ฤทธิ์ต้านภาวะเครียดออกซิเดชันในไตของสารสกัดจากดอกสะเดา ในหนูขาวที่ถูกเหนี่ยวนำความเครียด
Azadirachta indica Flower Extract Attenuates Kidney Oxidative Stress in Rat Exposed to Restraint Stress

นิพนธ์ต้นฉบับ **Original Article**

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ต้านภาวะเครียดออกซิเดชั่นในไตของสารสกัดจากดอกสะเดา *(Azadirachta indica)* ในหนูขาวที่ถูกเหนี่ยวนำให้เกิดความเครียดสะสม **วิธีการศึกษา:** แบ่งกลุ่มหนูขาวเป็น 5 กลุ่ม ได้แก่ กลุ่มควบคุม, กลุ่มถูกเหนี่ยวนำความเครียด, กลุ่มถูกเหนี่ยวนำความเครียดและได้รับสารสกัดจากดอกสะเดาในปริมาณ 250, 500, และ 1,000 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว หนูขาวจะได้รับสารสกัดเป็นเวลา 30 นาที ก่อนถูกเหนี่ยวนำความเครียด โดยการให้อยู่ในกล่องที่มีขนาดเท่ากับลำตัวเพื่อจำกัดการเคลื่อนไหวเป็นเวลา 3 ชั่วโมงต่อวัน ทำการทดลองติดต่อกันเป็นเวลา 30 วัน จากนั้น ทำการสลบหนูขาวด้วย Thiopental sodium 40 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว เก็บเลือดจากหัวใจเพื่อวัดระดับยูเรียไนโตรเจนและครีเอทินีน ทำการุณยฆาตและนำไตออกมาศึกษาภาวะเครียดออกซิเดชั่น โดยการวัดปริมาณ Malondialdehyde (MDA) และการทำงานของเอนไซม์ต้านสารอนุมูลอิสระ ได้แก่ Superoxide dismutase (SOD) **ผลการศึกษา:** หนูขาวที่ถูกเหนี่ยวนำความเครียดมีปริมาณ MDA และการทำงานของ SOD ในไตเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ การได้รับสารสกัดจากดอกสะเดาในปริมาณ 1,000 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว สามารถลดระดับ MDA และเพิ่มการทำงานของ SOD ในไตอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ หนูขาวที่ถูกเหนี่ยวนำความเครียดมีระดับครีเอทินีนในเลือดลดลง และสารสกัดจากดอกสะเดามีผลเพิ่มระดับครีเอทินีนในเลือด อย่างมีนัยสำคัญทางสถิติ **สรุป:** สารสกัดจากดอกสะเดามีฤทธิ์ยับยั้งภาวะเครียดออกซิเดชั่นในไตของหนูขาวที่ถูกเหนี่ยวนำให้มีความเครียดสะสม โดยมีผลเพิ่มการทำงานของเอนไซม์ต้านสารอนุมูลอิสระในไต

คำสำคัญ: สะเดา, เครียด, เครียดออกซิเดชั่น, การต้านอนุมูลอิสระ, ไต

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Abstract

Objective: This study aims to investigate a protective effect of extract from *Azadirachta indica* flower against oxidative stress and anti-oxidant activity in kidneys of rats exposed to restraint stress. **Methods:** Wistar rats were divided into five groups: control group, stress group, stress group with *Azadirachta indica* flower extract (AIFE) 250, 500, and 1,000 mg/kg body weight. AIFE were given daily to the rats by oral gavage, for 30 consecutive days. Stress rats were kept in a restrainer to induce restraint stress for 3 hours every day, for 30 days, after received AIFE 30 minutes. To evaluate oxidative stress and anti-oxidant activity, levels of malondialdehyde (MDA) and superoxide dismutase (SOD) activity were determined in kidney tissues. Blood urea nitrogen (BUN) and serum creatinine were measured to investigate kidney function. **Results:** Stress rats showed a significant increase of MDA level and SOD activity in the kidney tissues when compared to the control ($P < 0.05$). Administration with 1,000 mg/kg body weight of AIFE to the stress rats demonstrated a therapeutic effect to reduce MDA level in the kidneys ($P < 0.05$) associated with significant increasing of SOD activity ($P < 0.05$), compared to untreated stress rats. In addition, serum creatinine was significantly decrease in stress rats and AIFE supplementation can restore this effect ($P < 0.05$). **Conclusion:** This study indicates that AIFE exhibits a protective effect on kidney oxidative stress in the stress condition by improving an anti-oxidant activity.

Keywords: *Azadirachta indica*, restraint stress, oxidative stress, anti-oxidant, kidney

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Introduction

Stress is considered as a cause promoting psychological and physiological health. The body responds to this condition by increasing cellular metabolism which can develop a production of free radicals.[1] Overproduction of free radicals that exceed an efficacy of anti-oxidant mechanism leads to a condition called oxidative stress.[2] An excess of free radicals can cause cellular damage and mediate organs dysfunction.[3] Previous studies have demonstrated that oxidative stress is a main factor in several diseases progression such as diabetes, neurodegenerative diseases, cardiovascular disease, and chronic kidney disease.[4-7] Thus, long-term exposure to stress may be an important cause of increased morbidity rate in various disorders.

Restraint stress is one of the most widely used technique for inducing stress condition. It limits the movement of rodent which can promote oxidative stress by generating free radicals

and altering anti-oxidant enzymes activities in tissues.[8] There was a study has reported that restraint stress induced oxidative stress in brain, liver, and kidneys of rats by increasing oxidative stress marker, malondialdehyde (MDA), and decreasing levels of anti-oxidant enzymes including reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT).[9] Restraint stress is also found to alter kidney morphology and kidney function. Using a restrainer to inhibit the movement of rats for 2 hours over 5 weeks affects morphological changes of their kidneys.[10] Moreover, restraint stress induction to the rats could promote inflammation and apoptosis via endoplasmic reticulum stress in the kidneys, results to kidney injury and dysfunction.[11] These data reveal that restraint stress can facilitate kidney injury that may involve in an induction of oxidative stress.

A number of natural products have been demonstrated their anti-oxidant effect. *Azadirachta indica* A. Juss. var. *siamensis* Valeton (*A. indica*, neem) is one of the most medicinal plants, having a variety of biological activities, such as anti-oxidant, anti-inflammatory, anti-diabetic, and anti-cancer effects.[12] Edible parts of *A. indica* have been reported their pharmacological properties. *A. indica* leaf extract was found to ameliorate hyperglycemia and diabetic nephropathy[13], normalize altered oxidative stress and anti-oxidant system[14], attenuate nephrotoxicity[15], and relieve hypertensive effect and cardiovascular complications[16]. Furthermore, recent study has shown that *A. indica* flower extract demonstrated its effect on functional recovery of sciatic nerve crush injury in diabetic rats that the mechanism underlying is associated with an anti-oxidant effect.[17] However, an anti-oxidant effect of *A. indica* flower extract on kidney oxidative stress has not been elucidated. This study aimed to investigate a protective effect of *A. indica* flower extract on oxidative stress in the kidneys of rats exposed to restraint stress.

Methods

Preparation of *Azadirachta indica* flower extract

Flowers of *A. indica* in the stage of beginning of the blossom were collected during December 2015 to January 2016 from Maetumboonyong city, Phayao, Thailand. The flowers were placed as voucher specimen (No. 003805) in herbarium of Faculty of Biology, Naresuan University, Phitsanulok, Thailand. The flowers were washed and mixed with distilled water in a ratio of plant to water 1:3. The solution of flowers and distilled water were filtered and freeze-dried to obtain a powder extract. The powder extract of *A. indica* flower was stored at -20 °C until used.

Animals

Thirty male Wistar rats weighting between 200-250 grams were included in this study. Rats were purchased from the Nomura Siam International Co., Ltd. (Bangkok, Thailand). The animals were housed in a well-ventilated room with controlled temperature at 25 ± 1°C under artificial 12-h light-dark cycle. They were provided with standard rat food and water *ad libitum*. The rats were acclimatized for 2 weeks before starting experiment. All experiments in this study were conducted according to the protocol of the guidelines for laboratory animal care and use, and were approved by the animal ethics committee at Mae Fah Luang University, Chiang Rai, Thailand.

Experimental protocol

Rats were randomly divided into five groups with six animals in each group: control (C); stress (St); stress with 250 mg/kg BW of AIFE (St+AI250); stress with 500 mg/kg BW of AIFE (St+AI500); and stress with 1,000 mg/kg BW of AIFE (St+AI1000). The experiments were conducted for 30 consecutive days. *Azadiracta indica* flower extract (AIFE) was provided daily by orally gavage to the rats 30 minutes before inducing of restraint stress. AIFE was freshly prepared daily by dissolving in distilled water and gave to the rat with 0.5 mL of total volume per 300 grams of rat's body weight. For restraint stress induction, rats were restrained the movement by keeping in a rodent restrainer made of wood that can be adjusted diameters and length depending on the animal's weight and size. Immobilization was performed for 3 hours per day. As a control group, free movement is allowed for control rats during the period of restraint stress induction. At the end of experiment, the 30th day, all rats were anesthetized with 40 mg/kg BW injection of thiopental sodium. Blood was subsequently collected from cardiac puncture. Right and left kidneys were immediately dissected, cleared of capsule, and weighed. Cortex layer of the kidneys were removed and stored at -80 °C until used.

Relative kidney weight

Each rat was weighted at the end of experiment. The total kidney weight was average from the left and the right kidneys weight. The relative kidney weight was calculated by dividing total kidney weight by the body weight. Finally, percentage of the relative kidney weight was expressed.

Renal function measurement

Serum was separated to determine the levels of serum creatinine and blood urea nitrogen (BUN). The serum creatinine and BUN were measured using an automatic biochemical analyzer (COBAS INTEGRA® 400 plus analyzer, Roche, Germany) at clinical laboratory Meng Rai Lab, Chiang Rai, Thailand.

Renal protein extraction and quantification

Renal cortical tissues were weighted to 0.04 grams and homogenized on ice in mammalian cell lysis buffer (Sigma-Aldrich, USA) with 1% protease inhibitors cocktail (Roche Applied Science, USA). Homogenized tissue was then centrifuged at 1,600 x g for 10 minutes at 4 °C. Supernatant was collected and used as whole cell protein lysate. Total protein concentration was evaluated using colorimetric Bradford protein assay by commercially available kits (Bio-Rad, USA). Bovine serum albumin (BSA) was used as a standard control. Extracted protein was stored at -80 °C until used.

Measurement of kidney oxidative stress

To determine kidney oxidative stress, malondialdehyde (MDA) level was measured in renal cortical tissue. MDA is a product from an oxidation of lipids that used as a biomarker to measure the level of oxidative stress. The renal cortical MDA was determined using commercial thiobarbituric acid (TBAR) assay kit (Cayman Chemical, USA) according to the manufacturer's protocols. Briefly, MDA reacts with thiobarbituric acid (TBA) to form thiobarbituric acid reactive substances (TBARS), which absorbed at a wavelength of 540 nm. The level of TBARS is therefore proportionate to the amount of MDA. Concentration of TBARS was calculated using standard MDA. The amount of MDA was expressed as nmol/mg of protein.

Measurement of kidney anti-oxidant status

Renal anti-oxidant status was determined by measuring the activity of superoxide dismutase (SOD) enzyme using commercial SOD determination kit (Sigma-Aldrich, USA). The method was conducted according to the manufacturer instructions. SOD catalyzes the dismutation of superoxide anion into hydrogen peroxide and oxygen. To determine SOD activity, highly water-soluble tetrazolium salt (WST-1) produce ก a water-soluble formazan dye upon reduction with a superoxide anion. The absorbance was measured at a wavelength of 450 nm. The percent inhibition of the rate of WST-1 formazan formation was expressed as SOD activity.

Ethics approval

All experiments were approved by the Ethics Committee of the Laboratory Animal Research Center, Mae Fah Luang University, Chiang Rai, Thailand (Approval number AR04/62).

Statistical analysis

Data were showed as mean ± SEM. The differences between groups were analyzed using a one-way ANOVA followed by Fisher's least significant difference test (LSD). Statistically significant was considered at P values of less than 0.05.

Results

Effects of AIFE on kidney parameters are shown in Table 1. Comparing the relative kidney weight of rats exposed to restraint stress to the control, a significant kidney weight loss was found in stress rats ($P < 0.05$). Administration of AIFE 250, 500, and 1,000 mg/kg BW to the stress rats did not show a restoration of the relative kidney weight. For kidney function, level of BUN was found significant reduction in the stress rats, compared to the control rats ($P < 0.05$). However, AIFE treatment to the stress rats could not restore the level of BUN. In addition, serum creatinine has reduced significantly in rats exposed to restraint stress when compared to the control rats ($P < 0.05$). Pretreatment with 250, 500, and 1,000 mg/kg BW of AIFE to the stress rats significantly increase the level of serum creatinine ($P < 0.05$), compared to untreated stress rats.

The effects of AIFE on kidney oxidative stress and anti-oxidant status are shown in Figure 1. MDA level, an indicator for oxidative stress, shows marked elevation in the renal

cortical tissue of stress rats, compared to the control rats (P < 0.05, Figure 1A). Significant dose-dependent decreases kidney MDA level was seen in the stress group treated with AIFE at 1,000 mg/kg BW when compared to untreated stress group (P < 0.05). The restoration of kidney MDA level indicates an oxidative stress protective effect of AIFE. Additionally, there was a significantly increase in SOD activity in renal cortical tissue of the stress rats, compared to the control rats (P < 0.05, Figure 1B). Supplementation with 250 and 500 mg/kg BW of AIFE to the stress rats have no effect on SOD activity. However, there was a marked significant increase in SOD activity seen in the stress group treated with 1,000 mg/kg BW of AIFE when compared to the stress group (P < 0.05). These results indicate that SOD activity was increased in the stress condition and following 1,000 mg/kg BW of AIFE treatment.

Table 1 Effects of AIFE on kidney parameters.

Parameters	Control	Stress	St+AI250	St+AI500	St+AI1000
Body weight (g)	369.33 ± 10.47	372.17 ± 6.93	349.17 ± 9.29 †	352.67 ± 4.18	358.67 ± 6.82
Kidney weight (g)	1.36 ± 0.06	1.22 ± 0.02*	1.21 ± 0.03*	1.18 ± 0.04*	1.15 ± 0.03*
Relative kidney weight (%)	0.37 ± 0.01	0.33 ± 0.00*	0.35 ± 0.01	0.33 ± 0.01*	0.32 ± 0.00*
BUN (mg/dL)	26.82 ± 0.61	23.42 ± 0.71*	23.08 ± 1.18*	24.30 ± 1.32	23.93 ± 0.73*
Creatinine (mg/dL)	0.26 ± 0.01	0.23 ± 0.01*	0.25 ± 0.01 †	0.26 ± 0.01 †	0.26 ± 0.01 †

Data are mean ± SEM (n = 6 per group). One-way ANOVA followed by Fisher's least significant difference test was used for multiple comparisons. *P < 0.05 compared with control group. †P < 0.05 compared with stress group. St+AI250: stress group with 250 mg/kg BW of AIFE; St+AI500: stress group with 500 mg/kg BW of AIFE, and St+AI1000: stress group with 1,000 mg/kg BW of AIFE.

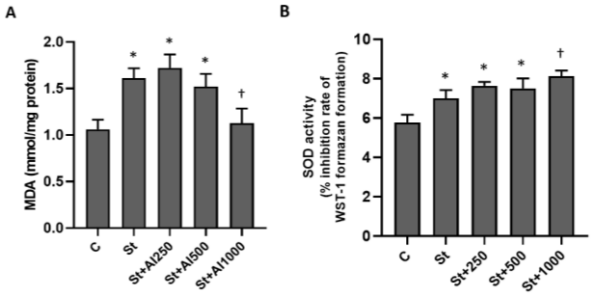

Figure 1 Effects of AIFE on kidney oxidative stress and anti-oxidant status. (A) Renal cortical tissue MDA level. (B) Renal cortical tissue SOD activity. Data are mean ± SEM (n = 5 per group). One-way ANOVA followed by Fisher's least significant difference test was used for multiple comparisons. *P < 0.05 compared with control group. †P < 0.05 compared with stress group. C: control group; St: stress group; St+AI250: stress group with 250 mg/kg BW of AIFE; St+AI500: stress group with 500 mg/kg BW of AIFE, and St+AI1000: stress group with 1,000 mg/kg BW of AIFE.

Discussions and Conclusion

The present study shows that using rat restrainer that closely fit to the rats' body for 3 hours per day, for 30 consecutive days, causes an increasing of MDA level, an oxidative stress marker, in the renal cortical tissues. This result is in accordance with the previous studies that have reported that stress condition induced by restraint stress was characterized by an increased production of MDA in the kidney tissues.[9, 18] Increased production of free radicals results to induce anti-oxidant mechanism against oxidative stress. This study also demonstrates that the stress rats show a significantly increase in activity of SOD, a natural cellular anti-oxidant enzyme, in the kidney tissues. This result in a higher activity of SOD is a compensatory mechanism to the increase in MDA level. It suggests that the mechanism of free radical scavenging activity is responded to the increased production of free radicals in the kidney of rats exposed to restraint stress. *A. indica* has been documented as an anti-oxidant agent which is shown to reduce oxidative stress in the kidney.[15] The present study demonstrates that *A. indica* flower extract (AIFE) has a protective effect on kidney oxidative stress in the condition of restraint stress. The results reveal that administration with AIFE 1,000 mg/kg BW to the stress rats ameliorates MDA level and enhances SOD activity in the renal cortical tissues. Recent study has reported that 750 mg/kg BW of AIFE could decrease MDA level and improve SOD activity in injured sciatic nerve tissues of diabetic rats.[17] Thus, it could postulate that 1,000 mg/kg BW of AIFE is an appropriate dosage to scavenge an increasing of free radicals in the kidney under stress condition. Previous study investigated that quercetin was identified as the primary constituent presented in the extract from *A. indica* flower.[19] This flavonoid has shown an anti-oxidative effect.[20] It is suggested that the quercetin may be an active component which attenuated kidney oxidative stress in the present study.

The decrease in kidney weight has been associated with glomerular hyperfiltration[21] and renal tubular atrophy[22] which is considered as a factor for promoting kidney damage and kidney dysfunction. Previous study has reported that chronic stress is an important factor that decreases the number of nephrons and glomerular volume.[10] It is suggested that chronic exposure to stress is the cause of decrease in kidney

weight in the present study that may encourage future kidney damage and kidney dysfunction. However, AIFE has no effect on an improving the kidney weight. Oxidative stress can damage kidney tissues and alter kidney function. This study shows that restraint stress causes a reduction in serum creatinine level. This result is in accordance with a recent study. The study reported that restraint stress promoted histological changes in the rats' kidney associated with glomerular hyperfiltration, leading to a decrease in serum concentration of creatinine.[23] It is proposed that the reducing of serum creatinine caused by chronic stress might be involved in the kidney histological changes-associated glomerular hyperfiltration. Furthermore, we found that decreasing of serum creatinine in the stress rats was restored by AIFE pretreatment. Previous study demonstrated that leaf extract of *A. indica* could restore an elevated serum creatinine and improved kidney histopathological damage in Cisplatin-induced nephrotoxicity rats.[15] Although histological parameters of the kidney are not investigated in the present study, it could be suggested that AIFE may restore renal histological changes by attenuating renal oxidative stress, leading to decrease in glomerular hyperfiltration and eventually improves serum creatinine level.

In conclusion, the present study reveals that AIFE provides a significant protective effect against restraint stress-induced kidney oxidative stress. The mechanism underlying of renoprotective effect by AIFE is associated with an improvement in anti-oxidant activity.

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this research.

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