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SCREENING OF PHYTOCHEMICALS AND ANTIDIABETIC ACTIVITY OF *Limonia elephantum* L.

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ABSTRACT

The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000. In India the prevalence rate of diabetes estimated to be 1-5% complication are the major cause of morbidity and mortality in diabetic mellitus. There is an increasing demand by the use of animal products of natural products due to the side effects associated with uses of insulin and oral hypoglycemic agents. The present study aimed to evaluate the phytochemical and in vitro anti diabetic activity of *Limonia elephantum* was analyzed. Based on the results of the present study it can be concluded that the methanolic extract of *Limonia elephantum* contain rich source of phytochemical. *Limonia elephantum* has potential antidiabetic activity. Antidiabetic activity may due to active compounds present in the extract. Further pre-clinical studies should be needed to confirm antidiabetic activity and isolation of antidiabetic compound...

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INTRODUCTION

Diabetes is a metabolic disorder of carbohydrate, fat and protein, affecting a large number of population in the world (Pareek *et al*., 2009). Diabetes mellitus is not a single disorder but it is a group of metabolic disorder characterised 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 leads 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.

Hyperglycaemia 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 containing natural antioxidants such as tannins, flavonoids, vitamin C and E 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, 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).

Many indigenous Indian medicinal plants have been found to be successfully used to manage diabetis. Plant drugs are frequently considered to be less toxic and free from side effects then synthetic ones. However, search for new anti-diabetic drugs continue. Keeping this view, the present study aimed to evaluate the phytochemical and *in vitro* anti diabetic activity of *Limonia elephantum* were analyzed.

MATERIALS AND METHODS

Plant materials:

The fully mature *Limonia elephantum* leaf was collected in January 2017 from Valuthur, Thanjavur District, Tamil Nadu, India.

Preparation of alcoholic extract

The leaf of *Limonia elephantum* was first washed well and dust was removed from the leaves. The leaf was dried at room temperature and coarsely powdered. The powder was extracted with methanol, 70% methanol and aqueous for 24 hours. The extract was stored in refrigerator until used.

Phytochemical screening

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

Quantitative analysis of phytochemicals

Determination of total phenols by spectrophotometric method:

Flavonoid determine by the method of Bohm and Kocipai-Abyazan (1994)

Alkaloid determine by the method of Harborne (1973)

Terpenoids were determined by the method of Indumathi *et al*. (2014)

Histochemical tests

The powders of *Limonia elephantum* were treated with specific chemicals and reagents. The treated plant powder further analysed in light microscope. The *Limonia elephantum* treated with phloroglucinol and diluted HCl gave red colour indicates lignin, treated with diluted ammonia and H_2SO_4 gave yellow colour indicates flavonoids and treated with Dragant draft reagent gave brown colour indicates alkaloids.

GC MS Analysis

GC MS analysis was carried out on Shimadzu 2010 plus comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column RTX 5Ms (Column diameter is 0..32mm, column length is 30m, column thickness 0.50µm), operating in electron impact mode at 70eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1.73 ml /min and an injection volume of 0.5 µI was employed (split ratio of 10:1) injector temperature 270 ºC; ion-source temperature 200 ºC. The oven temperature was programmed from 40 ºC (isothermal for 2 min), with an increase of 8 ºC/min, to 150ºC, then 8ºC/min to 250ºC, ending with a 20min isothermal at 280ºC. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time is 51.25min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver 5.2.0 (Srinivasan *et al*., 2013).

Identification of components

Interpretation on GCMS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained (Dr. Dukes, 2013).

***In vitro* antidiabetic activity**

In vitro α-amylase inhibition assay was carried out by the method of Apostolidis (2007). The α-glucosidase inhibitory activity was determined according to the method described by Apostolidis et al., (2007).

RESULTS AND DISCUSSION

Phytochemical simply means plant chemicals. "Phyto" is the Greek word for plant. Phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary metabolism is important for growth and development of plants include the common sugars, aminoacids, proteins, purines and pyrimidines of nucleic acids, chlrophyll's etc. Secondary metabolism in a plant plays a major role in the survival of the plant in its environment. Attractions of pollinators, natural defense system against predators and diseases, etc., are examples of the roles of secondary metabolites. (Sofowara, 1993).

In the present study was carried out on the plant sample revealed the presence of medicinally active constituents. The phytochemical characters of the *Limonia elephantum* investigated and summarized in Table-1 and fig- 2. The phytochemical screening aqueous extract of *Limonia elephantum* showed that the presence of tannin, steroids, saponins, terepenoids, phenolics, glycosides, anthriquinone and protein Flavonoids, triterpenoids while alkaloids, phlobatannins and carbohydrate were absent. Methanol extract of *Limonia elephantum* showed that the presence of alkaloids, steroids, saponins, triterpenoids, phenolics, carbohydrate, anthriquinone and glycosides while flavonoids, tannin, terepenoids, phlobatannins and protein (fig.2,3). Significant amount of falvonoids (60mg/gm), phenol (200mg/gm), alkaloids (60mg/gm) and terpenoids (26mg/gm).

Histochemical studies

Histochemistry is the branch of histology dealing with the identification of chemical components of cells and tissues, it is a powerful tool for localization of trace quantities of substances present in biological tissues (Krishnamurthy, 1998). Histochemical techniques have been employed to characterize structure and development, and to study time course of deposition and distribution of major storage compounds such as proteins, lipids, starch, phytin and minerals like calcium, potassium and iron (Krishnan *et al*., 2001). The importance of histochemistry in solving critical biosystematic problems is as popular as the use of other markers. According to botanical literatures, the use of histochemical characters in taxonomic conclusions is now a common practice. Table 2 and figure 5 represents histochemical studies of *Limonia elephantum* powder. This study further confirmed the presence of phytochemicals in *Limonia elephantum.*

Identification of bioactive compounds in *Limonia elephantum* leaves extract by GC MS analysis

Twenty compounds were identified in *Limonia elephantum* by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in (Table 3, 4 and Fig 6). The prevailing compounds like 1-Undecanol, Cyclotrisiloxane, Di-n-octyl phthalate Di-n-octyl phthalate, Z-4-Dodeceno, Methoxsalen and 1,5-Hexadiene were found in this plant. The presence of various bioactive compounds justifies the use of the plant for various ailments by traditional practitioners. However isolation of individual phytochemical constituents and subjecting its biological activity will definitely give fruitful results.

***In vitro* Antidiabetic activity**

A study of ancient literature indicates that diabetes (Madhumeha/Prameha) 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).

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). These inhibitors have been found useful in the control of diabetes mellitus over many years (Layer et al., 1986 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). In this present study, *in vitro* α-glucosidase inhibitor activity of ethanolic extract of *Limonia elephantum* 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 values show that *Limonia elephantum* has 84.83% and standard 92.84%

The intestinal digestive enzymes 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 *Limonia elephantum* well anti diabetic activity (Table 6). The percentage inhibition at 10, 20, 40, 60, 80 µg/ml concentration of crude plant extracts shown concentration dependent reduction in percentage inhibition. At a concentration of 10µ/ml of *Limonia elephantum* showed a % of inhibition 87.90% for 500 µg/ml extracts and standard showed inhibition of 84.65%.

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 and aqueous extract of *Limonia elephantum demonstrated* significant Alpha amylase inhibition activity as compared to standard drug acarbose.

Based on the results of the present study it can be concluded that the methanolic extract of *Limonia elephantum contain* rich source of phytochemical. *Limonia elephantum* has potential antidiabetic activity. Antidiabetic activity may due to active compounds present in the extract. Further pre-clinical studies should be needed to confirm antidiabetic activity and isolation of antidiabetic compound.

able: 1. Phytochemical screening of *Limonia elephantum*

S.No	Phytochemical analysis	Aqueous	70% Methanol	Methanol	Quantitative analysis (mg/gm)
1	Tannin	-	-	+	---
2	Phlobatannins	-	-	-	---
3	Saponin	+	+	+	---
4	Flavonoids	+	+	+	60
5	Steroids	+	++	-	---
6	Terpenoids	+	+	+	26
7	Tritrpenoids	+	+	+	---
8	Alkaloids	-	+	-	60
9	Carbohydrate	-	-	+	---
10	Protein	+	-	-	---
11	Anthroquinone	+	-	-	---
12	Polyphenol	+	-	+	200
13	Glycoside	+	+	-	---

(+) Presence (-) Absence

Table :2 . Histochemical studies of *Limonia elephantum* powder

S.No.	Secondary metabolites	Observation	Result
1	Lignin	Red/Pink	+
2	Flavonoids	Yellow	+
3	Alkaloids	Reddish Brown	+
4	Tannin	Dark Blue to Black	+
5	Steroids	Violet to Blue (or) Green	+
6	Poly phenol	Blue green/Red	+
7	Terpenoids	Orange	+
8	Saponin	Yellow	+

(+) Presence ; (-) Absence

Table: 3. Identification of bioactive compounds in *Limonia elephantum* leaves xtract by GC- MS analysis

Peak	R.Time	Area %	Height %	Molecular Formula	Name	Molecular Weight
1	9.952	9.33	11.74	$C_{10}H_{12}O$	Benzene, 1-methoxy-4-(2-propenyl	148
2	11.833	1.02	1.77	$C_{10}H_{14}O$	4-Hexenal, 3-ethenyl-5-methyl-2-m	150
3	15.556	2.93	4.52	$C_9H_5F_3O_3$	4-(Trifluoroacetyl)benzoi	218
4	15.644	2.93	4.28	C_9H_{14}	1,5-Hexadiene, 2,5-dimethyl-3-methylene-	122
5	18.236	3.35	6.37	$C_{12}H_{24}O$	Z-4-Dodeceno	184
6	18.724	4.05	7.47	$C_{30}H_{50}O_4$	1,2-Benzenedicarboxylic acid, diundecyl ester	474
7	19.739	1.72	2.88	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, dibut	278
8	20.843	4.86	7.24	$C_{11}H_{24}O$	1-Undecanol	172
9	20.843	16.91	11.46	$C_{12}H_8O_4$	Methoxsalen	216
10	21.033	4.60	6.62	$C_{14}H_{20}N_6O_6$	Leuciny lglycine hydrazine, N-[2,4-dinitrophnyl	368
11	21.092	1.63	4.16	$C_8H_{22}OSSi_2$	C[si](c)(c)occs[si](c)(c)c	222
12	21.226	13.31	8.27	$C_{12}H_8O_4$	5-Methoxy-2H-furo[2,3-h]chromen-	216
13	21.696	3.75	2.95	$C_{14}H_{22}O_4Si$	Benzeneacetic acid, 3-methoxy-4-[(trimethyls	282
14	21.992	2.10	2.54	$C_{16}H_{32}NO_8P$	Propionic acid, 3-[(diethoxymethyl)ethylphosphin	397
15	22.067	5.77	2.01	C_4H_9NS	Thiazolidine, 2-methyl-	103
16	25.067	0.90	1.47	$C_{13}H_{18}F_7NO_3$	N-(Heptafluorobutyryl)norleucine, propyl ester	369
17	27.858	3.61	1.32	$C_{13}H_{24}O_6$	Isobutyl propane-1,3-diyl dicarbonate	276
18	28.183	1.01	1.62	$C_6H_{18}O_3Si_3$	Cyclotrisiloxane, hexamethyl-	222
19	28.245	5.98	3.36	$C_{14}H_{26}O_2Si_2$	Trimethylsilyloxyethane, 2-(3-trimethylsilyloxy	282
20	28.719	10.24	7.96	$C_{24}H_{38}O_4$	Di-n-octyl phthalate Di-n-octyl phthalate Di-n-octyl phthalate	390

Table :4 .*In vitro* α-amylase inhibition of *Limonia elephantum*

Concentrations	Limonia elephantum	Standard Acarbose
	% of inhibition	
100µg/ml	11.70±0.21	22.45 ± 1.57
200µg/ml	25.59±15.22	43.61 ± 2.74
300µg/ml	74.83±0.09	65.74 ±4.67
400µg/ml	79.9±0.31	84.31 ± 5.48
500 µg/ml	84.83±0.03	97.84 ± 6.49

Values expressed as Mean ± SD for triplicates

Table: 5. *In vitro* α-glucosidase inhibition of *Limonia elephantum*

Concentrations	Limonia elephantum	Standard Acarbose
	% of inhibition	
100µg/ml	15.60±4.61	20.35 ± 1.43
200µg/ml	40.46±0.30	49.15 ± 2.41
300µg/ml	62.43±1.81	69.55 ± 4.36
400µg/ml	75.53±0.30	88.25 ± 5.35
500 µg/ml	87.90±0.36	95.65 ± 5.92

Values expressed as Mean ± SD for triplicates

Fig: 4. Histochemical studies of *Limonia elephantum* powder

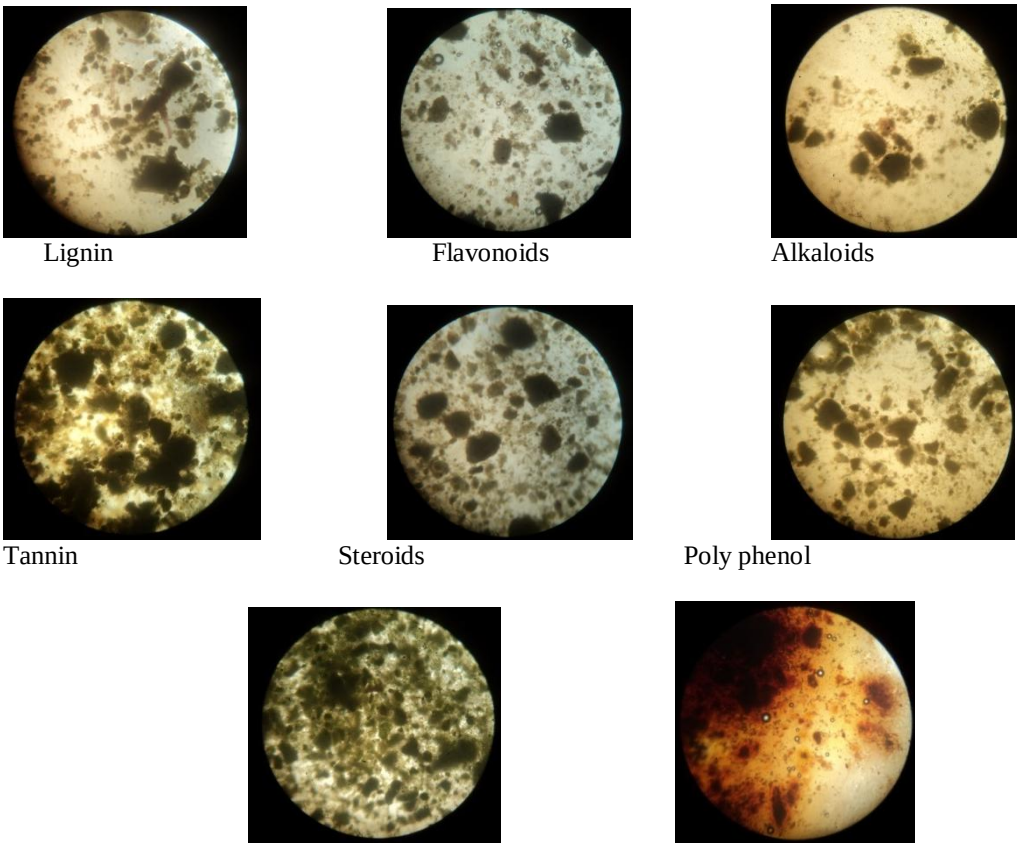
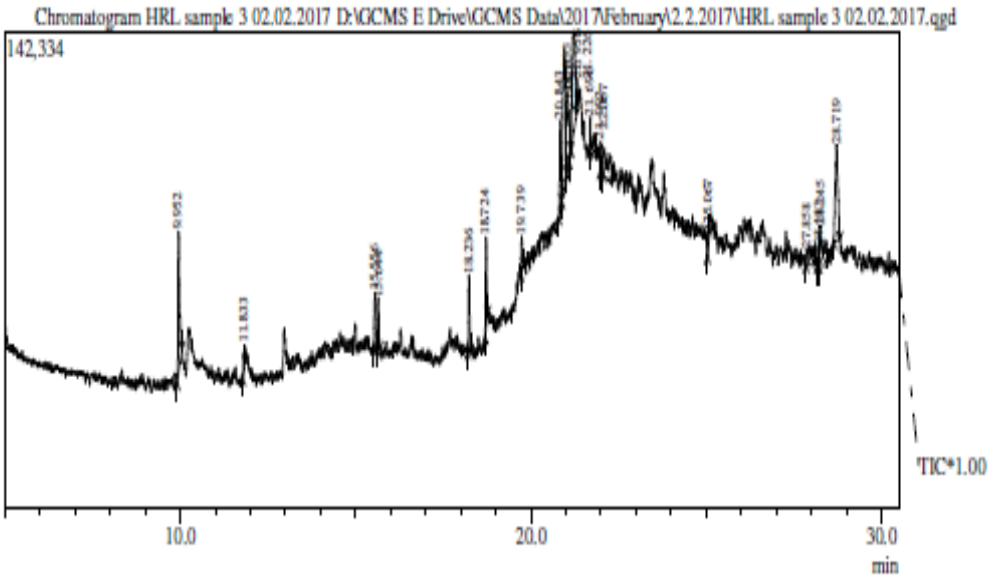


Fig:5. GC- MS analysis of leaves extract of *Limonia elephantum*



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