

PROTECTIVE EFFECT OF *PHYLLANTHUS EMBLICA* EXTRACT PREVENT CONTRAST-INDUCED ACUTE KIDNEY INJURY IN RATS

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ABSTRACT

Objective: The objective of the study was to investigate the anti-apoptosis effect of the extract from *Phyllanthus emblica* (PE) for the prevention of contrast-induced acute kidney injury (CI-AKI).

Methods: Male Sprague Dawley rats were given saline (control) or PE extracts (500 mg/kg/day) for 5 days before the induction of CI-AKI. Renal tissues were collected for an evaluation of gene expression and immunohistochemistry (IHC). To indicate anti-apoptotic effect, the expression levels of Bax, Bcl-2, and caspase in kidney were also determined, using real-time polymerase chain reaction (RT-PCR) and Western blot analysis.

Results: In the CI-AKI group, RT-PCR and Western blot analysis revealed that the expression levels of Bax and cleaved-caspase-3 were upregulated in the CI-AKI group, whereas the expression of Bcl-2 was downregulated. However, the pre-treatment with PE increased Bcl-2 expression. Moreover, decreased cleaved-caspases-3 activity was also detected using IHC.

Conclusion: These findings suggested that pretreatment with PE extract provided the anti-apoptotic effect against CI-AKI in the rat model.

Keywords: Anti-apoptosis, Contrast-induced acute kidney injury, *Phyllanthus emblica*.

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INTRODUCTION

Contrast-induced acute kidney injury (CI-AKI), a complication resulted from the administration of iodinated intravascular contrast medium, is the third most common cause of hospital-acquired AKI [1,2]. CI-AKI is defined as an impairment of renal function 48–72 h after receiving the contrast medium with an absolute increase of serum creatinine by 0.5 mg/dl or more or a relative increase by 25% or more in the serum creatinine level from baseline. Although the exact incidence of CI-AKI remains unclear, the incidence in a high-risk population (such as patients with chronic renal impairment and/or diabetes mellitus) has increased up to 25%, resulting in a significant rise of morbidity and mortality rate [2-5]. The pathogenesis of CI-AKI is due to the ability of the contrast agents that could induce renal vasoconstriction causing a decrease in renal blood flow and hypoxic milieu. In hypoxic tissue, the formation of reactive oxygen species (ROS) is increased, and the ROS that exceeds the cellular scavenging capacities can subsequently damage the surrounding cells and tissues [6]. The contrast media (CM) also have a direct toxic effect on tubular cells by directly inducing cellular apoptosis. Oxidative damage and apoptosis altogether contribute to the pathogenesis of CI-AKI. Thus, any intervention that can prevent apoptosis is important for improving nephropathy [7,8].

Although there are many CI-AKI preventative strategies which have been tested in various experimental studies and clinical trials, hydration with isotonic saline has been recommended as a mainstay of CI-AKI prevention [9]. Additional antioxidants agents such as *N*-acetylcysteine, ascorbic acid, Vitamin E, and other agents including statin and fenoldopam have been evaluated, but the prophylactic effects of these agents are still questionable, and they are not yet included in the recommendation for CI-AKI prevention [4,10,11].

Phyllanthus emblica (PE) Linn. (synonym. *Emblia officinalis* Gaertn.), a member of the Euphorbiaceae family, is native to the South and Southeast Asia. In Thailand, it is commonly known as Makhampom. Its fruit serves as a rich source of Vitamin C, gallic acid, minerals, and amino acid [12-14]. It also contains mixture of phenolic compounds (such as flavonoids, tannins, terpenoids, and alkaloids) which have potent various biological activities including anticancer [15-17], radioprotection [17], and anti-inflammatory [18]. Its antioxidant property has been shown in several injury-protection models of various organs including brain, heart, liver, kidney, and stomach [13,19-21]. Recently, PE has been reported that its antioxidant property accounts for the preventive effect in CI-AKI [22]. However, its anti-apoptotic effect of PE extract on CI-AKI has not been studied yet. Therefore, the main purpose of this study was to investigate whether the PE extract was able to attenuate apoptosis and to prevent CI-AKI in rat models.

METHODS

Sample preparations

In the present study, all samples were taken from the previous work [22]. Therefore, the preventive effect of PE against CI-AKI has also confirmed by renal function test and histopathology examination. The kidney samples were taken only three groups including Group 1, control rat administrated with 1 ml of normal saline; Group 2, rat administrated with CM; and Group 3 rats were orally administrated with PE (500 mg/kg/day) for 5 days before receiving CM on the following day. For protein study, small sections of the kidney tissues were frozen in liquid nitrogen and stored at -70°C before being analyzed by Western blot analysis. Separated sections were fixed in RNAlater Stabilization Solution (Ambion, Inc.) for gene expression studies using real-time polymerase chain reaction (RT-PCR).

Immunohistochemistry (IHC) staining of cleaved caspase-3 in kidney tissue

Paraffin-embedded tissues were cut into 4 μm thick slices for IHC. The sections were deparaffinized and rehydrated by passing through xylene, series of ethanol, and water. Antigen retrieval was performed by boiling the sections in sodium citrate buffer for 10 min and then steadily cooling at room temperature. Subsequently, the sections were blocked using 3.0% hydrogen peroxide in methanol for 15 min to inhibit endogenous peroxidase activity. Following washing in phosphate-buffered saline, the sections were incubated overnight at 4°C with a monoclonal rabbit anti-cleaved-caspase-3 primary antibody (Cell Signaling Technologies, Beverly, MA) at a dilution of 1:200, biotinylated secondary antibody, streptavidin peroxidase reagent, and 3, 3'-Diaminobenzidine. Finally, the sections were lightly counterstained with hematoxylin and observed under a light microscope (Olympus: $\times 400$).

Quantitative RT-PCR analysis

Total RNA was extracted from the kidney tissues using Trizol reagent (Invitrogen Carlsbad, CA, USA). The total RNA concentration was measured at 260 nm (OD_{260}). Pure RNA possessed an $\text{OD}_{260}/\text{OD}_{280}$ ratio of 1.6–1.9. The total RNA (0.25 μg) was reverse-transcribed to cDNA using random primer and High-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, California, USA). Transcript expression was analyzed using quantitative polymerase chain reaction (qPCR) analysis with a Step One Plus™ (Applied Biosystems) using Taqman EXPRESS qPCR SuperMix Universal (Invitrogen). Values for each gene were normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample. The primers (Applied Biosystems) used for the PCR reaction are as follows: Assay ID; Bax; Rn02532082_g1, Assay ID; Bcl-2; Rn99999125_m1, and Assay ID; GAPDH; Rn99999916_s1. The relative concentration of products was calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method, according to the instructions supplied by Applied Biosystems.

Western blot analysis

Total proteins were extracted from fresh frozen kidney tissues. The tissues were homogenized in 500 μl of RIPA buffer. Homogenates were treated with protease inhibitors and centrifuged in a pre-chilled rotor at 15,000 $\times g$ for 15 min. Supernatants were stored at -70°C . Protein content was measured using a BCA™ Protein Assay Kit (PIERCE, IL, USA). Protein equivalent to 40 μg was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were treated with Odyssey® blocking buffer followed by an overnight incubation at 4°C with primary antibody (rabbit anti-Bax, dilution 1:500; rabbit anti-Bcl-2, dilution 1:500; rabbit anti-cleaved caspase-3, dilution 1:500; rabbit anti-actin, dilution 1:2000; Cell Signaling Technology, Beverly, MA). Blots were then incubated with goat anti-rabbit LI-COR IRDye 680 antibody (dilution 1:5000). Densitometric analysis of bands was performed using LI-COR Odyssey®.

Statistical analysis

Data were expressed as mean \pm SD. The means of the different groups were evaluated by one-way analysis of variance or unpaired t-test. The level of statistical significances was defined as $p < 0.05$.

RESULTS

PE extract has been used for the renoprotective effect of CI-AKI which was demonstrated by our work. A previous study has demonstrated that antioxidant property of PE extract accounts for the preventive effect in CI-AKI, which all rats in the CM group exhibited a significant increase in blood urea nitrogen (BUN) and Cr concentrations when compared to control. However, rats treated with PE before receiving CM had an improved renal function. Addition, histological examination of the kidney tissues showed extensive tubular damages in the CM group and also was markedly different from those in the control kidney. In contrast, rats pretreated with PE demonstrated a significantly decreased histopathological alternation of the kidney tissues when compared with the CM group [22].

In the present study, an investigation of the anti-apoptotic effects of PE extract to ameliorate in CI-AKI was performed. It was found that the expression levels of Bcl-2 and Bax were assessed. In the CM group, the expression of Bcl-2 mRNA was significantly downregulated while the expression of Bax mRNA was upregulated. However, when pre-treated with PE, there was a significantly reduced expression of Bax and increased expression of Bcl-2 levels, as shown in Fig. 1a. Moreover, a significant increase of Bax protein level and a significant decrease of Bcl-2 protein level, as indicated by Western blot analysis, were found in the kidney tissues from the CM rats compared with the control rats. Interestingly, the PE pre-treated CM rats appeared to have the augmented expression of Bcl-2 and the reduced expression of Bax (Fig. 1b). Furthermore, Western blot analysis and IHC study revealed the expression of the apoptotic protein cleaved caspase-3 protein in the renal tissue. This protein was increased in the CM group and was reduced in the CM + PE group (Fig. 2).

DISCUSSION

The present study has demonstrated the renoprotective effect of PE on CI-AKI in rats, which was mediated through suppression of apoptosis. We found that pre-treatment with PE at 500 mg/kg/day for 5 days before the CI-AKI induction improved function and structure of renal tissues as well as markedly suppressed renal tubular injuries in the CI-AKI rats [22].

CI-AKI is a common clinical complication in patients receiving CM administration. The morbidity and mortality rates associated with AKI remain high, especially in the population at risks such as elderly, diabetes, and chronic kidney diseases [23]. Therefore, it is necessary to find an agent for preventing this serious complication. At present, there is no specific recommended therapy. Thus, the alternative therapy including nutritional intervention and supportive care which showed an improvement of the clinical outcomes of patients with AKI has been continuously encouraged [24]. The oxidative stress and apoptosis play important roles in the cellular pathogenesis of CI-AKI. The current body of evidence indicated that direct tubular toxicity of contrast agent, renal medullary ischemia, and generation of ROS leading to increased oxidative stress were responsible for the renal tubular cell apoptosis [8]. Therefore, many studies have focused on the identification of novel agents that can scavenge ROS and/or reduce apoptosis. Nowadays, several studies have discovered the antioxidant active constituents from natural plant extracts that could ameliorate CM-induced nephropathy in rats. These agents include magnolin [25], curcumin [7], and xuezhikang [26]. Recently, our previous study showed that pre-treatment with PE extract exhibited the antioxidant effect in CI-AKI rats as shown by the improvement of the oxidative-status parameters including lower renal malondialdehyde, higher renal Total antioxidant capacity (TAC), Superoxide dismutase (SOD) and catalase (CAT) activity, the improved renal function, and the attenuation of the severity of pathological damage [22]. However, its efficacy in preventing CI-AKI through anti-apoptotic has not been investigated.

P. emblica is widely used in Thai traditional medicine and Ayurvedic Medicine for the treatment of various diseases. The fruit of PE is known as a rich source of ascorbic acid [12,13] gallic acid [14], and also contains a mixture of phenolic compounds including flavonoids and tannins [15]. The major substrate which acts to be antioxidant in PE is gallic acid, Vitamin C, and others depending on the technique that separates the compound from PE [27,28]. In the present study, PE extract has been identified by high-performance liquid chromatography analysis. It was found that gallic acid was one of the main active constituents [22]. Because gallic acid is a polyphenolic compound with multiple hydroxyl groups which are able to donate its proton to break the chain reaction of free radicals [29,30], it was demonstrated as an excellent antioxidant with high free radical scavenging effect [29,31]. Moreover, gallic acid has been demonstrated to possess a wide range of pharmacological effects including antioxidant, anti-apoptosis, anticholestatic, anti-inflammatory, and immune-modulating activities as shown in nephroprotective, neuroprotective, and hepatoprotective animal models [32-34]. Furthermore, the renoprotective effect of

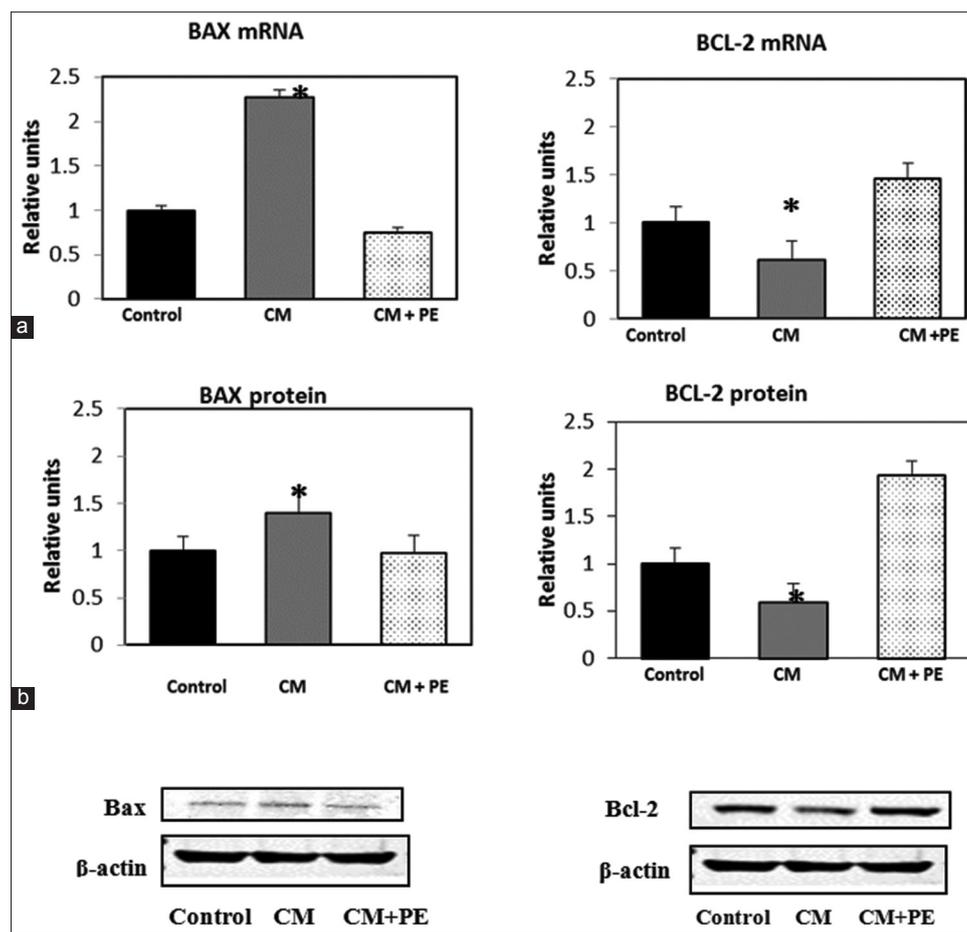


Fig. 1: Effect of PE on (a) the apoptotic gene and (b) protein expression in the study groups: ** $p < 0.05$; CM versus CM + PE

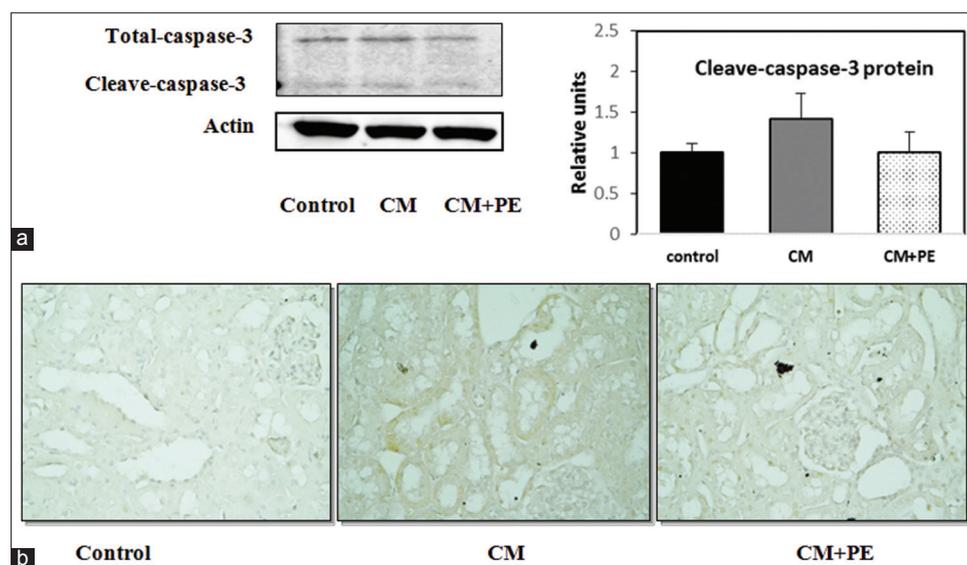


Fig. 2: Changes in cleaved caspase-3 activity in rat renal, Cleaved-caspase-3 expression as assessed by Western blotting (a) and by immunostaining (b); alteration in the histological feature of renal tubules and glomerulus in the CM group with strong immunostaining of cleaved caspase-3 ($\times 400$)

gallic acid from its antioxidant and anti-inflammation properties was demonstrated in many experimental studies in AKI [35].

The current study demonstrated that pre-administration of rats with PE extract ameliorated the deterioration of renal function and

histopathological changes found in kidney injuries in CI-AKI rats. After pretreating rats with PE (CM + PE group), the BUN and creatinine levels significantly decreased compared with the CM group. Overall, our results suggested that PE extract could prevent renal damages and could help to preserve the kidney function.

As apoptosis is one of the pathophysiological mechanisms of CI-AKI, the anti-apoptotic property of PE extract against CM has been investigated [36,37]. It has been reported that both mitochondrial and intrinsic apoptosis pathways participated in the tubular injuries of Contrast induced nephropathy (CIN) [38]. Suppressing apoptosis pathways may attenuate pathological alterations in CIN. Since Bcl-2 is an anti-apoptotic protein that can inhibit caspase-3, increased Bcl-2 levels and decreased caspase-3 activity might contribute to reduced apoptosis [39]. As expected, rats in the CM group exhibited the upregulating expression of Bax mRNA and protein levels, the downregulating expression of Bcl-2 mRNA and protein, and increased level of cleaved caspase-3. Interestingly, pretreatment with PE extract decreased the Bax and cleaved caspase-3 while increased Bcl-2 level. Our findings were consistent with the evidence of anti-apoptotic effect on the nephroprotection that has been previously demonstrated in a study using *E. officinalis* against cisplatin-induced nephrotoxicity [40]. Moreover, the anti-apoptotic activity of gallic acid has been demonstrated to ameliorate an ischemia-reperfusion injury on gastric mucosa [41]. Overall, this study using PE extract which contains gallic acid as one of the active compounds has demonstrated the renoprotective effect, and we convincingly proposed that this effect was mediated by the anti-apoptotic properties.

CONCLUSION

To our best knowledge, this study is the first in the literature demonstrating that the anti-apoptotic activities of PE extract could attenuate renal injury in the CI-AKI model. Thus, PE extract is a possible novel candidate for preventing kidney damages from the CM. This warrants an extensive exploration of its beneficial properties.

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COMPLIANCE WITH ETHICAL STANDARDS ETHICAL STATEMENT

The experimental protocol was approved by the Animal Ethics Committee of Faculty of Medicine, Thammasat University. Registration No.AE 007/2015

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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