

Full Length Research Paper

Persimmon leaf (*Diospyros kaki*), a potent α -glucosidase inhibitor and antioxidant: Alleviation of postprandial hyperglycemia in normal and diabetic rats

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This research aims to investigate the α -glucosidase (baker's yeast and rat intestinal acetone powder) inhibitory potential of *Diospyros kaki* Thunb. (Ebenaceae) leaf extract (DKLE) along with its antioxidant activity against metal-catalysed protein oxidation by pro-oxidant model (Fe^{2+} /ascorbate) in rat liver homogenates, *in vitro*. DKLE evidenced the outstanding inhibitory effect with IC_{50} values of 1.22 and 32 $\mu\text{g}/\text{ml}$ for baker's yeast and rat intestinal α -glucosidase enzymes. The inhibition mode of this extract was determined by measuring enzyme activity in different concentrations of substrate for Lineweaver-Burk plot analysis. Its inhibition was found to be noncompetitive. The addition of Fe^{2+} /ascorbate to the liver homogenate significantly increased the extent of lipid peroxidation which was significantly lowered with the extract treatment. Furthermore, DKLE ability to alleviate postprandial hyperglycemia in normal and streptozotocin (STZ)-induced diabetic rats was scrutinized. Oral administration of this leaf extract (250 to 1000 mg/kg body wt.) considerably decreased blood glucose in normal and diabetic rats after glucose and maltose loading in a dose-dependent manner. These results propose that DKLE might show an anti-diabetic effect by suppressing carbohydrate and glucose absorption from the intestine and can reduce the postprandial blood glucose rise.

Key words: *Diospyros kaki*, α -glucosidase, antioxidant, diabetic rats, postprandial hyperglycemia, streptozotocin.

INTRODUCTION

Postprandial hyperglycemia plays an important role in the development of type 2 diabetes and complications associated with the disease, such as micro- and macro-vascular diseases have been proposed as an independent risk factor for cardiovascular disease (Bonora and Muggeo, 2001; Ceriello, 2005). Hence, the control of postprandial hyperglycemia is recommended to be an important parameter in the treatment of diabetes and prevention of cardiovascular complications. α -glucosidase

(located in the brush border of the small intestine, required for the breakdown of carbohydrates to absorbable monosaccharides) inhibitors are among the available glucose-lowering medications for the treatment of postprandial hyperglycemia, since they slow the uptake of dietary carbohydrates (Stuart et al., 2004).

The role of oxidative protein damage in the pathophysiology of human diseases is currently a topic of considerable interest, as oxidized proteins have been implicated in a wide spectrum of clinical conditions including diabetes (Dalle-Donne et al., 2003; Telci et al., 2000).

Much effort has been extended in the search for effective and safe α -glucosidase inhibitors possessing

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strong antioxidant effects from natural source in order to develop a physiological functional food or lead compounds for use as antidiabetic agents. In the course of our ongoing program on identifying α -glucosidase inhibitors from natural medicines in Korea, we found the aqueous methanolic extract of the leaves of *Diospyros kaki* possessing potent α -glucosidase inhibitory effect. In Korea, the leaves of *D. kaki* Thunb., family Ebenaceae, have long been used as herb tea and were traditionally employed for the treatment of hypertension in patients with type 2 diabetes mellitus (Sa et al., 2005). The condensed tannins and flavonoids from the persimmon leaves were found to be mainly responsible for the antihypertensive, antimutagenic, anticarcinogenic and antioxidative actions (Choo et al., 2000). However, little studies have been performed on the antidiabetic properties of the *D. kaki* leaves. There is no available report on the pharmacological action of *D. kaki* leaf as a α -glucosidase inhibitor till date. Therefore, we carried out *in vitro* experiments including kinetics of enzyme inhibition, its antioxidant activity against metal-catalysed protein oxidation by pro-oxidant model (Fe^{2+} /ascorbate) in rat liver homogenates along with its *in vivo* monitoring for blood glucose level in normal and diabetic rats after glucose and maltose administration, in order to elucidate postprandial anti-hyperglycemic effect of *D. kaki* leaf extract.

MATERIALS AND METHODS

Plant material

The dried and matured leaves of *D. kaki* were obtained from "Korean Collection of Herbal Extracts", a Biotech company in Korea. A voucher specimen is available from the company (Korea Collection of Herbal Extracts, 2000).

Chemicals

α -glucosidase (from bakers yeast and rat intestinal acetone powder), 4-nitrophenyl α -D-glucopyranoside (PNPG), streptozotocin (STZ), acarbose, ascorbic acid, ferrous sulphate (FeSO_4), and quercetin were obtained from Sigma (St. Louis, MO).

Extraction of *D. kaki* leaves

The dried leaves (3 kg, dry weight) were extensively extracted with 80% aqueous MeOH for 2 days. The extract was dried using a rotary vacuum evaporator below 40°C. The vacuum-dried crude extract (198 g) was dissolved in distilled water and lyophilized and was used for the experiment.

Bakers yeast α -glucosidase inhibition assay

The enzyme inhibition activity for α -glucosidase was evaluated according to the method previously reported by Shibano et al. (1997) with minor modifications. The reaction mixture consisted of 50 μ l of 0.1 M phosphate buffer (pH 7.0), 25 μ l of 0.5 mM PNPG (dissolved in 0.1 M phosphate buffer, pH 7.0), 10 μ l of test

sample/and or standard (acarbose) (0.02 to 3 mg/ml) and 25 μ l of α -glucosidase solution (a stock solution of 1 mg/ml in 0.01 M phosphate buffer, pH 7.0, was diluted to 0.1 U/ml with the same buffer, pH 7.0, just before the assay). This reaction mixture was then incubated at 37°C for 30 min. The reaction was then terminated by the addition of 100 μ l of 0.2 M sodium carbonate solution. The enzymatic hydrolysis of the substrate was monitored based on the amount of *p*-nitrophenol released in the reaction mixture by observation at 410 nm using a microplate reader. All experiments were carried out in triplicate and the results are expressed as the mean \pm S.D. of three determinations.

Rat intestinal α -glucosidase inhibition assay

The method followed by Kim et al. (2009) was referred for rat intestinal α -glucosidase assay with slight modifications. 0.5 g of rat-intestinal acetone powder was suspended in 10 ml of 0.9% saline, and the suspension was sonicated twelve times for 30 s at 4°C. After centrifugation (10000 g, 30 min, 4°C), the resulting supernatant was used for the assay. The reaction mixture consisted of 50 μ l of 0.1 M phosphate buffer (pH 7.0), 30 μ l of 0.5 mM PNPG (dissolved in 0.1 M phosphate buffer, pH 7.0), 10 μ l of test sample and or standard (acarbose) (0.1 to 0.7 mg/ml) and 15 μ l of rat intestinal α -glucosidase solution. This reaction mixture was then incubated at 37°C for 30 min. The reaction was then terminated by the addition of 100 μ l of 0.2 M sodium carbonate solution. The enzymatic hydrolysis of the substrate was monitored based on the amount of *p*-nitrophenol released in the reaction mixture by observation at 410 nm using a microplate reader. All experiments were carried out in triplicate and the results are expressed as the mean \pm S.D. of three determinations.

Kinetics of inhibition against bakers yeast and rat intestinal α -glucosidase

To evaluate the inhibition type of DKLE against bakers yeast and rat intestinal α -glucosidase, increasing concentrations of PNPG (4-nitrophenyl α -D-glucopyranoside) were used as a substrate in the absence or presence of DKLE at different concentrations. The inhibition type of the data was determined by Lineweaver-Burk plot analysis.

Animal

Male Sprague Dawley rats weighing 180 to 200 g each were obtained from Daehan Biolink Co., Chungcheongbuk-Do, Korea. The animals were housed in polycarbonate cages under 12/12 h light/dark cycles at room temperature (20 to 22°C). The animals were allowed free access to a laboratory chow diet and water *ad libitum*. The animal protocol used in this study was approved by the University Animal Ethical Committee.

Induction of diabetes

For the induction of diabetes, 55 mg/kg body wt. of STZ was dissolved in citrate buffer, pH 4.5, and injected by a single intraperitoneal injection to rats previously fasted for 16 h. Three days after STZ induction, the development of diabetes was confirmed from tail vein blood glucose levels. Animals with blood glucose levels greater than 250 mg/dl were included in the study (Rafiq et al., 2009).

Experimental designs

Effect of DKLE on blood glucose levels after glucose loading in

normal and diabetic rats was examined. Glucose, acarbose and DKLE were dissolved in distilled water and administered orally by gauge needle in a final volume of 1 ml. Rats were divided into ten groups of six animals each, as follows: Normal groups (group 1 to 5): Group 1 was treated with 1 g/kg body wt. of glucose as the control group. Group 2 was treated with 1 g/kg body wt of glucose and 250 mg/kg body wt. of DKLE. Group 3 was treated with 1 g/kg body wt. of glucose and 500 mg/kg body wt. of DKLE. Group 4 was treated with 1 g/kg body wt. of glucose and 1000 mg/kg body wt. of DKLE. Group 5 was treated with 1 g/kg body wt. of glucose and 50 mg/kg body wt. of acarbose. Diabetic groups (group 6 to 10): They were treated in a similar fashion to the normal rats (group 1 to 5). Effect of DKLE on blood glucose levels after maltose loading in normal and diabetic rats was also examined. They were treated entirely similar to the normal and diabetic rats, as explained earlier, only the glucose load was replaced with maltose load (1 g/kg body wt.). Blood glucose levels were determined just before and 30, 60 and 120 min after the administration of food using an Accu-Chek Glucometer for all the experiments.

Preparation of liver homogenate and induction of oxidative stress by Fe²⁺/ascorbate

For liver homogenate preparation, male Sprague Dawley rats were anaesthetized using diethyl ether, and their livers were quickly removed. Each liver was then cut into small pieces and homogenized in phosphate buffer (50 mM, pH 7.4), to give a 10% (w/v) liver homogenate. Each homogenate was then centrifuged at 5000 g for 15 min at 4°C. The protein concentration of the supernatant was determined by the using standard assay kit (Asan Pharmaceutical, Seoul, Korea) using bovine serum albumin as standard. The oxidant pair Fe²⁺/ascorbate was used to induce oxidative stress in rat liver homogenate (Ardestani and Yazdanparast, 2007; Bahramikia et al., 2009). The reaction mixture was composed of 0.5 ml of each liver homogenate, 0.9 ml phosphate buffer (50 mM, pH 7.4), 0.25 ml FeSO₄ (0.01 mM), 0.25 ml ascorbic acid (0.1 mM), and 0.1 ml of different concentrations of DKLE (200, 500 and 1000 µg/ml) and/or quercetin (100 µg/ml) standard. The reaction mixture was incubated for 30 min at 37°C.

Determination of lipid peroxidation

The extent of lipid peroxidation of the rat liver homogenate in the presence and absence of DKLE was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS) using the method described by Ohkawa et al. (1979). The MDA (malondialdehyde)-TBA adduct formed by the reaction of MDA and TBA under high temperature and acidic conditions was measured calorimetrically at 532 nm. The amount of TBARS formed was calculated using the MDA standard curve.

Statistical analysis

Values are presented as the mean ± S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) and the Post-Hoc Tukey test; p<0.05 was considered to be statistically significant.

RESULTS

Inhibitory effects of DKLE on baker's yeast and rat intestinal α-glucosidase and their kinetics

DKLE exerted the most prominent inhibiting effect on

bakers yeast α-glucosidase. The optimal concentrations of DKLE required for the 50% inhibition (IC₅₀) against alpha-glucosidase was 1.22 µg/ml, when the PNPG was used as a substrate. Acarbose was used as positive control which showed low α-glucosidase inhibitory potential with IC₅₀ value of 133 µg/ml. DKLE showed potent inhibitory effect on rat intestinal α-glucosidase. The IC₅₀ value against rat intestinal α-glucosidase was 32 µg/ml, when the PNPG was used as a substrate. Acarbose was used as positive control (IC₅₀ = 10.7 µg/ml). The inhibition kinetics of DKLE was analyzed by Lineweaver-Burk plot analysis, which indicated that it is a non-competitive inhibitor with respect to PNPG for both the enzymes (baker's yeast and rat intestinal alpha glucosidase) (Figures 1 and 2).

Effects of DKLE on blood glucose levels in normal and diabetic rats after glucose loading

In Table 1, the results of the effect of graded doses of DKLE (250 to 1000 mg/kg) on the blood glucose levels of normal rats are presented. Acarbose at 50 mg/Kg was used as a reference standard. DKLE at all three doses (250, 500 and 1000 mg/kg) significantly reduced the postprandial blood glucose levels in a dose-dependent manner when compared to rats in the control group. In the control group, the blood glucose levels increased from 89.3 to 185.2 mg/dl 30 min after glucose administration and decreased thereafter. However, the elevation of blood glucose was significantly controlled by DKLE (1000 mg/kg) after 30 min and changed from 83.12 to 148.31 mg/dl. Acarbose did not show any positive effect on glucose loaded normal rats. The results for acarbose at 50 mg/kg were almost identical to those of control group.

Different doses of DKLE (250, 500 and 1000 mg/kg) were evaluated in diabetic group after glucose administration and compared with that of acarbose (50 mg/kg).

The results are detailed in Table 2. Thirty minute after a glucose load (1 g/kg), the blood glucose levels in all groups increased rapidly and gradually decreased thereafter. DKLE at 1000 mg/kg caused a significant attenuation of the rise of blood glucose levels after 120 min when compared to the other doses. The effect of DKLE (1000 mg/kg) was comparable to that of acarbose (50 mg/kg).

Therefore, it appears that DKLE at 1000 mg/kg body weight is the most effective dose, as it produced a significant hypoglycemic effect (Table 2).

Effects of DKLE on blood glucose levels in normal and diabetic rats after maltose loading

In Table 3, different doses of DKLE (250, 500 and 1000 mg/kg) and acarbose (50 mg/kg) are presented. Thirty

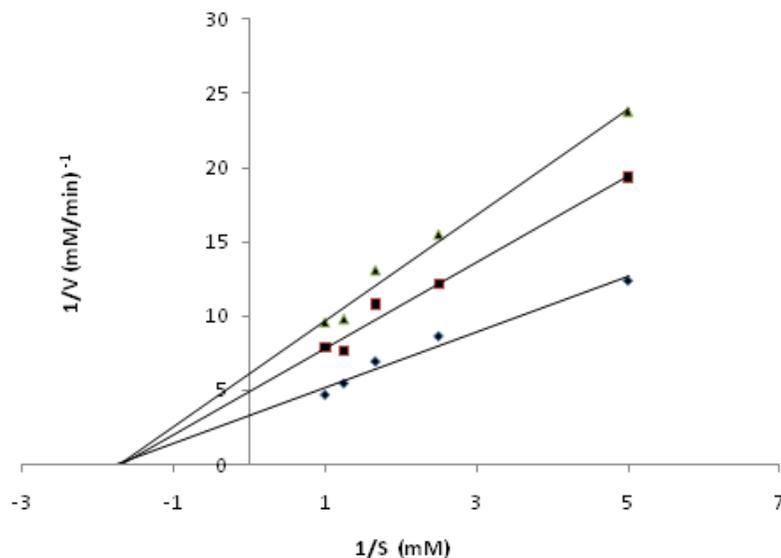


Figure 1: Lineweaver-Burk plot of baker's yeast α -glucosidase and PNPG without (\blacklozenge) and with DKLE [\blacksquare 150 and (\blacktriangle) 300 $\mu\text{g/ml}$].

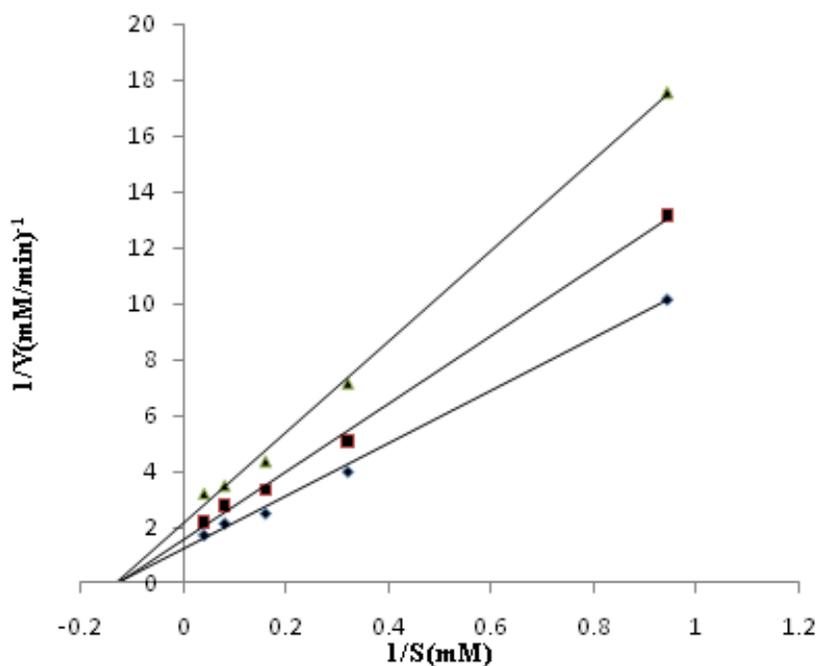


Figure 2: Lineweaver-Burk plot of rat intestinal α -glucosidase and PNPG without (\blacklozenge) and with DKLE [\blacksquare 400 and (\blacktriangle) 800 $\mu\text{g/ml}$].

minute after a maltose load (1 g/kg) the blood glucose levels in all groups increased rapidly and gradually decreased thereafter. DKLE at 1000 mg/kg caused a significant reduction of the rise of blood glucose levels after 30 min when compared to the other doses. The effect of DKLE at 1000 mg/kg was comparable to that of

acarbose at 50 mg/kg. Table 4 shows the effects of different doses of DKLE on fasting glucose levels in diabetic rats after maltose administration for a period of 120 min. As shown, DKLE induced significant blood glucose lowering effects in a dose-dependent fashion. In all doses, DKLE at 1000 mg/kg body weight produced

Table 1. Effect of DKLE and acarbose on blood glucose levels in normal rats after glucose administration (single dose administration).

Treatment	Time (min) blood glucose level (mg/dl) before and after glucose administration				
	0	30	60	90	120
Control (DW)	89.3 ± 3.11	185.2 ± 6.32	149.4 ± 2.11	115.2 ± 6.31	97.2 ± 4.43
250 mg/Kg DKLE	89.11 ± 3.25	233.61 ± 9.12*	151.12 ± 7.22*	109.12 ± 9.33	95.23 ± 4.22
500 mg/Kg DKLE	83.27 ± 2.42	162.91 ± 6.87	140.15 ± 6.22	99.24 ± 6.29*	89.12 ± 8.37**
1000 mg/Kg DKLE	83.12 ± 1.89	148.31 ± 8.67*	139.19 ± 9.21*	86.23 ± 4.21	82.26 ± 4.57*
50 mg/Kg acarbose	87.35 ± 2.36	186.82 ± 9.97*	155.20 ± 5.28**	113.29 ± 7.24**	95.5 ± 7.21**

Values are expressed as mean ± S.D. for six animals in each group. *P < 0.01 compared with control; **P < 0.05 compared with diabetic rats.

Table 2. Effect of DKLE and acarbose on blood glucose levels in diabetic rats after glucose administration (single dose administration).

Treatment	Time (min) blood glucose level (mg/dl) before and after glucose administration				
	0	30	60	90	120
Control (DW)	270.21 ± 7.21	441.28 ± 5.21	421.32 ± 3.29	373.25 ± 6.37	329.21 ± 8.2
250 mg/Kg DKLE	269.21 ± 5.32	424.51 ± 4.9*	438.24 ± 5.3*	395.24 ± 9.25*	343.29 ± 3.95**
500 mg/Kg DKLE	271.24 ± 6.31	362.91 ± 5.30*	401.28 ± 4.25**	353.29 ± 4.88**	312.72 ± 7.23**
1000 mg/Kg DKLE	262.27 ± 7.29	291.21 ± 4.32*	387.21 ± 5.25**	321.27 ± 4.82**	261.29 ± 7.49**
50 mg/Kg acarbose	272.21 ± 7.48	431.29 ± 6.98*	457.23 ± 4.25**	409.58 ± 7.54**	304.78 ± 4.54**

Values are expressed as mean ± S.D. for six animals in each group. *P < 0.0001 compared with control; **P < 0.05 compared with diabetic rats.

Table 3. Effect of DKLE and acarbose on blood glucose levels in normal rats after maltose administration (single dose administration).

Treatments	Time (min) blood glucose level (mg/dl) before and after maltose administration				
	0	30	60	90	120
Control (DW)	85.66 ± 3.65	207.33 ± 13.43	156.76 ± 12.42	115.89 ± 7.76	107.77 ± 6.93
250 mg/Kg DKLE	82. ± 5.78	163.43 ± 9.11*	150.21 ± 7.91*	133.34 ± 9.78*	115.32 ± 4.54
500 mg/Kg DKLE	83.89 ± 5.90	153.56 ± 6.89*	146.56 ± 4.89	119.67 ± 9.09*	99.56 ± 8.29**
1000 mg/Kg DKLE	84.87 ± 2.93	148.03 ± 8.99	137.92 ± 9.67*	116.67 ± 4.22**	98.67 ± 4.91*
50 mg/Kg acarbose	87.56 ± 4.91	132.22 ± 6.78*	123.56 ± 5.98**	113.39 ± 7.76*	95.23 ± 7.72*

Values are expressed as mean ± S.D. for six animals in each group. *P < 0.01 compared with control; **P < 0.05 compared with diabetic rats.

Table 4. Effect of DKLE and acarbose on blood glucose levels in diabetic rats after maltose administration (single dose administration).

Treatment	Time (min) blood glucose level (mg/dL) before and after maltose administration				
	0	30	60	90	120
Control (DW)	260.78 ± 10.3	425.28 ± 15.21	355.32 ± 13.32	325.25 ± 6.37	321.21 ± 8.2
250 mg/Kg DKLE	268.42 ± 8.28	399.65 ± 18.2	378.22 ± 7.27*	345.56 ± 8.82	319.29 ± 9.95*
500 mg/Kg DKLE	264.62 ± 5.86	372.82 ± 6.45	361.27 ± 8.53**	323.29 ± 4.88**	312.72 ± 5.82
1000 mg/Kg DKLE	268.72 ± 5.96	351.21 ± 7.26*	357.21 ± 7.58	328.22 ± 6.84**	289.69 ± 6.92**
50 mg/Kg acarbose	270.62 ± 4.89	333.22 ± 8.82*	315.35 ± 3.75*	304.57 ± 9.38*	284.84 ± 7.44**

Values are expressed as mean ± S.D. for six animals in each group. *P < 0.0001 compared with control; **P < 0.05 compared with diabetic rats.

Table 5. Effect of DKLE and quercetin on lipid peroxidation induced by the oxidant pair Fe²⁺/ascorbate, in rat liver homogenate.

Sample	Concentration (µg/ml)	MDA (µmole/L)
DKLE	200	20.05 ± 0.38
DKLE	500	17.03 ± 0.62
DKLE	1000	11.31 ± 0.52
Quercetin	100	16.34 ± 0.58
Oxidative stress	-	24.51 ± 0.24

Values are mean ± S.D. of three independent replicates.

maximum diminution of blood glucose after 120 min. The glucose levels in rats treated with acarbose at a dose of 50 mg/kg body weight showed significant changes in blood glucose levels. Interestingly, DKLE (1000 mg/kg) produced almost comparable effects as that of standard drug acarbose.

This suggests that DKLE possesses an important antihyperglycemic effect.

Effects of DKLE on lipid peroxidation

The addition of Fe²⁺/ascorbate to the liver homogenate significantly increased the extent of TBARS formation in comparison to the control sample. Table 5 show the oxidative damage measured in terms of MDA equivalents (µmole/L). Addition of DKLE at different concentrations (200, 500 and 1000 µg/ml) to the liver homogenates significantly reduced TBARS values to a remarkable extent. In this assay quercetin was used as positive standard.

DISCUSSION

In this study, we investigated the inhibitory potential of *D. kaki* leaf extract on α -glucosidase by two different sources (bakers yeast and rat intestinal), where it proved to possess intensive inhibitory effects on both α -glucosidases. Furthermore, kinetic studies were performed to determine the mode of inhibition by Lineweaver–Burk plot analysis. This showed non-competitive inhibition for both the enzymes, which indicates that DKLE binds to a site other than the active site of the enzyme and combines with either free enzyme or the enzyme-substrate complex, possibly interfering with the action of both (Figures 1 and 2).

In addition, the *in vivo* results of the present study indicate that DKLE reduces the glucose level in normal and diabetic rats and thus improves glucose tolerance after glucose and maltose administration. The dose of 1000 mg/kg showed a marked improvement in glucose tolerance in normal and diabetic rats.

Digestive enzymes play a vital role in the carbohydrate

hydrolysis. α -glucoside bonds of disaccharides are cleaved by α -glucosidase enzymes to yield monosaccharides. These monosaccharides, from the small intestine, are then further absorbed through Na⁺/glucose cotransporter (SGLT1). Disaccharides are not absorbed through the SGLT1 as they are not substrates against this transporter whereas the glucose and galactose are absorbed readily from the small intestine due to their high substrate specificity. Therefore, as SGLT1 is the primary step of glucose absorption, its inhibition can be the most important to suppress the postprandial hyperglycemia (Mizuma and Awazu, 1998; Ikumi et al., 2008). Interestingly, in the present study, DKLE showed potent antihyperglycemic effect not only after maltose administration but also after glucose load in normal and diabetic rats. This dual inhibition of DKLE seems to be by α -glucosidase (inhibits maltose load) and SGLT1 (inhibits glucose load) inhibition. Similar effects were reported in previous studies performed by Park et al. (2009) and Nakahara et al. (1994). Based on these results, we can affirm that DKLE induced potent hypoglycemic effects in both normal and STZ induced diabetic rats.

The increase of free radical mediated-toxicity is well documented in clinical diabetes (Nourooz-Zadeh et al., 1997). Oxidative stress induces the production of highly reactive oxygen radicals that are toxic to cells, particularly the cell membrane in which these radicals interact with the lipid bilayer and produce lipid peroxides (Haugaard, 1968). Furthermore, high levels of lipid peroxides have been found in the serum of patients suffering from liver disease, diabetes, vascular disorders, and tumors (Pezzuto and Park, 2002). Antioxidants can influence the oxidation process through simple or complex mechanisms including prevention of chain initiation, binding of transitional metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Ames et al., 1993). Therefore, the antioxidant activity of DKLE against metal-catalysed oxidation by pro-oxidant model (Fe²⁺/ascorbate) in rat liver homogenates on lipid peroxidation was determined at 200, 500 and 1000 µg/ml. Induction of oxidative stress in rat liver homogenate uniformly resulted in increased lipid peroxidation levels. Captivatingly, DKLE at all concentrations showed strong antioxidant activity. Also,

DKLE at 500 µg/ml showed potent antioxidant activity which was comparable to quercetin (positive standard) at 100 µg/ml (Table 5). Consequently, it can be concluded that DKLE decrease TBARS content and thus may be effective in preventing oxidative protein damage which is believed to occur during oxidation processes.

In conclusion, the present study revealed that the antihyperglycemic effect of DKLE might be associated with alpha glucosidase inhibition, Na⁺/glucose cotransporter (SGLT1) inhibition and its ability to scavenge free radicals. Our findings provide insight into the effects and action mechanism of DKLE as a suitable candidate for treating postprandial blood glucose rise. Therefore, it could be used as a dietary supplement for prevention or early treatment of diabetic disorders.

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