Trop J Nat Prod Res, December 2022; 6(12):1953-1956
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Protective Effect of Sa-khan (*Piper*, Piperaceae) Against High Glucose-Induced Cytotoxicity in Human Proximal Tubular Epithelial Cells

Daoyot Daorueang*

School of Allied Health Sciences, University of Phayao, Phayao, 56000, Thailand

ARTICLE INFO

Article history:
Received 16 October 2022
Revised 02 December 2022
Accepted 05 December 2022
Published online 01 January 2023

ABSTRACT

High glucose-induced cytotoxicity plays a critical role in the pathogenesis of diabetic kidney disease (DKD). The present study aimed to investigate the potential nephroprotective effect of Sa-khan (*Piper*, Piperaceae) against high glucose-induced cytotoxicity in a human proximal tubular epithelial cell line. The cytotoxicity induced by high glucose in HK-2 cells, a human proximal tubular epithelial cell line, was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The wound scratch assay was performed to evaluate the migration capability of HK-2 cells. Treating cells with high glucose significantly reduced the viability of HK-2 cells ($p < 0.001$). Sa-khan crude extract (SCE) at concentration of 0.031 mg/mL was found to increase the viability of high glucose-induced HK-2 cells ($p = 0.013$). In addition, high glucose significantly increased the migration capability of HK-2 cells ($p < 0.001$). Furthermore, SCE at concentrations of 0.016 and 0.031 mg/mL abolished the increased migration capability of high glucose-induced cells ($p < 0.001$). This study shows that Sa-khan crude extract displays a potential protective effect against high glucose-induced proximal tubular cell injury.

Keywords: Sa-khan, High glucose-induced cytotoxicity, HK-2, MTT, Wound scratch assay.

Introduction

Diabetic kidney disease (DKD) is a progressive microvascular complication of diabetes mellitus.[1] Epidemiological studies have shown that diabetes affects more than 425 million people.[1] According to the high prevalence of diabetes, the incidence of DKD is rising rapidly, with approximately 40% of diabetic patients developing DKD.[2] High glucose-induced proximal tubular injury plays an important role in the development and progression of DKD. Anatomically, the proximal tubule is the part of the nephron that is directly contiguous with the parietal epithelium of Bowman's capsule. The proximal tubule reabsorbs 100% of the glucose filtered by the glomeruli. In diabetic patients, a high glucose transport status in the proximal tubular cells may be the initial cause of proximal tubular injury.[3] This injury is generally caused by an inability of the cells to compensate for the increased glucose uptake during hyperglycemia.[4] In addition, glucose entry into proximal tubular cells occurs in an insulin-independent manner, which makes proximal tubular cells highly sensitive to hyperglycemia, leading to interstitial inflammation and fibrosis.[5] The de-differentiation of injured proximal tubular cells leads to structural changes and functional alterations. De-differentiated proximal tubular cells may migrate across the basement membrane to become myofibroblasts, key producers of the extracellular matrix.[6] Increasing evidence suggests that consistent de-differentiation of injured proximal tubule cells may play an important role in tubulointerstitial fibrosis and DKD progression.[7] Sa-khan (*Piper*, Piperaceae) is usually found in the northeastern and northern parts of Thailand.[8] It has been used in traditional medicine as a carminative and tonic element.[9] The previous study indicates that Sa-khan contains phenolic compounds and shows anti-oxidant properties.[10] However, the beneficial effect of Sa-khan on renal tubular cells in response to hyperglycemia remains incompletely elucidated. The main aim of this study was to evaluate the protective effects of Sa-khan extract against high glucose-induced cytotoxicity in a human proximal tubular epithelial cell line.

Materials and Methods

Preparation of the plant extract

Stems of Sa-khan were purchased in June 2016 from local markets (Phayao, Thailand). The plant specimen was identified by a plant taxonomist and deposited by a curator associated with Queen Sirikit Botanic Garden Herbarium (QBG number 111859). The plant extract was prepared as previously described.[10] Briefly, 800 g of air-dried Sa-khan was ground and macerated in 1,000 mL of absolute methanol for approximately 24 h at room temperature. The mixture was filtered through Whatman No. 1 filter paper and evaporated using a rotary evaporator at 55ºC. The Sa-khan crude extract (SCE) was weighed and stored at -20°C.

Cell culture

HK-2 cells, a normal human proximal tubular epithelial cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in low glucose Dulbecco's Modified Eagle's Medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum under sterile conditions at 37 °C and 5% CO_2 with 95% humidity. The cells were treated with 0.05% trypsin-EDTA (Gibco, NY, USA) for passaging when they reached 70–80% confluence.

Cytotoxic effect of SCE on HK-2 cells

The MTT assay was performed on HK-2 cells to evaluate the cytotoxicity induced by SCE. Briefly, HK-2 cells (2×10^3 cells/well) were seeded into a 96-well plate and allowed to adhere for 48 h at 37 °C. Four concentrations of SCE (0.004, 0.008, 0.016, and 0.031 mg/mL) were prepared by dissolving the SCE in the medium and incubated with the cells for 24 h. Then, cells were incubated with 0.5 mg/mL MTT at 37 °C for another 3 h. Subsequently, the medium was removed and dimethyl sulfoxide (DMSO) was added to each well.

*Corresponding author. E mail: daoyot.da@up.ac.th
Tel: +66-5-446-6697

Citation: Daorueang D. The Protective Effect of Sa-khan (*Piper*, Piperaceae) Against High Glucose-Induced Cytotoxicity in Human Proximal Tubular Epithelial Cells. Trop J Nat Prod Res. 2022; 6(12):1953-1956. http://www.doi.org/10.26538/tjnpr/v6i12.9

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Optical density (OD) was quantified using a microplate reader at 540 nm. The percentage of cell viability was calculated using the formula: percentage of cell viability = [(X-B)/Y] × 100, where X is the optical density of cells treated with SCE, B is the optical density of the blank, and Y is the optical density of cells without the plant extract (control).

Effect of SCE against high glucose-induced cell cytotoxicity

In order to investigate whether SCE can reduce high glucose-induced cytotoxicity, cells were treated with a high glucose concentration in the presence and absence of SCE. HK-2 cells were seeded at a density of 2×10^3 cells/well in a 96-well plate and pre-incubated with 5.5 mM glucose for 48 h. The cells were then treated with 60 mM glucose (high glucose; HG) alone or combined with various concentrations of SCE (0.004, 0.008, 0.016, and 0.031 mg/mL) and further incubated for 24 h, followed by the MTT assay. The cells were treated with 5.5 mM glucose alone as the low glucose control (LG).

Cell migration analysis

The effect of SCE on the migration capability of HK-2 cells was assessed using a wound scratch assay. HK-2 cells were seeded into 6-well plates at a density of 2×10^4 cells/well and cultured in medium containing 5.5 mM glucose until they formed a near-confluent cell monolayer. A horizontal wound was generated in the monolayer using a sterile 200 µL plastic pipette tip. Remnants of cell debridement were removed by washing three times with pre-warmed phosphate-buffered saline. Cells were treated with 5.5 mM glucose (LG), 60 mM glucose (HG), or 60 mM glucose plus different concentrations of SCE (0.016 and 0.031 mg/mL). Images of the horizontal wounds were captured at 0, 12, and 24 h after scratching to calculate cell migration as the percentage of wound closure as follows: percentage of wound closure = $[(A_0-A_t)/A_0] \times 100$, where A_0 represents the area of the initial wound, and A_t represents the remaining area of the wound at the point of measurement.

Statistical analysis

Statistical analyses were carried out using STATA version 14. Data were expressed as mean ± SD. The differences between the groups were determined using an independent t-test. A two-tailed value of $p < 0.05$ was considered statistically significant.

Results and Discussion

In Thailand, Sa-khan (*Piper*, Piperaceae) has been extensively used in Thai traditional medicine.[9] The stem of this plant has long been used as carminative, antiflatulent, and tonic element.[9] The plant extract has been demonstrated to exhibit various activities including anti-cancer,[11] anti-microbial,[12] anti-inflammation,[13] and anti-oxidant.[10] However, there are few reports on the protective activity of this medicinal plant in proximal tubular epithelial cell.[10] The cytotoxicity of SCE in HK-2 cells was assessed after treatment for 24 h with different concentrations of SCE. As shown in Figure 1, the viability of HK-2 cells was not affected by treatment with SCE ranging from 0.004 to 0.031 mg/mL. To determine whether high glucose-induced cell toxicity in HK-2 cells is modulated by treatment with SCE, the cell viabilities were measured using the MTT assay after 24 h of incubation. The results revealed that cell viability decreased under high glucose conditions compared with that in the low glucose control group ($p < 0.001$), as shown in Figure 2. However, cell viability was significantly increased in the high glucose-induced cells treated with 0.031 mg/mL of SCE compared with the high glucose group ($p = 0.013$). Proximal tubular injury plays an important role in the development and progression of DKD. Because of their functional and structural characteristics, the proximal tubular epithelial cells of the nephron can be easily injured by a variety of potentially damaging factors that can trigger an oxidative stress, inflammatory, and pro-fibrotic response.[14,15] High glucose induced-cytotoxicity is the major cause of proximal tubular cell injury in diabetic patients. In this study, the result revealed that SCE significantly ameliorated the loss of cell viability induced by high glucose. In recent years, increasing evidence supports that an elevated glucose transport state and local hypoxia in the proximal tubular epithelial cells of diabetic patients may be important factors in causing proximal tubular epithelial cell injury.[16,17] In diabetes, the proximal tubular epithelial cells need a large amount of ATP as an energy source to reabsorb excess glucose. In addition, ATP production generates superoxide, which can be converted into reactive oxygen species (ROS) and lead to proximal tubular epithelial cell injury.[18] Increasing evidence supports that oxidative stress is an important factor in the damage of proximal tubular epithelial cells by high glucose. For example, Coughlan *et al.* demonstrated that the increasing production level of ROS may exceed the capacity of the local antioxidants, a biomarker of renal mitochondrial dysfunction in diabetes.[19] In addition, Lee *et al.* noted that high glucose induced mitochondrial fragmentation in HK-2 cells.[20] Moreover, high glucose increased the level of the apoptotic markers such as Bax,[21] and cleaved-caspase-3.[22] Recent research indicates that the SCE contains phenolic compounds and shows anti-oxidant properties, which may have protective effects against oxidative stress.[10] The effect of SCE on the migration capability of HK-2 cells was assessed using a wound scratch assay. High glucose increased HK-2 cell migration compared with the low glucose control group as shown in Figure 3. Wound closure of high glucose-induced cells was significantly faster than that of low glucose control group at both 12 h and 24 h after scratching ($p < 0.001$).

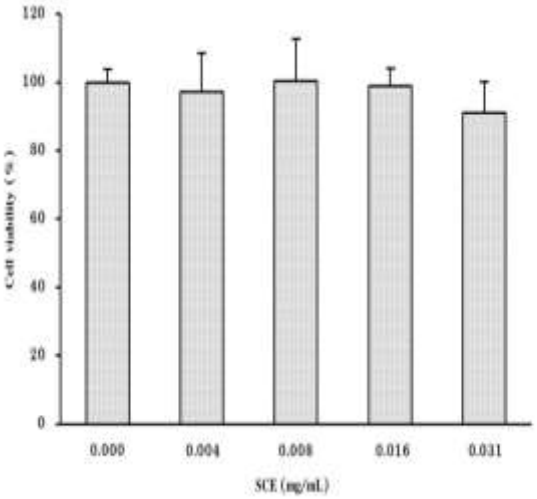

Figure 1: Viability of HK-2 cells. SCE: Sa-khan crude extract.

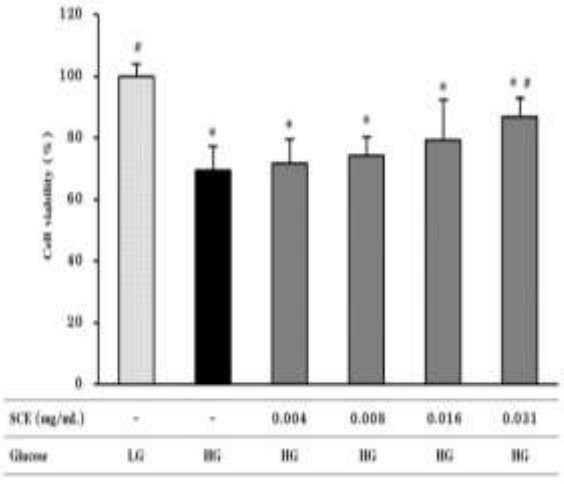

Figure 2: The effect of SCE against high glucose-induced cell cytotoxicity.

SCE: Sa-khan crude extract. LG: cells with 5.5 mM glucose. HG: cells with 60 mM glucose. * indicates significantly different from the low glucose group ($p < 0.05$), # indicates significantly different from the high glucose group ($p < 0.05$).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

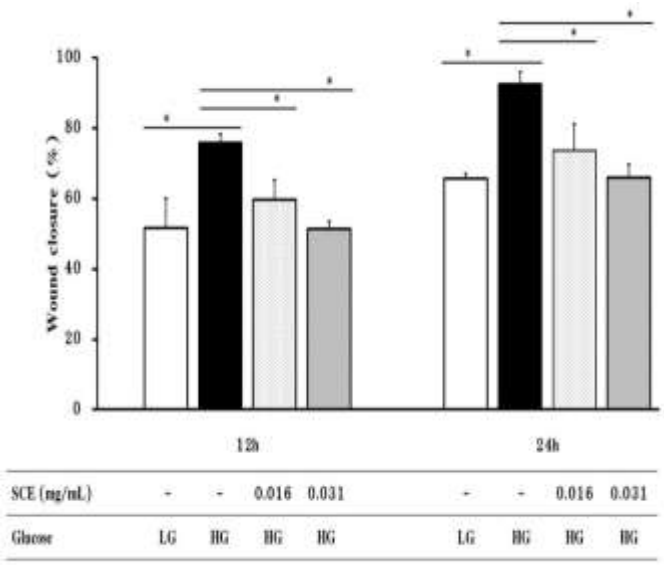

Figure 3: The effect of SCE on HK-2 cell migration. SCE: Sa-khan crude extract. LG: cells with 5.5 mM glucose. HG: cells with 60 mM glucose. * indicates significantly different from the high glucose control group ($p < 0.001$).

SCE treatment significantly abolished the increased migration capability of high glucose-induced cells ($p < 0.001$). High glucose stimulated de-differentiation and epithelial-mesenchymal transition (EMT) of proximal tubular epithelial cell, playing a critical role in initiating and progressing renal fibrosis in DKD. EMT is a process by which epithelial cells lose their cell-cell adhesion and cell polarity.[23] This process may lead to the gain of migratory and invasive properties in epithelial cells and transformation to mesenchymal cells. In this study, we revealed that high glucose increased migration of HK-2 cells. Many studies have indicated that high glucose induces the EMT process in renal tubular epithelial cells. For example, Zhang *et al.* demonstrated that a high concentration (60 mM) of glucose significantly induced EMT in HK-2 cells.[24] In addition, high glucose increased the expression of the mesenchymal marker α-SMA but deceased the epithelial marker E-cadherin in HK-2 cells.[25] The result of the present study demonstrated that SCE significantly reduced migration of high glucose-induced HK-2 cells. Overall data indicated the potential protective effect of SCE against high glucose-induced proximal tubular cell injury. This effect could be a new alternative explanation for the beneficial effects of SCE. However, further intensive studies are needed to identify the exact mechanism of action of Sa-khan crude extract.[26-27]

Conclusion

The present study provided novel evidence that SCE could increase cell viability and suppress cell migration of high glucose-induced HK-2 cells. Taking together, these findings demonstrate the potential protective effect of SCE against high glucose-induced proximal tubular cell injury.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This work was supported by the Thailand Science Research and Innovation Fund and the University of Phayao (Grant number FF65-RIM127).

References

1. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, Malanda B. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract. 2018; 138:271-281.
2. Gembillo G, Ingrasciotta Y, Crisafulli S, Luxi N, Siligato R, Santoro D, Trifirò G. Kidney Disease in Diabetic Patients: From Pathophysiology to Pharmacological Aspects with a Focus on Therapeutic Inertia. Int J Mol Sci. 2021; 22(9):4824.
3. Chang J, Yan J, Li X, Liu N, Zheng R, Zhong Y. Update on the Mechanisms of Tubular Cell Injury in Diabetic Kidney Disease. Front Med (Lausanne). 2021; 30:661076.
4. Zeni L, Norden AGW, Cancarini G, Unwin RJ. A more tubulocentric view of diabetic kidney disease. J Nephrol. 2017; 30(6):701-717.
5. Vallon V and Thomson SC. The tubular hypothesis of nephron filtration and diabetic kidney disease. Nat Rev Nephrol. 2020; 16(6):317-336.
6. Gewin LS. Renal fibrosis: Primacy of the proximal tubule. Matrix Biol. 2018; 68:248-262.
7. Garcia-Fernandez N, Jacobs-Cachá C, Mora-Gutiérrez JM, Vergara A, Orbe J, Soler MJ. Matrix Metalloproteinases in Diabetic Kidney Disease. J Clin Med. 2020; 9(2):472. doi: 10.3390/jcm9020472
8. Sudmoon R, Tanee T, Wongpanich V, Bletter N, Chaveerach A. Ethnobotany and species specific molecular markers of some medicinal sakhan (*Piper*, Piperaceae). J Med Plants Res. 2012; 6:1168–1175.
9. Panthong S and Itharat A. Effect of *Piper chaba* Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz. on natural killer cell activity and lymphocyte proliferation. J Med Assoc Thai. 2014; 8:S133-9.
10. Daorueang D. Antioxidant activity and effect of Sa-khan (*Piper*, Piperaceae) on the normal human proximal tubular epithelial cell line. Naresuan University Journal: Science and Technology (NUJST), 2020; 28(4):29-35.
11. Ruangnoo S, Itharat A, Sakpakdeejaroen I, Rattarom R, Tappayutpijam P, Pawa KK. *In vitro* cytotoxic activity of Benjakul herbal preparation and its active compounds against human lung, cervical and liver cancer cells. J Med Assoc Thai. 2012; 95Suppl1:S127-34.
12. Kondo S, Sattaponpan C, Phongpaichit S, Srijan A, Itharat A. Antibacterial activity of Thai medicinal plants Pikutbenjakul. J Med Assoc Thai. 2010; 93Suppl 7:S131-5.
13. Kuropakornpong P, Itharat A, Panthong S, Sireeratawong S, Ooraikul B. *In Vitro* and *In Vivo* Anti-Inflammatory Activities of Benjakul: A Potential Medicinal Product from Thai Traditional Medicine. Evid Based Complement Alternat Med. 2020; 2020:9760948.
14. Lash LH. Environmental and Genetic Factors Influencing Kidney Toxicity. Semin Nephrol. 2019; 39(2):132-140.
15. Sheng L and Zhuang S. New Insights Into the Role and Mechanism of Partial Epithelial-Mesenchymal Transition in Kidney Fibrosis. Front Physiol. 2020; 11:569322.
16. Packer M. Mechanisms Leading to Differential Hypoxia-Inducible Factor Signaling in the Diabetic Kidney: Modulation by SGLT2 Inhibitors and Hypoxia Mimetics. Am J Kidney Dis. 2021; 77(2):280-286.
17. Hesp AC, Schaub JA, Prasad PV, Vallon V, Laverman GD, Bjornstad P, van Raalte DH. The role of renal hypoxia in the pathogenesis of diabetic kidney disease: a promising target for newer renoprotective agents including SGLT2 inhibitors? Kidney Int. 2020; 98(3):579-589.
18. Higgins GC and Coughlan MT. Mitochondrial dysfunction and

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

mitophagy: the beginning and end to diabetic nephropathy? Br J Pharmacol. 2014; 171(8):1917-42.
19. Coughlan MT, Higgins GC, Nguyen TV, Penfold SA, Thallas-Bonke V, Tan SM, Ramm G, Van Bergen NJ, Henstridge DC, Sourris KC, Harcourt BE, Trounce IA, Robb PM, Laskowski A, McGee SL, Genders AJ, Walder K, Drew BG, Gregorevic P, Qian H, Thomas MC, Jerums G, Macisaac RJ, Skene A, Power DA, Ekinci EI, Wijeyeratne XW, Gallo LA, Herman-Edelstein M, Ryan MT, Cooper ME, Thorburn DR, Forbes JM. Deficiency in Apoptosis-Inducing Factor Recapitulates Chronic Kidney Disease via Aberrant Mitochondrial Homeostasis. Diabetes. 2016; 65(4):1085-98.
20. Lee WC, Chau YY, Ng HY, Chen CH, Wang PW, Liou CW, Lin TK, Chen JB. Empagliflozin Protects HK-2 Cells from High Glucose-Mediated Injuries via a Mitochondrial Mechanism. Cells. 2019; 8(9):1085.
21. Zhou J, Zhang S, Sun X, Lou Y, Yu J. Hyperoside Protects HK-2 Cells Against High Glucose-Induced Apoptosis and Inflammation via the miR-499a-5p/NRIP1 Pathway. Pathol Oncol Res. 2021; 27:629829.
22. Hou Y, Wang Q, Han B, Chen Y, Qiao X, Wang L. CD36 promotes NLRP3 inflammasome activation via the mtROS pathway in renal tubular epithelial cells of diabetic kidneys. Cell Death Dis. 2021; 12(6):523.
23. Cruz-Solbes AS and Youker K. Epithelial to Mesenchymal Transition (EMT) and Endothelial to Mesenchymal Transition (EndMT): Role and Implications in Kidney Fibrosis. Results Probl Cell Differ. 2017; 60:345-372.
24. Zhang H, Wei X, Lu S, Lin X, Huang J, Chen L, Huang X, Jiang L, Li Y, Qin L, Wei J, Huang R. Protective effect of DMDD, isolated from the root of *Averrhoa carambola* L., on high glucose induced EMT in HK-2 cells by inhibiting the TLR4-BAMBI-Smad2/3 signaling pathway. Biomed Pharmacother. 2019; 113:108705.
25. Zhang J, Jiang T, Liang X, Shu S, Xiang X, Zhang W, Guo T, Xie W, Deng W, Tang X. lncRNA MALAT1 mediated high glucose-induced HK-2 cell epithelial-to-mesenchymal transition and injury. J Physiol Biochem. 2019; 75(4):443-452.
26. Falodun A, Siraj R, Choudhary MI. GC-MS Insecticidal Leaf essential oil of *Pyrenacantha staudtii* Hutch and Dalz (Icacinaceae). Trop J Pharm Res. 2009; 8(2):139-143.
27. Okolie NP, Falodun A, Davids O. Evaluation of the antioxidant activity of root extract of pepper fruit (*Dennetia tripetala*), and its potential for the inhibition of Lipid peroxidation. Afr J Trad Compl and Altern Med. 2014; 11(3):221-227.