In-vitro Evaluation of anti-oxidant and anti-diabetic activity of leaves of *Tephrosia uniflora*

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Abstract

Natural anti-oxidants are being researched as prospective treatment modalities since oxidative stress is heavily involved in the physiopathology of diabetes. In this work, *Tephrosia uniflora* leaf extracts such as pet ether, chloroform, alcohol, water is investigated phytochemical, *in-vitro* for their anti-oxidant and inhibitory effects. The hydrogen peroxide assay (H2O2), ferric reducing anti-oxidant power (FRAP), and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) techniques were employed to assess anti-oxidant activity. A model created *in-vitro* was used to study the inhibitory effects of glucose diffusion and -amylase. With IC50 values of *Tephrosia uniflora* of various extracts for DPPH is 123.75 mg/ml, 77.28 mg/ml, 69.50 mg/ml, 62.362 mg/ml, FRAP is 96.463 mg/ml, 96.161 mg/ml, 86.47 mg/ml, 75.483 mg/ml and H2O2 is 54.433 mg/ml, 49.813 mg/ml, 45.000 mg/ml, 37.446 mg/ml. The diffusion of glucose and -amylase was nevertheless constrained by the extracts is 23.30 mg/ml, 17.58 mg/ml, 4.98 mg/ml, 0.85 mg/ml, and 55.59 mg/ml, 46.86 mg/ml, 34.21 mg/ml, 26.86 mg/ml. Aside from that, the outcomes demonstrated that the phenolic and flavonoid that exhibit the highest anti-oxidant and free radical scavenging potential. The findings suggested that this plant might be a substantial source of naturally occurring chemicals with pharmacological significance.

Key words: *Tephrosia uniflora*, anti-oxidant activity, diabetes, inhibitory activity, pharmacological significance.

Introduction

The prevalence of diabetes mellitus (DM) has increased recently in many countries, especially emerging ones, as a result of sedentary behaviour and nutritional changes. By 2025, there are expected to be 80 million cases of diabetes worldwide, which would increase the prevalence of this condition, which accounts for nearly 90% of all cases of DM.[1] Numerous people throughout the world suffer from type 2 diabetes (T2D). In addition to hyperglycaemia and decreased insulin action and/or secretion, T2D is a complicated diverse set of metabolic illnesses that affects many different organs and tissues. Current hypotheses for T2D include a failure in insulin-mediated glucose absorption in muscle, pancreatic -cell dysfunction, a disruption of adipocyte secretory function, and reduced insulin action in the liver. Postprandial hyperglycaemia caused by a weakened pancreas after meals is linked to type 2 diabetes in its early stages.[2]

Hyperglycaemic spikes that causes oxidative stress are a hallmark of the postprandial hyperglycaemia pathogenesis.[3] Reactive oxygen species (ROS) are produced as a result of hyperglycaemia, which is a major contributor to the secondary complications of diabetes mellitus like damage to the kidneys, eyes, blood vessels, and nerves. ROS are chemicals that harm membranes and lipids. Antioxidants have been shown to block the death of cells by inhibiting the peroxidation chain reaction, which suggests that they may prevent the onset of diabetes.[4]

Demand for natural antioxidants has recently increased due to the possibility that synthetic antioxidants may be linked to disease, and much attention has been focused on biologically active compounds found in plants and herbs for their safety and efficacy in prevention and/or treatment of human diseases.[5]

Tephrosia uniflora is Semi-erect perennial, up to 1 m tall, stem pubescent, hairs silky. Leaf imparipinnate, petiole 3-8 mm long, rachis 2 cm long; leaflets 5-9, up to 5.5 cm long, and 13 mm wide, oblanceolate or elliptic, acute or obtuse, glabrous or pubescent above, appressed pilose below; stipules up to 9 mm long. Flowers 2-3 in the leaf-axils. Pedicel up to 4 mm long. Calyx pubescent, tube c. 1.5 mm long, teeth up to 6 mm long. Corolla pink. Vexillum c. 10 mm long, pubescent externally. Ovary pubescent, style glabrous, stigma penicillate. Fruit c. 3.8-4.8 cm long, c. 4-4.5 mm broad, appressed pubescent, 7-8-seeded.6 *Tephrosia uniflora* used for the treatment of Syphilis, Dropsy, Diabetes, Spleenic and liver disorders, cough, dysponea, disorders of blood, chronic ulcers, fever, pain, poisoning, vata diseases, thirst, gingivitis, abdominal disorders, hepatomegaly and spleenomegaly, elephantiasis, asthama, scabies, itches, eczema, skin disorders, laxative.7,8

Therefore, in this study, to elucidate the biological activities of *Tephrosia uniflora*, we analyzed the total phenolic content (TPC) and the total flavonoid content (TFC) and determined the antioxidant activity from various extracts such as petroleum ether, chloroform, alcohol and water. The investigation of *in-vitro* glucose diffusion inhibition assay and α-amylase inhibition assays potential of *Tephrosia uniflora* as an antidiabetic agent is studied.[9]

Materials and methods

Collection and identification of the plant

The leaves of *Tephrosia uniflora* plant were collected. It is dried under shade and made into coarse powder. The plant material collected was identified and authenticated by Scientist (Dr) K Madhavachetty , M.Sc, M Ed, M.Phil, PhD, PGDPD, plant taxonomist ,Assistant professor Department of Botany ,Tirupathi, India.

Preparation of extracts

The previously powdered drug was used for preparing extract. Different extracts are prepared by extracting plant material with different solvents viz. Pet ether, Chloroform, Ethanol and Water with increasing polarity.

Phytochemical screening

Phytochemical screening was carried out as per the methods mentioned in standard pharmacopoeias. The secondary chemical constituents are selective and vary considerably from plant to plant and even within the species or varieties of the same genus. The secondary chemical constituents are chiefly responsible for the biological activities of plants or drugs. Different chemical tests were performed for detecting various chemical constituents.[10,11]

Total phenolic content

Total phenol content was determined with the Folin- Ciocalteu's assay using gallic acid as standard. In the procedure, 0.5 ml of plant extracts were mixed with 1.5 ml Folin- Ciocalteu's reagent (FCR) diluted 1:10 v/v then after 5 minutes 1.5 ml of 7% sodium carbonate solution was added. The final volume of the tubes was made upto 10 ml with distilled water and allowed to stand for 90 minutes at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. All the experiment was repeated three times for precision and values were expressed in mean ± standard deviation in terms of phenol content (Gallic acid equivalent, GAE) per g of dry weight.[12]

Total flavonoid content

Total flavonoid content was determined by Aluminium chloride method using quercetin as a standard. 1ml of test sample and 4 ml of water was added to a volumetric flask (10 ml volume). Add 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added after 5 minutes. After 6 mins incubation at room temperature, 1ml of 1 M Sodium hydroxide was added to the reaction mixture. The final volume was made up to 10 ml with distilled water. Absorbance of sample was measured against the blank at 510 nm using a spectrophotometer. All the experiment was repeated three times for precision and values were expressed in mean ± standard deviation in terms flavonoid content (Quercetin equivalent, QE) per g of dry weight.[13,14]

In-vitro antioxidant activity

DPPH reducing assay

The ability of *Tephrosia uniflora* extracts to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was determined according to method as described by Moraes-de-Souza et al (2008) with some modifications. To 0.5 mL of extracts 3 mL of methanol and 0.3 mL of 0.5 mM DPPH radical solution in methanol were added. The reaction mixture was incubated in the dark at room temperature for 30 mins and absorbance was measured at 517 nm in a spectrophotometer. The blank was performed using reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate.[15] The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula:

$$\text{Inhibitory activity } (\%) = \frac{\text{Abs Control } (AC) - \text{Abs Sample } (AS)}{\text{Abs Control } (AC)} \times 100$$

Where,

AC is the absorbance of the control

AS is the absorbance in the presence of the sample of *Tephrosia uniflora* extracts or standards.

Ferric reducing power assay

The ability of *Tephrosia uniflora* extracts to scavenge ferric ions was determined according to method as described by Patel et al., (2010); Patel et al., (2012). with some modifications. To the 2.5 ml of extract, 1ml of 0.2 M phosphate buffer pH 6.6 and 1 ml of 1 % potassium ferric cyanide was added. The reaction mixture was incubated in water bath at 50°C for 20 minutes. Afterward, the reaction mixture was rapidly cooled and 2.5 ml of 10 % trichloroacetic acid was added to stop the reaction and was centrifuged for 10 minutes. 2.5 ml of aliquots was pipetted out and 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride solution was added. The color changes to green. The mixture was allowed to stand for 10 minutes, and absorbance was measured at 593 nm in a spectrophotometer. The blank was performed using reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate.[16] The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula

$$\text{Inhibitory activity } (\%) = \frac{\text{Abs Control } (AC) - \text{Abs Sample } (AS)}{\text{Abs Control } (AC)} \times 100$$

Where,

AC is the absorbance of the control

AS is the absorbance in the presence of the sample of *Tephrosia uniflora* extracts or standards.

H2O2 reducing assay

The ability of *Tephrosia uniflora* extracts to scavenge hydrogen peroxide was determined according to the method as described by Ruch et al (1989) with some modifications. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). To 1 ml of extracts in distilled water, hydrogen peroxide solution (0.6 mL, 40mM) was added. The mixture was allowed to stand for 10 mins and absorbance was measured at 230 nm in a spectrophotometer. The blank was performed using reagent blank and solvent. Ascorbic acid was used as standard.

The extracts were performed in triplicate.[17] The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula:

$$\text{Inhibitory activity (\%)} = \frac{\text{Abs Control (AC)} - \text{Abs Sample (AS)}}{\text{Abs Control (AC)}} \times 100$$

Where,

AC is the absorbance of the control

AS is the absorbance in the presence of the sample of *Tephrosia uniflora* extracts or standards.

In-vitro anti-diabetic activity

Glucose diffusion method

In-vitro glucose diffusion was measured using a slightly modified version of the method of Rastogi *et al* with slight modifications. In brief, the model consisted of a one-sided sealed dialysis tube (cm × mm) into which 3 mL of glucose solution (22 mmol L−1) in NaCl (0.15 mol L−1) was mixed with 1 mL (5 mg/kg) of each of the following *Tephrosia uniflora*. The control group (without extract, with distilled water) was also used for comparison. The dialysis membrane of each sample was sealed at each end and was placed in a beaker containing 40 mL of NaCl solution (0.15 mol L−1) and 10 mL of distilled water. The beakers containing the resulting solutions were then placed on an orbital shaker at room temperature and the extent of diffusion across the dialysis membrane was determined after 30, 60, 90, and 120 mins. The absorbance reading was measured at 545 nm in a spectrophotometer. The extracts were performed in triplicate.[18] The α-amylase inhibitory activity (%) was calculated using the formula below.

$$\text{Inhibitory activity (\%)} = \frac{\text{Abs Control (AC)} - \text{Abs Sample (AS)}}{\text{Abs Control (AC)}} \times 100$$

Where,

AC is the absorbance of the control

AS is the absorbance in the presence of the sample of *Tephrosia uniflora* extracts or standards.

Alpha amylase inhibition assay

The pancreatic α-amylase inhibition assay was investigated according to the method described by Daoudi et al, with slight modifications. Briefly, 1 mL of 0.02 M phosphate buffer (pH = 6.9); 0.2 mL of α-amylase enzyme solution (13 IU); and 1 mL of *Tephrosia uniflora* extracts (0.1, 0.2, and 0.4, 0.6, 0.8, 1.0 µg/mL). Then, 1 mL of starch (1%) solution (dissolved in the above buffer) was added to test tubes and the mixture was incubated at 37 ◦C for 20 min. The reaction was terminated by adding 0.6 mL of di-nitro salicylic acid (DNSA) color reagent (2.5%) followed by incubation in a hot water bath at 100 ◦C for 8 min to inactivate the enzymes. Afterward, the tubes were put in a cold-water bath, after which 1 mL of distilled water was added. The absorbance was measured at 540 nm in a spectrophotometer. The extracts were performed in triplicate.[19] The α-amylase inhibitory activity (%) was calculated using the formula below.

$$\text{Inhibitory activity (\%)} = \frac{\text{Abs Control (AC)} - \text{Abs Sample (AS)}}{\text{Abs Control (AC)}} \times 100$$

Where,

AC is the absorbance of the control

AS is the absorbance in the presence of the sample of *Tephrosia uniflora* extracts or standards.

Result

Preliminary phytochemical screening

Preliminary phytochemical investigations of extracts were assessed to reveal the presence of different secondary metabolites the extracts indicated the presence of flavonoids, carbohydrates, saponins, proteins, alkaloids phenols, and tannins respectively.

Total phenolic content

The total phenolic content for aqueous, ethanol, chloroform, and petroleum ether extracts of *Tephrosia uniflora* were estimated by Folin-Ciocalteu's method using Gallic acid as standard. The reagent is formed from a mixture of phosphor-tungstic acid and phosphor-molybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 750 nm and proportional to the total quantity of phenolic compounds originally present. The Gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression co-efficient (R2) =0.9905. The plot has a slope (m) = 0.011 and intercept = 0.1017. The equation of standard curve is y = 0.011x+0.1017

Total flavonoid content

The total flavonoid content for aqueous, ethanol, chloroform and petroleum ether extracts of *Tephrosia uniflora* were measured with the Aluminium chloride colorimetric assay using quercetin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition, it also forms liable complexes with Ortho di-hydroxide groups in A/B rings of flavonoids. The quercetin solution of concentration (100-1000 ppm) conformed to Beer's Law at 510 nm with a regression co-efficient (R2) = 0.9973. The plot has a slope (m) = 0.0105 and intercept = 0.0891. The equation of standard curve is y = y = 0.0105x + 0.0891

Table 1: Total phenolic content and total flavonoid content of various extracts of *Tephrosia uniflora*

***Tephrosia uniflora* plant extract**	**Total phenolic content**	**Total flavonoid content**
Petroleum ether	0.24877±0.00012µg/ml	0.308267±0.002801µg/ml
Chloroform	0.3145±0.00014µg/ml	0.331233±0.031771µg/ml
Alcohol	0.71207±0.0026µg/ml	0.58±0.001µg/ml
Water	1.12373±0.00015µg/ml	1.714533±0.000462µg/ml

Anti-oxidant activity

The anti-oxidant activity of all extracts is measured by three methods viz. DPPH scavenging assay, Ferric reducing anti-oxidant power assay and H_2O_2 reducing assay and was compared with the standard Ascorbic acid. It was observed that the water extracts of *Tephrosia uniflora* has higher anti-oxidant activity than that of ethanol, chloroform, and pet ether extracts of *Tephrosia uniflora*. Though the scavenging activity of the extracts are less than that of Standard Ascorbic acid, the study shows that the extracts have the proton donating ability and could serve as free radical inhibitors acting possibly as primary antioxidants. The percentage inhibition of extracts of *Tephrosia uniflora* was given in the tables below (Tables 2,3,4).

Table 2: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using DPPH inhibition assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	2.8±0.017	4.28±0.02	7.14±0.01	12.85±0.015
20	8.57±0.015	11.42±0.01	15.17±0.01	21.42±0.017
40	14.28±0.01	21.42±0.01	32.85±0.0115	37.14±0.015
60	21.42±0.01	38.57±0.025	42.85±0.0115	45.71±0.005
80	32.85±0.01	48.57±0.015	54.85±0.01	61.42±0.020
100	38.57±0.152	60±0.005	72.85±0.005	77.14±0.015
IC 50	123.75	77.28	69.50	62.362

Data are expressed as mean ± SD (n = 3)

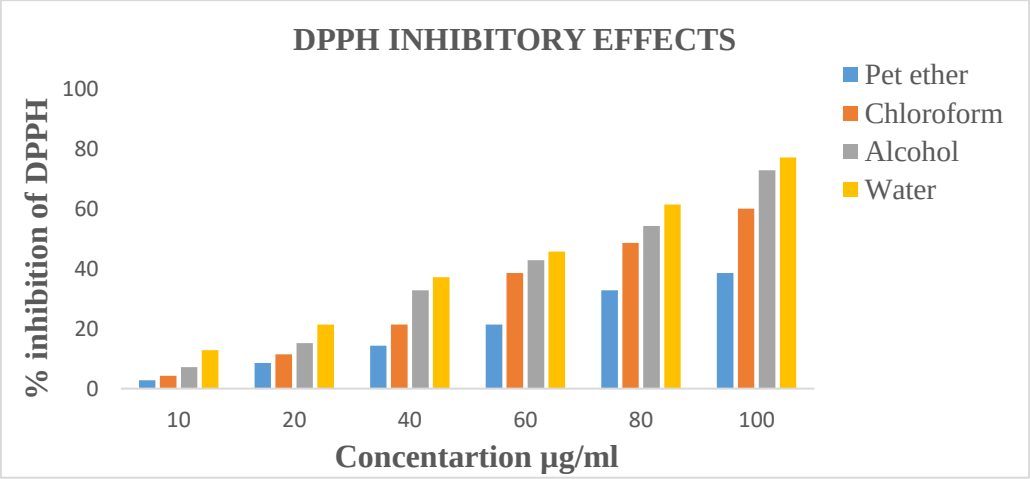

Figure 1: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using DPPH inhibition assay

Table 3: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using ferric chloride inhibition assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	1.666667±0.04	5±0.0556	10±0.0264	15±0.0115
20	8.333333±0.035	13.33333±0.0305	16.66667±0.0152	21.66±0.02
40	16.66667±0.0503	23.33333±0.0264	28.33333±0.0264	33.33±0.0057
60	26.66667±0.0152	31.66667±0.0321	36.66667±0.0264	43.33±0.01
80	36.66667±0.0416	41.66667±0.0360	48.33333±0.0251	53.33±0.01
100	46.66667±0.0321	51.66667±0.0251	55±0.0264	61.66±0.0208
IC 50	96.463	96.161	86.47	75.483

Data are expressed as mean ± SD (n = 3)

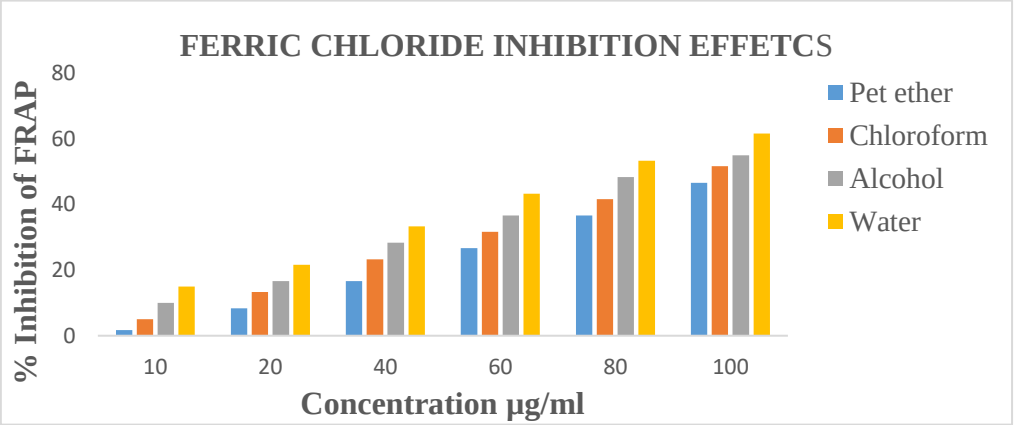

Figure 2: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using Ferric reducing inhibition assay

Table 4: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using H2O2 inhibition assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	10±0.01	11.42±0.0230	14.28±0.01	22.85±0.0152
20	21.42±0.0057	24.28±0.0057	30±0.0057	37.14± 0.01
40	37.14±0.01	41.42±0.01	47.14±0.0115	54.28±0.0251
60	57.14±0.0152	62.85±0.0173	65.71±0.02	71.42±0.0173
80	74.42±0.0115	78.57±0.01	82.85±0.0173	86.57±0.0152
100	85.71±0.0680	91.42±0.0173	95.71±0.0115	98.57±0.0015
IC 50	54.433	49.813	45.000	37.446

Data are expressed as mean ± SD (n = 3)

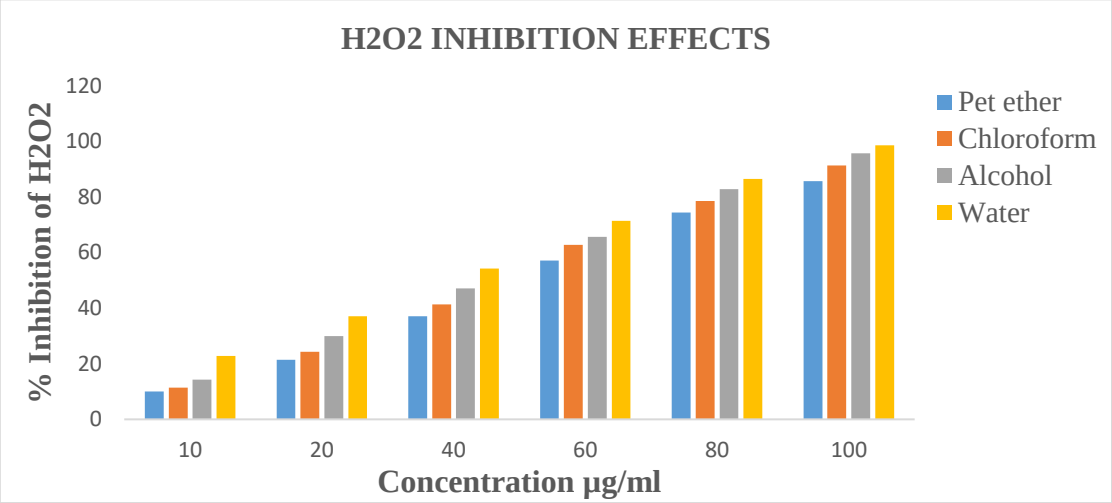

Figure 3: Anti-oxidant effects of various extracts of *Tephrosia uniflora* using H2O2 inhibition assay

Anti-diabetic activity

Anti-diabetic activity of leaf extracts of *Tephrosia uniflora* was carried out by alpha-amylase inhibition assay and glucose diffusion assay. The study shows that there was a significant increase of the percentage inhibitory activity in a concentration dependent manner. It was observed that the water extracts of *Tephrosia uniflora* has higher antioxidant activity than that of ethanol, chloroform, and pet ether extracts of *Tephrosia uniflora*. The percentage inhibition of extracts of *Tephrosia uniflora* was given in the tables below (Tables 5,6)

Table 5: Anti-diabetic effects of various extracts of *Tephrosia uniflora* using glucose diffusion assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	39.37±0.493	41.25±0.197	44.68±0.406	46.25±0.293
20	48.75±0.486	52.29±0.382	60.29±0.360	61.87±0.260
40	62.50±0.433	63.75±0.224	70.37±0.340	71.87±0.246
60	68.75±0.213	74.37±0.292	76.35±0.360	77.50±0.446
80	75.00±0.360	80.81±0.195	84.93±0.446	85.00±0.206
100	80.62±0.513	85.00±0.432	88.12±0.226	88.75±0.326
IC 50	23.30	17.58	4.98	0.85

Data are expressed as mean ± SD (n = 3)

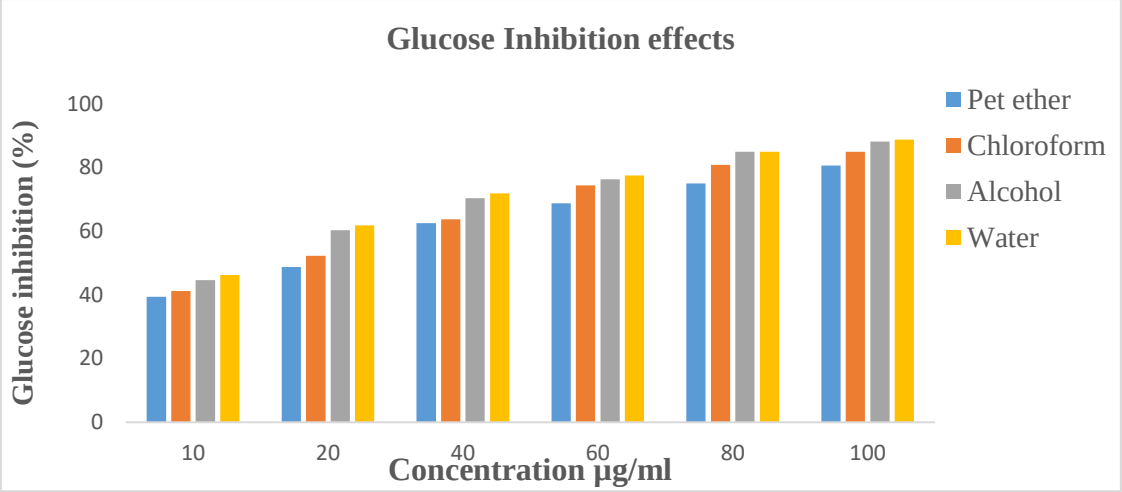

Figure 4: Anti-diabetic effects of various extracts of *Tephrosia uniflora* using Glucose diffusion assay

Table 6: Anti-diabetic effects of various extracts of *Tephrosia uniflora* using Alpha-amylase inhibition assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	25.05±0.245	29.54±0.340	35.55±0.440	39.77±0.220
20	31.88±0.142	33±0.406	42.88±0.353	45.88±0.140
40	46.22±0.286	52.55±0.401	54.55±0.546	60.33±0.286
60	52.77±0.376	56±0.360	66.11±0.246	67.88±0.113
80	60.11±0.117	66±0.513	71.11±0.201	72.11±0.273
100	72.77±0.117	76.55±0.406	77.55±0.223	81.55±0.173
IC 50	55.59	46.86	34.21	26.86

Data are expressed as mean ± SD (n = 3)

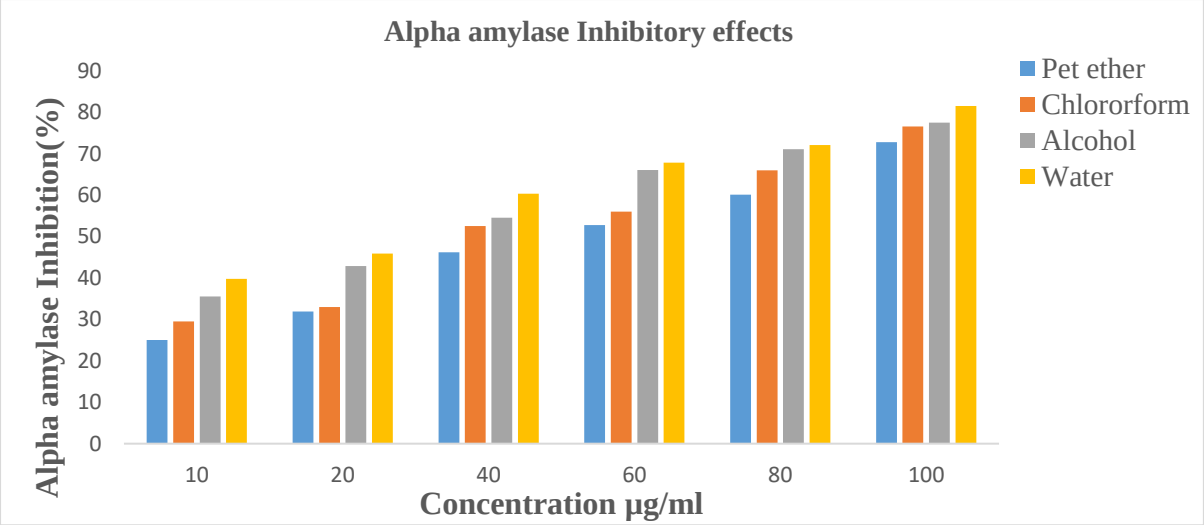

Figure 5: Anti-diabetic effects of various extracts of *Tephrosia uniflora* using Alpha-amylase Inhibition assay

Discussion

The results of this study explored the anti-oxidant capabilities of *Tephrosia uniflora* extracts and the *in-vitro* inhibition of major enzymes associated with type-2 *diabetes mellitus* (alpha-amylase and glucose diffusion). The phenolic and flavonoid contents of the leaves section were highly linked with their anti-diabetic and antioxidant effects. The traditional usage of *Tephrosia uniflora* leaves in the prevention and control of diabetes may have biological justification due to their combination enzyme inhibitory and antioxidant effects. However, this study found that *Tephrosia uniflora* leaves had the highest anti-oxidant and enzyme inhibitory effects.

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