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Original Article

Hypouricemia and nephroprotection of *Coix lacryma-jobi* L. seed extract

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Abstract

The *Coix lacryma-jobi* L. or Adlay seed has been used as a healthy food and herb in many Asian countries. A previous *in vitro* study found that it has an inhibitory effect on xanthine oxidase (XOD) activity but its use as a traditional medicine in the treatment of hyperuricemia and gout has a lacked scientific evidence. This study aimed to investigate the effects of Adlay seed extract on the level of plasma uric acid (Pua), reduction in fractional excretion of urate (%FEua), decreasing creatinine clearance (Ccr), and increasing plasma creatinine and urea nitrogen in hyperuricemic mice. The steps of the study comprised collecting and inducing the sample mice by giving potassium oxonate and uric acid and treating the hyperuricemic mice by giving Adlay seed extract at doses of 0.1 to 100 mg/kg for 10 days. The results showed that Adlay seed extract significantly reduced Pua in a dose-dependent manner, increased %FEua and Ccr to normal level at doses of 10 and 100 mg/kg and also inhibited XOD activity in liver and plasma in hyperuricemic mice. From this results indicated that Adlay seed extract has hypouricemia and nephroprotective action in hyperuricemic mice.

Keywords: *Coix lacryma-jobi* L, hypouricemia, nephroprotection, xanthine oxidase, potassium oxonate

1. Introduction

Hyperuricemia refers to a level of uric acid (UA) in blood serum that is above normal and results from over-production of uric acid or impairment of renal excretion of uric acid, or a combination of both. Hyperuricemia is a key risk factor of gout and it has been causally linked to renal dysfunction, cardiovascular diseases, hypertension, hyper-lipidemia, cancer, diabetes and metabolic syndrome (Chen *et al.*, 2001; Lu *et al.*, 2012; Lippi *et al.*, 2008; Feig *et al.*, 2006; Choi and Ford, 2007). A number of recent studies have shown an association between hyperuricemia and the development of chronic kidney disease. The role of uric acid in kidney disease has waxed and waned. (Kabul and Shepler, 2012)

Xanthine oxidase (XOD) plays as an important role in the catabolism of purines in humans. First, XOD catalyzes hypoxanthine to xanthine via oxidation reaction then catalyzes xanthine to uric acid via the same reaction (Hille, 2005; Harrison, 2002). This is the mechanism of over-production of uric acid in blood and acts a primary cause of hyperuricemia (Richette and Bardin, 2010). Allopurinol and Febuxostat, xanthine oxidase inhibitors, were considered to treat in this condition but allopurinol has life-threatening side effects such as hypersensitivity consisting of fever, skin rash, eosinophilia, hepatitis, and renal toxicity and having a fatality rate approaching 20% (Kumar *et al.*, 1996; Richette

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and Bardin, 2010). Many side effects of febuxostat, a new non-purine XOD inhibitor, have been reported (Yu, 2007). Benzbromarone and probenecid, uricosulic agents, also have some undesirable effects such as hepatotoxicity (Perez-Ruiz *et al.*, 1998; Schlesinger, 2004).

Adlay or Job's-tears (*Coix lacryma-jobi* L.) is a native plant of South East Asia countries such as China, Japan, Philippines, Burma and Thailand and it has been used as an alternative healthy food and a drug for thousands of years (Khongieamsiri *et al.*, 2011). In Thailand, Adlay seed is consumed by removing seed coats and may be boiled in water to produce a beverage. Not only has Adlay seed been used as food but also it has medicinal values in the treatment of anthrax, beriberi, diabetes, fever, headache and worms (Lu *et al.*, 2008), protecting from tumor stimulating compounds, protecting viral infection, reducing allergic reaction, reducing coronary artery disease and arthrosclerosis and reducing osteoporosis (Hung and Chang, 2003; Woo *et al.*, 2007; Yu *et al.*, 2011; Wang *et al.*, 2012). Furthermore, *in vitro* study found that Adlay seed extract had an inhibitory effect on xanthine oxidase (XOD) activity (Taejarernwiriyakul *et al.*, 2011) but its use as a traditional medicine in the treatment of hyperuricemia and gout has a lacked of scientific evidence (Fritsch and Sidoroff, 2000). Therefore, this study aimed to evaluate the hypouricemic effects of Adlay seed extract *in vivo* model by focusing on xanthine oxidase (XOD) activity, plasma creatinine (Pcr), blood urea nitrogen (BUN) levels and creatinine clearance (Ccr) in hyperuricemic mice.

2. Materials and Methods

2.1 Reagents

Potassium oxonate (Wako), allopurinol (Sigma), uric acid (Sigma), benzbromarone (Sigma), xanthine (Sigma), xanthine oxidase enzyme (Wako).

2.2 Plant material

The dried Adlay seed was purchased from a herbal drug store in Ubonratchathani province. Whole seeds were milled and percolated in methanol for 24 hr and a total of 3 times. Methanol was kept and the collected solution concentrated by rotary evaporation.

2.3 Animals

Male ICR mice (26–30 g) were purchased from the CLEA Company (Japan CLEA Co., Tokyo, Japan) and were housed in plastic cages. All of these animals were allowed one week to adapt to their environment before use, were maintained in 12 hr light/12 hr dark cycles at 25°C and were given the CLEA Rodent Diet CE-2 food from the CLEA Company (Japan CLEA Co., Tokyo, Japan) and water *ad libitum*. In the experiment, all of the procedures were conducted according to the PR Japan legislation on the use and care of laboratory animals and followed the guideline established by the institute for experimental animals of Dokkyo Medical University, School of Medicine, and approved by the university committee for animal experiments.

2.4 Animal model of hyperuricemia in mice

Animal models were induced by potassium oxonate, a uricase inhibitor, which has been used to study of drug action (Yonetani *et al.*, 1980). In this study, 40 mice were divided into 8 groups each of 5 mice. All groups received various agents for 10 days as following: normal control group (water and 0.9% saline), hyperuricemia control group (300 mg/kg of potassium oxonate suspended in 0.9 % saline and 100 mg/kg of uric acid suspended in 0.9 % saline and water), and six last groups. The last groups all received 300 mg/kg of potassium oxonate suspended in 0.9 % saline and 100 mg/kg of uric acid suspended in 0.9 % saline, with the addition, respectively of allopurinol (10 mg/kg suspended in water), benzbromarone (15 mg/kg suspended in water) or Adlay seed extract at a dose of 0.1, 1, 10 or 100 mg/kg. The potassium oxonate and uric acid were injected intraperitoneally while allopurinol, benzbromarone and Adlay seed extract were administered orally once daily from day 1 to day 10 in mice.

2.5 Blood and urine collection

At day 10 of treatment, 24 hr urine samples were collected and the volume recorded. Then whole samples were centrifuged at 2000×g for 10 min to remove particulate contaminants and the supernatant used to detect uric acid, creatinine and urea nitrogen. Blood samples were collected from tail bleeding and cardiac puncture in the tubes containing 200 units/ml heparin in saline and the samples were cooled in ice water immediately after collection. The plasma was separated as soon as possible by centrifuging at 3,000×g at 4°C for 10 min.

2.6 Determination of uric acid

Plasma uric acid level (Pua) and urine uric acid level (Uua) were determined by modified ferric reducing ability of plasma (FRAP) assay, using 96-well plates and following the method reported by Duplancic *et al.* (2011). Briefly, 2.5 µl of plasma, urine or uric acid standard were mixed with 0.5 µL of PBS (pH 7.4) or PBS-containing uricase enzyme (12.5 U/ml) and incubated at 25°C for 20 min. 100 µL of FRAP reagent (acetate buffer: 10 mmol/L TPTZ: 20 mmol/l $FeCl_3$ ratio 10:1:1) was then added and the samples kept at 37°C for 30 min, and then the absorbance at 590 nm measured. Uric acid was calculated as A-B, where A is the uric acid level of samples without uricase enzyme and B is the uric acid level of samples with uricase enzyme.

2.7 Determination of plasma creatinine (Pcr) and urea nitrogen (BUN) concentrations

Urine creatinine (Ucr) and the urea nitrogen (Un) concentrations were determined by using a standard diagnostic kit (Bioassay system, USA).

- Excretion of urate in 24 hr = volume of urine in 24 hr × Uua
- Urine urate clearance (Cua) = (Uua × Volume of urine in 24 hr)/(Pua × 24 × 60)
- Fractional excretion of uric acid (%FEua) = (Uua × Pcr)/(Pua × Ucr) × 100, expressed as a percentage.
- Creatinine clearance (Ccr) = (Ucr × Volume of urine in 24 hr)/(Pcr × 24 × 60)

2.8 Liver sample collection

After determination of urate, the animals were killed by decapitation under anesthesia via i.p. injection of pentobarbital. The liver was excised, frozen and stored at -80°C until use. Tissue samples were homogenized with 5 volumes of 50 mM potassium phosphate buffer (pH 7.4) contains 1 mM EDTA-Na. Each homogenate was centrifuged at 3000 ×*g* for 15 min, the lipid layer carefully removed and the supernatant kept. The supernatant was then further centrifuged at 10,000×g at 4°C for 60 min and used for detecting xanthine oxidase activity.

2.9 Assay of xanthine oxidase activity in liver and plasma

XOD activity was assayed by spectrophotometry in aerobic condition and followed the method reported by Stavric *et al.* (1975), Hall *et al.* (1990) and Yu *et al.* (2006). Briefly, the assayed mixture consisted of 50 µl of uric acid or test solution (plasma and liver of sample) and 145 µl of 50 mM potassium phosphate buffer (pH 7.4) and kept at 25°C for 15 min. 60 µl of substrate solution (150 mM xanthine with the same buffer) were then added and the sample incubated at 37°C for 30 min. The reaction was terminated by adding 25 µl of 1 N HCl and the XOD activity measured with the absorbance at 290 nm. A blank was prepared with the same direction but xanthine was added to the assayed mixture after adding 1 N HCl. Uric acid was used as a standard. One unit of XOD was defined as the amount of enzyme required to produce 1 nmol of uric acid per minute at 37°C pH 7.4. The XOD activity was expressed as units per milliliter and units per gram of protein for plasma and liver. Protein concentration was determined using the Bradford protein assay kit and bovine serum albumin as the standard (the Thermo Scientific Pierce Coomassie protein assay kit).

2.10 Assay of inhibition of xanthine oxidase activity, *in vitro* study

XOD activity, using xanthine as a substrate, was assayed by spectrophotometry with the absorbance at 290 nm and followed the method reported by Yu *et al.* (2006). The assayed mixture consisted of 145 µl of 50 mM potassium phosphate buffer (pH 7.5) and 5 µl of 1 U enzyme with or without the test samples and kept at 25°C for 15 min. 100 µl of substrate solution (150 mM xanthine in the same buffer) was then added and the sample incubated at 25°C for 30 min. The reaction was terminated by adding 50 µL of 1 N HCl and XOD activity measured with the absorbance at 290 nm. The IC_{50} value of samples was calculated from regression line of the percentage inhibition of XOD activity versus the concentration of the sample. The test sample solutions were dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted with phosphate buffer (pH 7.5) to a final concentration which contained less than 1% of DMSO (v/v). All of the determinations were performed in triplicate. This assay was carried out at five different concentrations ranging from 0.1-100.0 µg/ml and used allopurinol as a positive control at a final concentration of 10 µM in the assayed mixture.

2.11 Statistical analysis

The statistical analysis was performed using Student's t-test. The values were expressed as mean±S.E.M. and the significant difference was accepted with P-value less than 0.05.

3. Results

3.1 Adlay seed extract reduced plasma uric acid (Pua), plasma creatinine (Pcr) and blood urea nitrogen (BUN) levels in hyperuricemic mice

Mice were induced by given 300 mg/kg potassium oxonate and 100 mg/kg uric acid giving via intraperitoneal route for 10 days. Figure 1A shows a significant elevation of plasma uric acid level compared with normal control group ($P \leq 0.05$) and indicated that these animal models were successful in inducing hyperuricemic mice. Treatment of Adlay seed extract (0.1, 1, 10, and 100 mg/kg) has a significant effect of reducing plasma uric acid (Pua) comparable with allopurinol (10 mg/kg) and benzbromarone (15 mg/kg) in hyperuricemia group. Figure 1B shows that Adlay seed extract, allopurinol and benzbromarone each have a significant effect of reducing plasma creatinine (Pcr) in hyperuricemia group and also have a significant effect of reducing blood urea nitrogen (BUN) (Figure 1C).

3.2 Adlay seed extract enhanced the urinary urate excretion and improved renal function in hyperuricemic mice

Table 1, Treatment of Adlay seed extract at 10 and 100 mg/kg and benzbromarone each significantly increased Cua and %FEua in hyperuricemic mice. In addition, Adlay seed extract at 10 and 100 mg/kg and allopurinol each signifi-

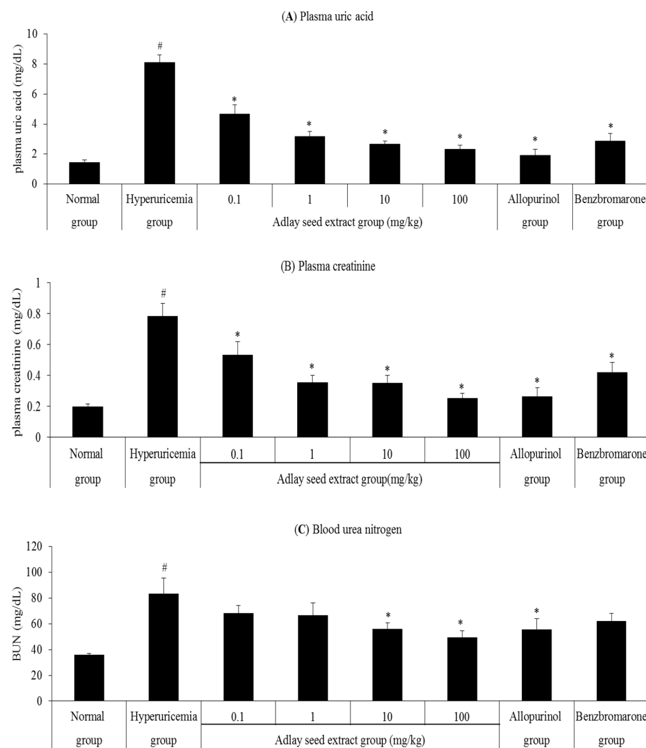

Figure 1. The effects of Adlay seed extract, Allopurinol and Benzbromarone on Pua (A), Pcr (B) and BUN level (C) in hyperuricemic mice. The values are expressed as mean ± S.E.M. (n=5). A value of #P<0.05 is considered statically significant compared with normal group and a value of *P<0.05 is considered statically significant compared with hyperuricemia group.

cantly elevated Ccr compared with the hyperuricemia group. This result indicated that Adlay seed extract has a nephro-protective effects in this animal model.

3.3 Inhibition of xanthine oxidase activity from Adlay seed extract, *in vivo* study

Allopurinol has a potent inhibitory effect on xanthine oxidase activity in plasma and liver compared with hyper-uricemia group (Figure 2A). Adlay seed extract has an inhibitory effect on xanthine oxidase activity in plasma in dose-dependent manner and also has the same effect in liver compared with hyperuricemia group as shown in Figure 2B.

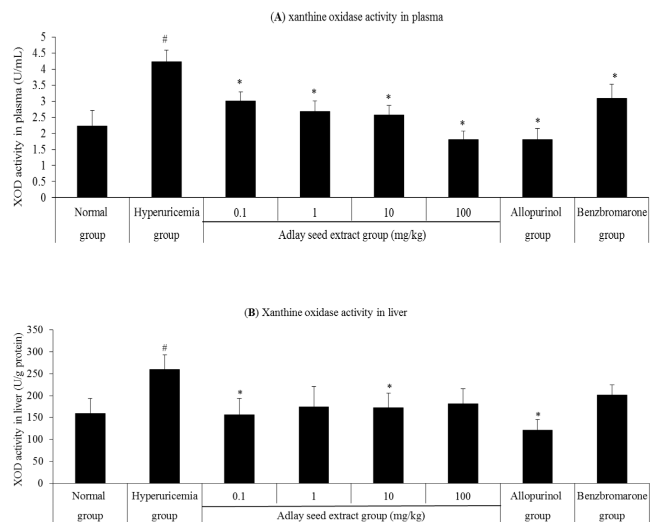

Figure 2. The effects of Adlay seed extract on xanthine oxidase activity in plasma (A) and liver (B) in hyperuricemic mice. The values are expressed as mean±S.E.M. (n=5). A value of #P<0.05 is considered statically significant compared with normal group and a value of *P<0.05 is considered statically significant compared with hyperuri-cemia group.

Table 1. The effects of Adlay seed extract, Allopurinol and Benzbromarone on the level of urine volume, Ccr, Cua and %FEua in hyperuricemic mice.

Group	Dose (mg/kg)	Urine volume (mL)	Ccr (mL/min)	Cua (mL/min)	%FEua
Normal group	-	0.91±0.22	0.08±0.03	0.013±0.01	18.15±7.79
Hyperuricemia group	-	2.33±0.59#	0.04±0.01#	0.004±0.01#	9.03±3.07#
Adlay seed extract group	0.1	1.39±1.02	0.03±0.03	0.003±0.01	13.59±7.32
	1	1.91±0.71	0.05±0.01*	0.007±0.01	13.54±5.36
	10	2.23±1.12	0.08±0.06	0.012±0.01*	18.82±11.92*
	100	1.71±0.94	0.08±0.03*	0.014±0.01*	18.48±10.66*
Allopurinol group	10	3.49±2.25	0.14±0.09*	0.025±0.02	15.16±8.89
Benzbromarone group	15	2.52±0.87	0.06±0.03	0.029±0.02*	56.84±55.04*

The values are expressed as mean ± S.E.M. (n=5). A value of #P<0.05 is considered statically significant compared with normal group and a value of *P<0.05 is considered statically significant compared with hyperuricemia group.

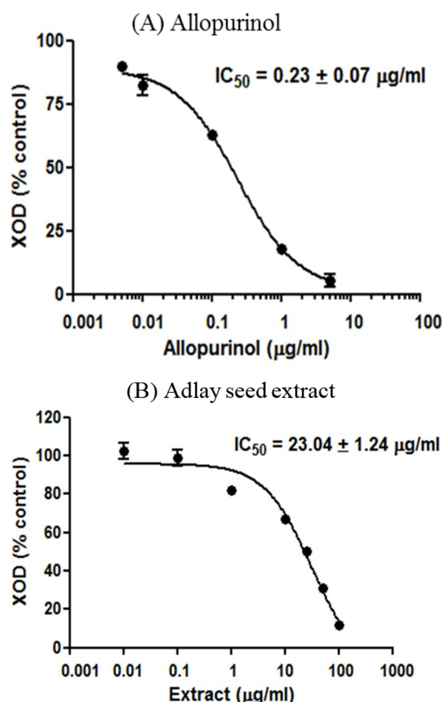

Figure 3. The effect of allopurinol (A) and Adlay seed extract (B) on inhibition of xanthine oxidase activity, *in vitro* study.

3.4 Inhibition of xanthine oxidase activity from Adlay seed extract, *in vitro* study

The IC_{50} value of allopurinol was 0.23±0.07 µg/ml as shown in Figure 3A, while the IC_{50} value of Adlay seed extract was 23.04±1.24 µg/ml as shown in Figure 3B.

4. Discussion

Potassium oxonate is a famous chemical that is used for inducing hyperuricemia in medical investigations (Stavric *et al.*, 1975; Hall *et al.*, 1990; Yu *et al.*, 2006). This chemical was given as a single injection or as an injection followed by intravenous infusion resulting in hyperuricemia and a concentration peak at 1.5 to 2 hours and duration of action at least 5 hours (Yonetani and Iwaki, 1983). However, potassium oxonate is metabolized or excreted rapidly. Thus, frequent injections are required to sustain uricase inhibitory activity. In this study, these animal models were intraperitoneally administered 300 mg/kg potassium oxonate and 100 mg/kg uric acid for 10 days with the result that uric acid level in plasma was significantly increased while urine urate clearance and %FEua decreased, which confirmed the development of hyperuricemia and impairment of renal function in mice.

Xanthine oxidase is an enzyme that catalyzes hypoxanthine and xanthine to produce uric acid via oxidation reaction (Reinders *et al.*, 2009) and has significantly activity in liver. Xanthine oxidase inhibitor e.g. allopurinol and febuxostat were used as a therapeutic approach for hyperuricemia by inhibiting the biosynthesis of uric acid from purine (Khanna *et al.*, 2012; Angelo and Kenneth, 2008). A number of clinical studies have found that allopurinol has a number of side effects e.g. hepatitis, nephropathy, allergic reaction and 6-mercaptopurine toxicity (Kumar *et al.*, 1996). Moreover, febuxostat, an effective drug that is our alternative to allopurinol also has common side effects e.g. liver function abnormalities, diarrhea, headache, nausea, vomiting, abdominal pain, arthralgia and musculoskeletal symptoms (Edwards, 2009). From the side effects of conventional drug therapy, the development of a new xanthine oxidase inhibitor, especially from less toxic of natural sources is an alternative approach to treatment of hyperuricemia. In *in vivo* study, Adlay seed extract has a significantly effect on inhibit xanthine oxidase activity in plasma and liver in hyperuricemic mice and this effect was similar to that of allopurinol. Adlay seed extract decreased xanthine oxidase activity in plasma in a dose-dependent manner; at a dose 100 mg/kg of Adlay seed extract had xanthine oxidase inhibitory activity similar to the allopurinol as shown in Figure 2A. The IC_{50} of this extract was 23.04±1.24 µg/ml which is higher than the IC_{50} of allopurinol (0.23±0.07 µg/ml). The cause of different value was the use of crude extract of Adlay seed. Allopurinol and Adlay seed extract also increased creatinine clearance in hyperuricemic mice which indicated that they have nephroprotective action. This action results from hypouricemic effect of Adlay seed by xanthine oxidase inhibition. Therefore, Adlay seed extract was confirmed to have good efficacy by inhibition xanthine oxidase activity for treatment of hyperuricemia.

In clinical practice, about 90% of gout patients are ascribed to renal urate under excretion (Wright *et al.*, 2003). Glucose transporter 9 (URATv1 or GLUT9 encoded by *SLC 2A9*) and urate anion transporter 1 (URAT1 encoded by *SLC 22A6*) mediate renal urate handling for regulation of uric acid levels and are considered as the promising therapeutic target for treatment of hyperuricemia and gout (El-Sheikh *et al.*, 2008; Preitner *et al.*, 2009; Shin *et al.*, 2011). Benzbromarone was confirmed to inhibit renal GLUT9 and URAT1 activity and expression *in vivo* and *in vitro* (El-Sheikh *et al.*, 2008). From the result as shown in Table 1, benzbromarone extremely increased %FEua and Cua in hyperuricemic mice and Adlay seed extract at doses of 10 and 100 mg/kg also increased %FEua and Cua but these values are less than the corresponding values for benzbromarone. This finding indicated that hypouricemic effect of Adlay seed extract might not be related to renal urate transporter inhibition. However, it should be further investigated.

In conclusion, the Adlay seed extract is a mediator of hypouricemic effect by inhibiting xanthine oxidase activity, resulting in decreased uric acid synthesis and increased urine uric acid in hyperuricemic mice. The active constituents of Adlay seed extract should be further investigated. Our study suggests that Adlay seed may have a considerable potential for development as a urate-lowering drug.

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