Original Research Article

In-vitro Antioxidant and DPP-IV Enzyme Assay of Ethyl acetate extract of *Enicostemma littorale* to investigate its Antidiabetic Potential

Abstract

Background: Pharmacological treatments for diabetes are based on increasing insulin availability and improving insulin sensitivity. Today, glucagon-like peptide 1 (GLP-1) -based therapies aim to control glucose through DPP-4 inhibitors. DPP-4 is a transmembrane glycoprotein belonging to the prolyl oligopeptidase family, with the specificity of eliminating the X-Pro or X-Ala dipeptides from the N-terminal end of the polypeptides. The effect of GLP-1 in stimulating the release of glucose-dependent insulin from pancreatic islets inhibits inappropriate glucagon release after meals and slow gastric emptying promotes intestinal permeability.

Study Design: The current study investigated the inhibitory activity of DPP-4 along with the antioxidant activity of *Enicostemma littorale* extract.

Place and Duration of Study: The present study was conducted at Anurag University, Hyderabad between June-2021 to Sept-2021.

Methodology: The extracts tested for a range of activities such as hydroxyl radical scavenging activity test, *In-vitro* DPP-IV enzyme test activity test, DPPH free radical scavenging.

Results: The extract shows dose-dependent DPPH and hydrogen peroxide radical scavenging activity. In *in-vitro* DPP-IV enzyme assay activity, *Enicostemma littorale* ethyl acetate extract showed greater inhibitory activity of DPP-IV compared to vildagliptin with IC_{50} values of 165.64µg/ml, respectively. Vildagliptin, based on the reference standard for DPP-IV inhibitor activity, has an IC_{50} value of 57.44 µg/ml. According to the *in-vitro* analysis, the *Enicostemma littorale* extract has a strong inhibitory activity of DPP-IV.

Conclusion: We concluded that the *Enicostemma littorale* will be a better source for further development as new antidiabetic drugs. To the best of our knowledge, there is no report and study on the bark part of this species.

Keywords: Enicostemma littorale, DPPH, Diabetics, vildagliptin, Ascorbic acid, BHT, *In-vitro* DPP-IV enzyme assay

Abbreviations

DPPH:	*2, 2-Diphenyl-1-Picrylhydrazyl*
BHT:	*Butylated hydroxytoluene*
E. littorale:	*Enicostemma littorale*
T2DM:	*Type 2 Diabetes Mellitus*

1. INTRODUCTION

In 2019, approximately 463 million adults (20-79 years old) were living with diabetes; by 2045, this number will rise to 700 million. The percentage of people with type 2 diabetes is increasing in most countries [1]. 79% of adults with diabetes lived in low and middle-income countries, 1 in 5 people over 65 have diabetes, and 1 in 2 people (232 million) with diabetes was undiagnosed. Diabetes caused 4.2 million deaths [2]. Diabetes caused at least $ 760 billion in health care spending in 2019, 10% of total adult spending. More than 1.1 million children and adolescents are living with type 1diabetes. More than 20 million live births (1 in 6 live births) have diabetes during pregnancy; 374 million people are at increased risk of developing type 2 diabetes [3, 4].

About 60% of the world's population uses traditional medicines derived from medicinal plants. This review focuses on Indian medicinal herbs and plants that are used in the treatment of diabetes, especially in India [2]. Diabetes is a major human disease that affects many people from different walks of life in different countries. In India, it is proving to be a serious health problem, especially in urban areas [3]. Although there are several approaches to reducing the damaging effects of diabetes and its secondary complications, herbal formulations are preferred due to their fewer side effects and low cost. A list of medicinal plants with proven anti-diabetic effects and related beneficial and herbal medicinal effects used in the treatment of diabetes is compiled [5]. These include *Allium sativum, Eugenia jambolana, Momordica charantia Ocimum sanctum, Phyllanthus amarus, Pterocarpus marsupium, Tinospora cordifolia, Trigonella foenum graecum,* and *Withania somnifera*. One of the etiological factors involved in the development of diabetes and its complications is the damage induced by free radicals, so an anti-diabetic compound with antioxidant properties would be more advantageous [1-5].

Enicostemma littorale contains several antioxidant phytochemicals, including catechins, alkaloids, sterols, saponins, triterpenoids, flavonoids, phenolic acids, and xanthones. It contains many minerals such as calcium, iron, magnesium, potassium, sodium, silica, chloride, phosphate, carbonate, and sulfate [1]. *Enicostemma littorale* is known to have antibacterial [5], anti-inflammatory [4], antitumor [3, 4], and

antidiabetic [4] activities and *E. littorale* has been found to improve glucose-dependent insulin release. *Swertiamarin*, a secondary compound present in *Enicostemma littorale* [6], has antispasmodic and anticholinergic activity [7]. The result of a single clinical study with *E. littorale* showed a significant effect on hypotensive, hypoglycemic and lipid-lowering drugs [2, 3]. *Swertiamarin* demonstrated antibiotic activity *in-vitro* [7].

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia caused mainly by insulin resistance or impaired insulin secretion, along with disorders of carbohydrate, fat, and protein metabolism [8]. Various therapeutic approaches have been used to treat diabetes, including improving insulin sensitivity, inhibiting gluconeogenesis, and decreasing glucose uptake from the gut. Recently, a new approach has emerged that uses dipeptidyl peptidase IV (DPP-IV) inhibitors as a possible agent for the treatment of type 2 diabetes without producing side effects, such as hypoglycemia and depletion of pancreatic cells [9]. DPP-IV inhibitors improve hyperglycemic conditions by stabilizing the postprandial level of intestinal hormones such as glucagon-like peptide-1 and glucose-dependent tropic insulin-dependent polypeptides, which function as incretins to help up-regulate insulin secretion and beta-cell mass [10].

DPP-IV enzyme inhibitors have provided a unique therapeutic perspective for the treatment of DM2 [8, 9]. Furthermore, mechanisms based on intestinal hormones, glucagon-like peptide 1 (GLP-I), glucose-dependent insulinotropic polypeptide (GIP), and gastric inhibitory polypeptide have been shown to reduce postprandial and fasting blood glucose in patients with DM2 and provide therapeutic potential for DM2. The role of these hormones is of considerable importance in the survival of beta cells and increasing beta-cell mass and insulin production. They also regulate glucose homeostasis by improving blood insulin levels [10, 11].

Incretin/intestinal hormones have increased rapidly around the world over the past few decades. Both GLP-1 and GIP are secreted by intestinal cells to improve pancreatic cell mass and function. The functions of these peptides are very effective in reducing glycosylated hemoglobin (HbA1c) and maintaining glucose homeostasis [9, 10]. DPP-IV inhibitors are also acceptable therapies and include vildagliptin, sitagliptin, and many others. Mechanically, DPP-IV inhibitors block enzyme activity to increase the half-life of GLP-1 to normal levels in the blood plasma and this helps restore beta-cell function, improve insulin secretion, and slow glucagon secretion from part of the α-cells. Previous research has revealed that the primary clinical approach for DPP-IV inhibitors with antioxidant capacity is the first-line treatment, due to its capacity, safety, and acceptability [11]. DPP-IV inhibitors also improve the metabolic system (measured by lowering blood sugar) without causing hypoglycemia. The antidiabetic effects of bioactive compounds of plant and animal origin can be associated with a mixture of phytochemicals or with individual compounds [8-11].

Pharmacological inhibitors of DPP-4 as a therapy of type 2 diabetes to achieve adequate glycemic control are firmly established and numerous inhibitors are in different stages of clinical development [12, 13]. Currently available DPP-4 inhibitors are saxagliptin, sitagliptin, alogliptin, and vildagliptin. Validgliptin is licensed in Europe and Latin America in 2007, alogliptin is licensed only in Japan in 2010 [12-14, 18]. At the same time, many studies have investigated the adverse effects of current DPP-4 inhibitors: nasopharyngitis, upper respiratory tract infection, urinary tract infection, severe allergic reactions, headache, anaphylaxis, angioedema, nausea, diarrhea and pain, sugar levels. Especially if taken in case of overdose [15-18]. But herbal products do not cause any side effects. In the present study, several solvent extracts of *Enicostemma littorale* were investigated to determine the inhibitory activity of DPP-4 and were followed by the antioxidant activity. This study is the first report on *Enicostemma littorale* extract showing strong inhibition of DPP-4, strongly supporting the possible potential of incretin-based antidiabetic therapy.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

Ascorbic acid, 2,2-diphenyl-1-picryl hydrazyl radical (DPPH), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloro acetic acid (TCA), and thio barbituric acid (TBA) were purchased from Lab Trading Laboratory Aurangabad. BHT, ascorbic acid and vildagliptin were purchased from Sigma Chemicals, India. All other chemicals and solvents used were of analytical grade available commercially.

2.2 Collection of Plant and Extraction

Whole plants of *Enicostemma littorale* collected were washed with tap water. The plants were cut into small pieces and air-dried completely in the shade (at room temperature) for 2-3 weeks to avoid direct loss of phytoconstituents from sunlight. Shade dried materials were sprayed using the sprayer and screened at 80 mesh. It was then homogenized into a fine powder and stored in an airtight container for subsequent analysis.

10 grams of powdered material was suspended in 150 ml of water and refluxed for three hours in a round bottom flask on a heating mantle. The crude extract was dissolved in 100 ml of ethyl acetate. The extract subjected to filtered, and evaporated to dryness on a rotary evaporator under a vacuum at 400°C. The dry crude aqueous extract was weighed and a 1 g portion was lyophilized and stored at 40°C for further investigation. [19].

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2.3 Preliminary Phytochemical Screening

The extract of *Enicostemma littorale* plant obtained during the extraction process was subjected to preliminary phytochemical examination for the presence of various phytoconstituents using reported methods [20-24].

Alkaloid test: 0.5 g of powder sample was extracted with 2.5 ml of methanol and 2.5 ml of 2N hydrochloric acid. Then the filtrate was treated with the Meyer and Wagner reagents. The sample was recorded as negative based on turbidity and color.

Flavonoid test: 0.5 g of powdered extract was heated with 5 ml of ethyl acetate, a water bath (40-50°C) for 5 min. The filtrate was then treated with 0.5 ml of diluted ammonia solution. A yellow color revealed the presence of flavonoids.

Saponin test: 1 g of powdered extract is boiled in 10 ml of distilled water and the solution is filtered. Then, 5 ml of the filtrate was mixed with 5 ml of distilled water and vigorously shaken to obtain stable and persistent foam. The foam was mixed with three drops of olive oil and shaken vigorously to form an emulsion, indicating the presence of saponins.

Glycoside test: Keller-Kiliani test: 1 ml of extract was added to 1 ml of glacial acetic acid. Then 1 ml of ferric chloride was added with 1 ml of concentrated sulfuric acid. The blue-green color of the solution specified the presence of glycoside.

Carbohydrate test: 1 ml of n-hexane extract was added to 1 ml of Molisch's reagent with agitation. 1 ml of concentrated sulfuric acid was added carefully from the side of the tube. The development of a red or purple color at the interface of the two layers is the symbol of a positive test that concludes the presence of carbohydrates.

Tannin test: About 10% alcoholic solutions of ferric chloride were added in 2 ml of extracts (1: 1). The development of the dark blue color of the solution indicated the presence of tannins.

Terpenoid test: Salkowski test: 2 ml of chloroform was added to 2.5 ml of n-hexane extract. Then, 2ml of concentrated sulfuric acid was slowly added to form a layer. A reddish-brown color at the edge indicated the presence of terpenoids.

Steroid test: To 1 ml of sample extract, add 2 ml of acetic anhydride and 2 ml of concentrated sulfuric acid, the color changes from blue to dark green showed the existence of steroids.

2.4 In-vitro Antioxidant Activity

2.4.1 DPPH Radical Scavenging Assay

The ability of the extracts to eliminate DPPH free radicals was determined using the standard method [25]. 100µl of various concentrations (25-200µg/ml) of each extract was mixed with 100µl of ethyl acetate and 50µl of 0.05 mM 1,1-diphenyl-2-picrylhydrazyl in ethyl acetate. Controls contained all reaction reagents except extract or positive control substance. After 20 min of incubation in the dark and at 25°C, the resulting absorbance was recorded at 517 nm. A line graph of concentration versus percentage inhibition was prepared and IC50 values were calculated from the line equation obtained by plotting

concentration versus percentage inhibition. Values are presented as the mean of the triplicate analysis. Butylated hydroxytoluene (BHT) was used as a reference. The% inhibition was calculated according to the following equation:

$$\%\text{DPPH radical scavenging activity} = [(A_0-A_1)/A_0] \times 100 \text{ ---------- (1)}$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the drugs/standard.

2.4.2 Hydrogen Peroxide Scavenging Activity

A solution of hydrogen peroxide (20 mM) was prepared in phosphate-buffered saline (PBS) (pH 7.4). Various concentrations (20-200µg/ml) of the extract or standard in ethyl acetate (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min, the absorbance was measured at 230 nm [25-26]. Ascorbic acid was used as a reference.

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2.5 DPP-IV Enzyme Assay

In-vitro inhibition of DPP-4 was tested as described method [26]. The varied concentration of plant extract (25, 50, and 200µg/ml) and Vildagliptin was used as the standard DPP-IV inhibitor in 1 ml of Tris-HCl buffer (pH 8.4) were pre-incubated with 50µl of DPP-4 (0.05U/mL) of enzyme for 30min at 37°C. After pre-incubation, 60µL of 10mM gly-pro-p-nitronilide was added and the final reaction mixture was made up to 1.5mL using Tris-HCl buffer (pH 8.4) and then incubated at 37°C for 30 minutes and absorbed was finally read at 410 nm. IC_{50} values were calculated from the line equation obtained by plotting concentration versus percentage inhibition [27-29].

3. RESULT AND DISCUSSION

3.1 Extractive value

In this present study, the percentage yield of ethyl acetate extractive value (36.3%) of *Enicostemma littorale*.

Table 1: Extractive Values of *Enicostemma littorale*

Sr. No	Extract Name	Extractive Value (%)
1	Ethyl acetate extract	36.3
2	Methanol extract	14.3
3	Water extract	11.2
4	N-hexane extract	4.6

In the present study and mentioned in **Table 1**, the percentage yield of the extraction value of ethyl acetate (36.3%) of *Enicostemma littorale.* The extraction values are valuable in estimating the specific soluble components present in the particular solvent. The water-soluble extraction value indicated the

presence of sugar, acids, and inorganic compounds, and the alcohol-soluble extraction values indicated the presence of polar constituents [30].

3.2 Preliminary Phytochemical Screening

The medicinal properties of the *Enicostemma littorale* plant are perhaps due to the presence of various secondary metabolites such as alkaloids, tannins, flavonoids, proteins, and mucilages. Therefore, preliminary screening tests can be useful in the detection of bioactive ingredients and can subsequently lead to drug discovery and development. Furthermore, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds [26]. Preliminary phytochemical screening with various qualitative chemical tests revealed the presence of antioxidant phytochemicals, including glycosides, alkaloids, sterols, saponins, triterpenoids, flavonoids, phenolic acids and xanthones in the *Enicostemma littorale* extract, presented in **Table 2** [31].

Table 2: Preliminary phytochemical screening of *Enicostemma littorale* extract

Sr. No.	Phytochemical	Test Performed	Observation
1	Alkaloids	Mayer's test and Wagner's test	Present
2	Flavonoids	Lead acetate test and Ferric chloride test	Present
3	Saponins	Foam test	Present
4	Carbohydrates	Molisch's test	Absent
5	Tannins	Ferric chloride test	Absent
6	Terpenoids	Salkowski test	Present
7	Glycosides	Keller–Kiliani test	Present
8	Steroids	Salkowski test	Absent

3.3 *In-vitro* Antioxidant Activity

3.3.1 DPPH Radical Scavenging Assay

Antioxidants are believed to affect DPPH due to their ability to donate hydrogen. Free radical scavenging activities are critical to preventing the damaging effects of free radicals in a variety of diseases, including cancer. Free radical scavenging with DPPH is an established method for determining the antioxidant activity of plant extracts. In the DPPH test, a purple solution of DPPH is converted into a yellow product, diphenylpicyrylhydrazine, by the addition of the concentration-dependent extract. Due to the relatively short time required for the analysis, this technique has been used frequently to identify antioxidant activity. The DPPH test was performed on the *Enicostemma littorale* extract using a BHT solution as a reference. The absorbance values were plotted against the chosen concentration. Percentage inhibition

curves of BHT extract and *Enicostemma littorale* were plotted for DPPH and IC50 values for percent inhibition of DPPH by BHT and *Enicostemma littorale* extract were determined using a regression equation. The absorbance and% removal values of BHT and *Enicostemma littorale* extract are tabulated in **Table 3.** The regression curve of BHT and *Enicostemma littorale* extract is shown in **fig. 1** [32].

Table 3: DPPH radical scavenging activity of *Enicostemma littorale*

Conc. (µg/ml)	Absorbance of Control (Ac)	Absorbance of Test (At)	% of Scavenging	Absorbance of Control (Ac)	Absorbance of Test (At)	% of Scavenging
	BHT			*Enicostemma littorale*		
25	0.587	0.498	20.98	0.0576	0.051	29.86
50		0.381	28.69		0.039	39.68
100		0.353	32.86		0.032	51.69
150		0.333	39.81		0.029	69.76
200		0.276	48.75		0.015	81.47

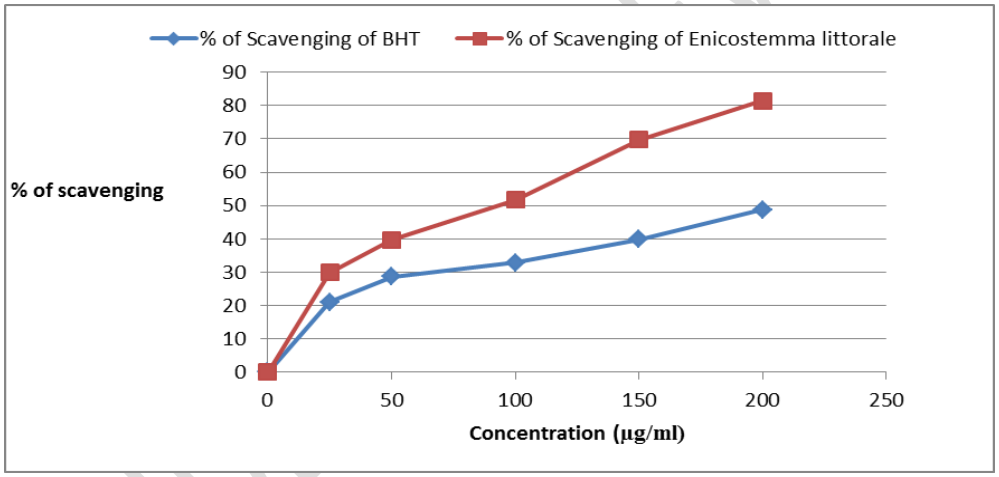

Fig. 1: The regression curve of DPPH Radical Scavenging Activity of *Enicostemma littorale*

3.3.2 Hydrogen Peroxide Scavenging Activity

Table 4 shows the hydroxyl radical scavenging activity of *Enicostemma littorale* and present drugs have shown a dose-dependent activity. The hydroxyl radical is a highly reactive oxygen-centered radical formed by the reaction of various hydroperoxides with transition metal ions. Attacks proteins, DNA, polyunsaturated fatty acids on membranes, and most biological molecules. At the concentration of 150µg/ml, the scavenging activity of *Enicostemma littorale* was 79.46±0.64, whereas at the same concentration ascorbic acid was 35.81±0.84µg/ml [33].

Table 4: Hydrogen Peroxide scavenging activity of *Enicostemma littorale*

Conc. (µg/ml)	Absorbance of Control (Ac)	Absorbance of Test (At)	% of Scavenging	Absorbance of Control (Ac)	Absorbance of Test (At)	% of Scavenging
	Ascorbic acid			*Enicostemma littorale*		
25	0.487	0.428	18.98	0.0456	0.049	31.86
50		0.351	22.69		0.032	42.68
100		0.323	29.86		0.026	54.69
150		0.303	35.81		0.019	79.76
200		0.256	51.75		0.011	98.47

3.4 DPP-IV Enzyme Assay

The extracts of *Enicostemma littorale* were tested in a DPP-IV inhibitory assay by an in vitro method. The inhibitory activity of DPP-IV is shown in **Table 5.** The results obtained in the inhibitory test of DPP-IV showed that the ethyl acetate extract of *Enicostemma littorale* showed greater activity in the inhibition of DPP-IV, approximately 2 times higher than vildagliptin as reference drugs of the DPP-IV inhibitor. [33, 34].

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Comment [K4]: Author failed to discuss the result properly

Table 5: DPP-IV inhibitory activity of *Enicostemma littorale* extracts and vildagliptin

Sample	Conc. (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Enicostemma littorale	25	36.17	165.64
	50	48.94	
	100	55.32	
	150	61.70	
	200	74.47	
vildagliptin	25	8.93	57.44
	50	16.07	
	100	37.50	
	150	46.63	
	200	60.71	

CONCLUSION

To characterize the antioxidant activity of *Enicostemma littorale* extracts, it is desirable to subject it to a battery of tests evaluating the range of activities such as the hydroxyl radical scavenging activity test, the *in-vitro* DPP-enzyme activity test IV, the free radical scavenger DPPH. The *in-vitro* antioxidant activities of the extract have indicated the effectiveness of the formulation as a source of natural antioxidants that will be used to reduce oxidative stress with consequent health benefits. Natural inhibitor such as *Enicostemma littorale* includes fractions such as alkaloids, phenolic acids, flavonoids, steroids, saponins and DPP-IV glycosides. These compounds play an important role in the suppression of oxidative stress due to their antioxidant potential. During the condition of diabetes, the oxidative stress generated to overcome this situation, DPP-IV inhibitors together with antioxidants play an important role in increasing insulin secretion by increasing the half-life of GLP-1 and molecules Antioxidants help to eliminate free radicals then the effect of oxidants on the beta cell will be minimized. *Enicostemma littorale* extract shows

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promising activity based on *in-vitro* DPP-IV inhibitors which, together with their antioxidant nature, can influence the immune system and its function; therefore, a longer duration is required for its safety and efficacy assessments. DPP-IV inhibitors will provide a better solution for the treatment of type 2 diabetes mellitus in our society. Further research is needed for the isolation and characterization of the phyto-constituents responsible for its antioxidant and anti-diabetic properties.

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