**Anti Diabetic Activity of Illupai Ver Chooranam on Streptozotocin Diabetic Rats**

P. Azhagarasi, A. Vijayalakshmi, C. Surya, T. Prabha, N. Kiruthiga, R. Senthil Kumar, P. Selvamani and S. Latha

*Department of Gunapadam, RVS Siddha Medical College and Hospital, Sulur, Coimbatore, Tamil Nadu, India.
2*Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-117, Tamil Nadu, India.
3Department of Biochemistry, Dhanalakshmi Srinivasan College of Arts and Science for Women, Perambalur, Trichy.
4Department of Pharmaceutical Chemistry, Nandha College of Pharmacy, Erode, Tamilnadu, India.
5Department of Pharmaceutical Chemistry, Swamy Vivekananda College of Pharmacy, Tiruchengode, Tamilnadu, India
6Department of Pharmaceutical Technology, Centre for Excellence in Nanobio Translational Research, Anna University, Tiruchirappalli, Tamilnadu, India

**Abstract:** Roots of Illupai (*Madhuca longifolia*) are mentioned for Madhumegam in Gunapadam-Mooligai (Siddha Materia Medica). The disease Madhumegam found in siddha text is correlated with Diabetes mellitus type-II. *Madhuca longifolia* known as Illupai in Tamil has been widely used for the treatment of diabetes. Hence, the present study aimed to demonstrate the antidiabetic effects of Illupai ver chooranam in normal and streptozotocin (STZ) induced diabetic rats. The qualitative phytochemical analysis of the chooranam showed the presence of flavonoids, glycosides, tannins, triterpenoids, polyphenols, carbohydrates, steroids, saponin and proteins. Acute toxicity studies revealed the nontoxic nature of the illupai ver chooran up to a dose level 2000 mg/kg body weight until the end of the study period. The illupai ver chooranam (500 and 1000 mg/kg) significantly reduced ($P**< 0.01$) glucose levels in diabetic rats. The other biochemical parameters like cholesterol, lipid, urea, protein, liver glycogen, serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT), were found to be reduced by the illupai ver chooranam. The histopathological results of treated groups showed the regenerative/protective effect on $\beta$-cells of pancreas in diabetic rats. In the oral glucose tolerance test, the illupai ver chooranam increased the glucose tolerance. The current study revealed the antidiabetic potential of illupai ver chooranam effective in hyperglycemia and that it can effectively protect against other metabolic aberrations caused by diabetes in rats, which seems to validate its therapeutic traditional use.

**Keywords:** Diabetes, Madhumegam, Iluppai ver chooranam, Siddha Medicine, Streptozotocin induced model.
1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis, with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both.1 Diabetes mellitus is represented by hyperglycemia, lipidemia and oxidative stress; it predisposes affected individuals to long term complications affecting the eyes, skin, kidneys, nerves, and blood vessels.2 Diabetes is prevalent in all parts of the world and rapidly increasing worldwide. The estimated number of adults living with diabetes has soared to more than 371 million, having a prevalence of 8.3%. India has the more than 63 million of diabetic persons.3 Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations and harmful effects.4,5 Therefore, managing diabetes without any side effects is still a challenging task for health care providers.6 The scientific paper reported an enormous number of medicinal plants to retain antidiabetic activity and used for the management of diabetes. Hence the medicinal plants/herbs/foods are good sources of alternative or complementary medicines for the management of diabetes. According to WHO, globally about 70-80% of the population preferring traditional medicines which are obtained from the plant material and products for the treatment of hyperglycemia. The use of herbal medicines has increased over the last decade due to low side effects, easy availability, inexpensive and higher therapeutic efficacy.7 Hence, the studies are being conducted for finding more efficient, safer, and less expensive hypoglycemic agents. Herbal medicines have ever been used and claimed as antidiabetic agents but very less are available on commercially formulated forms.8 India has a rich heritage of traditional medicine constituting with its different components like Ayurveda, Siddha and Unani. Traditional and indigenous methods have been employed in order to prevent diabetes mellitus in India since ancient times. Currently available treatment options fail to maintain tight glycemic control over time and are accompanied by various side effects. Therefore, there is a need to develop newer treatment strategies such as hypoglycemic agents of plant origin as they are known to have fewer adverse effects. Many herbs and plant products have been shown to have antihyperglycemic action.9 Madhuca longifolia, (synonym M. indica), belonging to the family Sapotaceae, known as iluppai in Tamil is an important economic tree growing throughout India. Traditionally, Madhuca longifolia bark has been used against diabetes, rheumatism, ulcers, bleeding and tonsillitis.10 The root, bark, leaves and flowers are used for snake bite. Externally, the seed oil massage is very effective to alleviate pain. In skin diseases, the juice of flowers is rubbed for ocleation. It is also beneficial as a nasya (nasal drops) in diseases of the head due to pitta, like sinusitis.11 Antidiabetic activity was reported with bark of this plant but not reported in the root of the plant. Hence, the present study aimed to demonstrate the antidiabetic effects of Illupai ver chooranam in normal and streptozotocin (STZ) induced diabetic rats.

2. MATERIALS AND METHODS

2.1. Procurement of the test Drug

The root of Madhuca longifolia is collected inside the campus of Pandit Jawharhal Nehru College for Agriculture (PAJANCOA), Karaikkal, Puducherry. After that the roots were cut into slices and dried in the sunlight. It was identified by a renowned botanist and confirmed by our professor and Head Department of Gunapadam, Arignar Anna Govt. hospital of Indian medicine, Arumbakkam, Chennai.

2.2. Preparation of Illupai ver Chooranam

The purified dried roots of Madhuca longifolia were finely powdered and was filtered by using a white cloth (Vasthira Kayam)

2.3. Purification of the Chooranam

A mud pot half filled with milk was taken and its mouth was tied with a cloth, in which the chooranam was placed. It was closed with the second mud pot. The gap between the two mud pots was tied with a wet cloth to avoid evaporation. Then this arrangement was put on fire, boiled until the milk in the mud pot boiled up to one fourth of the quantity of milk. Then the powder was taken and dried. Then it was grinded to fine powder and filtered by using white cloth (Vasthira Kayam) and preserved. The chooranam was stored in a clean, dry, tightly stoppered glass container. As the period of chooranam is for three months only, it was used within that period.

2.4. Preliminary Phytochemical Tests

The illupai ver chooranam was subjected to preliminary phytochemical analysis for the identification of various phytochemical constituents present using standard methods.12

2.5. Anti diabetic activity

2.5.1. Experimental Animals

Healthy adult male Wistar albino rats between 2-3 months of age and weighing 250-280 g were used for the study. Housed individually in polypropylene cages, maintained under standard conditions (12-h light and 12-h dark cycle; 25 ± 5°C; 35-60% humidity), the animals were fed with standard rat pellet diet (Hindustan Lever Ltd, Mumbai, India) and provided water ad libitum. Before conducting the experiment all the animals were acclimatized to laboratory condition for 7 days. The animals were housed in separate polypropylene cages inside a well ventilated room and their bedding changed from time to time. As per the guidelines of CPCSEA, all the animals were maintained with due approval from the Institutional Animal Ethics Committee (IAEC). The protocol was approved by the Institutional Animal Ethics Committee (IAEC) (Registration no. 294 CPCSEA dated 06.10.2008).

2.5.2. Preparation of Stock solution

The weighed quantity of siddha drug Illupai Ver Chooranam was suspended in 2% carboxymethyl cellulose and further it was diluted with the same to obtain 100 mg/ml solution.

2.5.3. Acute toxicity study

Acute oral toxicity study was performed for the illupai ver chooranam according to the guidelines of the Organisation for Economic Cooperation and Development (OECD).13 The rats were kept on fasting overnight, being provided only water prior to oral dosing. Then the chooranam was administered orally at different dose levels, 100, 200, 500, 1000, 1500, and 2000 mg/kg of body weight. The rats were observed continuously for 24h for behavioral and any adverse change and thereafter for any lethality.
2.5.4 Induction of diabetes in rats

The animals were fasted overnight prior to the induction of diabetes. Streptozotocin (Product code 1758) of Himedia Laboratories, India was dissolved in an ice cold 0.1 M citrate buffer, pH 4.5 and always prepared freshly for immediate use within 5 min. STZ injections were given i.p. in a single dose of 50 mg/kg body wt. and the doses were determined according to the body weight of animals. In the present study the blood glucose levels were evaluated on day 0 in all the rats prior to administration of streptozotocin. On day 3 i.e. after 72 hours, the blood glucose levels were evaluated and the rats with blood glucose level >250 mg/dl were considered as diabetic and taken up for the study.13

Experimental Design

The albino rats were divided into 5 groups, each consisting of 6 animals. Diabetes was induced by a single i.p. injection of streptozotocin at a dose of 50 mg/kg body weight. Except Group I all the other 4 groups were induced with diabetes. From 4th day onwards standard drug, glibenclamide and illupai ver chooranam was fed to the rats and it was continued till the end of the study. The animals included in the experiment were divided as follows:

Group I: Normal control group: Received normal saline in the dose of 10 ml/kg/day per orally. Group II: Diabetic control group: Received normal saline in the dose of 10 ml/kg/day per orally. Group III: Diabetic standard group: Received glibenclamide at a dose of 5 mg/kg/day per orally. Group IV: Diabetic test: Received illupai ver chooranam 500 mg/kg/day per orally. Group V: Diabetic test: Received illupai ver chooranam 1000 mg/kg/day per orally.

The fasting blood glucose levels were determined on days 0, 10, and 15. During the experimental period, the rats were weighed daily and the mean change in body weight was calculated.

2.5.5 Collection of blood sample and blood glucose determination

Blood samples were drawn from the tail tip of the rat during the study. Fasting blood glucose estimation was done on day 1, 4, 7 and 15 of the study. For the estimation of blood glucose level Accu-Chek Active glucometer (A product of Roche Diagnostics, Germany) was used where the blood glucose levels were expressed in mg/dl. This method has adequate sensitivity with the advantage that a small amount of blood (1-2 µL) can be used for blood glucose analysis. Blood sample was collected by cutting the tail tips with a sharp blade and put on the glucose test strip on the glucometer. After collection of blood, the tail tips were exposed to povidone iodine ointment to counteract the possibility of infection and inflammation.

2.5.6 Estimation of Biochemical parameter

Oral administration with illupai ver chooranam was started 72h after streptozotocin injection in diabetic rats while normal group and diabetic control group were administered only with vehicle. The rats were sacrificed after 15 days and blood was collected retro-orbitally from the eye under light ether anesthesia using capillary tubes with the diameter of 0.5mm (Micro Hematocrit Capillaries, Mucaps) in fresh microcentrifuge tube and plasma separated in an electric centrifuge (REMI, New Delhi) at 2000 rpm for 15 min. Serum glucose, serum cholesterol, serum total lipids, serum protein, serum urea, SGOT (Serum glutamate oxaloacetate transaminase) and SGPT (Serum glutamate pyruvate transaminase) were estimated by commercially available kits (Span Diagnostic Pvt. Ltd. Surat, India).

2.5.7 Histopathological Examination

Pancreas of animals were excised for histopathological investigations using Haematoxylin and Eosin (H&E) stains. Changes in morphology of pancreatic cells were observed under the microscope using 20x and 40x magnifications.16

2.5.8 Oral Glucose Tolerance Test

The oral glucose tolerance test was performed overnight (18-h) fasted normal rats. Rats were divided into four groups (n = 6) and were administered either with drinking water or illupai ver chooranam, 500 and 1000 mg/kg, respectively. Glucose (2 g/kg) was fed 30 min after the administration of the extract. Blood was withdrawn from the retro orbital sinus under ether inhalation anesthesia at 30, 60, and 120 min of glucose administration and glucose levels were estimated using glucose oxidase-peroxidase reactive strips and a glucometer (Accu-Chek, Roche Diagnostics, USA).

3. STATISTICAL ANALYSIS

All results were expressed as the mean ± Standard Error Mean (S.E.M.) Statistical analysis was performed with Graph Pad Instat software (version 3.00, Graph Pad Software, San Diego, California, USA) by using one way analysis of variance (ANOVA) comparison was done by using Dunnett’s test. P value <0.05 was considered as significant.

4. RESULTS

4.1 Preliminary Phytochemical Screening

The qualitative phytochemical analysis of the chooranam showed the presence of flavonoids, glycosides, tannins, triterpenoids, polyphenols, carbohydrates, steroids, saponin and proteins.

4.2 Effect of Illupai ver chooranam on acute toxicity study

Acute toxicity studies revealed the nontoxic nature of the illupai ver chooranam. There were no lethality or toxic reactions found up to a dose level 2000 mg/kg body weight until the end of the study period. All the animals were alive, healthy, and active during the observation period.

4.3 Effect of Illupai ver chooranam on fasting blood glucose level

The effect of Illupai Ver Chooranam (500 mg/kg, 1000 mg/kg) and glibenclamide (5 mg/kg) on serum glucose levels in normal, diabetic, and chooranam treated rats is presented in Table 1.
The percent reduction of hyperglycemia was significant (P**< 0.01) on the 1st day of the treatment by Illupai Ver Chooranam (1000 mg/kg) and of Glibenclamide (5 mg/kg) treatment. The percentage reduction of hyperglycemia was more significant (P**< 0.01) on 4th, 7th and 15th day after treatment of the 500 mg/kg of Illupai Ver Chooranam which was 33.3%, 43%, 65% respectively. The percent reduction of hyperglycemia was more significant (P**< 0.01) on 4th, 7th and 15th day after treatment with the 1000 mg/kg of Illupai Ver Chooranam which was 45.9%, 65.9%, 80.3% respectively and with glibenclamide 5 mg/kg, it was 48%, 65%, 81% respectively over the control.

### 4.4 Effect on serum urea, protein, cholesterol, total lipids, SGOT and SGPT, body weight and liver glycogen

At the end of 15 days of treatment the body weight of the untreated diabetic rats was found to be significantly decreased and significant increase in the liver glycogen compared with the control rats. The administration of Illupai Ver Chooranam and Glibenclamide to diabetic rats restored the changes in the body weight, liver glycogen to near normal levels. The normal function of the kidney was assessed as blood urea level in normal, diabetic and treated animals. The results of serum urea level indicates that the animals administered with illupai ver chooranam (500 and 1000 mg/kg) in diabetic rats reduced the urea levels dose dependently (P**<0.01). The percentage reduction of blood urea level was 29.7% and 52.7% with the treatment of illupai ver chooranam at the dose of 500 and 1000 mg/kg respectively and Glibenclamide 5 mg/kg was reduced 48%, 65%, 81% respectively over the control.

### Table 1: Effect of Illupai Ver Chooranam on Serum glucose on Streptozotocin Induced Model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum glucose level mg/dl</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>60.40 ± 4.20</td>
<td>60.20 ± 3.20**</td>
</tr>
<tr>
<td>Control</td>
<td>260.20 ± 4.40</td>
<td>266.00 ± 3.80</td>
</tr>
<tr>
<td>Standard Glibenclamide 5 mg/kg</td>
<td>254.40 ± 2.40</td>
<td>150.20 ± 4.40**</td>
</tr>
<tr>
<td>Illupai Ver Chooranam 500 mg/kg</td>
<td>261.83 ± 2.30</td>
<td>252.50 ± 2.09*</td>
</tr>
<tr>
<td>Illupai Ver Chooranam 1000 mg/kg</td>
<td>254.16± 1.18</td>
<td>230.83±1.32**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6). One Way ANOVA followed by Dunnett’s multiple comparison tests is done. The values were considered significant at *p<0.05; **p<0.01

### Table 2: Effect of Illupai Ver Chooranam on biochemical parameter in Streptozotocin induced diabetes

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Body Weight</th>
<th>Final Body Weight</th>
<th>Urea mg/dl</th>
<th>Protein mg/dl</th>
<th>Cholesterol mg/dl</th>
<th>Total Lipid mg/dl</th>
<th>Liver Glycogen mg/dl</th>
<th>SGOT mg/dl</th>
<th>SGPT mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>207.8±5</td>
<td>229.6±1.9</td>
<td>31.83</td>
<td>5.84</td>
<td>72.91</td>
<td>177.27</td>
<td>38.6</td>
<td>51.65</td>
<td>39.23</td>
</tr>
<tr>
<td>Diabetes</td>
<td>227.8±9</td>
<td>216.75±0.6</td>
<td>131.51</td>
<td>4.23</td>
<td>230.58</td>
<td>483.42</td>
<td>12.99</td>
<td>109.31</td>
<td>106</td>
</tr>
<tr>
<td>Glibenclamide 5 mg/kg</td>
<td>211.8±1</td>
<td>203.6±2.1</td>
<td>41.68</td>
<td>5.65</td>
<td>78.80</td>
<td>176.84</td>
<td>35.03</td>
<td>53.63</td>
<td>44.96</td>
</tr>
<tr>
<td>Illupai ver choorannam 500 mg/kg</td>
<td>220.33±2.3</td>
<td>223.83±3.1</td>
<td>92.33</td>
<td>4.5</td>
<td>80.33</td>
<td>175.5</td>
<td>29.83</td>
<td>69.33</td>
<td>51</td>
</tr>
<tr>
<td>Illupai ver choorannam 1000 mg/kg</td>
<td>±2.06</td>
<td>±4.30</td>
<td>±2.91</td>
<td>±0.42</td>
<td>±1.30**</td>
<td>±2.14**</td>
<td>±2.02**</td>
<td>±1.95*</td>
<td>±2.65**</td>
</tr>
</tbody>
</table>
4.5 Histopathological Observations

Histopathological evaluation of pancreas of normal rats showed normal islets cell morphology and architecture. Pancreas of rats in the diabetic group showed necrotic islets cells with dilated acini and shrinkage. Morphology of pancreas in Glibenclamide-treated rats showed increase in islets size and proliferating beta cells and normal pancreatic architecture. Morphology of pancreas in rats treated with 500 mg/kg of illupai ver chooranam showed improved islets structure with minimal necrosis and relatively proliferating cells while at 1000 mg/kg of illupai ver chooranam treated rats, large patches of highly proliferating beta cells with normal pancreatic architecture was observed (Figure 1).

![Histopathological Observations](image)

(A) Control  (B) Diabetic control  (C) Standard  (D) Illupai ver chooranam (500 mg/kg)  (E) Illupai ver chooranam (1000 mg/kg)

4.6 Effect of Illupai ver chooranam on oral glucose tolerance test

The effect of illupai ver chooranam (500mg/kg, 1000 mg/kg) on glucose tolerance test is shown in Table 3. The supplementation of illupai ver chooranam improved the glucose tolerance in the fasted normal rats. Illupai ver chooranam at 500 mg/kg lowered the serum glucose level significantly ($P<0.05$) at 30, 60 and 120 mins. Illupai ver chooranam at 1000 mg/kg showed a significant reduction ($P**<0.01$) in plasma glucose levels from 30 min onwards (Table 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time (min)</th>
<th>Initial</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>67.20 ± 2.20</td>
<td>68.40 ± 3.40</td>
<td>67.20 ± 2.60</td>
<td>67.20 ± 2.40</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>65.40 ± 2.40</td>
<td>120.40 ± 2.40</td>
<td>102.40 ± 3.60</td>
<td>98.20 ± 3.40</td>
</tr>
<tr>
<td>Illupai Ver Chooranam 500 mg/kg</td>
<td></td>
<td>66.16 ± 0.87</td>
<td>104.83 ± 1.62*</td>
<td>71.33 ± 0.49*</td>
<td>68.00 ± 0.57*</td>
</tr>
<tr>
<td>Illupai Ver Chooranam 1000 mg/kg</td>
<td></td>
<td>71.16 ± 1.42</td>
<td>59.16 ± 0.79**</td>
<td>56.50 ± 0.42**</td>
<td>59.00 ± 0.73**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6). One Way ANOVA followed by Dunnett's multiple comparison tests is done. The values were considered significant at *p<0.05; **p<0.01.
5. DISCUSSION

Diabetes mellitus (DM) is an endocrine disorder responsible for approximately 3 million deaths worldwide. It is caused due to an abnormality in metabolic processes either due to reduced insulin production, suppressed insulin actions or both happening at the same time. Treatment of diabetes with conventional drugs is very expensive and chances of side effects are high. Phytochemicals obtained from medicinal plants offer a promising alternative for the development of new therapeutic agents against DM. The STZ induced diabetic rat is one of the animal models of human diabetes mellitus. It is well known for its selective pancreatic islet beta cell cytotoxicity and has been extensively used to induce type 1 diabetes in experimental rat models. Glibenclamide is often used as a standard antidiabetic drug in STZ induced diabetes to compare the efficacy of variety of hypoglycemic drugs. Diabetes arises from irreversible destruction of pancreatic β cells, causing reduction of insulin secretion. The observed increased blood glucose level in the study is in agreement with reports by several workers that STZ induced diabetes mellitus leads to increased blood glucose. It has been reported that STZ at lower doses produce partial destruction of pancreatic β cells with permanent diabetes conditions and there is possibility of many surviving β cells. Since a low dose of STZ (50 mg/kg body wt. i.p.) was chosen for this study there might have been many surviving β cells, capable of undergoing regeneration. Glibenclamide, a standard hypoglycemic agent was taken for comparison of the glucose lowering effectiveness of the Illupai ver chooranam. For the estimation of blood glucose level Accu-Chek active glucometer (A product of Roche Diagnostics, Germany) was used. This method has adequate sensitivity with the advantage that a small amount of blood (1-2 µL) can be used for blood glucose analysis. The present work has detected the antidiabetic effect of the illupai ver chooranam in streptozotocin induced type-II diabetic rats. Streptozotocin injection caused diabetes mellitus, probably due to destruction of the β-cells of the islets of Langerhans of the pancreas. Over-production of glucose and decreased utilization by the tissues form the fundamental basis of hyperglycemia in diabetes mellitus. It has been proposed that sulphonylurea produce their hypoglycemic effect primarily through increased release of insulin in pancreatic β cells. Thus any plant secondary metabolite or chemical constituent which is capable of affecting the insulin secretion from pancreatic β cells will be a good mimicker of sulphonylureas. The illupai ver chooranam was able to significantly lower the blood glucose level. Therefore, the chooranam might have been able to potentiate the release of insulin from pancreatic islets similar to that of results observed after Glibenclamide administration. Also there is a possibility that there might be the presence of some constituents with insulin like action which directly lowered the blood glucose level independent of insulin secretion. Induction of diabetes with STZ is associated with a characteristic loss of body weight, which is due to increased muscle wasting and loss of tissue proteins. Diabetic rats treated with the illupai ver chooranam showed an increase in body weight as compared to the diabetic control, which may be due to its effect in controlling muscle wasting, i.e., by reversal of antagonizing. Diabetic rats treated with illupai ver chooranam significantly decreased the serum cholesterol and lipid level since insulin is a major hormone regulating lipid metabolism. Illupai ver chooranam facilitated stimulation of insulin secretion in STZ induced rats will help to overcome lipid metabolism abnormalities and increase of glucose uptake in the presence of insulin suggests the possibility of increased binding of insulin to receptors in STZ induced rats. Histopathological observation of the pancreas reveals that the pancreatic islets of the disease animal were degenerated, with their cell size being shrunken and reduced islets of pancreas was observed. In non diabetic control group animals, the cells appeared normal and being proliferated. Pancreas of rats treated with Illupai Ver Chooranam showed reversal of damages caused by streptozotocin induced hyperglycemia and resulted in normal pancreatic architecture. When illupai ver chooranam was administered to glucose-loaded normal rats, hypoglycemia was observed after 30 min, with the maximum effect being seen at 2 h. Our investigations also indicate the efficacy of the illupai ver chooranam in the maintenance of blood glucose levels in normal and streptozotocin induced diabetic rats.

5. CONCLUSION

The findings of the experimental animal study indicate that 14 days daily treatment with test drug illupai ver chooranam possesses antidiabetic property and thus lead pharmacological support to the traditional Siddha medical use of illupai ver chooranam for the management of Mathumegam. The antidiabetic activity of illupai ver chooranam may be attributed to the active ingredients present in the drug. However, additional pharmacological, toxicological and histopathological studies are necessary to determine the probable mechanisms of action of Illupai ver chooranam.

6. AUTHOR CONTRIBUTION STATEMENT

Dr. P. Azhagarasi designed and performed the experiments. Dr. A. Vijayalakshmi contributed to the design and writing of the manuscript. Dr. C. Surya and T. Prabha contributed to biochemical parameters. All other authors contributed to the study conception, design and necessary inputs were given towards the designing of the manuscript. All authors read and approved the final manuscript.

7. ACKNOWLEDGEMENTS

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8. CONFLICT OF INTEREST

Conflict of interest declared none

9. REFERENCES


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