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Antidiabetic potential of aqueous extract of *Citrus limonia* leaves on experimental animal models

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Abstract
Several plants have been scientifically evaluated to be beneficial for diabetes mellitus management world over. Plants are important for pharmacological research and drug development, not only when bioactive phytocompounds are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. The present study is therefore an expansion towards developing a novel oral antidiabetic medicine of superior quality with negligible or no side effects. This study therefore, examined the glycemic power of *Citrus limonia* L. (Rutaceae) leaves aqueous extract in normal as well as diabetic models and the possible role of its phytocompounds in generating this bioactivity. The variable doses of 100, 200, 300, and 400 mg kg^{-1} body weight (bw) of the extract were administered orally to normal and streptozotocin (STZ) induced sub and mild diabetic rats. The dose of 300 mg kg^{-1} bw was ascertained as the most effective dose capable of reducing the blood glucose level (BGL) by 35.4% (p<0.001) at 6 h and 19.5% (p<0.001) at 3 h during fasting blood glucose (FBG) and glucose tolerance test (GTT) studies in normal rats, respectively. Whereas, the maximum fall observed in case of sub and mild diabetic rats during GTT studies was 24.4, and 33.8% (p<0.001), respectively, at the same interval of time. Moreover, chemical tests were carried out on the leaf extract using standard procedures to identify the main classes of phytoconstituents present viz. terpenoids, flavonoids, alkaloids, tannins, carbohydrates and glycosides, saponins as well as steroids. This verification clearly proves that the aqueous extract of *Citrus limonia* leaves holds potent hypoglycemic effect as well as strong antidiabetic potential which may be due to the presence of certain class of compounds in the extract as evident from preliminary phytochemical screening.

Keywords: Antidiabetic, *Citrus limonia*, glibenclamide, glucose tolerance test, streptozotocin, phytoconstituents

Introduction
Diabetes today is one of the most challenging diseases facing clinicians, researchers, medical and healthcare professionals world over. Its increasing prevalence puts a large burden on society and the public health sector. This disease is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. India leads the world with the largest number of diabetic patients earning the dubious distinction of being termed the "diabetes capital of the world" [1].

Plants have been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. It is reported that about 800 plants may possess antidiabetic potential [2]. A number of Indian medicinal plants have been used for thousand of years in the traditional system of medicine for treating various diseases. The medicinal property of plants has now been investigated scientifically throughout the world, due to their potent pharmacological activities, low toxicity and economic viability[3-6]. In recent years, a number of plants commonly used to treat diabetes in the traditional system of medicine have been explored scientifically by our research group for the investigation of their chemical constituents, elemental analysis, their role in diabetes management and other pharmacological activities [7-10].

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [11]. Many of these indigenous medicinal plants are used as spices and food plants [12, 13].

Citrus limonia (*C. limonia*) (Family: Rutaceae), commonly known as 'Lime' or 'Lemon' in English and 'Nimbu' in Hindi, is native to Asia and cultivated in large numbers throughout India. This plant is of high medicinal value and known to be used from ancient times for therapeutics. Its mildness makes it beneficial for children. *C. limonia* has been reported to be

rich in polyphenols, viz. flavonoids. As dietary compounds, they are known as antioxidants that inhibit the oxidation of low-density lipoproteins and reduce thrombotic tendencies [14]. Lemon fruit acts as a powerful antioxidant and hence slows down the aging process. The fruits are known to be used for urinary infections, kidney stones, bronchitis, constipation and heartburn. Lemon oil is used in sore throat, anxiety, blood pressure and respiratory infections. Its oil also acts as antiinflammatory, antidepressant and antimicrobial. Lemon peels provide relief from joint and nerve pains and are also known for their antiviral activity [15]. Lemon leaves reduce fever, cramps, coughs, asthma and insomnia. They stimulate pancreas and liver function and reduce cholesterol. They are used as a teeth whitener as well. It is rich in vitamins A and C. It also contains biflavonoids and terpenes [15, 16].

Thus, the present investigation was carried out in order to assess the glycemic potential of the aqueous extract of *C. limonia* leaves on BGL of normal and STZ-induced sub- and mild-diabetic rats so that a unique oral antidiabetic remedy could be proposed. Phytochemical screening of the aqueous extract of *C. limonia* leaves was also determined with the help of chemical tests using standard procedures in order to identify the major class of constituents that might be responsible for the glycaemic potential of the aqueous extract and thus, play a pivotal role in management of diabetes.

Material and Methods

Chemicals

Streptozotocin was purchased from Sigma-Aldrich, Seelze, Germany. Blood glucose level (BGL) was assayed using kits, from Bayer Diagnostics, New Delhi, India and a one touch Accu-Chek sensor from Roche Diagnostics, Mannheim, Germany. The solvents were purchased from E. Merck, Darmstadt, Germany.

Plant material and preparation of extract

Fresh leaves of *C. limonia*, about 5 kg, were purchased in April from the local market of Allahabad, U. P. (India), and authenticated by Prof. Satya Narayan, Taxonomist, Department of Botany, University of Allahabad, Prayagraj, India. A voucher specimen has been submitted to the University herbarium. The leaves were first washed well, then shade-dried and mechanically crushed. The crushed leaves were then extracted with distilled water (65°C) using soxhlet upto 72 h. The extract was filtered and concentrated in rotary evaporator at 45-50°C under reduced pressure, to obtain a semisolid material (150 g), which was then lyophilized at -40°C to get a powder (30 g, 20%, w/w). This powder was dissolved in distilled water and used for experimental study.

Experimental animals

More than 100 albino Wistar rats of the same age group and body weight 150-200 g were selected for all the experiments. Animals obtained from the Indian Institute of Toxicology Research (IITR), Lucknow, India were housed in spacious polypropylene cages at an ambient temperature of 25-30°C and 45-55% relative humidity with a 12 h each dark and light cycle. Animals were fed pellet diet (Pashu Aahar Kendra, Varanasi, India) and water *ad libitum*. The study was approved by the Institutional Ethical Committee (83 a/a/04/CPCSEA).

Induction of diabetes

Diabetes was induced to overnight fasted rats by a single intraperitonial injection of freshly prepared streptozotocin (STZ) 50 mg kg^{-1} bw (Sigma-Aldrich Chemicals Pvt. Ltd., New Delhi, India) in 0.1 M citrate buffer (pH = 4.5) [17]. After 3 days of STZ administration, rats with marked hyperglycemia were selected for the study [18]. The rats with hyperglycemia were divided into two groups of 36 rats each:

- sub-diabetic animals with FBG 80-120 $mgdL^{-1}$ PPG >210 $mgdL^{-1}$
- mild-diabetic animals with FBG 150-200 mg $dL^{-1}$and PPG>250 $mgdL^{-1}$

Estimations

BGL was estimated by the glucose oxidase method [19] using standard kit of Bayer Diagnostics India Ltd., New Delhi, India.

Experimental design

Initial screening of the aqueous extract of *C. limonia* leaves for the hypoglycemic activity was done with a range of variable doses in normal healthy rats by conducting FBG and GTT studies. The antidiabetic effect was assessed in sub- as well as mild-diabetic models with the same range of doses based on GTT studies conducted by our research group only [18, 4, 20].

Evaluation of glycemic management in normal healthy rats – FBG Studies

Six groups of six rats each fasted overnight were used in the experiment; group I served as untreated control received vehicle (distilled water only), whereas the animals of groups II, III, IV and V received the aqueous extract suspended in distilled water at doses 100, 200, 300 and 400 mg kg^{-1}, respectively. FBG was checked by collecting the blood samples from the tail vein at 2, 4 and 6 h after administering the extract.

Assessment of hypoglycemic activity in normal healthy rats - GTT Studies

The extract was given orally to different groups of overnight fasted normal healthy animals in the same fashion as above and the FBG was checked at 90 min and treated as 0 h value for GTT. The animals were then orally treated with 2 g kg^{-1} bw of glucose and their glucose tolerance was studied at 1 h interval for another 3 h. Thus, the total period of blood collection was up to 5 h.

Study of antidiabetic activity in sub- and mild-diabetic rats - GTT Studies

The antidiabetic effect of *C. limonia* leaf extract in sub and mild diabetic rats was also assessed by improvement in glucose tolerance. The overnight fasted rats were divided into six groups of six rats each. Group I was control, received vehicle (distilled water only), whereas variable doses of 100, 200, 300 and 400 mg kg^{-1} of the extract were given orally to groups II, III, IV and V, respectively. BGLs were first checked after 90 min of treatment, considered as 0 h value, and then 2 g kg^{-1} glucose were given orally to all the groups. BGLs were further checked up to 3 h at regular intervals of 1 h each, considered as 1, 2, and 3 h values. The results were compared with group VI rats, treated with 2.5 mg kg^{-1} of glibenclamide, a reference drug (considered as positive control).

LD_{50} experiment

Toxic effect of the *C. limonia* leaf extract was also studied by an LD_{50} experiment. Two groups of rats of both sexes (six

animals per group, three females and three males), weighing about 180-200 g, were orally treated with a single dose of 2 and 3 g of the leaf extract. No toxic effect was observed at doses up to 10 and 15 times the effective dose of the extract. The rats were then, observed for their gross behavioral, neurologic, autonomic, and toxic effects at short intervals of time up to 48 h. Food consumption, feces and urine were also examined at 2 h and then at 6 h intervals for 48 h.

Phytochemical screening of *C. limonia* leaves

Chemical tests were carried out on the aqueous extract of *C. limonia* leaves using standard procedures to identify the main classes of phytoconstituents present, as described below:

Test for Terpenoids

Salkowski test: 5 ml of the extract solution in distilled water was mixed in 2 ml of chloroform and then, 3 ml of conc. H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface confirmed the presence of terpenoids [21].

Test for Flavonoids

Small amount of the extract was dissolved in 50 ml of ethanol and filtered. 2 ml of this filtrate was mixed with equal volume of conc. HCl followed by addition of Magnesium ribbon. The appearance of tomato red colour confirmed the presence of flavonoids [22].

Test for Alkaloids

About 500 mg of the extract was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. Appearance of brownish green or a blue-black colouration confirmed the presence of alkaloids [21].

Test for Tannins

Small amount of the extract was stirred with 5 ml of distilled water. It was then filtered, and the filtrate was treated with ferric chloride reagent. The appearance of blue black colour precipitate would confirm the presence of tannins [23].

Test for Carbohydrates and Glycosides

Molisch test: 2 ml of the extract solution in distilled water was placed in a test tube and two drops of the Molisch reagent (a solution of α-napthol in 95% ethanol) was added to it. The solution was then poured slowly into a tube containing two ml of conc. H_2SO_4 so that two layers were formed. The formation of a purple product at the interface of the two layers confirmed the presence of carbohydrate/glycosides [24].

Test for Saponins

About 2 g of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion [21]. Absence of emulsion confirmed the absence of saponins.

Test for Steroids

2 ml of acetic anhydride was added to 0.5 g of extract followed by 2 ml of conc. H_2SO_4. The colour did not change from violet to blue or green indicating the absence of steroids [21].

Statistical Analysis

Data were statistically evaluated using two-way analysis of variance (ANOVA), followed by a *post hoc* % considered to be significant when $p<0.05$.

Results

Effect on FBG in normoglycemic rats

Table 1 shows the effect of the leaf extract on FBG in normoglycemic rats. Hypoglycemic effect of a single oral treatment of variable doses of 100, 200, 300 and 400 mg kg^{-1} of *C. limonia* leaf extract in normal healthy rats was observed showing a regular fall of 10.2, 17.1 and 35.4% from the doses of 100, 200 and 300 mg kg^{-1}, respectively, after 6 h. Whereas, a lesser fall of 31.8% was observed with an increased dose of 400 mg kg^{-1} after the same interval of time.

Table 1: Effect of variable doses of leaf extract on FBG of normoglycemic rats (Mean ± S.D.)

Blood Glucose Levels (mg/dl)					
Experimental Groups	**Treatment (mg kg^{-1} bw)**	**Pre Treatment**	**Post Treatment (h)**		
		FBG	**2**	**4**	**6**
Control	Distilled Water	99.2 ± 3.1	97.6 ± 3.7	96.7 ± 4.5	94.9 ± 4.3
Treated 1	100	97.4 ± 3.4	95.1± 3.6	91.0 ± 4.1	87.5 ± 3.9*
Treated 2	200	95.6 ± 3.2	84.8 ± 4.0	81.5 ± 3.9*	79.3 ± 3.0*
Treated 3	300	96.5 ± 2.9	75.2 ± 4.4	70.6 ± 3.5*	62.3 ± 3.7**
Treated 4	400	94.8 ± 3.3	78.4 ± 3.8	75.4 ± 3.4	64.7 ± 3.4

* $P<0.05$, ** $P<0.01$ as compared with initial values

Effect on FBG in normal rats during GTT

Table 2 demonstrates the effect of the leaf extract on FBG in normal rats during GTT studies. The effect of *C. limonia* leaf extract on GTT of normal healthy rats with different doses of 100, 200, 300 and 400 mg kg^{-1} of the extract were given orally to overnight fasted healthy rats. The FBG was checked after 90 min considered as 0 h value and then 2 g kg^{-1} of glucose was given .The fall was observed up to 3 h after glucose administration at 1 h intervals and the results reveal that, the percentage fall in BGLs was regular up to the dose of 300 mg kg^{-1} and reaches to its maximum of 19.5%. Moreover, a lesser fall of only 18.3% was observed with an increased dose of 400 mg kg^{-1} at the same time interval.

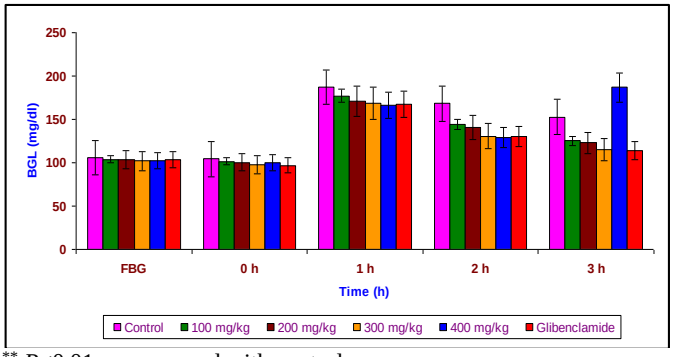
Table 2: Effect of variable doses of aqueous extracts on GTT of normoglycemic rats (Mean ± S.D.)

Blood Glucose Levels (mg/dl)						
Experimental Groups	**Treatment (mg kg^{-1} bw)**	**Pre Treatment**	**Post Treatment (h)**			
		FBG	**0**	**1**	**2**	**3**
Control	Distilled Water	92.9 ± 3.0	93.5 ± 2.5	106.1±3.7	105.8±3.2	106.2±2.4
Treated 1	100	92.7±2.7	92.5±3.0	100.9±3.4	95.1±3.1	93.2±4.3
Treated 2	200	92.2±3.1	91.6±3.6	100.6±4.1	93.0±3.8	91.2±3.8*
Treated 3	300	92.1±3.5	90.2±4.0	95.5±3.7	88.4±2.6**	85.5±3.9***
Treated 4	400	92.2±4.1	91.1±4.2	96.1±4.6	89.6±3.9**	86.8±4.4***

* P<0.05, ** P<0.01, *** P<0.001 as compared with control values

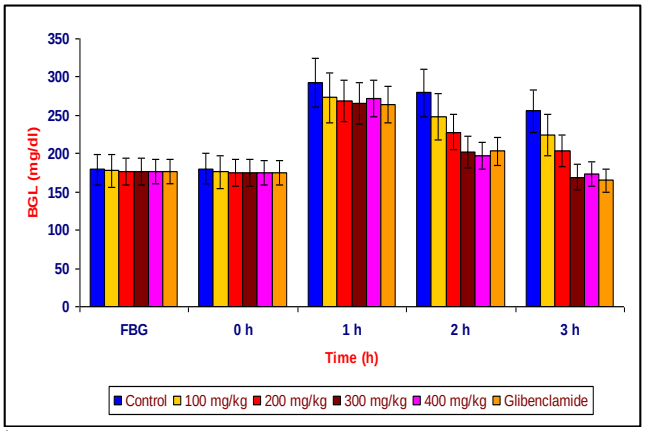
Effect on diabetic rats during GTT

Figures 1 and 2 reveal the antidiabetic effect of the *Citrus limonia* leaf extract on sub- and mild-diabetic animals, respectively. Different doses of the extract as mentioned above and the standard drug, glibenclamide (2.5 mg kg^{-1} bw) were given orally to the groups as defined in the experimental design. A fall of 18.1, 19.6, 24.4, and 22.3% in BGLs of sub-diabetic animals was observed after 3 h of glucose administration with doses of 100, 200, 300 and 400 mg kg^{-1} respectively. However, the dose of 2.5 mg kg^{-1} of glibenclamide reduced BGL by 25.5% at 3 h during GTT in sub-diabetic rats. Moreover, the fall observed after 3 h of glucose administration was 12.2, 20.4, 33.8, and 32.1% in BGLs of mild-diabetic animals with the doses of 100, 200, 300 and 400 mg kg^{-1}, respectively. However, the dose of 2.5 mg kg^{-1} of glibenclamide lowered BGL by 38.1% at 3 h during GTT in mild-diabetic rats.



** P<0.01 as compared with control

Fig 1: Effect of variable doses of leaf extract on GTT of sub-diabetic rats



** P<0.01 as compared with control

Fig 2: Effect of variable doses of leaf extract on GTT of mild-diabetic rats

LD_{50} studies

The experiment was carried out on normal healthy rats and the behavior of the treated rats appeared normal throughout the study. No toxic effect was reported at doses up to 10 and 15 times the most effective dose of the extract and there was no death in any of these groups.

Phytochemical screening for different classes of compounds

Table 3 reveals the phytochemical screening carried out on a small portion of the plant extract by various chemical tests. The presence of terpenoids, flavonoids, alkaloids, tannins and glycosides as medicinally active constituents was confirmed in the aqueous extract of *C. limonia* leaves.

Table 3: Qualitative analysis of the phytochemicals of aqueous extract of *C. limonia* leaves

Phytoconstituents tested	Results observed
Terpenoids	+
Flavonoids	+
Alkaloids	+
Tannins	+
Carbohydrates/ Glycosides	+
Saponins	-
Steroids	-

+ Present; - Absent

Discussion

The results demonstrate that the aqueous extract of leaves of *Citrus limonia* is able to reduce the blood glucose level in normal rats significantly and also cuts down on the high blood glucose levels in sub- and mild-diabetic models. The maximum hypoglycemic effect in case of normal rats was obtained by a fall of 35.4% in BGL at 6 h during FBG studies with the dose of 300 mg kg^{-1}. The effect was dose-dependent up to 300 mg kg^{-1}. However, the response decreases at higher dose of 400 mg kg^{1}, although it showed a substantial reduction in the BGL level. Such an occurrence of less hypoglycemic response at higher doses is common with indigenous plants and has already been scientifically proven by our research group in several plants viz. *Murraya koenigii* [25], *Psidium guajava* [26], *Cynodon dactylon* [5, 27], *Trichosanthes dioica* [18], *Emblica officinalis* [20], *Ficus bengalensis* [28], *Aegle* marmelos [29] and *Raphanus sativus* [4]. The dose of 300 mg kg^{-1} exhibited a noticeable improvement of 19.5, 24.4, and 33.8% in glucose tolerance of normal, sub- and mild-diabetic animals at 3 h during GTT, respectively. Whereas, glibenclamide which was used as a reference drug in diabetic models as positive control, produced fall of 25.5 and 38.1% in sub- and mild-diabetic animals respectively. The results therefore, reveal that the dose of 300 mg kg^{-1} of the extract was almost comparable with the dose of 2.5 mg kg^{-1} of glibenclamide during GTT in diabetic rats. It is therefore, interesting to note that the extract was significantly effective as compared to the reference drug. A comparatively higher concentration of the extract used, against the reference drug, glibenclamide could be possibly due to only a small

amount of active substance present in the extract. It was reported that streptozotocin can cause necrosis of β-cells of the islets of Langerhans, thus causing hypoinsulin and hyperglycemia [30]. The extract may thus increase insulin secretion from the β-cells of the islets of Langerhans in type 2 (Non Insulin Dependent Diabetes Mellitus) diabetic rats because only partly damaged β-cells of the islets of Langerhans remain active [31]. Therefore, the mechanism of hypoglycemic action of the extract may be similar to that of the reference drug, glibenclamide.

These results suggests that the antidiabetic effect of *C. limonia* leaf aqueous extract at least in part, is likely due to improvement in glucose metabolism or enhancement in insulin secretion from the β-cells of islets of Langerhans or by ameliorating insulin sensitivity. Though, the true mechanism of the antidiabetic effect of the extract still needs to be further investigated and scientifically proven. Thus, the multiple beneficial effects of aqueous extract of *C. limonia* leaves cannot be overlooked for managing hyperglycemia caused by streptozotocin.

Phytochemicals which possess many ecological and physiological roles are widely distributed as plant constituents. Woody plants can synthesize and accumulate in their cells a great variety of phytochemicals including alkaloids, flavonoids, tannins, cyanogenic glycosides, phenolic compounds, saponins and lignins [32]. Over 50% of all modern clinical drugs are of natural product origin [33]. Natural products play an important role in drug development programmes in the pharmaceutical industry [34]. There are a few reports on the use of plants in traditional healing by either tribal people or indigenous community [35]. Over 150 plants extracts and some of their active principles including flavonoids are known to be used for the treatment of diabetes [36]. Moreover, tannin-containing drug demonstrated antidiabetic activity [37]. Similarly, several phenolic compounds and flavonoid possess marked antidiabetic activity [38]. Possibly, the insulin-like activity of these bioactive compounds inherent in *C. limonia* is responsible for its hypoglycaemic effects.

C. limonia leaves aqueous extract can thus, be seen as a potential source of antidiabetic drugs. Further studies are in progress in order to isolate, identify, characterize and elucidate the structures of the different classes of phytocompounds found in the extract by preliminary phytochemical investigation.

Conclusion

In summary, the *in vivo* model of diabetes used herein confirm the antidiabetic potential of the aqueous extract of *C. limonia* leaves, with a vision that greater effects may be obtained in severe case of diabetes mellitus. Conclusively, it is suggested that the extract is worth for further study, in order to develop antidiabetic drug that can be used clinically for controlling type 1 and type 2 diabetes. Demand of interdisciplinary research is increasing in the new millennium. The present study therefore, provides scientific evidence that traditional medicinal plants do have the potential to possess significant antidiabetic activity. It also deals with the role of different phytoconstituents that may be responsible for the antidiabetic potential of the extract. Elucidation of the mechanism of action of antidiabetic action of *C. limonia* leaves at molecular level is in progress.

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