ABSTRACT

Objective: (i) To study the efficacy of Cassia kleinii leaf (ethanol extract) in streptozotocin diabetic rats. (ii) To isolate the active fraction from the ethanol extract. (iii) To evaluate acute and short-term general toxicity in male mice.

Methods: (i) The alcohol extract of the plant leaf was tested for its efficacy in streptozotocin-induced diabetic rats. (ii) The extract was evaluated for its acute and short term general toxicity in male mice. (iii) The aqueous suspension of ethanol extract was successively extracted with petroleum ether, chloroform, ethyl acetate and butanol. Each fraction was tested for antihyperglycemic activity using glucose tolerance test in rats. The active chloroform and ethyl acetate fractions were subjected to qualitative chemical analysis.

Results: In streptozotocin-diabetic rats the alcohol extract (200 mg/kg) showed significant antidiabetic property as judged from body weight, serum glucose, lipids, cholesterol and urea, and liver glycogen levels. However, the extract did not significantly influence the levels of serum insulin in both diabetic and normoglycemic rats. The alcohol extract was devoid of any conspicuous acute and short term general toxicity in mice. The antihyperglycemic activity was found predominantly in the chloroform fraction of alcohol extract, which contained terpenoids, coumarins and saponins.

Conclusion: The active fraction of Cassia kleinii leaf extract is very promising to develop standardized phytomedicine for diabetes mellitus.

KEY WORDS Antihyperglycemic activity C. kleinii diabetes mellitus

INTRODUCTION

Screening plants used for the treatment of diabetes mellitus in folk medicine in remote villages of Trivandrum and Kanyakumari Districts, India, revealed the antihyperglycemic property of Cassia kleinii leaves. This is a diffuse under-shrub found in partly shaded and moist places. The alcohol extract of the plant exhibited concentration dependent antihyperglycemic effect in glucose loaded rats. But, the extract did not show hypoglycemic effect in fasted normal rats. In alloxan-induced diabetic rats the extract (200 mg/kg) showed remarkable efficacy. The efficacy of this herbal extract was found to be almost equal to that of insulin (5 U/kg) in alloxan-induced diabetic model in lowering blood glucose levels. In view of these interesting observations there is an urgent need to carry on follow up studies leading to drug development. Alloxan diabetic model resembles type I diabetes (insulin dependent diabetes mellitus) without significant insulin resistance whereas streptozotocin induced diabetic animals exhibit reduced response to insulin in hepatic and peripheral tissues. Further, rats treated with streptozotocin display many of the features seen in human subjects with un-controlled diabetes mellitus. Therefore, in the present study the efficacy of C. kleinii leaf (alcohol extract) was evaluated in
streptozotocin diabetic rats and it was fractionated and analyzed chemically. Acute and short-term general toxicity evaluation of the extract was also carried out in male mice.

MATERIALS AND METHODS

Plant materials and preparation of alcoholic extracts: Cassia kleinii was collected in the month of June and July from Kanyakumari District, Tamil Nadu. A voucher specimen was deposited in the herbarium of TBGRI (No. 47600).

The plant leaves were dried in the laboratory at room temperature and powdered in a mixer grinder. To prepare the extract, 25 g of the plant leaf powder in 250 ml of ethyl alcohol was stirred magnetically for 4 h at room temperature. The residue was removed by filtration and it was once again extracted as above and filtered. The combined filtrate was evaporated to dryness at 40-50°C under reduced pressure in a rotary evaporator. (The yield of ethyl alcohol extract was approximately 10%).

The extract was suspended in 5% Tween –80 and used for oral administration.

Animals: Male Wistar rats weighing 150-200 g and male Swiss albino mice (25-30 g) were used with the approval of the Institute Animal Ethics Committee. Animals were fed a standard pellet (Lipton India, Ltd) and water ad-libitum and maintained at 24-28°C temperature, 60-70% relative humidity, and 12 h day and night cycle. Animals described as fasted were deprived of food for 16 h but had free access to water.

Streptozotocin-induced diabetic rats: Diabetes was induced in rats by tail vein injection of streptozotocin (50 mg/kg, i.v.) dissolved in normal saline. (One group of 6 identical rats was kept without streptozotocin administration as normal control, group I). Forty eight hours after streptozotocin administration blood samples were drawn by retro-orbital puncture and glucose levels determined to confirm diabetes. The diabetic rats exhibiting blood glucose levels in the range of 275 and 300 mg/100 ml were selected for the studies. These diabetic rats were sub-divided into 3 groups as follows: Group II, (untreated rats) given 0.5 ml of 5% Tween 80; Group III, diabetic rats given (200 mg/kg) Cassia kleinii leaf (alcohol extract) in 0.5 ml 5% Tween 80; Group IV, diabetic rats given 0.5 ml of 5% Tween 80 containing glibenclamide (500 µg/kg). The dose (500 µg/kg) of glibenclamide was selected based on previous reports. The normal control group of rats (group I) were given 0.5 ml of 5% Tween 80. Each group consisted of 6 animals.

The treatments were continued daily for 15 days. (In the untreated control (diabetic) rats 3 animals died on the 8th day and one on the 9th day). Blood was collected by retro-orbital puncture for glucose estimation just before drug administration on the 1st day and 1 h after drug administration on days 4, 7 and 10 as described earlier. The animals were sacrificed after blood collection, under chloroform anesthesia on the 15th day and liver was removed for glycogen estimation.

Determination of serum insulin: To determine the effect of C. kleinii extract on serum insulin levels, 3 groups of animals were used. They were over-night normal fasted rats (group I), normal fed rats (group II) and streptozotocin –induced diabetic rats (group III). Immediately after the collection of blood samples the extract (200 mg/kg; 1 ml/rat) was administered (p.o.). One hr after the extract administration, blood samples were again, collected. Serum was separated from the samples and insulin and glucose levels were determined. Insulin levels were measured by radio-immuno-assay method using kits obtained from Board of Radiation and Isotope Technology, Mumbai, India.

Isolation of an active fraction: The alcohol extract was subjected to glucose tolerance test guided fractionation. Briefly, the alcohol extract was suspended in water and successively extracted with petroleum ether, chloroform, ethyl acetate and butanol. Each fraction was dried free of solvent and the yield was determined. The dose selected for glucose tolerance test in each case was based on the yield from the alcohol extract. Since 400 mg/kg body weight is double the optimum dose the yield from 400 mg alcohol extract in the case of each fraction was administered per kg, body weight.

Analysis of the active fraction: Since the activity was found in the chloroform fraction and, to some extent in ethyl acetate fraction, these two fractions were tested for the presence of alkaloids, coumarins, flavonoids, steroids, saponins and terpenoids following standard methods.
**Glucose tolerance test:** This was done as described recently. Briefly, overnight fasted rats were divided into 5 groups. One group was kept as control which received 5% Tween 80 (0.5 ml, p.o.), group 2 received petroleum ether fraction (160 mg/kg) in 0.5% Tween 80 (0.5 ml, p.o.), groups III, IV and V received chloroform fraction (25 mg/kg), ethyl acetate fraction (75 mg/kg) and butanol fraction (100 mg kg) respectively in 5% Tween 80 (0.5 ml, p.o.). The rats of all the groups were loaded with glucose (3 g/kg, p.o.) 30 min after the administration of the drugs (fractions). Blood samples were collected at 30, 90 and 150 min after glucose loading. Serum was separated and glucose levels were measured immediately. Six rats were used in each group.

**Estimation of biochemical parameters:** Serum glucose was estimated spectrophotometrically using a commercial assay kit (Monozyme, India, Ltd.), 10 µl serum was used for each assay. Liver glycogen was estimated by the method of Carroll et al. Serum cholesterol and urea levels were determined following standard methods. Total lipid in the serum was estimated by phosphovanillin method using reagent kit (Span Diagnostic Ltd.). Protein was estimated using Folin's phenol reagent by the method of Lowry et al.

**Toxicity evaluation in mice:** The alcohol extract was tested for its acute and short-term toxicity (if any) in mice. To determine acute toxicity of a single oral administration of the herbal drug, different doses of the drug (0.5, 1.0, 1.5 and 2 g/kg) were administrated to different groups of mice (2 mice were used for each group, control mice received Tween 80). Mortality and general behavior of the animals were observed periodically for 48 h. The animals were observed continuously for the initial 4 h and intermittently for the next six h and then again at 24 h and 48 h following drug administration. The parameters observed were grooming, hyperactivity, sedation, loss of righting reflex, respiratory rate and convulsion.

To study short-term toxicity, 3 groups of mice each containing 6 male mice (20-25 g, body weight) were used. Group I was kept as control and group II and III received 200 and 400 mg/kg alcohol extract respectively in 5% Tween 80. The drug was administrated daily for 14 days (p.o.). Control group received 5% Tween 80 in an identical manner.

The behaviour of the animals was observed daily for 1h in the forenoon (10 to 11 A.M. ) for 14 days. Initial and final body weights, water and food intake, state of stool and body temperature were observed. The animals were killed on the 15th day. Hematological and serum biochemical parameters such as hemoglobin content, WBC and peritoneal macrophage count, serum protein, glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), urea, cholesterol and alkaline phosphatase were determined. Liver, spleen, kidneys and brain were dissected out and observed for pathological and morphological changes. Weights of these organs were determined.

Hemoglobin was measured using hemoglobinometer with comparison standards. GPT and GOT were measured by the method of Reitman and Frankel and alkaline phosphatase by the determination of hydrolyzed phenol with antipyrine. The peritoneal macrophages and total leucocytes were counted as described elsewhere.

**Statistical evaluation:** Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Dunnett's test, except for the data in Table 4 which were evaluated using Student's 't' test (400 mg/kg drug treated group was compared with control). A P value <0.05 was considered significant.

**RESULTS**

**Effect of the herbal extract on streptozotocin-induced diabetic rats:** The alcoholic extract exhibited antidiabetes property in streptozotocin-induced diabetic rats as evident from serum glucose and hepatic glycogen levels. The effect of the extract on body weight and liver glycogen content in the streptozotocin-induced diabetic rats is given in Table 1. In the untreated control (diabetic) group out of the 6 animals 3 died of diabetes on the 8th day and one died on the 9th day. The body weight was slightly increased in the normal control rats compared to initial body weight whereas in the diabetic control rats there was a significant decrease in the body weight (of the 2 animals which survived up to the 15th day). Glibenclamide (500 µg/kg) as well as the herbal extract (200 mg/kg) treatment significantly prevented this reduction in body weight. Although there is a
Table 1. Effect of *Cassia kleinii* leaf (ethanol extract) on body weight, liver glycogen and serum glucose, urea, total lipid, cholesterol and protein in streptozotocin-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bodyweight (g)</th>
<th>Liver glycogen (mg/g wet tissue)</th>
<th>Serum glucose (mg/dl)</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (1st day)</td>
<td>Final (15th day)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Normal control</td>
<td>208.4 ± 5.6</td>
<td>216.2 ± 3.0</td>
<td>50.0 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Diabetic control (5% Tween 80, p.o.)</td>
<td>210.3 ± 4.8</td>
<td>187.0 ± 5.7</td>
<td>7.3 ± 1.0</td>
<td>298.0 ± 6.3</td>
</tr>
<tr>
<td>Diabetic + <em>C. kleinii</em> (200 mg/kg, p.o.)</td>
<td>209.6 ± 4.6</td>
<td>204.5 ± 4.8</td>
<td>44.0 ± 2.8*</td>
<td>285.0 ± 6.3</td>
</tr>
<tr>
<td>Diabetic+ glibenclamide (500 µg/kg, p.o.)</td>
<td>211.0 ± 6.4</td>
<td>202.6 ± 5.5</td>
<td>41.6 ± 3.6*</td>
<td>286.0 ± 5.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>F</th>
<th>22</th>
<th>104.5</th>
<th>1048</th>
<th>1413</th>
<th>583</th>
<th>833</th>
<th>8.3</th>
<th>102.4</th>
<th>27.2</th>
<th>1.03</th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>3.16</td>
<td>3.16</td>
<td>3.20</td>
<td>3.20</td>
<td>3.16</td>
<td>3.16</td>
<td>3.16</td>
<td>3.16</td>
<td>3.16</td>
<td>3.16</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=6 in each group except in diabetic control group where n=2 on the 10th and 15th day (3 animals died on the 8th day). *P<0.01 (compared to diabetic control or normal control). **P<0.001 (compared to diabetic control or normal control). *P<0.01 (compared to normila control) and **P<0.001 (compared to diabetic control). NS—not significant.

Values in parentheses represent % change (loss or gain) in body weight compared to initial body weight. Herbal drug or glibenclamide was administered daily for 14 days and the animals were sacrificed on the 15th day. For glucose estimation blood was collected just before the drug administration on the 1st day and 1 h after the drug administration on the 4th 7th and 10th day.

A marginal reduction in the weight of animals in these groups, compared to initial body weights, it fell short of statistical significance. However, the reduction in body weight was significant when compared to the final weight of normal control rats (Table 1).

There was a marked reduction in liver glycogen level (in 15 days) in streptozotocin diabetic animals. The herbal extract treatment remarkably attenuated this reduction in glycogen content. This effect of the herbal drug (200 mg/kg) is equal to, if not better than, that of glibenclamide (500 µg/kg).

The effect of the herbal drug on serum glucose levels in streptozotocin induced diabetic rats is shown in Table 1. The initial blood glucose levels of the diabetic rats selected for the study were in the range of 280 to 300 mg/100 ml. In the untreated control (diabetic) rats the blood glucose levels increased to 410 mg/100 ml on the seventh day. Thereafter, 4 animals died in this group, and the glucose levels on the 10th and 15th day in the two animals, which survived, were 360 and 362 mg/100 ml respectively. In the herbal drug treated rats, the blood glucose levels steadily decreased and it was 118 mg/100 ml on the 15th day. Thus the drug treatment restored the serum glucose levels almost nearer to normal values. The effect of the herbal drug is comparable to that of glibenclamide (a standard drug).
Table 2. Effect of *Cassia kleinii* leaf (alcohol extract) on serum insulin levels in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum insulin (µ IU/ml)</th>
<th>Before extract administration (0 h)</th>
<th>After extract administration (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal fasted rats</td>
<td>12.5±2.4 (56±3)</td>
<td>15.0±3.2 (58±4.2)</td>
<td></td>
</tr>
<tr>
<td>Normal fed rats</td>
<td>24.2±3.8 (101±3.9)</td>
<td>28.0±3.4 (98.5±5.1)</td>
<td></td>
</tr>
<tr>
<td>Streptozotocin diabetic rats</td>
<td>9.6±3.1 (302±8.0)</td>
<td>10.7±2.8 (220±9.8)</td>
<td></td>
</tr>
</tbody>
</table>

Insulin values at one hr after drug administration are not significantly different compared to respective 0 h (before drug treatment) values. N=6 in each group. Serum glucose values (mg/dl) are given in brackets.

As shown in Table 1 in the untreated diabetic rats serum levels of total lipids, cholesterol and urea were significantly increased. These complications of diabetes were attenuated with the administration of the alcohol extract. The effects of the standard drug (glibenclamide) on serum urea, total lipid and cholesterol in the diabetic rat were comparable to those of the herbal extract.

**Effect of the extract on serum insulin levels:** The serum insulin levels at 0 and 1 h after the extract administration is shown in Table 2. The extract (200 mg/kg) did not significantly influence serum insulin levels in fasted normal rats, fed normal rats and streptozotocin diabetic rats.

**Isolation of an active fraction:** Successive extraction of the aqueous suspension of alcohol extract with solvents resulted in petroleum ether, chloroform, ethyl acetate and butanol fractions. The yields of different fractions as a % of alcohol extract were as follows: Petroleum ether, 39; chloroform, 6; ethyl acetate, 15; butanol, 24; water (remaining), 14 (values are averages of 3 separate determinations). As shown in Table 3, when different fractions were tested for their antihyperglycemic activity using glucose tolerance test in normoglycemic rats, the chloroform fraction was found active at 25 mg/kg level. Ethyl acetate fraction also showed activity to a lesser extent at 75 mg/kg level. The petroleum ether and butanol fractions were inactive. Qualitative tests for the presence of various classes of compounds showed the presence of coumarins, terpenoids and saponins in the chloroform fraction. This fraction was devoid of alkaloids, steroids and flavonoids. Coumarins, terpenoids and flavonoids (but, not saponins) were detected in the ethyl acetate fraction. Thus both chloroform and ethyl acetate fractions contained terpenoids and coumarins.

**Toxicity evaluation:** In the acute toxicity study, the alcohol extract of *C. kleinii* did not show any mortality up to a dose of 2 g/kg body weight in male mice. Even at this high dose there was no gross behavioural changes.
Daily feeding for 14 days with the alcohol extract (200 or 400 mg/kg) did not result in any change in general behaviour of the animals. Body temperature and state of the stool were also not influenced by the drug treatment. Body weight, weight of liver, kidneys and spleen and food and water intake were not significantly altered by the drug administration (data not shown). Hemoglobin content, total leucocyte count, serum urea, cholesterol, total lipid, glutamate pyruvate transaminase, glutamate oxalate transaminase and alkaline phosphatase were unchanged by the treatment (Table 4). However, the number of peritoneal macrophages was marginally increased by the drug at 400 mg/kg (Table 4).

**DISCUSSION**

The antidiabetic activity of the folk medicinal plant *C. kleinii* was brought to light by our previous report\(^1\). This plant leaf (alcohol extract) is active against both alloxan and streptozotocin-induced diabetic rats. Increase in glycogen in liver can be brought about by an increase in glycogenesis and/or a decrease in glycogenolysis. So the *C. kleinii* extract could have stimulated glycogenies and/or inhibited glycogenolysis in the diabetic rat liver. Insulin and sulfonylurea drugs (e.g., glibenclamide) cause hypoglycemia when taken in excessive doses and overt hypoglycemia is the most worrisome effect of these drugs\(^12\). However, another group of OHAs, biguanides do not cause hypoglycemia even when taken in excessive doses\(^13\). *C. kleinii* also did not cause hypoglycemic effect\(^1\). One of the biguanides, metformin is currently a top selling OHA in USA\(^12\). Metformin does not stimulate insulin secretion and acts by reducing hepatic glucose production through inhibition of gluconeogenesis and, to a lesser extent, by enhancing insulin sensitivity in the tissues. In the present study, like metformin *C. kleinii* extract did not influence serum levels of insulin in diabetic as well as normo-glycemic rats. Further, like metformin the extract reduced serum lipids. Detailed studies are in progress in this laboratory to understand the mechanism of action of the herbal drug.

In the present study, an active chloroform fraction has been isolated from the alcohol extract which is very effective at a dose of 25 mg/kg as against 200 mg/kg of original alcohol extract. It is likely that the active principle(s) could be one or more terpenoids and/or coumarins. Studies are in progress to identify the active principles.

The leaves of *C. kleinii* is ground into a paste with water and used for diabetes mellitus in remote villages in Kanyakumari and Trivandrum districts in folk medicine without any known or recorded adverse effects. Such herbal drugs may be used directly for clinical trials. However, when an extract or active fraction is used it is better to evaluate for possible toxicity. In this connection, in the present study the alcohol extract was evaluated and it did not exhibit any toxic symptoms in the limited toxicity evaluation in male mice. A perusal of literature shows that studies were not carried out on this plant with regard to other pharmacological properties and phytochemistry. In the present study at 400 mg/kg level the extract increased the number of the peritoneal macrophages. Since macrophages have crucial roles in immune function, further studies are warranted in this area.

---

**Table 4.** Effect of short term (15 days) *Cassia kleinii* (alcohol extract) administration on the hematological and serum biochemical parameters of male mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Treated 200 mg/kg</th>
<th>Treated 400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (mg/100 ml)</td>
<td>19.2±1.8</td>
<td>18.3±1.1</td>
<td>18.2±1.3</td>
</tr>
<tr>
<td>WBC (10⁶/mm³)</td>
<td>9.6±1.3</td>
<td>9.2±1.1</td>
<td>8.6±1.2</td>
</tr>
<tr>
<td>Peritoneal macrophages (10⁶/mouse)</td>
<td>9.5±1.1</td>
<td>10.3±0.9</td>
<td>11.7±0.8*</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>24.6±2.1</td>
<td>23.3±1.2</td>
<td>23.4±2.7</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>104±11.1</td>
<td>105±7.2</td>
<td>97±1.8</td>
</tr>
<tr>
<td>GPT (1 U/L)</td>
<td>39.1±1.3</td>
<td>38.5±1.0</td>
<td>39.4±0.8</td>
</tr>
<tr>
<td>GOT (1 U/L)</td>
<td>59.6±3.0</td>
<td>62.4±3.7</td>
<td>63.0±2.9</td>
</tr>
<tr>
<td>AP (KA Unit)</td>
<td>21.0±1.7</td>
<td>20.9±1.6</td>
<td>19.6±1.0</td>
</tr>
</tbody>
</table>

Values are mean±SD, n= 6, *P<0.5 compared to control (Student’s ’t’ test)
Although it is the normal practice to determine the \( \text{LD}_{50} \) value, now it is acceptable to limit the study with an acute toxicity test using several doses including reasonably high doses of the drugs. In the present study, acute toxicity was tested up to a high concentration of 2 g/kg (ten times more than the therapeutic dose). Even at this dose the herbal extract did not exhibit any sign of toxicity. Since the main purpose of the preliminary acute toxicity study is to get some idea on conspicuous behavioural changes and death, if any, only 2 animals were used for each group considering Institute Animal Ethics Committee views to minimize the use of animals.

In the untreated streptozotocin diabetic mice (control group) only 2 animals survived till the end of the experimental period (15 days). However, there were no mortality for eight days in this group. The glucose values were recorded for this period. The serum glucose, lipid and cholesterol values for the 2 rats are in agreement with those expected for streptozotocin diabetic rats. Further, the individual value for the parameters studied did not differ much between these two rats in the same group. Therefore, in substance the small \( n \) in the diabetic control group does not affect the interpretation of the data in the present case.

Development of phytomedicines is relatively inexpensive and less time consuming. It is more suitable to our economic conditions. However, ecotype and genotype variations, seasonal variations, etc. in efficacy and safety have to be addressed in phytomedicine development.

\textit{C. kleinii} is a herb which grows as a weed. It can be easily cultivated. Therefore, there cannot be shortage of raw materials for phytomedicine development. The growth conditions of this herb for optimum efficacy and safety are being standardized at our Institute.

ACKNOWLEDGEMENTS

One of the authors (V. Babu) is grateful to University Grants Commission, Regional Office, Bangalore for the award of Teacher Fellowship under Faculty Improvement Programme. Dr. G. M. Nair, Director, TBGRI, Palode, Thiruvananthapuram, is gratefully acknowledged for having provided laboratory facilities and other support for this work. Our thanks are also due to Dr. D. A. Evans (scientist) and G. Santhoskumar (Animal house technician) for their valuable assistance.

REFERENCES


