the *Tribulus terrestris* leaf extract.

Table 1 represents the qualitative analysis of phytochemicals in ethanolic extract of *Tribulus terrestris* leaves. *Tribulus terrestris* ethanolic leaf extract showed the presences of tannin, saponin, flavonoids, steroids, terpenoids, anthroquinone, polyphenol, glycoside and coumarins were present while triterpenoids and alkaloids were absent.

Phytochemicals are chemicals produced by plants through primary or secondary metabolism. They generally have biological activity in the plant host and play a role in plant growth or defense against competitors, pathogens, or predators (Molyneux *et al*., 2007).

Phytochemicals generally are regarded as research compounds rather than essential nutrients because proof of their possible health effects had not been established yet (Heneman *et al.,* 2008). Flavonoids can be further divided into groups based on their similar chemical structure, such as anthocyanins, flavones, flavanones, and isoflavones, and flavanols further are classified as catechins, epicatechins, and proanthocyanidins (Heneman *et al*., 2008).

Phytochemists study phytochemicals by first extracting and isolating compounds from the origin plant, followed by defining their structure of testing in laboratory model systems, such as cell cultures, *in vitro* experiments, or *In vivo* studies using laboratory animals (Molyneux *et al*., 2007). Challenges in that field include isolating specific compounds and determining their structures, which are often complex, and identifying what specific phytochemical is primarily responsible for any given biological activity (Molyneux *et al*., 2007).

These compounds are known to exhibit bioactive properties as triterpenoids display analgesic and anticancer properties (Ali *et al*., 2008). Saponins are reported to have hypocholesterolemic and antidiabetic properties, while triterpenoids display analgesic and anticancer properties (Ali *et al.,* 2008). So, these secondary metabolites contribute to potent use of plants in pharmacological industries.

Differences were observed in current and previous studies. In current study, terpenoids, tannins, coumarins, saponins and cardiac glycosides were present in both. extracts while alkaloids and

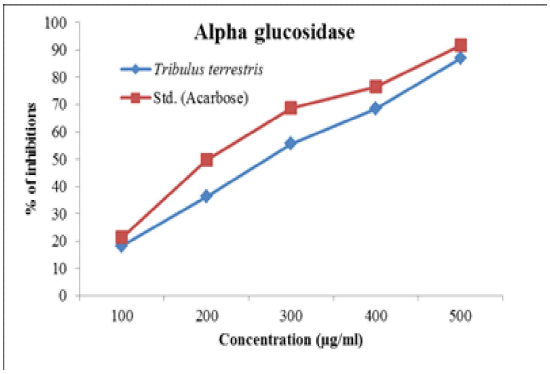

Fig.3. *In vitro* ant-diabetic activity (Alpha glucosidase) of ethanolic leaf extract of *Tribulus terrestris*

tritetrapenoids were not found. In previous studies alkaloids were found to be present in aq. extracts of the *Punica granatum* while terpenoids were not detected (Wadood *et al*., 2013). Similarly, in current study terpenoids and tannins were detected while previous studies in *Ficus microcarpa* alkaloids and steroids have also been reported (Shripad *etal*., 2012).

UV-Visible analysis of ethanolic leaf extract of *Tribulus terrestris*

Absorption spectroscopy is usually performed with molecules dissolved in a transparent solvent. The absorbance of a solute depends linearly on its concentration and therefore absorption spectroscopy is ideally suited for quantitative measurements. The wavelength of absorption and the strength of absorbance of a molecule depend not only on the chemical nature but also on the molecular environment of its chromophores. Absorption spectroscopy is therefore an excellent technique for following ligand-binding reactions.

The data is typically plotted as extinction as a function of wavelength. Each spectrum is background corrected using a "blank" - a cuvette filled with only the dispersing medium to guarantee that spectral features from the solvent are not included in the sample extinction spectrum. The UV-VIS spectroscopic studies revealed the presence of peaks in the range 400 and 650nm (Fig 1). On comparison of the spectra of leaves showed that the extract has some similar phenolic and flavonoid compounds reported by Jasper *et al*., (1958); Sofowora, (1993).

The UV-visible spectra were performed to identify the compounds containing ó-bonds, ð-bonds and lone pair of electrons, chromophores and aromatic rings. UV-VIS spectroscopic is simple, cost effective and rapid tests for detecting phytocomponents. UV-visible spectroscopy uses light in the visible ranges or its adjacent ranges. The colour of the chemicals involved directly affects the absorption in the visible ranges. Molecules undergo electronic transitions in these ranges of the electromagnetic spectrum (Gunasekaran, 2003).

In vitro* ant-diabetic activity ethanolic leaf extract of *Tribulus terrestris

A study of ancient literature indicated that diabetes was fairly well known and well-conceived as an entity in India. Regulation of glucose level in the blood of the diabetic patient can prevent the various complications associated with the disease. The maintenance of plasma glucose concentration for a long term under a variety of dietary conditions is one of the most important and closely regulated processes observed in the mammalian species (Raghavendra *et al.,* 2010).

The intestinal digestive enzyme alpha-amylase plays a vital role in the carbohydrate digestion. One antidiabetic therapeutic approach reduces the post prandial glucose level in blood by the inhibition of alpha-amylase enzyme. These can be an important strategy in management of blood glucose (Latha *et al*., 2009). The *in vitro* α-amylase inhibitory studies demonstrated that *Tribulus terrestris has* well anti diabetic activity (Table 2). The percentage inhibition at 100, 200, 300, 400 and 500 µg/ml concentration of crude plant leaf extract showed concentration dependent percentage inhibition. *Tribulus terrestris* showed an inhibition of 87.30% at 500 µg/ml extract and the standard showed an inhibition of 92.46%.

Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide in to mono and disaccharide. In our experimental study it was observed that ethanolic extract of *Tribulus terrestris* demonstrated significant Alpha amylase inhibition activity as compared to standard drug acarbose.

α-glucosidase catalyzes the final step in carbohydrate digestion which leads to postprandial hyperglycemia. Inhibitors of α-glucosidase are useful in the control of hyperglycemia as they delay carbohydrate digestion and causing reduced glucose absorption rate which consequently reduce the postprandial plasma glucose rise (Tarling *et al*., 2008).

In this present study, *in vitro* α-glucosidase inhibitor activity of ethanolic extract of *Tribulus terrestris* was evaluated. The retardation and delay of carbohydrate absorption with a plant-based α-glucosidase inhibitor offers a prospective therapeutic approach for the management of type-2 *Diabetes Mellitus*. The leaf extract of *Tribulus terrestris* has 86.88% while standard has

91.65% inhibition.

These inhibitors have been found useful in the control of *Diabetes mellitus* over many years (Tundis et al., 2010) Many scientists have investigated the plants containing various phytochemicals that exhibit additive and synergistic interaction in antidiabetic properties which exert positive health-promoting effects (Samad *et al*., 2009).

CONCLUSION

From the above results, it is concluded that, *Tribulus terrestris* leaves are rich source of phytochemicals. This study clearly indicated that these phytochemicals might be responsible for their therapeutic effects. In this present study, we evaluated *in vitro* alpha amylase, and alpha glucosidase activities of methanolic leaf extract of *Tribulus terrestris*. The plant extract showed significant inhibition activity. So further research on isolation, purification, and characterization compounds which are responsible for inhibiting activity, has to be done for their usage as antidiabetic agent.

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