Antioxidant Activity and Effect of Sa-khan (*Piper*, Piperaceae) on the Normal Human Proximal Tubular Epithelial Cell Line

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Abstract

The oxidative damage to proximal tubular epithelial cells may lead to renal dysfunction. This research aimed to investigate antioxidant activity of the Sa-khan crude extract (SCE) and evaluated its effect on cell viability of proximal tubular epithelial cells. The total phenolic content of the SCE was determined using Folin-Ciocalteu method. The free radical scavenging activity of the SCE was investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The effect of the SCE on HK-2 cells, the human proximal tubular epithelial cell line, was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The SCE showed the total phenolic content of 17.35 ± 0.40 mg GAE/g extract. In addition, the SCE exhibited scavenging activity on DPPH (IC_{50} = 1.15±0.071 mg/mL). Moreover, eleven spots of antioxidant compounds were detected when the two-dimensional thin-layer chromatography combined with the DPPH spray technique was performed. Furthermore, the SCE at the concentration of 0.125 mg/mL could promote the proliferation of HK-2 cells over various durations. Together, these results indicate that the SCE may contain compound(s) with potential health benefits.

Keywords: Sa-khan, normal human proximal tubular epithelial cell, antioxidant activity

Introduction

An imbalance between free radicals and antioxidants plays an important role in a pathological process of many types of diseases such as diabetes mellitus (Ceriello & Motz, 2004), cancer (Sullivan & Chandel, 2014), cardiovascular disease (Steven et al., 2019), neurodegenerative disease (Singh, Kukreti, Saso, & Kukreti, 2019), and kidney diseases (Ratliff, Abdulmahdi, Pawar, & Wolin, 2016). Free radicals attack macromolecules such as DNA, proteins, lipids, and carbohydrates leading to cell damage. In addition, the role of free radicals is considered an important factor involved in the initiation and progression of renal injuries and diseases including diabetic nephropathy, drug-induced nephropathy, ischemia-reperfusion injury (IRI), and hypertension-related kidney injury (Ratliff et al., 2016). The proximal tubule is a major site of free radical generation and it is sensitive to oxidative stress. Renal tubular epithelial cells take part in the secretion and reabsorption of substances. The damage of tubular cells may lead to renal dysfunction.

Plant-based antioxidants scavenge free radicals to inhibit cell/tissue damages and reduce the risk of chronic diseases. Antioxidant compounds have abilities to scavenge free radicals by donating hydrogen ion to neutralize both exogenous and endogenous free radicals. The search for nontoxic natural compounds with effective antioxidant activity has been intensified in recent years.

Sa-khan (*Piper*, Piperaceae) is commonly found in the northern and northeastern parts of Thailand. It has been used for traditional medicine as a carminative, and tonic element. The plant extract has been shown to exhibit various activities including antimicrobial activity (Kondo, Sattaponpan, Phongpaichit, Srijan, & Itharat, 2010), anti-inflammation (Sireeratawong et al., 2012), and anti-cancer (Ruangnoo et al., 2012). Nevertheless, there has been no scientific research regarding its biological activity on human renal cells.

Therefore, the main aims of this research were to evaluate antioxidant activity of Sa-khan extract and investigate its effect on cell viability of the human proximal tubular epithelial cell line.

Methods and Materials

Preparation of the plant extract

Stems of Sa-khan were purchased from local markets (Phayao, Thailand). A plant specimen was identified by a plant taxonomist and deposited by a curator associated with Queen Sirikit Botanic Garden Herbarium (QBG number 111859). A total mass of 800 g of air-dried Sa-khan was ground and soaked with 1,000 mL of absolute methanol. The extraction was conducted at room temperature for approximately 24 h in dark. The mixture was filtered through Whatman filter paper and evaporated using a rotary evaporator apparatus. The Sa-khan crude extract (SCE) was weighed and stored at -20 °C.

Determination of the total phenolic content

The total phenolic content of SCE was spectrophotometrically determined using the Folin-Ciocalteu method. Briefly, 0.1 mL of SCE (5 mg/mL) was mixed with 0.1 mL of Folin-Ciocalteu reagent then 0.3 mL of 2% sodium carbonate was added. The volume was adjusted to 4.6 mL with distilled water. After shaking, the mixture was left for 2 h at room temperature in a dark place. The absorbance was measured at 760 nm. A calibration curve was plotted for different concentrations of gallic acid. The total phenolic content was expressed in mg of gallic acid equivalent per g of extract (GAE/ g extract).

Evaluation of the total antioxidant activity by the DPPH method

The free radical scavenging activity of SCE was investigated by DPPH assay. In brief, 20 µL of different concentrations of SCE were mixed with 100 µL of 0.1 mM DPPH solution. The mixtures were then incubated for 30 min at room temperature in dark. The absorbance of a sample was measured at 540 nm against a methanol control. Percentage inhibition was calculated using the following formula; % Inhibition = $[(A_C-A_S)/A_C] \times 100$

Where A_c is the absorbance of the control and A_S is the absorbance of the sample. The antioxidant activity is expressed as IC_{50}, which is calculated by plotting inhibition percentages against the concentration of the sample.

Characterization of antioxidant compounds by two-dimensional thin-layer chromatography (2D-TLC)

SCE (200 µg) was spotted onto a TLC plate 1 cm from the left bottom corner. The plate was developed to distance of 7 cm in a chamber with methanol-DW (1:1 v/v) as a first developing solvent. Then, the solvent was evaporated to dryness at room temperature. The previously developed TLC plate was turned 90° and then placed into a chamber with DW-methanol-ethyl acetate (3:4:2 v/v) as a second developing solvent. After development, the TLC plate was evaluated under ultraviolet light of 365 nm then the antioxidant activities of antioxidant compounds were observed after spraying with 0.2% DPPH solution as yellow spots on a purple background.

Cell culture

HK-2 cells, the human proximal tubular epithelial cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA). HK-2 cells were grown in keratinocyte serum-free medium (Gibco, NY, USA) supplemented with epidermal growth factor (5 ng/mL) and bovine pituitary extract (0.05 mg/mL) in

95% humidified incubator with 5% CO_2 at 37 °C. The cells were treated with 0.05% trypsin-EDTA (Gibco, NY, USA) for passaging when they reached 70-80% confluence.

Investigation of the effect of SCE on the human proximal tubular epithelial cell line by MTT assay

The effect of SCE on HK-2 cells was investigated by MTT assay. Briefly, HK-2 cells (4×10^3 cells/well) were seeded in to a 96-wells plate and permitted to adhere for 48 h at 37°C. Seven concentrations of SCE (0.004, 0.008, 0.016, 0.031, 0.062, 0.125, and 0.250 mg/mL) were prepared by dissolving the SCE in the medium and incubated with cells for 24, and 48 h. After the incubation period, cells were incubated with 0.5 mg/mL MTT for 3 h at 37 °C. Subsequently, medium was removed and DMSO was added to dissolve formazan crystals. The plate was placed in a microplate reader and the absorbance was measured at 540 nm. The percentage of cell viability was calculated by the formula; cell viability (%) = [(X-B)/Y] x 100, where X is the optical density of cells treated with plant extract, B is the optical density of blank, and Y is the optical density of cells without plant extract (control).

Statistical analysis

Cell viability measured by MTT assay was expressed as percent viability relative to the control. Two independent experiments were performed and data were expressed as mean± SD. Statistical analysis was performed using IBM SPSS Statistics version 24. Differences in cell viability between treatments and control were determined using a one-way ANOVA statistical test followed by post-hoc tests. A p-value of less than 0.05 was considered statistically significant.

Results

Total phenolic content of SCE

Natural phenolic compounds benefit human health. In this study, the total phenolic content of the SCE was determined spectrophotometrically (Folin-Ciocalteu method) and expressed as mg of gallic acid equivalent per g of extract (mg GAE/g extract). Total phenolic content of SCE was 17.35 ± 0.40 mg GAE/g extract.

DPPH radical scavenging activity of SCE

DPPH assay was widely used for evaluating the antioxidant activities of bioactive compounds and plant extracts. The SCE exhibited concentration-dependent antiradical activity by inhibiting DPPH radical with IC_{50} value of 1.15±0.071 mg/mL (Figure 1A) while IC_{50} value of ascorbic acid was 0.08±0.001 mg/mL (Figure 1B).

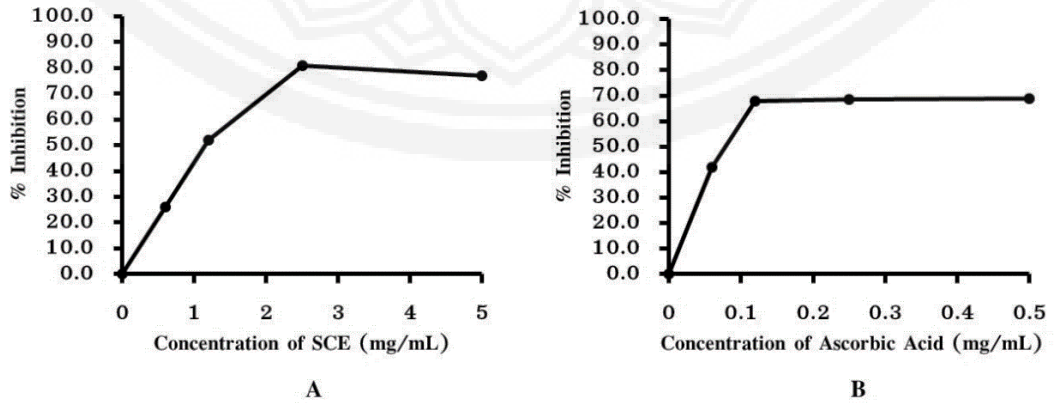

Figure 1 DPPH radical scavenging activity of SCE (A) and ascorbic acid (B)

Characterization of antioxidant compounds by 2D-TLC

In this context, characterization of antioxidant compounds from SCE was performed by 2D-TLC. 2D-TLC chromatogram of SCE isolated 11 spots of constituents under visible light (Table 1). Twelve spots were also detected under ultraviolet light of 365 nm. Moreover, 11 spots showed DPPH radical scavenging activity with R_f values of (0.02, 0.17), (0.05, 0.05), (0.12, 1.00), (0.45, 0.42), (0.45, 0.85), (0.45, 0.98), (0.81, 0.85), (0.81, 0.93), (0.90, 0.85), (0.93, 0.98), and (0.95, 0.05).

Table 1 R_f values of chemical compositions in SCE investigated by 2D-TLC

Solvent system	R_f value		
	Visible light	UV 365 nm	DPPH
First developing solvent	**11 spots**	**12 spots**	**11 spots**
Methanol-DW (1:1 v/v)	(0.02, 0.14),	(0.02, 0.14),	(0.02, 0.17),
Second developing solvent	(0.02, 0.05),	(0.02, 0.05),	(0.05, 0.05),
DW-Methanol-Ethyl acetate (3:4:2 v/v)	(0.12, 1.00),	(0.12, 1.00),	(0.12, 1.00),
	(0.40, 0.76),	(0.40, 0.76),	(0.45, 0.42),
	(0.45, 0.98),	(0.45, 0.42),	(0.45, 0.85),
	(0.48, 0.76),	(0.45, 0.85),	(0.45, 0.98),
	(0.81, 0.85),	(0.45, 0.98),	(0.81, 0.85),
	(0.81, 0.93),	(0.48, 0.76),	(0.81, 0.93),
	(0.90, 0.85),	(0.81, 0.85),	(0.90, 0.85),
	(0.93, 0.98),	(0.81, 0.93),	(0.93, 0.98),
	(0.95, 0.05)	(0.90, 0.85),	(0.95, 0.05)
		(0.93, 0.98)	

Effect of SCE on the human proximal tubular epithelial cell line

HK-2 cells were treated with different concentrations of SCE for 24 and 48h then cell viability was assessed using MTT assay. When cells were treated for 24h, the viability of HK-2 cells was not affected by the SCE treatments until a concentration of 0.062 mg/mL (Figure 2A). In addition, there was significant increase in cell viability in the presence of 0.125 mg/mL of SCE.

The viability of HK-2 cells was not affected by the SCE treatments until a concentration of 0.031 mg/mL when cells were treated for 48h, whereas, it was significantly increased in response to a concentration of 0.062 and 0.125 mg/mL (Figure 2B). At 0.250 mg/mL of SCE, the cell viability decreased to 80%, indicating that SCE might produce a toxic effect on HK-2 cells at a high dose (Figure 2B).

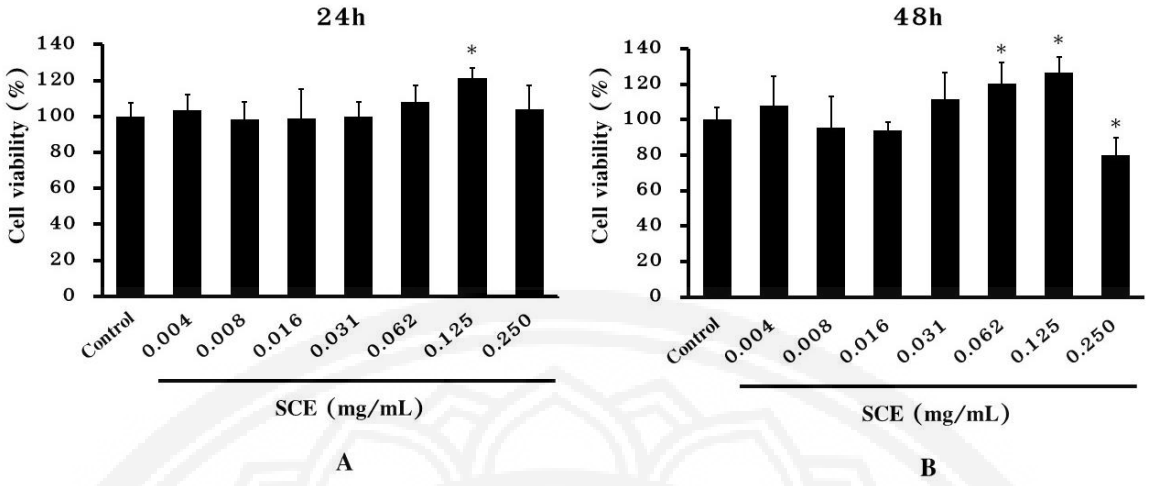

Figure 2 Effect of SCE on the human proximal tubular epithelial cell line at 24h (A) and 48h (B). * indicates significantly different from the control (p-value less than 0.05).

Discussion

Oxidative stress is a state of an imbalance between the production of free radicals and antioxidant defense mechanisms. Persistent oxidative stress is known to play a crucial role in the pathogenesis of many types of renal disease such as acute kidney injury (AKI) and chronic kidney disease (CKD) (Kao, Ang, Pall, & Struthers, 2010). The proximal tubule is a major site of free radical generation as a result of its massive production of ATP and oxygen consumption. Highly active mitochondrial electron transport chain for ATP production in the proximal tubular epithelial cells renders these cells sensitive to oxidative stress. The enhanced oxidative stress causes DNA, protein, and lipid damages, leading to cell dysfunction and/or cell death by either apoptosis or necrosis. Oxidative stress offers a potential target for disease prevention and therapeutic intervention. Free radical scavengers reduce DNA damage, lipid peroxidation, and protein damage (Small, Coombes, Bennett, Johnson, & Gobe, 2012). This property closely associated with improved renal function and inflammation. In this study, the SCE exhibited scavenging activity on DPPH radicals. The results postulated that the compounds of the SCE might contribute to its scavenging activity according to the analysis of composition in this plant extract using 2D-TLC.

In Thailand, Sa-khan (*Piper,* Piperaceae) has been broadly used for Thai traditional medicine. Benjakul, a Thai polyherbal formulation, has been used for controlled abnormal of an element in the body, balanced health, and relief of flatulence. It is composed of five plants including Sa-khan (*Piper interruptum* Opiz.), *Piper sarmentosum* Roxb., *Piper longum* L., *Plumbago indica* L., and *Zingiber officinale* Roscoe. The ethanolic extracts of Benjakul showed anti-allergic activity and exhibited potent nitric oxide inhibitory effect (Makchuchit, Rattarom, & Itharat, 2017). In addition, the ethanolic extract of Benjakul showed cytotoxicity against many types of cancers such as small cell lung cancer cell line (Rattarom, Sakpakdeejaroen, Hansakul, & Itharat, 2014), large cell lung cancer cell line, cervical cancer cell line, and liver cancer cells (Ruangnoo et al., 2012). Moreover, ethanolic extract of Sa-khan alone significantly stimulated lymphocyte proliferation (Panthong & Itharat, 2004).

Proximal tubule has a high ability to repair after injury. A main aspect is a proliferative behavior of proximal tubular epithelial cells. Recently, cell fate tracing studies have shown that repair of injured tubules does not

involve progenitors but the key to renal repair is proliferation of proximal tubular epithelial cells itself (Humphreys et al., 2011). In this study, the SCE could significantly promote the proliferation of the normal human proximal tubular epithelial cell line in a dose- and time-dependent manner. This result provides information that the SCE may contain compound(s) with potential health-promoting properties. However, further researches on identification and molecular mechanism of the phytochemicals are necessary to figure out the potent bioactive compounds and understand their pharmacological activity.

Conclusion and Suggestions

In this study, the phenolic content, antioxidant activity, and effect on the normal human proximal tubular epithelial cell line of the SCE were investigated. The result indicated that the SCE contained phenolic compounds and showed antioxidant property. In addition, 2D-TLC chromatogram of the SCE revealed eleven spots with antioxidant activity. Moreover, experimental evidence demonstrated that the SCE could significantly promote the proliferation of HK-2 cells. These results provide information that the SCE contained antioxidants with potential health benefits. However, further investigation of the mechanism of action and toxicity are required before this medicinal plant or its active compounds become a new option for the prevention or the treatment of kidney diseases.

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References

Ceriello, A., & Motz, E. (2004). Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol, 24*(5), 816-823.

Humphreys, B. D., Czerniak, S., DiRocco, D. P., Hasnain, W., Cheema, R., & Bonventre, J. V. (2011). Repair of injured proximal tubule does not involve specialized progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(22), 9226-9231.

Kao, M. P., Ang, D. S., Pall, A., & Struthers, A. D. (2010). Oxidative stress in renal dysfunction: mechanisms, clinical sequelae and therapeutic options. *Journal of Human Hypertension*, *24*(1), 1-8.

Kondo, S., Sattaponpan, C., Phongpaichit, S., Srijan, A., & Itharat, A. (2010). Antibacterial activity of Thai medicinal plants Pikutbenjakul. *The Journal of Medical Association of Thailand*, *93*, 131-135.

Makchuchit, S., Rattarom, R., & Itharat, A. (2017). The anti-allergic and anti-inflammatory effects of Benjakul extract (a Thai traditional medicine), its constituent plants and its some pure constituents using in vitro experiments. *Biomed Pharmacother*, *89*, 1018-1026.

Panthong, S., & Itharat, A. (2014). Effect of *Piper chaba* Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz. on natural killer cell activity and lymphocyte proliferation. *The Journal of Medical Association of Thailand*, *8*, 133-139.

Ratliff, B. B., Abdulmahdi, W., Pawar, R., & Wolin M.S. (2016). Oxidant Mechanisms in Renal Injury and Disease. *Antioxid Redox Signal, 25*(3), 119-146.

Ruangnoo, S., Itharat, A., Sakpakdeejaroen, I., Rattarom, R., Tappayutpijam, P., Pawa, K. K. (2012). *In vitro* cytotoxic activity of Benjakul herbal preparation and its active compounds against human lung, cervical and liver cancer cells. *The Journal of Medical Association of Thailand*, *95*, 127-134.

Singh, A., Kukreti, R., Saso, L., & Kukreti, S. (2019). Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. *Molecules, 24*(8), 1583. doi: 10.3390/molecules24081583.

Sireeratawong, S., Itharat, A., Lerdvuthisopon, N., Piyabhan, P., Khonsung, P., Boonraeng, S., & Jaijoy, K. (2012). Anti-Inflammatory, Analgesic, and Antipyretic Activities of the Ethanol Extract of *Piper interruptum* Opiz. and *Piper chaba* Linn. *ISRN Pharmacol*, *2012*, 480265. doi: 10.5402/2012/480265.

Small, D. M., Coombes, J. S., Bennett, N., Johnson, D. W., & Gobe, G. C. (2012). Oxidative stress, anti-oxidant therapies and chronic kidney disease. *Nephrology*, *17*(4), 311-321.

Steven, S., Frenis, K., Oelze, M., Kalinovic, S., Kuntic, M., Bayo-Jimenez, M. T., Daiber, A. (2019). Vascular Inflammation and Oxidative Stress: Major Triggers for Cardiovascular Disease. *Oxid Med Cell Longev, 2019,* 7092151. doi: 10.1155/2019/7092151.

Sullivan, L. B., & Chandel N. S. (2014). Mitochondrial reactive oxygen species and cancer. *Cancer Metab, 2,* 17. doi: 10.1186/2049-3002-2-17.