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Effect of 6-hydroxy-7,4’-dimethoxyflavone on antidiabetic effects in normal and streptozotocin-induced diabetic rats

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Kaempferia parviflora is a famous endemic Thailand species of the family *Zingiberaceae*. MeOH extract of *Kaempferia parviflora* was consecutively partitioned with organic solvents such as $CHCl_3$ EtOAc and BuOH. $CHCl_3$ fractions were diluted with distilled water and extracted with n-hexane and CH_2Cl_2. Five methoxyflavone compounds were isolated from the CH_2Cl_2 fractions with column chromatography. The structures of the isolated compounds were identified as 3,5,7,3’,4’-pentamethoxyflavone (KP1), 5,7-dimethoxyflavone (KP2), 5,7,4’-trimethoxyflavone (KP3), 3,5-dihydroxy-3’-methoxyflavone (KP4) and 6-hydroxy-7,4’-dimethoxyflavone (KP5) by ^{1}D H-NMR spectral analysis and comparison of spectral data with literature values. KP5 showed significant hypoglycemic activity in streptozotocin-induced diabetic rats for 28 days. It significantly decreased the serum glucose and triglycerides while it increased the serum insulin in diabetic rats but not in normal rats ($p < 0.05$; at doses of 50, 100 and 150mg/kg for 28 days). It had no effect on C-peptide (ECLIA). Further structure-activity relationships of position 6 in the aromatic ring B will be reported in due course.

Keywords: *Kaempferia parviflora*, α-glucosidase inhibitory activity, streptozotocin-induced diabetic rats, methoxyflavone

INTRODUCTION

Kaempferia parviflora (KP) is a species of the genus *Kaempferia* which involves 35 species and belongs to the family of *Zingiberaceae*. There are indigenes in Thailand which are spread in Loei, Pitsanulok and Phetchabun. They are called kra-chai-dum or Thai black ginger [1]. KP is a plant that grows relatively low to the ground. It is a understory plant which has short fleshy rhizomes dark purple in colour, tuberous roots, straight and curved leave ends and white with violet small blooms under the leaves [2]. KP extracts in ethanol or methanol are effective to visceral fat accumulation, hyperinsulinemia, glucose intolerance, hypertension, diabetes, antiobesity, peripheral neuropathy, anti-gastric ulcer and Alzheimer's disease [3]. 5,7,4’-Trimethoxyflavone and 5,7,3’4’-tetramethoxyflavone display antiplasmodial activity; 3,5,7,4’-methoxyflavone has antifungal and low-level antimycobacterial activity [4]; 5,7-dimethoxyflavone affects multidrug resistance associated proteins (MRP) - mediated transport in A549 cells [5]. Among the structures, the 5,7-methoxy groups lead to significant inhibitory activity while the 5-hydroxy group decreases the inhibitory activity. Non-insulin-dependent diabetes mellitus (NIDDM) is one of the main adult diseases [6]. Type 1 leads to destruction of the pancreatic langerhans β-cells producing insulin. Type 2 is a secretory decrease in insulin from pancreatic langerhans β-cells or lowering of insulin resistance due to excess glucose absorption [7]. Hyperglycemia and hyperlipidemia are involved in the development of microvascular and macrovascular complications of diabetes, which are the major causes of morbidity and mortality due to diabetes [8]. The current study describes the isolation of five methoxyflavones from *Kaempferia parviflora*. In the context of our natural product chemistry program dealing with the development of new potent antidiabetic agents, we examined the 6-hydroxy-7,4’-dimethoxyflavone as a lead for novel antidiabetic agents.

MATERIALS AND METHODS

General experimental procedures

^{1}H NMR spectra were recorded with an Agilent MR 400 DD2 400MHz spectrometer in $CDCl_3$ and DMSO-d_6 solution and chemical shifts (δ) are reported as parts per million (ppm). Coupling constant (J) is reported in hertz (Hz). Melting points were measured using a Fisher-Johns melting point

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apparatus and are uncorrected. Column chromatography was conducted using silica gel 60 (40-63 and 63-200 μm particle size, Merck) and RP-18 (40-63 μm particle size, Merck)

Plant Material

Kaempferia parviflora rhizomes were kindly provided by Professor Kim Soo-Ki from the Animal Sciences Department of Konkuk University. The rhizomes of *Kaempferia parviflora* were identified by Prof. Hyung-In Moon. A voucher specimen (No.2012-0405) has been deposited in the Herbarium of the Dong-A University (Busan, South Korea).

Extraction and fractionation

The rhizomes of *Kaempferia parviflora* (5 kg) were sliced. Reflux extraction was performed with 9 L of MeOH, three times at 5℃. The MeOH extracts (198 g) were combined and concentrated *in vacuo* at 40℃. The MeOH extract was diluted with distilled water (0.9 L) and then partitioned with $CHCl_3$ (0.6 L×3). $CHCl_3$-soluble part was evaporated (84.3 g) and diluted with distilled water (1L). $CHCl_3$-soluble part, diluted with distilled wáter, was partitioned with n-hexane (0.9 L×3), CH_2Cl_2 (0.7 L×3), EtOAc (0.8 L×3) and BuOH (0.8 L). The CH_2Cl_2 extract (49 g) was separated by silica gel column chromatography. A mobile gradient (from 30:1 to 0:1 v/v) was used and 12 fractions were obtained (K1-K12). The mobile phase for K1 to K8 was $CHCl_3$:MeOH (30:1 v/v). For K9 $CHCl_3$:MeOH (10:1 v/v) was used. For K10 and K11 $CHCl_3$:MeOH (5:1 v/v) was used. For K12 $CHCl_3$:MeOH (1:1 v/v) was used. All fractions were recrystallized at -20℃ from MeOH. Each recrystallized fraction was separated by filtering. The recrystallized compounds were dried at room temperature and the MeOH-soluble fractions were evaporated at 40℃. KP1 (5.6 g) was recrystallized from the K5 fraction. KP2 (3 g) was recrystallized from the K6 fraction. KP3 (2.7g) was recrystallized from the K4 fraction. K2 crystal fraction was separated by silica gel column chromatography. KP4 (2.16 g) was isolated from the K2 crystal fraction (6 g) with silica gel column chromatography using $CHCl_3$ as mobile phase. K8 crystal fraction (288 mg) was separated by Sephadex-LH20 column chromatography using 100% MeOH as mobile phase. KP5 was obtained from the K8 crystal fraction.

KP1: Bright yellow powder, ^{1}H-NMR ($CDCl_3$, 300MHz) δ: 7.82 (1H, d, 5'-H), 6.99(1H, d, 6'-H), 6.62 (1H ,s , 2'-H), 6.56 (1H, s, 6-H), 6.37 (1H, s, 8-H), 3.95 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.49 (3H, s, OCH_3)

KP2: Bright yellow powder, ^{1}H-NMR ($CDCl_3$, 300MHz) δ: 7.84 (2H, d, 2' and 6'-H), 7.51 (2H, t, 4'-H), 7.00 (2H, t, 3' and 5'-H), 6.70 (H, s, 3-H), 6.57 (H, s, 6-H), 6.39 (H, s, 8-H), 3.96 (3H, s, OCH_3), 3.92 (3H, s, OCH_3)

KP3: Bright yellow powder, ^{1}H-NMR ($CDCl_3$, 300MHz) δ: 7.71 (2H, d, 2' and 6'-H), 6.98 (2H, d, 3' and 5'-H), 6.77 (1H, s, 3-H), 6.51 (1H, s, 6-H), 6.35 (1H, s, 8-H), 3.96 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.88 (3H, s, OCH_3)

KP4: Yellow powder, ^{1}H-NMR ($CDCl_3$, 300MHz) δ: 12.75 (1H, s, OH), 12.60 (1H, s, OH), 8.09 (1H, t, 5'-H) 7.92, (1H, d, 6'-H), 7.57 (1H, s, 2'-H), 7.54 (1H, d, 4'-H), 6.53 (1H, d, 8-H), 6.48 (1H, d, 6-H), 6.40 (1H, t, 7-H), 3.90(3H, s, OCH_3)

KP5: Yellow powder, ^{1}H-NMR ($CDCl_3$, 300MHz) δ: 10.20 (1H, s, OH), 7.88 (2H, d, 2' and 6'-H), 6.90 (2H, d, 3' and 5'-H), 6.83 (1H, s, 3-H), 6.58 (1H, s, 5-H), 6.50 (1H, s, 8-H), 3.89 (3H, s, OCH_3), 3.82 (3H, s, OCH_3)

α-Glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity of compounds KP 1~5 was measured. The inhibitory assay was done by the chromogenic method with a slight modification. α-Glucosidase from *saccharomyces cerevisiae* (10 μg/ml, 14 U/mg, sigma) was dissolved in 100 mM phosphate buffer (PB, pH 7.0) containing 0.2% BSA and 0.02% NaN_3 and was used as an enzyme solution. 4-Nitrophenyl-α-D-glucopyranoside (Sigma, 5 mM) in the same buffer (pH 7.0) was used as a substrate solution. The enzyme solution (90 μl) contained concentrations of 0, 20, 40, 80, 100 μM of KP compounds, respectively, (18 μl) of the tested materials was mixed in a well of a microtiter plate and measured for titer (415 nm) at zero min with a microplate reader. After incubation for 5 min, the substrate solution (90 μl) was added and the reaction was carried out at room temperature for 5 min. The increase in absorbance from zero time was measured.

Animals

Adult male Wistar rats with body weights of 200-250 g were purchased from the Korea SAMTAKO CO., LTD (Kyunggido, South Korea), housed in specific pathogen-free facilities and provided with autoclaved water and standard food. The animals were housed at a controlled temperature (20-22ºC) and relative humidity (55%-

59%), with a normal 12-hour light-and-dark cycle. All experiments conducted on mice were in accordance with the guidelines for the care and use of laboratory animals approved by Dong-A University.

Experimental induction of diabetes in rats

Male adult Wistar rats were injected with streptozotocin (STZ). Streptozotocin was dissolved in saline immediately before use and injected intraperitoneally (i.p.) in a single dose of 250 mg/ kg. Five days after injection, rats with fasting blood glucose higher than 180 mg/dL were used for the experiments. Ten rats were used in each experiment. Each animal was used once. The food was removed from the cages 12 hours before testing. Streptozotocin (purity 99%) was purchased from Sigma Co. The volume of the above three doses was kept constant at 1 mL.

Drug administration

KP1~5 were suspended in acacia gum with saline and administered orally through orogastric tubes at doses of 50, 100 and 150 mg/kg body wt. The volume of the above three doses was kept constant at 1 mL.

Experimental design

In the experiment, a total of 60 rats (50 diabetic rats, 10 normal rats) were used. Diabetes was induced in rats 5 days before starting the treatment. The rats were divided into nine groups, each group involving six rats.

Group I: Normal control rats administered i.p.1 mL acacia gum with saline daily, for 28 days using an intragastric tube.

Group II: Control drug rats administered i.p. 1 mL acacia gum with Metformin (250 mg/kg) daily, for 28 days using an intragastric tube.

Group III: Diabetic control rats administered i.p. 1 mL acacia gum with STZ (250 mg/kg), for 28 days using an intragastric tube.

Group IV: Diabetic control rats administered i.p. KP5 (50 mg/kg bw) in 1 mL acacia gum with saline daily, for 28 days using an intragastric tube.

Group V: Diabetic rats administered i.p. KP5 (100 mg mg/kg bw) in 1 mL acacia gum with saline daily, for 28 days using an intragastric tube.

Group VI: Diabetic rats administered i.p. KP5 (150 mg mg/kg bw) in 1 mL acacia gum with saline daily, for 28 days using an intragastric tube.

Biochemical assays

Biochemical assays of male adult Wistar rats were performed using a modified method of Eidi [9]. After 28 days of treatment, blood samples were drawn from the heart. Serum glucose, insulin, triglycerides and C-peptide levels were determined. The serum insulin was estimated by using a radioimmunoassay kit (Diasorin, Italy) and the triglycerides by the method of Rifai [10].

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was carried out using one way ANOVA followed by the Tukey post hoc test. The criterion for statistical significance was $p < 0.05$.

RESULTS AND DISCUSSION

The current study describes the isolation of five methoxyflavones from *Kaempferia parviflora*. Molecular structures were determined through a combination of spectroscopic analyses, including ^{1}H nnuclear magnetic resonance (NMR), mass spectrometry (MS) data, and literature data. The effects of the isolated compounds (0-100 μM) on the inhibition of the α-glucosidase based assay are reported here. Compound KP1 was obtained as a bright yellowish amorphous powder. It was detected as a bright yellow color on TLC when visualized with 5% sulfuric acid spray reagent. In the ^{1}H NMR ($CDCl_3$) spectra of KP1, typical aromatic ring signals were observed, as well as five methoxy singlet signals of the 3,5,7,3',4' position methoxy groups. The five singlet signals at δ 3.49 to δ 3.95 indicated methoxy groups. Compound KP2 was obtained as a yellow amorphous powder. It was detected as a bright yellow color on TLC when visualized with 5% sulfuric acid spray reagent. In the ^{1}H NMR ($CDCl_3$) spectra of KP2 aromatic ring signals and two methoxy signals were observed. Two methoxy signals were indicated at δ 3.92 and δ 3.96. Compound KP3 was obtained as a yellow-white amorphous powder. It was detected as a bright yellow color on TLC when visualized with 5% sulfuric acid spray reagent. 1H NMR ($CDCl_3$) spectra of KP3 indicated the presence of 5,7,4' substituted methoxy group protons at δ 3.88, δ 3.91 and δ 3.96 signals and typical aromatic ring signals were observed at δ 8.1 to δ 6.3. Compound KP4 was obtained as a bright yellow amorphous powder. It was detected as a yellow color on TLC when visualized with 5% sulfuric acid spray reagent. ^{1}H NMR (CDCl3) spectra of KP4 indicated aromatic ring signals, two hydroxyl and one methoxy signal. Two hydroxy signals and one methoxy signal were indicated at δ 12.60, δ 12.75 and δ 3.90. Two hydroxy groups were indicated at 3,5 position and one methoxy group at 3' position. Compound KP5 was obtained as a white amorphous powder. It was

detected as a bright yellow color on TLC when visualized with 5% sulfuric acid spray reagent. In the ^{1}H NMR ($CDCl_3$) spectra of KP5, typical aromatic ring signals, as well as two methoxy singlet signals at the 7,4' position and one hydroxyl singlet signal at the 6 position were observed. Two methoxy singlet signals and one hydroxyl singlet signal were indicated at δ 3.82, δ 3.89 and δ 10.20. KP1~5 compounds were dissolved in DMSO and the total volume was 196 μl for all concentrations (Fig. 1).

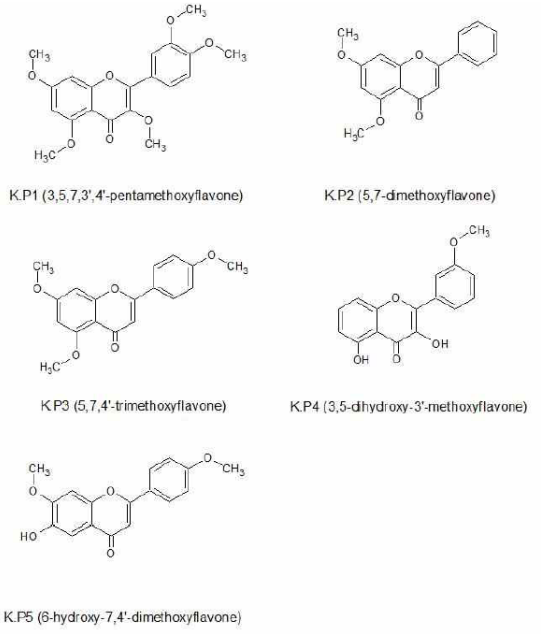

Fig. 1. Structures of KP1~5.

KP2~5 were confirmed to cause a dose dependent increase in α-glucosidase inhibitory activity. KP1 did not cause a dose dependent increase in α-glucosidase inhibitory activity. KP2 and KP3 showed lower dose inhibitory activity from 30 to 40% at 100 μM concentration, KP4 and KP5 showed a high inhibitory activity to 55% and 78%, respectively. KP5 inhibitory activity was confirmed to rapidly increase from 80 μM concentration, and the inhibitory activity of KP4 was highest (57%) at the 80 μM concentration (Fig. 2).

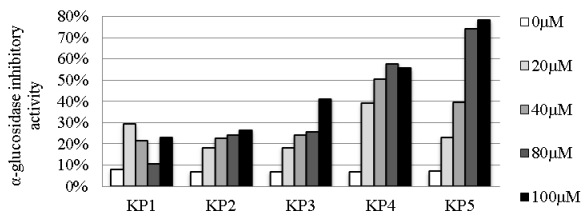

Fig. 2. α-Glucosidase inhibitory activity of KP1~5 with the inhibitory assay done by the chromogenic method with a slight modification. The increase in absorbance from zero time was measured.

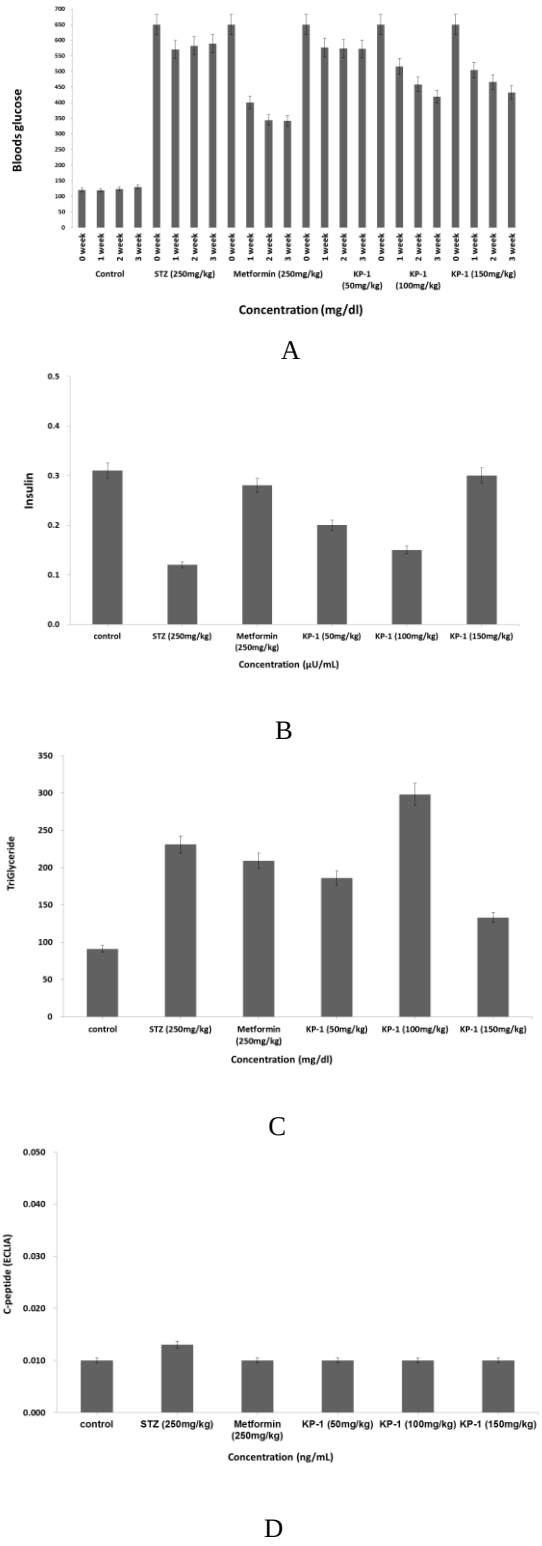

Fig. 3. Effect of 6-hydroxy-7,4'-dimethoxyflavone (KP-5) on the mean values of serum glucose (A), insulin (B), triglycerides (C) and C-peptide (D) after 28 days of treatment (50, 100, 150 mg/kg bw/day, i.p.). *The criterion for statistical significance was $p < 0.05$.

The 6-hydroxyl group of baicalein (5,6,7-trihydroxyflavone) was important to α-glucosidase inhibitory activity; flavones which lack a hydroxyl group on any of positions 5, 6, or 7, showed no activity [11]. KP4 and KP5 seem to be depending on the presence of the hydroxyl position in the flavone structure. We have found that the MeOH extract of *Kaempferia parviflora* has α-glucosidase inhibitory activity. KP5 significantly inhibited the α-glucosidase inhibitory activity in a dose‑dependent manner. Figure 3 shows the effects of KP5 on serum glucose, insulin, triglycerides and C-peptide in diabetic rats.

The results showed that serum glucose and triglycerides of diabetic rats increased while serum insulin decreased, when compared with normal rats. The administration of KP5 at doses of 50, 100 and 150 mg/kg body wt tended to bring serum glucose ($p < 0.05$), insulin ($p < 0.05$), triglycerides ($p < 0.05$) and C-peptide ($p<0.05$) significantly toward normal values, while normal rats did not exhibit any significant alterations in these parameters during the experiment. The 150 mg/kg bw treatment groups were found to be more effective than the 50 and 100 mg/kg bw. The administration of KP5 did not change C-peptide levels in normal and diabeic rats. The results indicated that KP-5 (150 mg/kg bw) treatment significantly decreased serum glucose and triglycerides while it increased the serum insulin levels in treated diabetic rats compared with the control diabetic rats. The results also showed that KP-5 (150 mg/kg bw) treatment caused a significant decrease in the level of serum in diabetic rats. This may be due to a metabolic disturbance in diabetes reflected in the high activities of xanthine oxidase, lipid peroxidation and increased triglycerides. As a result, KP-5 (150 mg/kg bw) is a potential antidiabetic agent for the tested streptozotocin-induced diabetes.

CONCLUSION

We have found that KP-5 (150 mg/kg bw) showed decreasing antidiabetic potency *in vivo*. Further study on the mode of action of position 6 on ring B is under progress. Taken together, our findings provide important information on the structural features that influence the functional activities of this class of compounds and offer new possibilities for further explorations to improve potency from damage of STZ-induced diabetic rats. It is shown that KP-5 may be of use as an anti-diabetic agent.

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ЕФЕКТ НА 6-ХИДРОКСИ-7,4'-ДИМЕТОКСИФЛАВОН ВЪРХУ АНТИДИАБЕТИЧНИТЕ ПРОЯВИ ПРИ НОРМАЛНИ ПЛЪХОВЕ И ТАКИВА С СТРЕПТОЗОТОЦИН-ИНДУЦИРАН ДИАБЕТ

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(Резюме)

Kaempferia parviflora е известно ендемично тайландско растение от семейство *Zingiberaceae*. Неговите метанолови екстракти последователно се разделят с органични разтворители, като $CHCl_3$ EtOAc и BuOH. Хлороформените фракции се разреждат с дестилирана вода и се екстрахират с n-хексан и метиленхлорид. От фракцията с метилен хлорид са изолирани пет метоксифлавонови съединения с помощта на колонна хроматография. Структурите на изолираните съединения са идентифицирани като 3,5,7,3',4'-пентаметоксифлавон (KP1), 5,7-диметоксифлавон (KP2), 5,7,4'-триметоксифлавон (KP3), 3,5-дихидрокси-3'-метоксифлавон (KP4) и 6-хидроксиу-7,4'-диметоксифлавон (KP5) чрез ^{1}D H-ЯМР спектрален анализ и сравнение с литературни данни.

KP5 проявява значителна хипогликемична активност при плъхове със стрептозотоцин-индуциран диабет в продължение на 28 дни. Той намалява значително серумната глюкоза и триглицеридите, докато повишава серумния инсулин при диабетичните плъхове, но не и при нормалните плъхове ($p < 0.05$, при дози от 50, 100 и 150mg/kg за 28 дни). Препаратът няма ефект върху С-пептидите (ECLIA). Следващата зависимост между структура и активност на позиция 6 в ароматния пръстен В ще бъде съобщен в близко време.