INTRODUCTION

The plant kingdom holds many species of plant containing substances of medicinal values. Traditional medicine is the backbone of primary health care and act as an important global item due to the demand. Safety, efficacy and fewer side effects are the reasons increased of the demand [1, 2]. L. microphyllum are classified as pteridophytes and estimated about 10,500 to 11,300 species have been described and recorded in the tropical rain forest of Malaysia [3]. L. microphyllum was used as a traditional medicine with a different mode of uses. It is the one of the plant potentially useful in traditional medicine practice in the management of diabetes, but the scientific basis for this action has not yet been explored. Diabetes mellitus (DM) is a chronic endocrine disorder involving most common metabolic disorders of carbohydrate, fat, and protein [4, 5]. In Malaysia, Type II DM is the most prevalent and became a growing problem. The Fourth Malaysian National Health and Morbidity Survey (NHMS IV, 2011) reported that the increased to 20.8% (10.7% known and 10.1% undiagnosed) compared to the Third Malaysian National Health and Morbidity Survey (NHMS III, 2006) which is 14.9% from the population [6]. DM management is very costly because Malaysia Ministry of Health (MOH) spent a calculated amount of RM386, 531.21 as RM2, 684.24 direct costs per patient for a 6-month period [7]. Nowadays, DM becomes a major and much bigger problem in Malaysia. Satisfactory of DM care of patients still not achieving the clinical goal and the rate of complications being still high [8]. Therefore, treatments or management of DM is very important. Plant and plant products played an important role in DM treatment and had been used throughout the world since ancient times. Folk and traditional medicine healing system have been used globally as an antioxidant, antihyperglycemic, and anti diabetic properties from plants.

In the laboratory, experimental DM is commonly induced by using alloxan monohydrate (ALX) or streptozotocin (STZ). Alloxan is a β-cyto toxic glucose analogue [9]. The chemical is selectively destroyed the pancreatic beta cells through the reactive oxygen species (ROS) production. Damage of the cells is the results of L. microphyllum, the antihyperglycemic effect of L. microphyllum is still lacking. Therefore, present study was aimed at evaluating the effect of aqueous extract of L. microphyllum as an antihyperglycemic and antioxidative by determining the fasting blood glucose, biochemical assays and the histopathological effect on the alloxan-induced diabetic rat pancreas.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): 1,2-Dithio-bis-nitro benzoic acid (DTNB), alloxan monohydrate, 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (BSA), disodium hydrogen phosphate dehydrate (NaHPO₄), ethylene diamine tetraacetic acid (EDTA), folin-ciocalteu reagent (FCR), glutathione (oxidized form, GSSG), glutathione reductase, hydrogen peroxide (H₂O₂), reduced glutathione (GSH), sodium dihydrogen phosphate (NaH₂PO₄), sodium hydroxide (NaOH), sulfosalicylic acid (SSA), thio barbituric acid (TBA), trichloroacetic acid (TCA) and β-Nicotinamide adenine dinucleotide phosphate reduced (NADPH). All other solvents and chemicals used were either of analytical grade or of the highest purity commercially available.

Preparation of plant extract

Fresh leaves of L. microphyllum (voucher number: Syahidah 001) were collected locally at Universiti Malaysia Sabah (UMS), Kota Kinabalu, Sabah, Malaysia identified and authenticated by Mr. Johnny Gisil (botanist), Biology Tropical and Conservation Institute, Universiti Malaysia Sabah. Voucher specimens have been submitted to the universiti herbarium. Leaves were washed with tap water to remove unwanted dirt or any other contaminant. The leaves...
related to a drying method for up to 14 d until no moisture left. Then, the dried leaves were grind using heavy duty blender to coarse smaller samples. Briefly, to prepare the extract, the coarse smaller sample was boiled in 1:10 volume of water in 10 min [11]. After 10 min, the mixture stops heating and cooling at room temperature for 1 h. Then, the products filter using Whatman No. 1 filter paper. Filtered product frozen at -80°C for three days and freeze-dried for also three days. The extract ready to use in the process of animal treatment.

Animals and treatment

The study protocol was approved by the Animal Ethics Committee (AEC) of the University Malaysia Sabah reference number AEC-01/2015. Adult Sprague-Dawley male rats (8-12 w old), weighing 120-150 g were purchased from the animal house facility of Animal Health Campus, Universiti Sains Malaysia. In this experiment, 36 healthy male Sprague Dawley rats were divided into 6 groups (n = 6) and kept under standard laboratory environmental conditions (25 °C, 12 h light/12 h dark cycle). A standard pelit diet and water were available ad libitum throughout the experimental period. After 6 h to 8 h fasting, four groups of rats were injected intravenously with alloxan monohydrate freshly dissolved in normal saline at dose 100 mg/kg of body weight i. v. whereas the control animals received a normal saline intravenous injection. Diabetes was induced in groups II, IV, V, and VI. Group I and group III served as the normal control groups. Three days after treatment, rats with diabetes (blood glucose level>7.0 mmol/l) were used for the experiment. Group treatments as shown below:

Group I: Normal control rats received normal saline
Group II: Diabetic control rats alloxan treated with normal saline
Group III: Normal control rats treated with plant extract 400 mg/kg of body weight
Group IV: Diabetic control rats alloxan treated with plant extract 400 mg/kg of body weight
Group V: Diabetic control rats alloxan treated with plant extract 200 mg/kg of body weight
Group VI: Diabetic control rats alloxan treated with plant extract 100 mg/kg of body weight

The rats have received treatment daily via orogastric intubation for 14 d. Blood glucose level was measured using a glucometer machine on the pre and post (day-3, 0th, 7th, and 14th) of the treatments. Blood samples were taken from the tail by puncturing the caudal vein using a sterile needle. Blood drop was put in contact with the glucometer machine to measure the blood glucose level.

After 14 d of treatment (day 15), the animals were anesthetized using diethyl ether. The technique of cervical dislocation was performed after the animals were deeply anesthetized. The whole pancreas carefully dissected out. Pancreas was removed quickly and cleaned free from extraneous materials. Ice cold saline (0.85% w/v, sodium chloride) was used to perfuse the pancreas. Then, it was dipped in 0.1 M, pH 7.4 phosphate buffer. The pancreas cut into a portion for biochemical analysis and another portion for histopathological investigation. The portion for biochemical analysis was weighed and stored at -80°C, while the balance for histopathological investigations was fixed in 10% buffered formalin. Before PMS were prepared, rat’s pancreas rinsed twice with phosphate buffer saline, PBS (0.1 M, pH 7.4). The pancreases were homogenized in 1:10 PBS (0.1 M, pH 7.4) with KCl (1.17% w/v, potassium chloride) using a homogenizer. The homogenate was centrifuged at 1,200 × g for 10 min at 4°C. The supernatant transferred to clean centrifuge tubes and the pellet was discarded. The resulting supernatant (aliquot) centrifuged at 10,000 × g for 10 min at 4°C. The PMS obtained were kept at -80°C for further analysis.

Biochemical assays

Reduced glutathione was measured by the method of follow et al. [12]. The assay for LPO was determined by the method of Buenger and Aust [13]. Glutathione reductase was determined according to the method of Carlberg and Mannervik [14]. Glutathione-S-transferase activity was measured by the method of Habig et al. [15]. Catalase activity was assayed by the method of Clairolne [16]. Protein determination was carried out according to the method of Lowry et al. [17].

Histopathological assessment

The excised pancreas tissues were processed for light microscopy study to substantiate the biochemical findings and to ascertain the cause of pancreatic cell injury. The pancreas tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin blocks, sectioned, trimmed into 5-6 µm in thickness sections in a rotator microtome and stained with hematoxylin and eosin (H and E). Section was examined by a pathologist who had no prior knowledge of the sample assignment to experimental groups for pathological symptoms of pancreatic toxicity.

Statistical analysis

The data were expressed as mean±SEM of three independent experiments. Significant differences between groups mean were analyzed using one-way analysis of variance (ANOVA). A result of P<0.05 is regarded as statistically as significant. All the statistics were performed using SPSS statistical analysis software.

RESULTS

Effect of L. microphyllum aqueous extract on body weight

Animal body weights were determined at day-3 for pre-treatment, 0th, 7th, and 14th. The result was shown in the table 1. There are 6 groups and 6 experimental rats in each group. Based on the data, the normal control group has maintained their weight with a slightly increment in day-3, 7th, and 14th. While the normal rats treated with plant extract gained their body weight in the 0th day but to some extend days, it was decreased. All the alloxan treated with aqueous plant extract groups showed weight loss compared to their corresponding control except the alloxan treated with plant extract 400 mg/kg dose.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>0 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>244.1±13.31</td>
<td>251.6±14.12</td>
<td>241.3±10.74</td>
<td></td>
</tr>
<tr>
<td>Alloxan treated with normal saline</td>
<td>251.6±14.12</td>
<td>273.1±16.20**</td>
<td>251.6±14.12</td>
<td></td>
</tr>
<tr>
<td>Plant extract 400 mg/kg body weight</td>
<td>219.5±7.97**</td>
<td>223.8±12.00**</td>
<td>219.3±7.27**</td>
<td></td>
</tr>
<tr>
<td>Alloxan treated with plant extract 400 mg/kg body weight</td>
<td>223.8±12.00**</td>
<td>223.8±12.00**</td>
<td>223.8±12.00**</td>
<td></td>
</tr>
<tr>
<td>Alloxan treated with plant extract 200 mg/kg body weight</td>
<td>231.2±29.45**</td>
<td>223.9±26.69**</td>
<td>223.8±12.00**</td>
<td></td>
</tr>
<tr>
<td>Alloxan treated with plant extract 100 mg/kg body weight</td>
<td>273.1±16.20**</td>
<td>223.8±12.00**</td>
<td>223.8±12.00**</td>
<td></td>
</tr>
</tbody>
</table>

Data represented as mean±SEM. *Significant value at P<0.05 compared normal saline, **Significant value at P<0.05 compared to the alloxan treated with normal saline

Table 1: Effect of L. microphyllum aqueous extract on body weight

Effect of L. microphyllum aqueous extract on fasting blood glucose levels

Fasting Blood Glucose (FBG): table 2 shows the effect of L. microphyllum aqueous extract on FBG of experimental animals.

There was a significant increase in FBG level in alloxan-induced diabetic rats (P<0.05), compared to the treatment groups.

The extract proved to be effective in lowering FBG levels as the reading significantly lowered over time.
study. Alloxan induced diabetes caused a marked decrease in the oxidative stress, which is consistent with the results obtained in this study. Diabetes is closely associated with reactive oxygen species and LPO, GR, GST, and CAT. 

Data represented as mean±SEM, *Significant value at P<0.05 compared normal saline, **Significant value at P<0.05 compared to the alloxan treated group.

Table 2: Effect of *L. microphyllum* aqueous extract on fasting blood glucose (FBG)

<table>
<thead>
<tr>
<th>Treatment (n = 6)</th>
<th>Fasting blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day-3</td>
</tr>
<tr>
<td>I Normal saline</td>
<td>5.0±0.65</td>
</tr>
<tr>
<td>II Alloxan treated with normal saline</td>
<td>3.9±0.05*</td>
</tr>
<tr>
<td>III Plant extract 400 mg/kg of body weight</td>
<td>4.6±0.43**</td>
</tr>
<tr>
<td>IV Alloxan treated with plant extract 400 mg/kg of body weight</td>
<td>2.8±0.46**</td>
</tr>
<tr>
<td>V Alloxan treated with plant extract 200 mg/kg of body weight</td>
<td>4.4±0.80**</td>
</tr>
<tr>
<td>VI Alloxan treated with plant extract 100 mg/kg of body weight</td>
<td>3.4±0.64**</td>
</tr>
</tbody>
</table>

Data represented as mean±SEM, *Significant value at P<0.05 compared normal saline, **Significant value at P<0.05 compared to the alloxan treated group.

Effect of *L. microphyllum* aqueous extract on pancreatic GSH, LPO, GR, GST, and CAT

Diabetes is closely associated with reactive oxygen species and oxidative stress, which is consistent with the results obtained in this study. Alloxan induced diabetes caused a marked decrease in the antioxidant enzyme activities (GSH, GR, GST, and CAT) of the diabetic rats. Furthermore, the MDA level in the diabetic rats was slightly increased in comparison to the normal control group. In contrast, *L. microphyllum* aqueous extract significantly increased the activities of the antioxidant enzymes and decreased the pancreatic levels of MDA in the treated diabetic rats as summarized in the table 3.

Table 3: Effect of *L. microphyllum* aqueous extract on pancreatic GSH, LPO, GR, GST, and CAT

<table>
<thead>
<tr>
<th>Treatment (n = 6)</th>
<th>Biochemical assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH (µmol reduced GSH/g tissue)</td>
</tr>
<tr>
<td>I Normal saline</td>
<td>11.1±0.53</td>
</tr>
<tr>
<td>II Alloxan treated with normal saline</td>
<td>6.7±0.54*</td>
</tr>
<tr>
<td>III Plant extract 400 mg/kg of body weight</td>
<td>10.8±3.05**</td>
</tr>
<tr>
<td>IV Alloxan treated with plant extract 400 mg/kg of body weight</td>
<td>10.7±2.08**</td>
</tr>
<tr>
<td>V Alloxan treated with plant extract 200 mg/kg of body weight</td>
<td>9.3±0.59**</td>
</tr>
<tr>
<td>VI Alloxan treated with plant extract 100 mg/kg of body weight</td>
<td>7.2±0.50**</td>
</tr>
</tbody>
</table>

Data represented as mean±SEM, *Significant value at P<0.05 compared normal saline, **Significant value at P<0.05 compared to the alloxan treated group.

Fig. 1-6: Histopathological changes occurred in rats by the pretreatment with aqueous extract of *L. microphyllum* (I: Islet of Langerhans, BV: Blood vessel)

Effect of *L. microphyllum* aqueous extract on pancreatic histopathological findings

Assessment of histopathological alteration of H and E staining pancreatic tissue sections were observed to support the biochemical studies and demonstrate the morphological changes between the control group and group treated with plant extract with different doses. The histopathology of rat pancreas from the different experimental group was shown in fig. 1-6. The pancreas section of normal rats treated with normal saline microscopic investigation.
showed normal cellular architecture which means that the normal appearance of the islet of Langerhans. The acinar cells are formed of pyramidal cells as shown in fig. 1. The islet cells are seen interspersed between the acinar cells. The islet appeared lightly stained than the surrounding acinar cells. However, fig. 2 the diabetic rats treated with normal saline showed pathomorphological changes. The islet displayed marked atrophy. No significant lesion in the exocrine tissue was observed. The group of normal rat treated with plant extract does not show any changes (fig. 3). The islet is similar to the normal cells. On the other hand, the diabetic group treated with plant aqueous extract 400 mg/kg and 200 mg/kg (fig. 4 and 5) showed that the pancreas has a depicted evidence of cellular regeneration among the islet of Langerhans. No significant changes in cells architecture compared to the normal control group. B-cells were evenly distributed. Since a dose of 100 mg/kg is the lowest dose, it shows mild atrophy of the islet of Langerhans (fig. 6).

DISCUSSION

This present study focused on the evaluation of the anti-hyperglycemic and antioxidative effects of *L. microphyllum* aqueous extract in alloxan-induced diabetic rats. Diabetes which is synonymous to hyperglycemia as a result of insulin deficiency or insensitivity of the body to insulin or both. The defect in the metabolism and energy regulation of the body due to the insulin resistance or deficit leading to high blood sugar levels is the main cause of various complications developed in diabetes. Alloxan induced diabetes resulting in a decrease in endogenous insulin release. This happened as the damage of insulin-secreting pancreatic beta cells. The destruction of beta cells of the islets of Langerhans inducing hyperglycemia. Reduction in FBC levels in rats treated with the plant extract was observed and indicates that the plant has antidiabetic and antihyperglycemic potentials. It could improve the utilization of glucose in alloxan-induced diabetic rats. The plant extract is also possibly protecting the cells by preventing the decrement of insulin secretion and competing for glucose associated receptors on beta cells [20, 21]. The results of the study indicate that aqueous extract of *L. microphyllum* has anti-hyperglycaemic, antioxidative, and tissue protective properties.

Diabetes is marked by the increased production of free radicals or impaired antioxidant defenses [19]. Glucose oxidation and dismutation to hydrogen peroxide were a generation of superoxide anion radicals. This was leads to the formation of reactive hydroxyl radicals. In the present study, LPO levels were increased and reduced in GSH, GST, GR, and CAT levels in alloxan-induced diabetic rats compared to the control group. The changes might be due to the glucose oxidation and formation of free radical generation. Administration of the plant extract significantly showed a positive result on the levels of LPO, GSH, GR, GST, and CAT levels in alloxan-induced diabetic rats compared to the control group. The plant extracts functionally restoring the altered antioxidant enzymes in alloxan-induced diabetic rats. This indicates that the plant has its free radical scavenging potential.

Beta cells were responsible for producing insulin, and the depletion of beta cells will cause insulin deficiency and hyperglycemia. In this study, the positive control group showed normal structure while the negative control diabetic group showed destruction which obvious atrophy is seen. Reduction in size could alter the release of insulin and hinder glucose uptake [20, 21]. The groups administered with the plant extract showed the normal cell architecture and no atrophy. This may be caused by the regeneration and rejuvenation of beta cells in the pancreas. The results obtained from this study revealed the effectiveness of the plant extract in pancreatic cell damage.

CONCLUSION

The aqueous extract of *L. microphyllum* showed a decrease in fasting blood glucose level and effectively counter back the reaction of alloxan-induced changes in the beta cell population of the pancreas. The extract also showed in vivo efficacy against the mediated manifestation of oxidative stress in the pancreas. Significant restoration in non-enzymatic (GSH) and enzymatic (LPO, GR, GST, CAT) antioxidant was clearly seen in a dose-dependent manner. In the light of the above experimental results, the plant may become a potential anti-diabetic drugs derivative. However, further investigation is necessary with respect to the toxicological evaluation of this plant.

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AUTHORS CONTRIBUTIONS

Dg Syahidah Nadiah binti Abdull Majid-Conducted the experiment and prepared the manuscript. Dr. Mohamad Iqbal-Helped in designing and conducting the experiment.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

REFERENCES


