Evaluation of Anti-Hyperglycemic Activity of Adiantum Philippense Linn, a Pteridophyte in Alloxan Induced Diabetic Rats

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Abstract
To evaluate the potential of Adiantum philippense Linn in hyperglycemia induced by alloxan monohydrate, ethanolic and aqueous extracts of the fern, Adiantum philippense were assessed at two dose levels of 500 mg/kg and 250 mg/kg (p.o) respectively. Both the extracts treated groups at two doses exhibited significant hypoglycemic effect when compared to standard drug, glibenclamide and disease control group at an interval of 14 days which is evident from Plasma Glucose levels and Oral Glucose Tolerance Test (OGTT) values.

The serum parameters for kidney functioning like Urea, Creatinine, and lipid profile like Triglycerides, Cholesterol and HDL-cholesterol along with liver enzymes like SGPT and SGOT were assessed which favoured the potential of the fern in causing anti-hyperglycemia. At the end of 14 days study, the levels of liver glycogen of the rats of all the groups were estimated in vitro which showed higher glycogen content in the extract treated groups as compared to the diabetic rats.

To further potentiate the role of this fern in producing hypoglycemia the antioxidant property was evaluated in the extracts through ABTS radical scavenging assay and in the pancreatic tissue by Superoxide Dismutase (SOD), Catalase (CAT), Reduced glutathione (GSH) and Lipid peroxidation (LPO) assays. Among the parameters estimated there was an increase in the levels of SOD, CAT, GSH whereas decrease in the LPO levels in the extracts treated groups which further support the role of Adiantum philippense Linn, in lowering the hyperglycemic state of experimentally induced diabetic rats. Purification of its active principles will be carried out to focus on its lead component that may be helpful in current therapy for treating Diabetes and its complications by rescuing tissues from free radicals and oxidative stress.

Keywords: Adiantum philippense Linn; Hyperglycemia; Alloxan; Liver glycogen

Introduction
Diabetes is a multifactorial disease which has become pandemic nowadays in India and worldwide with the highest population engulfed by this disease causing enormous health problem [1]. The free radicals generated due to oxidative stress pose life-threatening state which is common in hyperglycemic condition [2]. Alloxan monohydrate (2, 4, 5, 6-tetraoxypyrimidine; 2, 4, 5, 6-pyrimidinetetrone) being a toxic glucose analogue, selectively destroys insulin-producing pancreatic β-cells and causes Diabetes Mellitus in experimental animal [3].

Nowadays, Tolbutamide, Metformin, Gliclazide etc. that are highly preferred as anti-diabetic drugs are mainly sulfonylureas and biguanides which are known to cause various adverse effects [4]. Hence in the recent years medicinal plants have become a subject of interest for drug development owing to their phytochemical constituents and their therapeutic potential. In particular, the angiosperms are given more emphasis but the pteridophytes which are significant group of plant kingdom with 12000 species are not much studied for their antidiabetic activity.

Adiantum philippense Linn is commonly known as ‘Hanspadi’ or ‘Walking Maiden hair fern’. It is widely distributed in many parts of India and used as an ornamental plant. It is ethnomedically used in bleeding diseases, burning sensation, erysipelas, epileptic fits, dysentery, and elephantiasis [5]. It has been reported that the dried whole plant has been used as a medicine for bronchitis and cough [6]. Adiantum philippense is also reported to have anti-bacterial [7] and anti-fungal activity [8].

Adiantum capillus veneris, a fern of the same genus as that of Adiantum philippense Linn possess antidiabetic potential which is marketed with brand name “Avenca TM”. Belonging to the same genus and family of Pteridophytes, insufficient information was available regarding anti diabetic potential of Adiantum philippense Linn (Adiantum lunulatum Burm f.) which led us to investigate its anti-hyperglycemic potential on alloxan induced diabetic rats.

Materials and Methods
Collection and authentication
The fern Adiantum philippense Linn (AP) was collected from ChandraprabhaVanrai, Dapoli in the Ratnagiri district of Maharashtra in the end of monsoon and was authenticated by Dr. P.G Dwarkan from Botanical Survey of India, Pune under the number BSI/ WC/Tech./2011/306. The animal usage protocol was approved by the Institutional Animal Ethical Committee (IAEC) for National Toxicology Centre through Protocol no.120 on 30/03/2011 and...
executed by following the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments in Animals) guidelines.

**Preparation of plant extracts**

The fronds were cleaned and shade dried in a dryer for 48 hours and percent weight loss was determined. The dried fronds obtained were crushed and kept in the Soxhlet apparatus for obtaining ethanolic and aqueous extract by their respective solvents. The ethanol and aqueous extracts obtained were concentrated in rotary evaporator under vacuum and their percent yields were determined.

**Animals**

The Male Sprague Dawley rats weighing 200-250 grams were procured from in-house animal facility of National Toxicology Centre, Pune. They were housed under standard conditions of temperature and relative humidity with 12 hr light/dark cycle. Animals were fed on standard commercial pellet diet and water ad libitum.

**Acute oral toxicity study**

The Acute oral toxicity test of the extracts was determined prior to the experimentation on animals according to the OECD (Organisation for Economic Co-operation and Development) guidelines no 423. Female Swiss Albino mice (18-20 g) were taken for the study and dosed once with 2000 mg/kg of both the extracts. The treated animals were monitored for 14 days [9], for general clinical signs and symptoms as well as mortality. No mortality was observed till the end of the study revealing the 2000 mg/kg dose to be safe. Thus, 1/4th and 1/8th doses of 2000 mg/kg i.e. 500 mg/kg and 250 mg/kg were chosen for subsequent experimentation.

**Induction of diabetes**

Male Sprague Dawley rats were kept on fasting overnight and injected Alloxan monohydrate (Sigma-Aldrich) intraperitoneally (i.p.) for the induction of diabetes at a dose of 150 mg/kg [10]. The animals were bled through the retro orbital plexus and blood was collected in heparinised tube. The plasma glucose was measured in a biochemical analyzer by Glucose oxidase-peroxidase (GOD/POD) method. The rats were monitored for 14 days [9], for general clinical signs and symptoms as well as mortality. No mortality was observed till the end of the study revealing the 2000 mg/kg dose to be safe. Thus, 1/4th and 1/8th doses of 2000 mg/kg i.e. 500 mg/kg and 250 mg/kg were chosen for subsequent experimentation.

**Liver glycogen analysis**

The assessment of liver glycogen was undertaken by following a protocol described by Sheth et al. [13]. In brief, the rats were fasted overnight and the baseline blood glucose was determined next day with the help of an automated glucometer (Accuchekactive). The rats were loaded with 2 g/kg of glucose 30 minutes after administration of drug at their respective doses. The blood glucose was monitored for 30, 60 and 120 minutes thereafter.

**Oral glucose tolerance test**

The Oral Glucose Tolerance Test (OGTT) was done according to the protocol described by Sheth et al. [13]. In brief, the rats were fasted overnight and the baseline blood glucose was determined next day with the help of an automated glucometer (Accuchek active). The rats were loaded with 2 g/kg of glucose 30 minutes after administration of drug at their respective doses. The blood glucose was monitored for 30, 60 and 120 minutes thereafter.

**Pancreatic antioxidant enzymes**

a. Superoxide dismutase (SOD; EC: 1.15.1.1): Superoxide dismutase activity was estimated according to the method of Kakkar et al. [17]. The developed blue colour in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of Nitro Blue Tetrazolium by 50% and the activity was expressed as U/mg protein.

b. Catalase (CAT; EC: 1.11.1.6): The Catalase activity was determined according to the method given by Aebi [18]. The rate of \(H_2O_2\) decomposition was followed by monitoring absorption at 240 nm. One unit of enzyme Catalase activity is defined as the amount of enzyme required to decompose 1 mol of hydrogen peroxide in 1 min. One unit of enzyme activity was expressed as \(\mu\text{mol }H_2O_2\text{ consumed}/\text{min/mg protein}\) [19].

c. Lipid Peroxidation (LPO): Lipid peroxidation in the pancreatic tissue was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) which were homogenized in the incubation medium using a Teflon homogenizer and 1.0 ml of homogenate was mixed with 2.0 ml of TBA-TCA-HCl reagent [20]. The contents were incubated
in a boiling water bath for 15 minutes and the pink colour developed was estimated at 535 nm against a reagent blank, in a spectrophotometer [21]. The MDA equivalents of the samples were calculated using the extinction coefficient 1.56×10^5 M^−1cm^−1.

d. Reduced glutathione (GSH): Reduced glutathione on reaction with DTNB (5,5′-dithiobis-2-nitro benzoic acid) produces a yellow colored product that absorbs at 412 nm [22]. Briefly a homogenate was prepared with 0.1 g of the pancreas with 0.5 ml of 5% TCA. The precipitated protein was centrifuged at 1000 rpm for 10 minutes. The supernatant (0.1 ml) was made up to 1 ml with phosphate buffer and mixed with 2 ml of DTNB for the estimation of GSH. The values were expressed as n moles of GSH/g of tissue [23].

e. Protein analysis in pancreas: Protein concentrations were measured using the method of Lowry et al. [24]. Bovine serum albumin was used as a standard.

**Histopathology**

At the end of the study animals were dissected out and the organs were fixed in 10% formalin for monitoring necrosis or adverse effect of the treatment. The tissues were processed for histopathology starting from trimming, graded alcohol dehydration, embedding in paraffin, sectioning, spreading, fixing in the slides and staining with hematoxylin and eosin. The slides were seen under the microscopy (200 X) for abnormalities and photomicrographs were taken.

**Statistical analysis**

The results were analyzed for statistical significance by one way ANOVA and were expressed as Mean ± SD by using Graph pad Prism 5 version (Dunnett’s multiple comparison tests). P<0.001 were considered as statistically significant.

**Results**

The body weights were assessed at an interval of 7 days. The control animals showed a linear proportion of growth in terms of weight whereas the untreated diabetic rats were found to have significant weight loss of 23.96%. However, administration of AP ethanolic and aqueous extract at two different doses and standard drug (Glibenclamide) to diabetic rats restored the body weight to a normal level (P<0.001; Table 1).

Alloxan induced diabetic rats showed a 74.45% rise in the plasma glucose levels as compared to normal control group. Treating those hyperglycemic rats with two doses of ethanolic and aqueous extract of *Adiantum philippense* (250 mg/kg and 500 mg/kg) and standard drug (5 mg/kg) resulted in significant reduction in the plasma glucose level (P<0.001) which came at par with the normal control animals at the end of 14 days study. On the other hand the diabetic control animals which received no treatment continued to show high plasma glucose throughout the study (Figure 1).

In alloxan induced diabetic animals, elevated levels of serum urea and creatinine were observed. On treating the animals with both standard and test drugs the elevated levels decreased to almost normal range. However, the untreated diabetic control group showed high levels of serum urea and creatinine till the end (P<0.001; Table 2).

In the present study the lipid profile was assessed by monitoring the parameters like triglycerides, cholesterol and HDL-cholesterol. It was found that hypertriglyceridemia and hypercholesterolemia occurred with the induction of diabetes when compared with the normal control group (P<0.001). Treatment with standard drug and both the extracts of AP at different doses resulted in a reduction of those levels significantly (P<0.001) when compared with the diabetic control group. On the contrary, the HDL-cholesterol level was found to be lower in the beginning but later a significant increase in the levels were observed when supplemented with two extracts of AP and standard drug while the disease control group continued to remain low (P<0.001; Table 3).

SGPT levels were augmented at the induction of diabetes but later on with the administration of plant extracts it was able to lower the SGPT levels which were comparable to that of standard drug but was unable to bring down the levels to normal (P<0.001). Both the ethanolic and aqueous extract of AP at dose of 500 mg/kg and 250 mg/kg when

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (in grams)</th>
<th>Initial</th>
<th>Final</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td></td>
<td>222.00 ± 2.44</td>
<td>230.33 ± 4.08</td>
<td>3.75↑</td>
</tr>
<tr>
<td>DC</td>
<td></td>
<td>216.00 ± 2.28</td>
<td>171.83 ± 5.84***</td>
<td>23.96↓</td>
</tr>
<tr>
<td>Std</td>
<td></td>
<td>225.00 ± 4.73</td>
<td>233.16 ± 4.16***</td>
<td>3.62↑</td>
</tr>
<tr>
<td>AP 250 eth</td>
<td></td>
<td>221.50 ± 2.86</td>
<td>227.66 ± 3.44***</td>
<td>2.78↑</td>
</tr>
<tr>
<td>AP 500 eth</td>
<td></td>
<td>222.30 ± 1.40</td>
<td>233.50 ± 4.23***</td>
<td>5.03↑</td>
</tr>
<tr>
<td>AP 250aq</td>
<td></td>
<td>224.33 ± 2.30</td>
<td>231.83 ± 3.76***</td>
<td>3.34↑</td>
</tr>
<tr>
<td>AP 500aq</td>
<td></td>
<td>223.33 ± 1.89</td>
<td>233.83 ± 4.16***</td>
<td>4.70↑</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD
NC: Normal Control; DC: Diabetic Control; Std: Standard Drug; AP: *Adiantum philippense*; aq: Aqueous; eth: Ethanolic Extract

**Table 1: Effect of AP on the body weight of the diabetic animals.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NC</th>
<th>DC</th>
<th>Std</th>
<th>AP 250 eth</th>
<th>AP 2500 eth</th>
<th>AP 250 aq</th>
<th>AP 500 aq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dl)</td>
<td>20.83 ± 1.7</td>
<td>61.33 ± 2.7***</td>
<td>37.17 ± 2.8***</td>
<td>37.83 ± 2.2***</td>
<td>34.00 ± 2.1***</td>
<td>42.17 ± 2.4***</td>
<td>39.83 ± 2.4***</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.35 ± 0.05</td>
<td>1.7 ± 0.21***</td>
<td>0.8 ± 0.09***</td>
<td>0.70 ± 0.11***</td>
<td>0.60 ± 0.09***</td>
<td>0.6 ± 0.08***</td>
<td>0.67 ± 0.12***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD
NC: Normal Control; DC: Diabetic Control; Std: Standard Drug; AP: *Adiantum philippense*

**Table 2: Effect of AP on serum urea and creatinine of experimental animals.**
given to diabetic rats lowered the serum SGOT levels at par with the normal control group. The SGOT levels were found to be significantly low when compared with the Diabetic control group (P<0.001; Table 4).

Initially when 2 gm/kg of glucose was given orally to the animals, 23% rise in the blood glucose levels were observed in the diabetic control group but approximately 18% increase in the blood glucose levels were observed in normal control, standard drug and both the extracts of AP at two different doses (250 and 500 mg/kg) treated animals. At an interval of 120 minutes the blood glucose levels of the above mentioned groups came to the baseline level except the diabetic control group which continued to show an elevated level (Figure 2).

There was a significant decrease in the hepatic glycogen levels of the diabetic control group as compared to the normal control group (P<0.001). A significant increase was also found in the liver glycogen content of both the test extract treated groups as well as standard drug group when compared with the diabetic control group (P<0.001). Both the test extracts treated groups were able to show more glycogen content as compared to standard drug (Figure 3).

In the ABTS antioxidant assay, the ethanolic extract of AP at 500 µg/ml glucose level showed the highest percent inhibition of 86.51 with a concentration equivalent to 45.86 µg/ml of ascorbic acid. However, the aqueous extract of AP at 500 µg/ml showed 78.34 percent inhibition with a concentration equivalent to 41.57 µg/ml of ascorbic acid (R²=0.995).

The SOD, CAT, GSH levels in pancreas of alloxan treated diabetic rats showed significant decreased levels (P<0.001) when compared to normal control group. While animals treated with AP 500 mg/kg p.o. and 250 mg/kg p.o. of both the extracts and Glibenclamide (5 mg/kg) showed significant increase (P<0.001) when compared to alloxan treated diabetic rats. Whereas the pancreatic MDA values were higher in the diabetic control group but the values obtained from all the treated groups (Extract and standard drug) and normal control groups showed significant reduced levels (Table 5).

The rounded islets of Langerhan’s were found embedded in between the acinar cells which were arranged in lobules with prominent nucleus in the normal control animals. The number, size and architecture of the islets were found to be normal. The islet cells of the disease control animals treated with alloxan monohydrate elicited severe injury by reduction in their number and diameter. However, the standard drug treated rats along with the test drug treated rats showed restoration of the pancreatic islets cells from the damage caused by alloxan monohydrate (Figure 5).

Discussion

Diabetes and its complication goes hand in hand, primarily uncontrolled hyperglycemia followed by decrease in body weight which can be attributed to a phenomenon known as muscle wasting due to rapid catabolism of fat and protein [25]. In the present study the diabetic animals showed 26.96% decrease in body weight whereas the standard and the treated groups gained 3-4% body weight in duration of 14 days.
The condition of hyperglycemia which was prominent in diabetic group of animals was also brought to normal levels with a daily dosing of the two extracts namely ethanolic and aqueous at 500 and 250 mg/kg doses. The ethanolic extract at a dose of 500 mg/kg lowered the elevated plasma glucose level by 66.30% while the same extract at 250 mg/kg was able to lower the plasma glucose by 63.50%. The aqueous extract treated groups at 500 mg/kg and 250 mg/kg showed a reduction of plasma glucose by 61.46% and 58.69% respectively. The reduction percent in case of standard drug treated group was found to be 62.16% and the diabetic control group continued to show a 2.21% increase in their plasma glucose concentration.

Plasma urea and creatinine are believed to be specific markers in diabetes for proper functioning of kidney [26]. Elevated levels of urea and creatinine were observed in diabetic animals owing to the adverse effect of alloxan on kidney [27,28]. These elevated levels came down to a normal level after treating the animals with standard drug and both the extracts of AP at two different doses which was evident from the results.

Induction of Diabetes to animals by alloxan monohydrate often shows elevated triglyceride and cholesterol levels [29] and reduced HDL-cholesterol values [30]. Similarly in this study the lipid parameters were performed where diabetic control animals showed higher triglycerides and cholesterol levels and lower HDL-cholesterol values which when treated with standard drug and test extracts resulted in lowered serum cholesterol and serum triglyceride levels and an increase in the HDL-cholesterol level.

During diabetic condition, high levels of SGPT and SGOT were found in rats [31]. In the present study normal levels of hepatic biomarkers like SGPT and SGOT were obtained after administration of the extracts for 14 days suggesting restoration of proper hepatic function.
Oral Glucose Tolerance Test being a potent diagnostic test during a diabetic study was carried out which showed a rise in the blood glucose level after administering glucose load which later came to the baseline at an interval of two hours but the diabetic group took more time to come to the baseline indicating damage of pancreas caused by alloxan. Moreover, while monitoring the hepatic glycogen concentration, a lower concentration of glycogen was found in the liver of the diabetic animals which can be attributed to the high amount of glucose circulating in their blood due to insulin insufficiency. The situation was however reversed in case of both the extracts treated and standard drug, Glibenclamide treated animals. Similar results were obtained in our previous study which was undertaken with another species of fern namely Pterisvittata [32] indicating a pivotal role of pteridophytes in diabetic condition.

As put forth by a recent research, free radicals being highly reactive in nature alters cellular morphological and functional integrity, damages membrane proteins leading to cell inactivation and hence body cells develop certain mechanisms to scavenge them either through enzymatic or non-enzymatic means [33].

Histopathological photomicrographs of Pancreas in adult rats at 200X magnification and stained with Hematoxylin and Eosin. Circle indicates area of the islets and arrow shows the number of islet at a particular field. (A) Normal rat showing normal acini and normal cellular population in islets of Langerhan’s (b) Diabetic control rat showing damaged islets and reduced islet size. (C) Diabetic rats treated with glibenclamide (5 mg/kg) showed almost normal sized islet cells. (D) AP ethanolic extract treated diabetic rats at 250 mg/kg showed normal islets (E) AP ethanolic extract treated diabetic rats at 500 mg/kg showed large islet cells (F) AP Aqueous extract treated rats at 250 mg/kg showed normal islets cells (G) AP Aqueous extract treated rats at 500 mg/kg showed many large islets of Langerhan’s

Figure 5: Histopathological photomicrographs of Pancreas.
Thus in the present study Superoxide Dismutase (SOD), Catalase (CAT), and reduced glutathione which are known to be involved in the direct elimination of active oxygen species [34] were evaluated along with lipid peroxidation that is often used as a marker for free radical induced tissue injury [35].

The ABTS radical scavenging activity of the both the extracts showed increasing antioxidant ability at increasing concentrations (Figure 4). In the in vivo study as well, the results obtained suggested that treating the diabetic animals with the two extracts of AP at two doses and standard drug can enhance the levels of SOD, CAT and GSH which otherwise in experimentally induced diabetic rats remains suppressed. LPO being one of the cellular features of severe diabetes [36], its level remain quite high in case of diabetic animals which in the present study reverted to normal in animals receiving the extracts of AP as well as standard drug. The histological observation of the pancreatic tissue from figure 5A shows the islet cells which are normal in their architecture whereas the figure 5B indicates that the Alloxan pancreatic tissue from figure 5A shows the islet cells which are normal of AP as well as standard drug. The histological observation of the pancreatic tissue because larger and prominent islets were seen at the end of 14 days treatment of the diabetic rats.

As an outcome, it can be concluded that the results indicate Adiantum philippense Linn possess anti-hyperglycemic as well as antioxidant potential and thus further studies on bioactive or fractions shall be evaluated for control and management of diabetes mellitus.

Acknowledgement

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