ANTIDIABETIC ACTIVITY OF AQUEOUS AND ETHANOLIC EXTRACTS OF PASSIFLORA FOETIDA L. IN ALLOXAN INDUCED DIABETES RATS

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ABSTRACT
The aim of the present investigation was to evaluate the antidiabetic efficacy of aqueous and ethanolic extracts of Passiflora foetida L. against alloxan induced diabetes rats. Wistar albino rats were used for the present study and the animals were divided into 12 groups. Diabetes was induced by a single intraperitoneal injection of alloxan monohydrate (100 mg/kg) in saline. Aqueous and ethanolic extracts of Passiflora foetida L. 200 mg/kg (leaf, unripe (UR) peel, full ripens (FR) peel, seed and root) was administered orally for 14 days. All the animals were sacrificed after 14 days treatment and its effect on blood glucose, Glucose tolerance test (GTT), hepatic marker enzymes such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum marker like urea, creatinine and protein in alloxan induced diabetic rats were examined. The results showed that the aqueous extract of P. foetida L. showed maximum percent reduction of glucose when compare to ethanolic extract of P. foetida L. The aqueous and ethanolic extracts of P. foetida L. also have the ability to decrease the level of serum SGOT, SGPT, urea and creatinine significantly in diabetic condition. The present study proved that the aqueous and ethanolic leaf extract of P. foetida L. had the best antidiabetic activity with a maximum percent reduction of glucose.

KEYWORDS: Passiflora foetida L., Antidiabetic, Alloxan, GTT, Urea, Creatinine.

INTRODUCTION
Diabetes is the most common endocrine disorder and poses a serious challenge to healthcare and it is considered as third greatest cause of death in all over the world[1]. It is a non
Communicable disease result from shortage or lack of insulin secretion or reduced sensitivity of the tissue to insulin[2]. During Diabetes mellitus (DM), persistent hyperglycemia causes an increased production of free radicals via auto oxidation of glucose and non-enzymatic protein glycation which may lead to disruption of cellular functions and oxidative damage to membranes[3].

Diabetes is a chronic disorder affecting the population on epidemic level. Diabetes results from abnormal metabolism of insulin wherein insulin action is impaired or absolute insulin deficiency results in imbalance of glucose metabolism and leads to a syndrome called diabetes mellitus[4]. It is associated with impaired glucose metabolism that leads to an increase in free radical production and increase in triglyceride and lipoprotein levels[5]. It is also associated with long-term complications, including retinopathy, nephropathy, neuropathy and angiopathy and several others. India has today become the diabetic capital of the world with over 20 million diabetics and this number is set to increase to 57 million by 2025[6]. Many synthetic remedies like sulfonylurea, insulin injection are used which are associated with various undesirable side effects[7]. Management of diabetes without any side effect is still a challenge to the medical community. There is continuous search for alternative drugs. Therefore it is prudent to look for options in herbal medicines for diabetes[8]. Thus due to an increase in demand by patients to use natural products with antidiabetic activity investigations on hypoglycemic agents derived from medicinal plants have gained popularity in recent years[9]. Compared to synthetic drugs, herbal preparations are frequently considered to be less toxic with fewer side effects[10].

Traditional medicines are used to cure many diseases like diarrhea, intestinal tract, throat, ear infections, fever and skin diseases. *Passiflora* is the largest genus in the Passifloraceae family and comprises nearly 500 species. It is commonly known as bush passion fruit or wild passion fruit, or love-in-a-mist or passion flower or stinking passion flower (English); welodam or padawal (Sinhala), siruppunaikkali (Tamil), loliloli ni kalavo (Fijian), passiflore or passiflore fétide (French)[11]. *Passiflora foetida* is a herbaceous climber the analgesic activity of the hydro-alcoholic extract in mice of *P. foetida* was already reported[12]. Furthermore, the extract from the callus of *P. foetida* leaf has been demonstrated to exert promising hepatoprotective effect in CCl₄ induced hepatic injury rats[13]. The plant is said to be used for curing itches[14]. It has been widely used in Mexican traditional medicine for the treatment of different central nervous system (CNS) disorders[15]. The methanolic extract of
*P. foetida* root has been reported to have anticancer activity in a conventional animal model of liver cancer[16]. The traditional and folklore uses, pharmacological, clinical and toxicological reports of the prominent species of the genus *Passiflora* was already established[17],[18]. Flavonoids, glycosides, alkaloids, phenolic compounds and volatile constituents have been reported as the major phyto-constituents of the *Passiflora* species [19]. Present study was performed to evaluate the antidiabetic potential of aqueous and ethanolic extracts of *Passiflora foetida* L. against alloxan induced diabetic rats.

**MATERIALS AND METHODS**

**Collection of plant material**

The leaves, root, fruit peel (both ripened & un ripened) & seed of *Passiflora foetida* L. were collected from in and around Karpagam University, Coimbatore, Tamilnadu and authenticated by Dr.M. Palanisamy, Botanical Survey of India, Tamilnadu Agricultural University Campus, Coimbatore. The Voucher No is BSI/SRC/5/23/2012-13/Tech-108. All the parts of plant were washed well with water. They were air dried at 25°C for 10 days in the absence of sunlight and powdered coarsely using a mixer. They were then weighed and kept in an airtight container and stored in refrigerator for future use.

**Extraction of samples**

100 g of dried plant powder was extracted in 500 mL of ethanol and aqueous solution kept in an orbitory shaker for 72 h. Repeated extraction was done with the same solvent till clear colorless solvent is obtained. Obtained extract was evaporated and stored at 0-4°C in an air tight container.

**Experimental animals**

Adult male albino rats weighing about 150-200 g were obtained from the animal house of Karpagam University, Coimbatore and used for the study. Rats were housed at constant temperature of 22±5°C with a 12-hour light, 12-hour dark cycle and fed on pellets with free access to tap water. All the experiments were carried out according to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and the study was approved by Institutional Animal Ethical Committee (IAEC) of Karpagam University.
Induction of Diabetes

Diabetes was induced by a single intraperitoneal injection of alloxan monohydrate (100 mg/kg) in saline. Before the induction of diabetes body weight and blood glucose levels were measured in all the experimental animals. Hyperglycemia in diabetes rats were confirmed after 72 hrs by the elevated blood glucose and the behavioural changes (Excess thirst and frequent urination). The rats with blood glucose level more than 240 mg/dl were used for the study.

Experimental design

In the experiment, the animals were divided into 12 groups (n=3) as follows. Group I served as a control; group II consisted of alloxan induced diabetic rats; group III consisted of alloxan – induced diabetic rats treated with ethanolic extract of leaf of *P.foetida* (200 mg/kg bw/d/rat); group IV consisted of alloxan – induced diabetic rats treated with ethanolic extract of unripe peel of *P.foetida* (200 mg/kg bw/d/rat); group V consisted of alloxan – induced diabetic rats treated with ethanolic extract of full ripe peel of *P.foetida*(200 mg/kg bw/d/rat); group VI consisted of alloxan – induced diabetic rats treated with ethanolic extract of seed of *P.foetida* (200 mg/kg bw/d/rat); group VII consisted of alloxan – induced diabetic rats treated with ethanolic extract of root of *P.foetida*(200 mg/kg bw/d/rat); group VIII consisted of alloxan – induced diabetic rats treated with aqueous extract of leaf of *P.foetida* (200 mg/kg bw/d/rat); group IX consisted of alloxan – induced diabetic rats treated with aqueous extract of unripe peel of *P.foetida*(200 mg/kg bw/d/rat); group X consisted of alloxan – induced diabetic rats treated with aqueous extract of full ripe peel of *P.foetida* (200 mg/kg bw/d/rat); group XI consisted of alloxan – induced diabetic rats treated with aqueous extract of seed of *P.foetida*(200 mg/kg bw/d/rat); group XII consisted of alloxan – induced diabetic rats treated with aqueous extract of root of *P.foetida* (200 mg/kg bw/d/rat). The animals were weighted and treated with prepared drugs through oral intragastric tube everyday under controlled room temperature and photoperiod. Standard food in form of pellets, and water were provided ad libitum. After the 14 days treatment period the animals were sacrificed under mild chloroform anesthesia.

Glucose Tolerance Test (GTT) in diabetic rats

After 14 days treatment with the respective aqueous and ethanolic extracts, the diabetic animals were overnight fasted and blood glucose was measured. Then the GTT was performed in all the experimental animals by the method of Bonner Weir,\(^{[20]}\) in the ethanol &
aqueous extract of *Passiflora foetida* on diabetic rats. The animals were fed with glucose (4g/kg) and blood glucose was determined, 30 minutes prior to the administration of plant extract. Blood was withdrawn from the orbital puncture and the glucose level was estimated using one touch electronic glucometer using heamoglucostrips (Lifescan, Johnson and Johnson Ltd) and it was also confirmed by the results of O-Toludine method.

**Biochemical studies**

The blood was drawn from the ventricles, centrifuged and the serum was immediately used for various biochemical parameters as follows. Urea was estimated by uric acid UV kinetic method using diagnostic reagent kit manufactured by Span diagnostics Ltd. Creatinine was estimated by alkaline picric acid UV kinetic method using a reagent kit manufactured by Beacon diagnostics Pvt Ltd. AST was estimated by 2, 4-DNPH Reitman and Frankel method²¹, ALT was estimated by the method of King’s²² two reagent chemistry method and Protein was estimated by Biuret method using diagnostic reagent kit manufactured by Span diagnostics Ltd.

**Statistical analysis**

Results are expressed as the Mean ± SD. Statistical significance was evaluated by One Way Analysis of Variance (ANOVA) using SPSS version (10.0) and the individual comparisons were obtained by the Duncan multiple range test (DMRT)²³. A value of p<0.05 was considered to indicate a significant difference between groups.

**RESULTS**

**Effect of aqueous and ethanolic extract of *Passiflora foetida* L. on changes in body weight**

The effects of the aqueous and ethanolic extract of *P. foetida* L. on body weight in the alloxan induced diabetic rats are shown in figure 1 & 2. The results of the body weight analysis indicate that the body weight of the untreated diabetic rats was found to be significantly decreased when compared with the normal control group. Treatment of diabetic rats with aqueous and ethanolic extract of *P. foetida* L. significantly improved the body weight.
Figure 1 Changes in body weight of ethanolic extract of *P. foetida* L. treated diabetic rats
Values are expressed as Mean ± S.D three individual experiments

Figure 2 Changes in body weight of aqueous extract of *P. foetida* L. treated diabetic rats:
Values are expressed as Mean ± S.D three individual experiments

Effect of aqueous and ethanolic extract of *Passiflora foetida* L. on changes in blood glucose

The aqueous and ethanolic extract of *P. foetida* L. had significantly reduced the blood glucose level of hyperglycemic animals when compared to untreated group shown in figure 3 & 4. The results showed that aqueous and ethanolic extract of leaf had better antidiabetic activity with a glycemic index of 63.3 & 57% respectively (Figure 5), than the other parts of *P. foetida* L.
Figure 3 Changes in blood glucose of ethanolic extract of *Passiflora foetida* L. treated rats:
Values are expressed as Mean ± S.D three individual experiments

Figure 4 Changes in blood glucose of aqueous extract of *Passiflora foetida* L. treated rats.
Values are expressed as Mean ± S.D three individual experiments

Figure 5 Effect of aqueous and ethanolic extract of *P. foetida* L. on Glycemic Index of treated groups.
GTT of aqueous and ethanolic extract of *Passiflora foetida* L. treated rats
The oral GTT was determined to evaluate the efficacy of antidiabetic effect of aqueous and ethanolic extract of *Passiflora foetida* L. Blood glucose level was assessed in overnight fasted rats treated with ethanolic and aqueous extract of *Passiflora foetida* L. fruit peel (both ripen & un ripen), leaf, seed and root with a concentration of 200mg/kg for a period of 14 days. GTT was performed after glucose load, there was a marked increase in blood glucose level upto 2 hour.

This was followed by the subsequent fall in blood glucose level and the amount of blood glucose was found to be normal after 4th hour of administration of glucose load. The results are represented in figure 6, 7. At dose of 200mg/kg of aqueous and ethanolic extract of *P. foetida* L. leaf (13.9%, 9.5% respectively) and root (11.1%, 8.9% respectively) shows maximum percent reduction of glucose. The altered glucose tolerance observed here is due to earlier diabetic induction in the rats and the subsequent treatment of diabetic rats with aqueous and ethanolic extract of *Passiflora foetida* L. before the GTT experiment. Among the two extracts of *P. foetida* L., the aqueous extract was showed maximum percent reduction of glucose which is represented in figure 8.

![Oral GTT of ethanolic extract of P. foetida](image)

**Figure 6** Glucose tolerance test of ethanolic extract of *P. foetida* L. treated rats:
Values are expressed as Mean ± S.D three individual experiments
Figure 7 Glucose tolerance test of aqueous and extract of *P. foetida* L. treated rats.
Values are expressed as Mean ± S.D three individual experiment

Figure 8 % reduction of glucose with aqueous and ethanolic extract of *P. foetida* L. in GTT

**Effect of aqueous and ethanolic extract of *Passiflora foetida* L. on serum hepatic markers**

The level of SGOT, SGPT were significantly increased (p<0.05) in diabetic group. The treatment with various parts of aqueous and ethanolic extract of *P. foetida* L. significantly reversed these values to that of control rats. The results of hepatic markers are represented in table 1& 2. In aqueous extract unripe (UR) peel and ethanolic extract full ripe (FR) peel and seed preserved the liver marker enzymes better than the other parts of aqueous and ethanolic extracts.

**Effect of aqueous and ethanolic extract of *Passiflora foetida* L. on serum renal markers**

Urea and creatinine were significantly increased (p<0.05) in diabetic group and on treatment with aqueous and ethanolic extract of *P. foetida* L. for 14 days significantly reversed and the
renal markers were well maintained in the normal range in both aqueous and ethanol extract of *P. foetida* L. treated diabetes rats these values to that of control rats (Table 1 & 2).

**Effect of aqueous and ethanolic extract of *Passiflora foetida* L. on serum protein**

The level of protein in serum was decreased in alloxan induced diabetic group when compared to normal control group and *P. foetida* L. treated groups. Treatment of diabetic rats with various parts of aqueous and ethanol extracts of *P. foetida* L. results in improvement of body weight (Table 1 & 2).

**Table 1 Concentration of urea, creatinine, protein, SGOT & SGPT in serum of control and aqueous extract of *P. foetida* treated rats.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic control</th>
<th>UR peel</th>
<th>FR peel</th>
<th>Leaf</th>
<th>Seed</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dL)</td>
<td>27.4±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.6±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.4±0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.5±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.4±0.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatine (mg/dL)</td>
<td>0.73±0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.63±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49±0.36&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.53±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35±0.41&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>8.4±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4±0.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.9±0.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.1±0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2±0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4±0.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>29.3±61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.4±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.6±0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.4±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.5b±0.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.8±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>27.4±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.7±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.6±0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.4±0.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>26.6±0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.7±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.4±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±S.D of three individual experiments
Values not sharing a common superscript letter differ significantly (DMRT)

**Table 2 Concentration of urea, creatinine, protein, SGOT & SGPT in serum of control and ethanolic extract of *P. foetida* treated rats.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic control</th>
<th>UR peel</th>
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<th>Seed</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dL)</td>
<td>27.4±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.6±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.3±0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.1±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.3±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.5±0.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatine (mg/dL)</td>
<td>0.73±0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.71±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43±0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29±0.39&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>8.4±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.2±0.89&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>7.62±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2±0.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>29.3±61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.4±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.7±0.31&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>27.4±0.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.4±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.1±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>27.4±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.7±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
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Values not sharing a common superscript letter differ significantly (DMRT)
DISCUSSION

The ethanolic & aqueous extract of *P. foetida* L. prevented the loss in body weight in alloxan induced diabetic rats and from this it is evident that the extract possesses a significant beneficial effect on body weight. This may be due to the protein sparing effect of *P. foetida* L. which prevent protein degradation those results in improvement of body weight. Lack of insulin in diabetics makes the cell to starve for glucose, so the cell uses proteins as an alternative energy source which ultimately result in weight reduction. Numerous studies have shown an association between hyperglycemia and decreased body weight of diabetic animals. It was observed that alloxan induced diabetes is associated with the reduction in the weight of animals and the relative weight of kidney, liver and spleen in rats\(^{[24]}\). The beneficial effect of *Passiflora* species leaf in diabetes mellitus was reported earlier\(^{[25]}\). The reduction of glucose might be brought by the stimulation of pancreatic β- cells of Langerhans to produce insulin or it prevent the absorption of glucose from the intestine or it may enhance the glucose uptake in peripheral tissues\(^{[26]}\). It is indicated that *P. foetida* L. contains a lot of bioactive compounds that preserve the integrity of liver cells which prevent the leakage of SGOT and SGPT in serum. Measurement of aminotransferases (SGOT and SGPT) and phosphatases (acid and alkaline) is of clinical and toxicological importance as changes in their activities is indicative of tissue damage\(^{[27]}\). A rise in aspartate transaminase activity is almost always due to hepatocellular damage followed by cardiac tissue damage and is usually accompanied by a rise in alanine transaminase activity\(^{[28]}\). When the cells and tissues in which they are found damaged, enzymes are released or rate of cell turnover is increased during tissue repair or in diabetes or duct obstruction. When cell membranes get damaged, the enzymes SGOT and SGPT, which are normally located in the cytosol, leak into the blood stream thus manifesting damage to liver and other tissues by toxicants or in disease conditions\(^{[29]}\). In our study, the liver markers were maintained in their normal range in diabetic treated rats supported the hepatoprotective effect of *P. foetida* L. aqueous & ethanolic. Increase in the level of urea and creatinine are markers of renal dysfunction in the diabetic groups\(^{[30]}\). Upon treatment with aqueous & ethanolic extracts of *P. foetida* L. the renal markers were significantly reduced, this explains the renal protection afforded by aqueous & ethanolic extract of *P. foetida* L. in diabetes. Reduction of protein level is an indicator of diabetic condition\(^{[31]}\). Decreased serum protein level in alloxan induced diabetic rats is presumed to be due to increased protein catabolism and gluconeogenesis in diabetes\(^{[32]}\). This implies that *P. foetida* L. enhance glucose utilisation and prevent protein degradation that normalised the altered protein level in *P. foetida* L. treated groups.
CONCLUSION
From the present study it can be concluded that, the oral administration of *P. foetida* L. aqueous & ethanolic extract (200mg/kg) significantly decreased the blood glucose level in hyperglycemic animals. The results showed that aqueous and ethanolic extract of leaf had the best antidiabetic activity with a maximum percent reduction of glucose. According to OGTT analysis, the aqueous extract of *P. foetida* L. showed maximum percent reduction of glucose when compared to ethanolic extract of *P. foetida* L. The aqueous and ethanolic extracts of *P. foetida* L. also have the ability to decrease level of serum SGOT, SGPT, urea and creatinine significantly in diabetic condition. On other hand, it increases serum protein level significantly in diabetes.

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CONFLICT OF INTEREST
We declare that we have no conflict of interest.

REFERENCES


