Original Research Article

HYPOGLYCEMIC EFFECT OF ANDROGRAPHIS ECHIOIDES ON STREPTOZOTOCIN INDUCED EXPERIMENTAL RATS
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ABSTRACT

The present study aims to study the hypoglycemic effect of methanol extract of Andrographis echioides (MEAE) in streptozotocin (STZ)-induced diabetic Wistar rats. Hyperglycemia was induced in rats by single intraperitoneal injection of STZ (55 mg/kg bodyweight). Three days after STZ induction, the hyperglycemic rats were treated with MEAE orally at the doses of 200, 500, and 800 mg/kg body weight daily for 21 days. Glibenclamide (1 mg/kg, orally) was used as reference drug. The fasting blood glucose levels were measured on each 7th day during the 21 days of treatment. Serum biochemical parameters including lipid content were estimated.

MEAE at the doses of 200, 500 and 800 mg/kg orally significantly (P < 0.01) and dose dependently reduced and normalized blood glucose levels as compared to that of STZ control group; the dose 800 mg/kg being the most potent showing complete normalization of blood glucose levels. Serum biochemical parameters including lipid profile were significantly (P < 0.01) restored toward normal levels in MEAE-treated rats as compared to STZ control animals. This study concludes that Andrographis echioides demonstrated promising hypoglycemic action in STZ-induced diabetic rats substantiating its ethno medicinal use.

Keywords: Andrographis echioides, Hypoglycemic, glibenclamide, streptozotocin, diabetes.

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INTRODUCTION

Diabetes mellitus (DM) may be a syndrome defined by chronic hyperglycemia and disturbances of saccharide, fat and macromolecule metabolism relating to absolute or relative deficiencies in endocrine secretion and/or endocrine action. There are a unit Associate in Nursing calculable 143 million folks within the world with diabetes and this variety will most likely double by the year 2030 [1]. In developing countries 80% of population are using traditional medicine in primary medical problems. By screening of anti-diabetic drugs, a large number of plant materials including phytoconstituents were found to possess potent anti-diabetic activity [2, 3]. Hemalatha et al. reported the pharmacologically tested anti-diabetic plant materials in steptozotocin induced diabetic animal model [4].

Andrographis echioides is a species of Andrographis commonly known as kalmegh in Hindi, belonging to the family Acanthaceae, is a dense herb found in plains and waste lands which fruits throughout the year. Andrographis echioides has been reported anti-Inflammatory, antimicrobial, anthelmintic, antioxidant and larvicidal activities [5-8]. Andrographis panniculata has been reported to possess anti-diabetic activity [9]. Pharmacological study on
MATERIALS AND METHODS

Drugs and chemicals

STZ was from Sigma Chemical Co., USA. Glibenclamide was from Hoechst, India. All other reagents used were of analytical grade obtained commercially.

Collection and extraction

The fresh aerial parts of *Andrographis echioides* were collected from SKM Siddha and Ayurvedic medicines India Pvt. Ltd., Erode Dist., Tamilnadu, India, in the month of August 2013 and identified by GVS Murthy, Botanical Survey of India, Coimbatore, India. A voucher specimen has been deposited in the laboratory for future reference (BSI/SC/5/23/13-14/TECH.835). The aerial parts of the plant were shade dried and pulverized. The powder was defatted with petroleum ether. Later, it had been subjected to continuous hot extraction with 95% aqueous methanol in a Soxhlet apparatus. The extract (MEAE) was concentrated under vacuum and dried in desiccators (yield 71 gm, 7.1% w/w). The dry extract was kept in vacuum desiccators until use. Preliminary phytochemical analysis [10] revealed the presence of flavonoids, alkaloids, and steroids in MEAE Plant material.

Animals

Adult male Wistar albino rats weighing 150–200 g were procured from Venkateshwara Enterprises, Bangalore, Karnataka, India and used throughout the study. All the animals were under the age of 8–12 weeks. They were housed in a very clean polypropylene cage and maintained under standard laboratory conditions (temperature 25 ± 2°C with dark/light cycle 12/12 h). They were fed with standard pellet diet and water *ad libitum*. The animals were acclimatized to laboratory conditions for one week before experiment. Experiments were performed complied with the rulings of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi, India. (Registration No: 1135/a/07/CPCSEA) and the study was permitted by the Institutional Animal Ethics Committee (IAEC).

Acute toxicity

Acute toxicity studies were performed as per OECD-423 guidelines [11]. Male Wistar albino rats selected by random sampling technique were utilized during this study. The animals were fasted for 4h with free access to water only. The plant extract was administered orally at a dose of 5 mg/kg initially and mortality if any was observed for 3 days. If mortality was ascertained in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was ascertained in only one animal out of three animals then the identical dose was repeated again to confirm the toxic effect. If no mortality was ascertained, then higher (50,300 and 2000 mg/kg) doses of extract were utilized for further toxicity studies.

Oral glucose tolerance test (OGTT)

The OGTT was performed in overnight fasted normal rats. Rats were divided into five groups (*n* = 6). Group I served as normal control (NC) and received distilled water (5 ml/ kg b.w.,
p.o.) and groups II, III, and IV received MEAE at the doses of 200, 500, and 800 mg/kg b.w., p.o., respectively. Group V received glibenclamide 1 mg/kg b.w. p.o. 30 min after these treatments, all groups received glucose (4 g/kgb.w.) orally. Blood was withdrawn from the tail vein just prior to and 30, 60, and 120 min after the oral glucose administration [12, 13]. Blood glucose levels were measured using glucose oxidase-peroxidase reactive strips and a portable glucometer (AccuSure blood glucose monitoring system).

**Induction of experimental diabetes mellitus**

The rats were rendered diabetic by a single intraperitoneal dose of 55 mg/kg b.w. STZ freshly dissolved in ice cold 0.1 M citrate buffer (pH 4.5). After 72 h, fasting blood glucose (FBG) levels were measured and only those animals showing blood glucose level ≥ 225 mg/dl were used for the subsequent investigation. The day on which hyperglycemia had been confirmed was designated as day 0[14, 15].

**Treatment schedule and estimation of FBG level**

Normal and hyperglycemic rats were divided into seven groups (n = 6) receiving the following treatment [16]:

Group I: Non-diabetic or normal control, received the vehicle (distilled water) 5 ml/kg b.w., p.o. (NC).

Group II: Non-diabetic control, received MEAE 500 mg/kg b.w., p.o.

Group III: Diabetic control, received the vehicle (distilled water) 5 ml/kg b.w., p.o. (DC).

Group IV: Diabetic treatment, received MEAE 200 mg/kg b.w., p.o.

Group V: Diabetic treatment, received MEAE 500 mg/kg b.w., p.o.

Group VI: Diabetic treatment, received MEAE 800 mg/kg b.w., p.o.

Group VII: Diabetic treatment, received glibenclamide 1 mg/kg b.w., p.o.

The above treatment was continued daily for 21 days. Fasting blood glucose concentrations were measured with a portable glucometer (AccuSure blood glucose monitoring system) at days 0, 7, 14, and 21.

**Body weight**

The body weights of rats of each group were recorded on 1st, 7th, and 15th day of MEAE treatment.

**Estimation of serum biochemical parameters**

After twenty one days treatment, blood samples were drawn from overnight fasted rats by retroorbital vein puncture technique from light-ether anesthetized animals. The nonheparinized blood was allowed to coagulate before being centrifuged (4000 rpm for 20 min) and the blood serum separated. Blood serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC), glycosylated hemoglobin (HbA1C), aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were estimated enzymatically using commercially available reagent kits (Erba Diagnostics and Span diagnostics Ltd.).

**Statistical analysis**

The data were expressed as mean ± standard error of mean (SEM). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s *post hoc* test of
significance using Graph Pad (Instat) software version 4.0. *P* values of < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Pharmacological treatment with of diabetes is based on oral hypoglycaemic agents and insulin. However, long term treatment with these drugs is expensive and poses life threatening adverse effects. Management of diabetes without any side effects continues to be a challenge to the medical community [17]. Despite considerable progress made in the conventional anti-diabetic management strategies, the search continues for plant-based products for the management of diabetes, which are deemed safe [18]. Phytotherapy has been extremely accepted worldwide in the health care system for DM [19].

Acute toxicity

The MEAE did not show any toxic effect or death up to the dose of 2000 mg/kg, b.w., p.o. in mice.

Oral glucose tolerance (OGTT)

Effects of the MEAE on glucose-loaded rats are shown in Table 1. Results of the OGTT strongly supported the improved ability of glucose tolerance with treatment of MEAE and glibenclamide. Among the groups, the concentrations of blood glucose baseline (0 min) were not considerably different. Though plasma glucose levels were increased after loading with glucose, animals treated with MEAE at 500 and 800 mg/kg showed slight increase when compared with the NC group at 30, 60, and 120 min during OGTT. Glibenclamide significantly blocked (*P* < 0.01) the rise in blood glucose levels after glucose administration at 120 min.

**Table 1:** Effect of methanol extract of *Andrographis Echioides* (MEAE) on oral glucose tolerance in normal rats.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control glucose (4 g/kg)</th>
<th>MEAE 200 mg/kg + glucose (4 g/kg)</th>
<th>MEAE 500 mg/kg + glucose (4 g/kg)</th>
<th>MEAE 800 mg/kg + glucose (4 g/kg)</th>
<th>Glibenclamide (1 mg/kg) + glucose (4 g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82.83±3.60</td>
<td>75.52±4.25</td>
<td>89.33±1.17</td>
<td>86.16±2.53</td>
<td>76.16±5.01</td>
</tr>
<tr>
<td>30</td>
<td>132.65±3.94</td>
<td>129.16±2.27</td>
<td>115.16±2.28</td>
<td>100.83±1.91</td>
<td>78.05±3.31</td>
</tr>
<tr>
<td>60</td>
<td>118.79±4.42</td>
<td>112.50±3.38¹</td>
<td>100.13±3.12</td>
<td>89.50±1.33</td>
<td>67.50±2.93</td>
</tr>
<tr>
<td>120</td>
<td>113.66±2.89</td>
<td>98.31±3.05²</td>
<td>93.27±1.43²</td>
<td>82.91±2.56²</td>
<td>65.29±4.05²</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (*n* = 6). ¹*P* < 0.05, ²*P* < 0.01 when compared with control group at corresponding time.

MEAE exhibited significant reduction in blood sugar in diabetic rats at the doses of 200, 500, and 800 mg/ kg. Though MEAE at 200 and 500 mg/kg reduced the hyperglycemia significantly as compared to the DC group, it did not restore the FBG level to that of the NC.
group, while with MEAE at 800 mg/kg the blood sugar levels of diabetic rats were reduced to NC group level. Blood glucose levels in normal rats treated with MEAE 500 at mg/kg were insignificant from that of the NC group, indicating that MEAE maintained glucose homeostasis. The hypoglycaemic action of MEAE could also be because of promotion of insulin release from existing β cells of the islets of Langerhans. The plasma glucose lowering activity was compared with that of glibenclamide, the reference oral hypoglycemic that has been used for several years to treat diabetes, to stimulate pancreatic β cells [20].

**Table 2**: Effect of methanol extract of *Andrographis Echioides* (MEAE) on fasting blood glucose levels in normal and streptozotocin (STZ) - induced diabetic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fasting blood glucose level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Normal control</td>
<td>80.43±4.30a</td>
</tr>
<tr>
<td>NC+MEAE (500 mg/kg)</td>
<td>93.16±5.12a</td>
</tr>
<tr>
<td>STZ control (55 mg/kg)</td>
<td>365.80±8.36b</td>
</tr>
<tr>
<td>STZ+MEAE (200 mg/kg)</td>
<td>311.23±22.8b</td>
</tr>
<tr>
<td>STZ+MEAE (500 mg/kg)</td>
<td>422.16±22.3b</td>
</tr>
<tr>
<td>STZ+MEAE (800 mg/kg)</td>
<td>425.66±22.7b</td>
</tr>
<tr>
<td>STZ+Glibenclamide (1 mg/kg)</td>
<td>375.18±16.1b</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6). *P < 0.01 when compared with STZ control group; bP < 0.01 when compared with normal control group.

**FBG levels**

Fasting blood glucose levels measured in normal and STZ-induced diabetic rats after a single day and at the end of 7, 14, and 21 days of treatment are given in Table 2. Here, diabetic rats had a significant effect on blood sugar response after treat for 21 days. NC rats did not show any significant variation in the blood sugar throughout the experimental period.

Administration of STZ (55 mg/ kg, i.p.) led to several fold elevation of blood glucose levels relative to that of the NC group, indicating stable hyperglycemia during the experimental period. FBG level of normal animals treated with MEAE at 500 mg/ kg (Group II) did not vary significantly from that of the NC group. Although MEAE at 200 and 500 mg/ kg reduced hyperglycemia significantly (P < 0.01) as compared to the diabetic control (DC) group, it failed to restore the level to that of the NC group; while MEAE at 800 mg/ kg or glibenclamide (1 mg/kg) significantly (P < 0.01) reduced the blood glucose levels close to NC group level.

**Effect on body weight**

The effect of MEAE on body weight of normal and diabetic animals is presented in Table 3. NC animals were found to be stable in their body weight but diabetic rats showed significant reduction in body weight during 21 days. STZ caused body weight reduction, which was significantly (P < 0.01) reversed by MEAE treatment.
Table 3: Effect of methanol extract of *Andrographis Echioides* (MEAE) on body weight in normal and streptozotocin (STZ)-induced diabetic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 day</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>162.16±1.66</td>
<td>175.66±3.57</td>
<td>184.00±2.30</td>
<td>193.17±2.98</td>
</tr>
<tr>
<td>NC + MEAE (500 mg/kg)</td>
<td>153.83±2.89</td>
<td>180.00±2.85</td>
<td>188.83±1.30</td>
<td>195.16±2.54</td>
</tr>
<tr>
<td>STZ control (55 mg/kg)</td>
<td>158.33±1.77</td>
<td>149.50±1.68</td>
<td>136.87±2.08</td>
<td>122.00±1.71</td>
</tr>
<tr>
<td>STZ + MEAE (200 mg/kg)</td>
<td>150.82±3.15</td>
<td>158.16±1.40</td>
<td>167.12±2.67</td>
<td>179.33±1.94</td>
</tr>
<tr>
<td>STZ + MEAE (500 mg/kg)</td>
<td>159.87±2.90</td>
<td>167.33±1.87</td>
<td>175.16±2.15</td>
<td>185.71±2.67</td>
</tr>
<tr>
<td>STZ + MEAE (800 mg/kg)</td>
<td>156.28±3.20</td>
<td>168.29±1.85</td>
<td>180.27±1.56</td>
<td>190.56±2.58</td>
</tr>
<tr>
<td>STZ+Glibenclamide (1 mg/kg)</td>
<td>160.50±3.01</td>
<td>175.50±1.61</td>
<td>183.61±2.78</td>
<td>192.16±1.13</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).<sup>a</sup>P < 0.01 when compared with STZ control group; <sup>b</sup>P < 0.05 and <sup>c</sup>P < 0.01 when compared with normal control group.

Diabetic rats treated with the MEAE showed significant improvement in body weight as compared to the STZ control animals; thus, MEAE exhibited marked effect in controlling the loss of body weights of diabetic rats. Oral administration of MEAE decreased the level of HbA1C. Lower levels of total haemoglobin observed in diabetic rats may be because of the exaggerated formation of HbA1C. Glycohemoglobin is made throughout the circulatory life of red blood cells (RBCs) by the addition of glucose to the N-terminal of the haemoglobin β chain. This process, which is nonenzymatic, reflects the average exposure of haemoglobin to glucose over an extended period.

**Serum biochemical parameters**

Results of biochemical parameters are represented in Table 4. MEAE had a significant (<0.01) effect in lowering HbA1C. After 21 days, the effect of MEAE on groups II, VI, and VII was not significant as compared to the NC group. Treatment with MEAE at 500 and 800 mg/kg and glibenclamide (1 mg/kg) decreased HbA1C significantly (<0.01) in the diabetic rats. There was a significant (<0.01) decrease in the level of serum HDL-cholesterol and significant (<0.01) increase in the levels of TC, LDLC, and TGs in diabetic rats when compared to NC rats. Administration of MEAE at 500 and 800 mg/kg and glibenclamide (1 mg/kg) significantly (<0.01) brought their levels toward normal. The activities of serum enzymes AST, ALT, and ALP were found to be significantly (<0.01) increased in diabetic rats compared to normal rats. Oral administration of MEAE at 500 and 800 mg/kg and glibenclamide at 1 mg/kg for 21 days significantly (<0.01) normalized the enzymatic activities in diabetic rats.

Lipids play a significant role in the pathogenesis of DM. It is documented that in uncontrolled diabetes mellitus, there is a rise in TC in blood, which can contribute to coronary artery diseases [23]. The foremost common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia. In this study, elevated levels of serum lipids such as TC, LDLC, and TGs were found in diabetic rats. STZ produced various cardinal symptoms of diabetes mellitus including hypoinsulinemia, a condition that is probably responsible for the elevation.
of serum cholesterol levels because the insulin has an inhibitory action on HMG-CoA reductase, a key enzyme that acts as rate limiting in the metabolism of cholesterol rich LDL particles [24].

Table 4: Effect of methanol extract of Andrographis Echioides (MEAE) on serum biochemical parameters in normal and streptozotocin (STZ)-induced diabetic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GH (%)</th>
<th>TG (mg/dl)</th>
<th>TC (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>4.42</td>
<td>±0.29b</td>
<td>50.98</td>
<td>74.49</td>
<td>29.92</td>
<td>34.32</td>
<td>52.54</td>
<td>24.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±2.83b</td>
<td>±2.86b</td>
<td>±1.65b</td>
<td>±2.40b</td>
<td>±2.36b</td>
<td>±1.57b</td>
<td>±2.33b</td>
</tr>
<tr>
<td>NC + MEAE (500 mg/kg)</td>
<td>3.75</td>
<td>±0.12b</td>
<td>40.36</td>
<td>69.6</td>
<td>32.98</td>
<td>31.29</td>
<td>49.31</td>
<td>22.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±1.29b</td>
<td>±3.72b</td>
<td>±1.39b</td>
<td>±1.77b</td>
<td>±1.88b</td>
<td>±1.23b</td>
<td>±2.06b</td>
</tr>
<tr>
<td>STZ control (55 mg/kg)</td>
<td>11.34</td>
<td>±0.47d</td>
<td>95.22</td>
<td>178.17</td>
<td>18.85</td>
<td>139.9</td>
<td>86.8</td>
<td>61.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±3.18d</td>
<td>±3.81d</td>
<td>±1.04d</td>
<td>±2.02d</td>
<td>±2.26d</td>
<td>±2.56d</td>
<td>±2.64d</td>
</tr>
<tr>
<td>STZ + MEAE (200 mg/kg)</td>
<td>9.96</td>
<td>±0.49d</td>
<td>86.07</td>
<td>120.06</td>
<td>23.21</td>
<td>78.79</td>
<td>80.46</td>
<td>51.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±2.57d</td>
<td>±4.37bd</td>
<td>±2.11</td>
<td>±1.77bd</td>
<td>±1.95d</td>
<td>±1.94bd</td>
<td>±1.09d</td>
</tr>
<tr>
<td>STZ + MEAE (500 mg/kg)</td>
<td>6.66</td>
<td>±0.35bd</td>
<td>63.15</td>
<td>103.59</td>
<td>26.48</td>
<td>64.20</td>
<td>65.63</td>
<td>42.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±4.03bd</td>
<td>±3.51bd</td>
<td>±2.14a</td>
<td>±2.07bd</td>
<td>±2.32bd</td>
<td>±1.88bd</td>
<td>±1.74bd</td>
</tr>
<tr>
<td>STZ + MEAE (800 mg/kg)</td>
<td>4.77</td>
<td>±0.42b</td>
<td>48.72</td>
<td>90.94</td>
<td>30.33</td>
<td>49.74</td>
<td>55.09</td>
<td>30.03</td>
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<td></td>
<td></td>
<td>±1.55b</td>
<td>±3.06b</td>
<td>±2.46b</td>
<td>±2.80bd</td>
<td>±2.75b</td>
<td>±2.25b</td>
<td>±2.23bd</td>
</tr>
<tr>
<td>STZ + Glibenclamide (1 mg/kg)</td>
<td>3.42</td>
<td>±0.17b</td>
<td>38.95</td>
<td>85.48</td>
<td>30.14</td>
<td>45.82</td>
<td>58.12</td>
<td>27.27</td>
</tr>
<tr>
<td></td>
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<td>±1.33bc</td>
<td>±2.45b</td>
<td>±2.95b</td>
<td>±2.77bd</td>
<td>±2.33b</td>
<td>±1.73b</td>
<td>±1.44bd</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6). *P < 0.05 and bP < 0.01 when compared with STZ control group; *P < 0.05 and bdP < 0.01 when compared with normal control group.

In insulin-deficient diabetes, the concentration of serum fatty acids is elevated as a result of free fatty acid out flow from fat depots, wherever the balance of the free fatty acid esterification-TG lipolysis cycle is displaced in favour of lipolysis. High-density lipoprotein (HDL) is an antiatherogenic lipoprotein. It transports cholesterol from peripheral tissues into the liver and thereby acts as a protective factor against coronary cardiovascular disease. The amount of HDLC, which increased after MEAE administration, may be due to the increase in the activity of lecithin cholesterol acyl transferase (LCAT), which can contribute to the regulation of blood lipids [25]. Oral administration of MEAE reduced the elevated serum lipids such as TC, LDLC, and TGs toward normal in diabetic rats. Elevation of serum biomarker enzymes such as SGOT, SGPT, and SALP was observed in diabetic rats indicating impaired liver function, which was obviously due to hepatocellular necrosis. It has been reported that liver necrosis occurred in STZ-induced diabetic rats [26]. Therefore, increase in the activities of AST, ALT, and ALP gives an indication on the hepatotoxic effect of STZ. Twenty-one days of treatment with MEAE restored all the above-mentioned serum hepatic
biochemical parameters toward the normal values in a dose-dependent manner, thereby alleviating liver injury caused by STZ-induced diabetes.

CONCLUSION
In this study, administration of MEAE to STZ-induced hyperglycaemic rats incontestable distinguished reduction in blood glucose level, standardization of serum biochemical profiles including lipid contents, comparing to STZ control rats. Therefore, it can be concluded that the MEAE is remarkably effective against STZ-induced diabetes in Wistar rats thereby validating its ethnomedicinal usage. From the ascertained oral hypoglycemic activity of MEAE in STZ-induced diabetic rats, it can be further inferred that *Andrographis echioides* will function a motivating candidate in complementary and alternative medicine for the effective management of diabetes.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES