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ANTIDIABETIC EFFECTS OF KNEMA GLAUCA LEAF EXTRACT TOWARD INHIBITIONS OF A-AMYLASE AND A-GLUCOSIDASE ASSAYS

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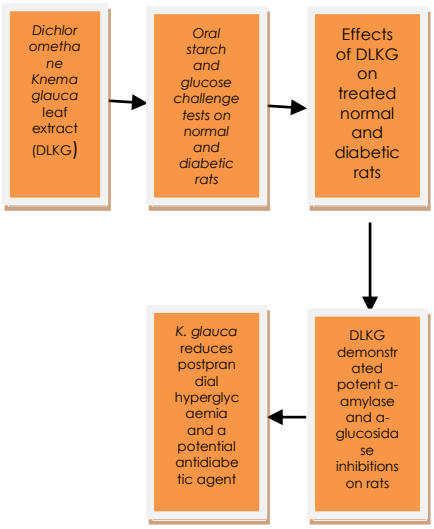
Graphical abstract



Abstract

This study was part of our preliminary work in the search for biologically active compounds from endemic plants of Kuala Keniam National Park, Pahang, Malaysia. Postprandial hyperglycaemia is linked to the development of diabetes complications such as micro and macro vascular diseases. Inhibitions of carbohydrate digestive key enzymes, a-amylase and a-glucosidase using plant extracts are possible therapeutic strategies to suppress postprandial hyperglycaemia. The aim of this study was to investigate the effects of the dichloromethane leaf extract of *Knema glauca* (DLKG) *in vitro* and *in vivo* a-amylase and a-glucosidase inhibitory activities. The antidiabetic property of DLKG was investigated by using *in vitro* a-amylase and a-glucosidase inhibitory activity assays. The effects of DLKG on oral starch and sucrose challenge tests were investigated by administering 125, 250 and 500 mg/kg of the extract to normal and diabetic rats. DLKG demonstrated potent a-amylase and a-glucosidase inhibitory activities with IC_{50} values of 1.26 and 4.09 µg/ml, respectively. In the oral starch challenge test, the extract significantly ($p<0.05$) reduced blood glucose levels in both normal and diabetic rats. On the other hand, DLKG showed significant ($p<0.05$) reduction of blood glucose levels in normal rats in the oral sucrose challenge test. The results showed that the leaves of *K. glauca* possessed a beneficial effect in reducing postprandial hyperglycaemia and have potential as an alternative antidiabetic agent.

Keywords: *Postprandial hyperglycaemia, Knema glauca leaf,* a-amylase, a-glucosidase

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1.0 INTRODUCTION

Uncontrolled consumption of dietary carbohydrates is the major source of glucose that may contribute to the most common metabolic disorder of diabetes *mellitus* worldwide which is characterized by defects in insulin production and/ or action. The International Diabetes Federation (IDF) estimates more than 371 million people around the world have diabetes in 2012 and the number of people with diabetes is increasing in every country. A typical symptom of this metabolic disorder is postprandial hyperglycaemia

which is associated to diabetes complications such as diabetic neuropathy, retinopathy, nephropathy and cardiovascular diseases [1]. Therefore, by controlling postprandial hyperglycaemia may help in the management of diabetes *mellitus* and its complications.

One of the strategies to treat postprandial hyperglycaemia involves inhibition of the carbohydrate-digesting key enzymes, a-amylase and a-glucosidase. a-Amylase which is present in saliva and pancreatic juice acts as a catalyst in the breakdown of polysaccharides to disaccharides [2]. The a-glucosidase that can be found in the brush-border surface membrane of intestinal cells functions in the hydrolysis of a disaccharide to the monosaccharide, glucose [3]. Inhibition of these key enzymes may delay the absorption of glucose resulting in the suppression of postprandial hyperglycaemia [4]. Examples of commercial drugs that were used as a-amylase and a-glucosidase inhibitors are acarbose, miglitol and voglibose [5]. However, these modern drugs have undesired side effects such as flatulence, diarrhea and abdominal cramping [6]. Alternative antidiabetic medicines from plant extracts involve in retarding a-amylase and a-glucosidase activities have been widely reported as an attractive strategy to reduce postprandial hyperglycaemia. For example, *Anthocleista djalonensis* shows strong *in vitro* a-amylase inhibitory activity with percentage inhibitions ranging from 27.5 to 73.7% and suppresses blood glucose levels in alloxan-induced diabetic rats [7]. *Garcinia mangostana* shows potent *in vitro* a-glucosidase inhibitory activity with IC_{50} value of 3.2 µg/ml and effectively reduces postprandial blood in steptozotocin-induced diabetic rats [8]. Phenolic compounds from the seed coats of *Eleusine coracana* L. show strong inhibition against *in vitro* a-glucosidase and pancreatic amylase with IC_{50} values of 16.9 and 23.5µg, respectively [9].

2.0 MATERIALS AND METHODS

2.1 Plant Materials

Knema glauca was collected at Sungai Keniam National Park, Pahang, Malaysia. The plant was identified by Mr. Shamsul Khamis, the resident taxonomist of the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia. The leaves were cut into small pieces and dried for 1 week at room temperature. The dried leaves were ground finely using a milling machine with pore size 10 mm.

2.2 Preparation of Sample

The sample was soaked in dichloromethane at room temperature for 72 hours. The extract was filtered through a porous plug of absorbable cotton and evaporated with a rotary evaporator at 40°C. The DLKG yield was around 1.9%.

2.3 a-Amylase and a-glucosidase Inhibitory Activity Assays

The assay procedures were performed according to the methods described in earlier works with modifications [11], [12]. The DLKG were weighed and prepared for two-fold serial dilutions at concentrations ranging from 6.25 to 100 µg/ml in dimethylsulfoxide. For the a-amylase inhibitory activity assay, 10 µl of extract, 40 µl of distilled water and 50 µl of porcine pancreatic a-amylase enzyme solution (4 U/ml in cold water) were pre-incubated at 25°C for 5 minutes. (For control, the plant extract was replaced with distilled water and for positive control, the extract was replaced with acarbose. For blank, the enzyme solution was replaced with distilled water). Then, 100 µl of starch soluble ACS reagent (0.5% w/v in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride at pH 6.9) was added into the mixtures to initiate the reaction and incubated at 25°C for 7 minutes. After incubation, 100 µl of dinitrosalicylic acid reagent (96 mM 3,5-dinitrosalicylic acid and 5.31 M sodium potassium tartrate in 2 M NaOH) was added to quench the reaction. The plate was placed in a water bath at 85°C for 15 minutes. The absorbance of the mixture was measured at a wavelength of 540 nm. For the a-glucoside inhibitory activity assay, a 10 µl of extract, 20 µl a-glucosidase enzyme type 1 from Baker's yeast (0.125 U/ml in cold phosphate buffer saline at pH 6.5), 40 µl buffer at pH 6.5 and 20 µl distilled water were pre-incubated at 37°C for 10 min. After pre-incubation, 10 µl of 20 mM p-nitrophenyl-a-D-glucopyranoside dissolved in a buffer were added to initiate reaction. The absorbance of the reaction mixtures were measured immediately at wavelength 405 nm and incubated at 37°C for 30 minutes. After incubation, the absorbance was measured again. The final control ($A^{control}$) and test sample ($A^{test\ sample}$) absorbance values were obtained by subtracting their corresponding sample blank readings ($A^{reaction} - A^{blank}$). The percentage inhibitions of the a-amylase and a-glucoside inhibitory activities were calculated as

$$(A^{control} - A^{test\ sample}) / A^{control} \times 100$$

Where A is the absorbance

2.4 Oral Starch and Sucrose Challenge Tests in Normal and Diabetic Rats

Male 8 to 9 weeks old *Sprague Dawley* rats weighing from 180 to 250 g were used in this study. Diabetes was induced in the rats by a single intraperitoneal injection of streptozotocin at a dose of 55 mg/kg body weight dissolved in sterile distilled water. Diabetes was confirmed in the rats by measuring the fasting blood glucose concentration after 120 hours

post injection using Accu-Chek® Advantage–II Glucose meter. The rats with blood glucose level ranging from 14.5 to 20.5 mmol/l were used in this experiment.

This experiment was carried out separately in normal and diabetic rats. Rats were fasted overnight with free excess to tap water. The rats were equally divided into 5 groups of treatment with each group consisting of 6 rats. The blood samples were collected from the tail vein to measure blood glucose levels at 0 time before treatment. Group 1 rats served as untreated control and were orally administered with 5 % (v/v) Tween-80 solution (vehicle) at a dose of 10 ml/kg body weight. Group 2 rats which served as positive control were orally administered acarbose tablet at a dose of 10 mg/kg body weight [13]. Groups 3, 4 and 5 rats were orally administered DLKG at doses of 125, 250 and 500 mg/kg body weight respectively. After 30 minutes, all rats were orally administered starch at dosage 3 g/kg body weight. Blood samples were collected from the tail vein 30, 60 and 120 minutes after the starch had been administered. The results were compared with those of Group 1 rats and the percentage changes in blood glucose levels were calculated for each group. The oral sucrose challenge test was conducted in the same manner as that of the oral starch challenge test but the starch was replaced with sucrose at a dose of 4 g/kg body weight.

2.5 Statistical Analysis

Data were express as mean ± standard deviation (SD). The IC_{50} values were calculated from plots of log concentration of sample concentration versus percentage enzyme inhibition curves by using GraphPad Prism 5. The statistic Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnette's multiple comparison test were calculated using SPSS 15.0. *P*-value lower than 0.05 ($p<0.05$) was considered as statistically significant difference.

3.0 RESULTS AND DISCUSSION

Natural α-amylase and α-glucosidase inhibitors from plant extracts are believed to be one of the most effective strategies to suppress postprandial hyperglycaemia in diabetic management [14]. In this study, DLKG was screened using *in vitro* α-amylase and α-glucosidase assays. As shown in Table 1, the extract shows potent α-amylase and α-glucosidase inhibitory activities with IC_{50} values of 1.26 µg/ml and 4.09 µg/ml respectively. The dose responds between concentrations of the extract and both enzymes inhibitory activities are linear. In comparison with IC_{50} values of positive controls (acarbose for α-amylase inhibitory activity assay and 1-deoxynojirimycin for α-glucosidase inhibitory activity assay) each test assay is found to be significantly ($p<0.05$) higher than the extract. These findings demonstrate that the extract has potent *in vitro* α-amylase and α-glucosidase inhibitory activities than acarbose and 1-deoxynojirimycin, respectively. Thus, the extract may contain inhibitors that bind to the enzyme or substrate and blocks access to the substrate-enzymatic reaction and possibly deforms the shape of the enzyme that it becomes unrecognizable to the substrate [15].

Table 1 Inhibitory effects of DLKG against α-amylase and α-glucosidase activities

Sample	IC_{50} (µg/ml) ± SD	
	α-Amylase	α-Glucosidase
DLKG	1.26 ± 0.16*	4.09 ± 0.17*
Acarbose	11.42 ± 0.02	NT
1-Deoxynojirimycin	NT	115.0 ± 15.74

The data presented as mean IC_{50} (µg/ml) ± standard deviation (SD) duplicate measurements in each three independent experiments. IC_{50} refers to the concentration of inhibitors that produces 50% inhibition of the initial rate of reaction. Acarbose and 1-deoxynojirimycin were used as positive controls in these assays. The statistical significant differences between DLKG and positive control were compared using Student's *t*-test. Statistical significant difference ($p<0.05$) as compared with positive control is represented by *. NT represents not tested.

DLKG was further tested with the oral starch and sucrose challenge tests in normal and diabetic rats to confirm the *in vitro* findings. Figure 1 (A) shows the effects of the extract on blood glucose levels after starch loading in normal and diabetic rats in order to excess α-amylase inhibitory activity. The extract significantly ($p<0.05$) reduces blood glucose levels in normal rats at all doses (125, 250 and 500 mg/kg body weight) as compared to the control group at time 30 minutes after starch loading. The doses of 125, 250 and 500 mg/kg body weight give percentage reductions of blood glucose levels of 36.4, 23.7 and 19.4%, respectively. At dose 500 mg/kg body weight, the extract also significantly ($p<0.05$) reduces blood glucose level after 60 minutes of starch loading with a percentage reduction of 17.6%. In diabetic rats, the leaf extract at all doses (125, 250 and 500 mg/kg body weight) demonstrates significant ($p<0.05$) reduction of blood glucose levels after 30 minutes of starch loading when compared to the control group (Figure 1 (B)). The percentage reduction at doses of 125, 250 and 500 mg/kg body weight are 29.1, 20.4 and 22.3%, respectively. At doses of 250 and 500 mg/kg body weight, the DLKG extract significantly ($p<0.05$) reduces blood glucose levels after 60 minutes of loading with percentage reduction of 20.3 and 13.8%, respectively.

The effects of acarbose at a single dose of 10 mg/kg body weight on blood glucose levels after starch loading in normal and diabetic rats are shown in Figure 1 (A) and (B). Acarbose exhibits significant ($p<0.05$) decrease of blood glucose level at 30 and

60 minutes after starch loading in normal rats when compared to the control group with percentage reduction of 32.5 and 18.8%, respectively (Figure 1 (A)). In diabetic rats, acarbose significantly (p<0.05) decreases the blood glucose levels at 30, 60 and 120 minutes with percentage inhibitions of 31.0, 29.6 and 20.6%, respectively (Figure 1 (B)). These data are in accordance with a previous study that reported that acarbose significantly suppressed blood glucose levels in normal and STZ-induced diabetic mice after starch loading [16].

DLKG demonstrates antidiabetic properties by reducing postprandial blood glucose levels in the starch challenge test in normal and diabetic rats as shown in Figure 1 (A) and (B). The results obtained are consistent with *in vitro* a-amylase inhibitory activity. The DLKG extract has potential to retard the hydrolysis process of starch by pancreatic a-amylase enzyme and prolong glucose absorption by the small intestine and prevents fluctuation of blood glucose levels in normal and diabetic rats. This mechanism of action may help in controlling the blood glucose level that is useful in the treatment of postprandial hyperglycaemia linked to diabetes [17].

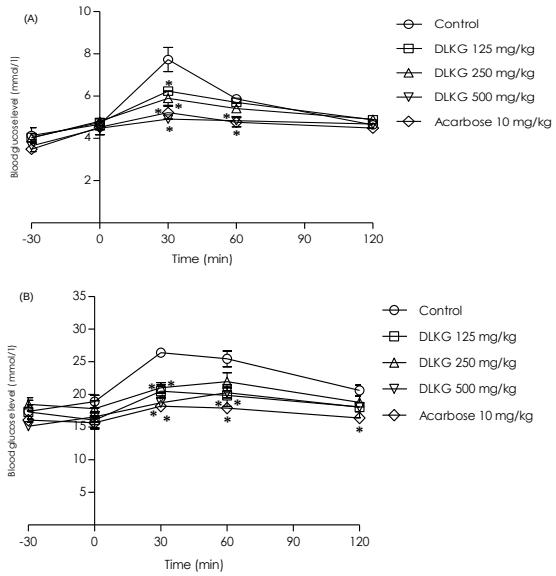

Figure 1 Effect of DLKG on oral starch challenge test in (A) normal rats (B) diabetic rats. The data presented as mean ± standard deviation (SD) (n=6). * represents statistical significant difference (p<0.05) as compared with control group at each time-point (ANOVA followed by Dunnett's Multiple Comparison test)

Figure 2 (A) shows the changes of blood glucose levels in normal rats orally treated with DLKG after sucrose loading. The blood glucose levels of normal rats treated with the extract at dose of 500 mg/kg body weight significantly (p<0.05) reduce after 30, 60 and 120 minutes of sucrose administration when compared with those of the control group. The extract shows percentage reduction of approximately 15.0%, 15.0% and 14.9% in the blood glucose levels at 30, 60 and 120 minutes, respectively. In diabetic rats, the extract did not effectively reduce blood glucose level (Figure 2 (B)). As shown in Figure 2 (A), acarbose at a single dose of 10 mg/kg body weight causes significant (p<0.05) reduction of blood glucose levels at time 30, 60 and 120 minutes after sucrose loading in normal rats with percentage inhibitions of 28.0, 13.9 and 13.7%, respectively. Acarbose also significantly (p<0.05) reduces the blood glucose levels in diabetic rats at times 30 and 60 minutes with percentage inhibitions of 28.0, 13.9 and 13.7% (Figure 2 (B)). A previous study by Subramaniam *et al*. (2008) also showed acarbose effectively suppressed blood glucose levels after sucrose loading in normal and diabetic rats. Acarbose is well known as a strong a-glucosidase inhibitor clinically used in treatment of Type 2 diabetes mellitus to reduce postprandial hyperglycaemia with increase time of glucose production that prevent diabetes complications [18].

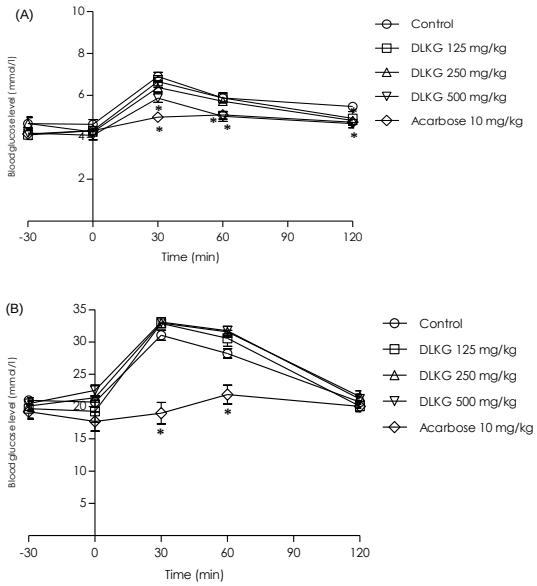

Figure 2 Effects of DLKG on oral sucrose challenge test in (A) normal rats (B) diabetic rats. The data presented as mean ± standard deviation (SD) (n=6). * represents statistical significant difference (p<0.05) as compared with control group at each time-point (ANOVA followed by Dunnett's Multiple Comparison test)

According to the *in vitro* results, the extract demonstrates potent a-glucosidase inhibitory activity. This finding correlates with the oral sucrose challenge test in normal rats but not in diabetic rats. The failure of the extract to suppress blood glucose level in the diabetic state could be due to the abnormal increase of a-glucosidase enzyme (sucrase) activity, the increase in absorption of intestinal glucose and enhances the glyconeogenesis activity which is related to enhanced glucose uptake from the liver and skeletal muscle by sensitization of insulin receptors [19],[20],[21],[22]. However, the higher oral

dosage of the extract possibly affects the blood glucose levels in diabetic rats after sucrose loading. The extract has a-glucosidase inhibitor to inhibit a-glucosidase activity from digestion of oligosaccharide to monosaccharide. This action will limit the production of glucose, slowing the absorption of glucose by the small intestine and control fluctuation of postprandial blood glucose level in diabetic patients [23], [24], [25].

DLKG is found to exhibit promising antidiabetic activity through inhibitory activity against a-amylase and a-glucosidase. The *in vivo* results show the extract is more active towards a-amylase inhibitory activity and reduce fluctuation of blood glucose levels after starch loading in both normal and diabetic rats. To the best of our knowledge, this is the first report on *in vitro* and *in vivo* antidiabetic activities of this plant extract. In a previous phytochemical study, the compound myristinin A, a flavanoid was isolated from *Myristica cinnamomea and Knema elegans* demonstrates anti-inflammatory activity through inhibition of cyclooxygenase-2 (COX-2) and antifungal activity. In addition, synthesis of myristinin A exhibits potent DNA-damaging agent and DNA polymerase beta inhibitor . [26], [27]. However, there is no antidiabetic report about the compound and phytochemical study on DLKG. Further comprehensive phytochemical and pharmacological studies are required to isolate the compounds responsible for the antidiabetic effects of the extract.

4.0 CONCLUSION

DLKG possesses potent in vitro a-amylase and a-glucosidase inhibitory activities. It also correlates with the in vivo findings by reducing blood glucose levels after starch loading in normal and diabetic rats. Furthermore, the extract effectively reduces blood glucose levels after sucrose loading in normal rats. This study demonstrates that the leaf of K. glauca is potentially beneficial for the development of antidiabetic agents that will help suppress postprandial hyperglycaemia and be useful in the treatment of diabetes mellitus.

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