

The potency of chitosan-*Pinus merkusii* extract nanoparticle as the antioxidant and anti-caspase 3 on lead acetate-induced nephrotoxicity in rat

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ABSTRACT

The current study was carried out to evaluate the antioxidant and anti-caspase 3 activity of chitosan-*Pinus merkusii* nanoparticle in against lead acetate-induced nephrotoxicity in rats. chitosan-*P. merkusii* nanoparticle was characterized by dynamic light scattering (DLS) and scanning electron microscope (SEM). The male rats were divided into control group (rats were given with distilled water), lead acetate group (rats were injected with lead acetate 15 mg/kg BW i. p), and the treatment group (rats were given the chitosan-*P. merkusii* nanoparticle 150 mg, 300 mg, 600 mg/kg BW orally and were injected with lead acetate 15 mg/kg BW). The rats blood samples were measured levels of blood urea nitrogen (BUN) and creatinine. The kidney tissues were collected to evaluate the malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Histological to evaluate renal damage, and immunohistochemical to analyze the expression of caspase 3. The results showed that DLS showed the size of chitosan-*P. merkusii* nanoparticle was 165.9 ± 24.18 nm. SEM images of the chitosan-*P. merkusii* nanoparticles showed an irregular shape and its the rough surface. Administration of lead acetate resulted in a significant increase in levels of the BUN, creatinine, MDA level, caspase 3 expression, and a decrease in SOD and GPx were compared with the control group. Treatment with the chitosan-*P. merkusii* nanoparticle 600 mg/kg BW significantly decreased the elevated BUN, creatinine, MDA levels, caspase 3 expression and also increase in SOD and GPx as compared to lead acetate group. The lead acetate induced loss of the normal structure of renal cells and necrosis, whereas treated with chitosan-*P. merkusii* nanoparticle improved renal cell necrosis. This study indicates that chitosan-*P. merkusii* nanoparticles appeared to be a promising agent for protection against lead-induced nephrotoxicity through increasing antioxidant and inhibiting caspase 3 expression.

Key words: Antioxidant, biochemical serum, caspase 3, chitosan-*Pinus merkusii* nanoparticle, lead acetate

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INTRODUCTION

Lead (Pb) is one of the heavy metals which can cause damage to several organs such as the liver,^[1] reproductive

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organs,^[2] immune system,^[3] and kidneys^[4] in both humans and animals.

Oxidative stress has been proposed as a possible mechanism involved in lead-induced nephrotoxicity.^[5,6] Oxidative stress develops when there is an imbalance between the reactive oxygen species (ROS) and the antioxidants in the nephron cells.^[6,7] It has been reported that overproduction of ROS or free radicals such as superoxide ion (O_2^-), hydroxyl radical (OH^\cdot), and nitric oxide and consequently can enhance lipid peroxidation, the decrease of antioxidant enzymes activities, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx).^[7,8] In addition, free radicals are highly reactive to membrane lipids, protein, DNA, and to be the major contributing factors to stress injuries and to cause rapid renal cell damage.^[3,8] It was also reported that malondialdehyde (MDA) is a product of lipid peroxidation which may be used as a biomarker of renal cell damage.^[4]

In living systems, the kidney is considered to be highly sensitive to toxic agents. The study of lead acetate in biochemical changes in blood such as blood urea nitrogen (BUN) and creatinine has been found to be of great value in experimental kidney damage.^[9] Various molecular, cellular, and intracellular mechanisms of lead nephrotoxicity to explain the toxicological profile of lead that includes generation of oxidative stress, necrosis, and apoptosis.^[3,7,8]

The herbal antioxidants have been reported to provide protection against lead-induced oxidative stress and emerged as a potential therapeutic to prevent free radical generated damage in the body. It has been reported that *Pinus merkusii* was one of the herbals, which have a strong antioxidant effect.^[10-12] Recent research activities have shown that *Pinus* plant is an important source of pycnogenol that contains proanthocyanidins.^[13-15] Proanthocyanidins are potent, antioxidant (free radical scavengers), antibacterial agents, anticancer, anti-inflammatory, immune-stimulating, antidiabetes, and anti-atherosclerosis.^[16-18]

In recent years, synthesis of natural product nanoparticles is an interesting issue of the nanoscience and nanobiotechnology.^[19] Chitosan nanoparticles have been studied extensively by researchers for their controlled drug release properties and are used for both *in vitro* and *in vivo* applications.^[20] It is also nontoxic and has many biological activities such as antibacterial, antioxidant, antihyperlipidemic, antidiabetic, anti-HIV, anti-inflammatory, drug delivery, and immunoenhancing, make it an ideal delivery agent for applications in medicine.^[20-22] The present study was carried out to evaluate the antioxidant and anti-caspase 3 activity of chitosan-*P. merkusii* extract nanoparticle in against lead acetate-induced nephrotoxicity.

MATERIALS AND METHODS

Preparation of chitosan-*Pinus merkusii* extract nanoparticles

The chitosan-*P. merkusii* extract nanoparticle was prepared according to the procedure first reported by based on the ionic gelation of chitosan-*P. merkusii* extract with sodium tripolyphosphate (TPP) anions.^[22,23] Briefly, concentrations of chitosan solutions (0.2% w/v) were prepared in 0.1% v/v glacial acetic acid and filtered. The TPP solution (0.1% w/v) was prepared in deionized water. *P. merkusii* extract 0.4% w/v in ethanol 70% was added to chitosan solution (0.2% w/v) under constant stirring. The mixture was then sonicated for 5 min and TPP solution was added dropwise under constant stirring. The ratio of chitosan: *P. merkusii*: TPP solution was maintained at 2:4:1 throughout the experiment. The supernatant obtained was subjected to ultracentrifugation at 25000 rpm for 20 min to sediment the chitosan-*P. merkusii* conjugated nanoparticles, which were then subjected to further characterization.

Characterization of nanoparticles by scanning electron microscopy and dynamic light scattering

The surface morphological features such as particle size, shape, and topography of the chitosan-*P. merkusii* extract nanoparticle were observed using scanning electron microscope (SEM).

The particle size of the chitosan-*P. merkusii* extract nanoparticle was determined using dynamic light scattering (DLS) (Horiba LA 900, Japan).

Experimental animal

Male Wistar rat weighing approximately 200–250 g (2.5–3 months) were obtained from the Gadjah Mada University, Yogyakarta, Indonesia, for experimental purpose. They were housed in plastic cages in an air-conditioned room with a temperature maintained at $26 \pm 2^\circ\text{C}$ and 12 h alternate light and dark cycles. The rats were given *ad libitum* with tap water and fed with a standard commercial rat.

Experimental design

The sample used 50 male rats were divided into five groups: Control group (rats were given daily with distilled water), lead acetate group (rats were injected with lead acetate solution, i.p., at a dose of 15 mg/kg BW for the 7 consecutive days), and the treatment group (rats were given the chitosan-*P. merkusii* extract nanoparticle 150 mg, 300 mg, and 600 mg/kg BW orally once in a day for 11 days, and on the 4th day, were injected with lead acetate solution, i.p., at a dose of 15 mg/kg BW 1 h after the chitosan-*P. merkusii* extract nanoparticle). On day 11, the rats blood samples were taken by cardiac puncture to be measured levels of BUN and creatinine. Measurement of BUN was conducted by the diacetyl monoxime method^[24] and measurement of creatinine was conducted by the alkaline picrate method.^[25]

Furthermore, rats were sacrificed and kidney tissues were used for the analyzes of MDA and antioxidant enzymes (SOD and GPx). MDA was determined in the supernatant of homogenate kidney tissue by the thiobarbituric acid method.^[4] MDA is expressed as nanomoles MDA/g tissue. The activity of SOD was measured with SOD detection kit according to the manufacturer's instructions. The level of SOD was measured at 505 nm and through a standard curve and expressed as U/mg protein.^[2] The activity of GPx was measured with GPx detection kit according to the manufacturer's instructions. The GPx was evaluated spectrophotometrically against blank at 340 nm. The GPx activity was expressed as U/mg protein.^[2]

The kidney was also fixed in a 10% neutral-buffered formalin solution for immunohistochemical evaluation of the expression of caspase 3^[8] and histopathological evaluation of the kidney damage.^[1]

Statistical analysis

Data were presented as means \pm standard deviation. One-way ANOVA has carried *post hoc* test and the statistical comparisons among the groups were performed with an LSD test using a statistical package program SPSS version 17.0 (SPSS Inc, Chicago, USA).

RESULTS

Characterization of chitosan-*Pinus merkusii* extract nanoparticles by scanning electron microscopy

SEM images of the chitosan-*P. merkusii* nanoparticles prepared using ionic gelation revealed that the nanoparticle surface showed the rough surface morphology and an irregular shape [Figure 1].

Characterization of chitosan-*Pinus merkusii* nanoparticles by dynamic light scattering

The average particle size of the chitosan-*P. merkusii* extract nanoparticles by DLS was 165.9 ± 24.18 nm as shown in Figure 2.

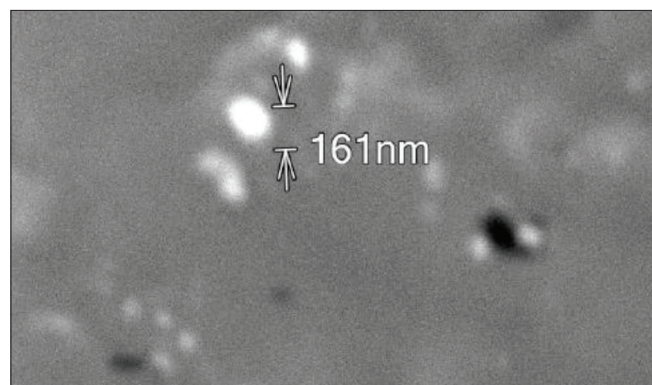


Figure 1: Scanning electron microscope images of chitosan-*Pinus merkusii* extract nanoparticles

Effects of chitosan-*Pinus merkusii* extract nanoparticles to lead acetate-induced changes in the renal serum enzymes

The lead acetate group showed a statistically significant ($P < 0.05$) increase in renal serum enzymes (BUN and creatinine) compared with the control group. In contrast, the chitosan-*P. merkusii* extract nanoparticles at dose 600 mg/kg BW showed significantly ($P < 0.05$) decreased renal serum enzymes (BUN and creatinine) levels were compare with the lead acetate group [Table 1].

Effects of chitosan-*Pinus merkusii* extract nanoparticle on lead acetate-induced changes in malondialdehyde, superoxide dismutase, and glutathione peroxidase of kidney tissue

Table 2 showed in the lead acetate group, the level of MDA of kidney tissue was significantly increased compared to the control group ($P < 0.05$). Treatment with chitosan-*P. merkusii* extract nanoparticle at dose 600 mg/Kg BW markedly reduced kidney tissue MDA which was significantly different from the lead acetate group ($P < 0.05$). Table 2 also showed the results of the lead acetate group, the level of SOD, and GPx of kidney tissue were significantly decreased compared to the control group ($P < 0.05$). Treatment with chitosan-*P. merkusii* extract nanoparticle at dose 600 mg/Kg BW markedly enhanced kidney SOD and GPx which was significantly different from the lead acetate group ($P < 0.05$).

Effects of chitosan-*Pinus merkusii* extract nanoparticle on the expression of caspase 3 of lead acetate

Figure 3 showed that in the lead acetate group, the expression of caspase 3 of the kidney tissue was significantly increased compared to the control group ($P < 0.05$). Treatment with chitosan-*P. merkusii* extract nanoparticle at dose 600 mg/Kg BW markedly reduced kidney tissue caspase 3 expression which was significantly different from the lead acetate group ($P < 0.05$).

Effects of chitosan-*Pinus merkusii* extract nanoparticle on lead acetate-induced kidney cell damage

Histological observations on the control group showed that in kidney cell appear normal architecture of the renal cell. In

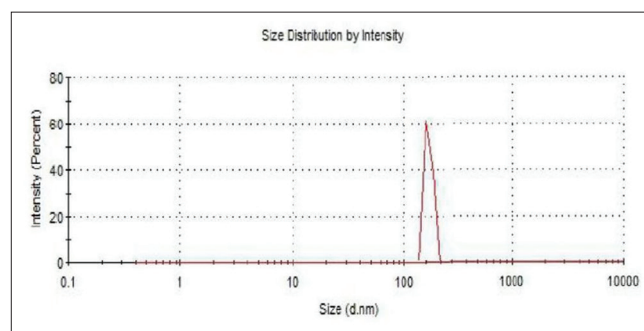


Figure 2: Size distribution of chitosan-*Pinus merkusii* extract nanoparticles by dynamic light scattering

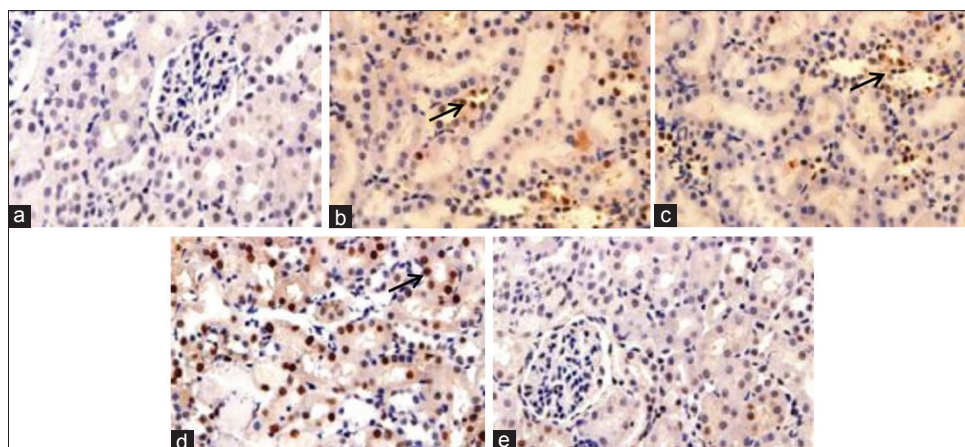


Figure 3: Immunohistochemical study of chitosan-*Pinus merkusii* extract nanoparticle on caspase 3 expression of lead acetate-induced nephrotoxicity. The lead acetate-treated group showed caspase 3 expression (indicated by red arrows). Negative control Group (a); Positive control Group (b); Rats treated with chitosan-*Pinus merkusii* extract nanoparticle 150 mg/kg BW; 300 mg/kg BW and 600 mg/kg (c-e)

the lead acetate group showed renal cell damage (necrosis). The treated chitosan-*P. merkusii* extract nanoparticles can inhibit renal cell damage caused by lead acetate [Figure 4].

DISCUSSION

In this study, we made *P. merkusii* extract was encapsulated into chitosan nanoparticle with the use of sodium TPP (STPP) as a cross-linking agent on ionotropic gelation method, which has more advantages over *P. merkusii* extract. This modification can improve biodistribution and increase specificity and sensitivity and reduced pharmacological toxicity.^[18,23] The *P. merkusii* extract is dissolved in chitosan

solution and when the STPP solution with negative charge is added to the positively charged chitosan solution, the nanoparticles are formed rapidly and the *P. merkusii* extract is surrounded by a polymeric network due to interaction between two polymers. The nanoparticles obtained in the present study had small particle size (165.9 ± 24.18 nm), which may increase the antioxidant and anti-caspase 3 activity. The SEM images of the chitosan-*P. merkusii* extract nanoparticles showed an irregular shape and its the rough surface.

In our study, administration of lead acetate can increase MDA and inducing oxidative damage in the kidney. MDA may be used as an indicator of cell membrane injury. The increase in MDA levels in the kidney suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals.^[4,5] Treatment of rats with chitosan-*P. merkusii* extract nanoparticle at a dose of 600 mg/kg BW prevented the levels of MDA to rise when the rats were challenged with lead acetate in the kidney, which might be due to the ability of chitosan-*P. merkusii* extract nanoparticle to reduce the accumulation of free radicals. It is known that chitosan-*P. merkusii* extract nanoparticle, which behaves as a powerful antioxidant and free radical scavenger, can decrease the MDA level perturbed by lead acetate in rats kidney. The findings of this study suggest

Table 1: Effects of Chitosan-*Pinus merkusii* extract nanoparticle on lead acetate induced changes in BUN and Creatinin

Groups	Means \pm Standard deviation	
	BUN (mmol/L)	Creatinin (mmol/L)
Control	8.37 ^a \pm 2.23	35.12 ^a \pm 3.18
Lead Acetate	25.33 ^b \pm 3.72	63.33 ^b \pm 6.24
Chitosan- <i>P. merkusii</i> 150 mg/kg	26.51 ^b \pm 3.64	65.27 ^b \pm 5.99
Chitosan- <i>P. merkusii</i> 300 mg/kg	19.43 ^b \pm 3.91	56.93 ^b \pm 4.82
Chitosan- <i>P. merkusii</i> 600 mg/kg	13.09 ^c \pm 1.87	42.16 ^c \pm 3.71

^{a-c}Different superscript within each column indicate significant difference between the means ($P < 0.05$)

Table 2: Effects of Chitosan-*Pinus merkusii* extract nanoparticle on lead acetate induced changes in MDA, SOD and GPx

Groups	Means \pm Standard Deviation		
	MDA (nmol/mg)	SOD (u/mg)	GPx (u/mg)
Control	52.81 ^a \pm 6.15	41.18 ^a \pm 4.26	53.42 ^a \pm 5.23
Lead Acetate	73.45 ^b \pm 8.92	23.22 ^b \pm 3.91	33.63 ^b \pm 3.97
Chitosan- <i>P. merkusii</i> 150 mg/kg	76.32 ^b \pm 7.24	21.31 ^b \pm 2.85	35.63 ^b \pm 4.09
Chitosan- <i>P. merkusii</i> 300 mg/kg	69.52 ^b \pm 5.72	26.13 ^b \pm 3.79	38.63 ^b \pm 3.42
Chitosan- <i>P. merkusii</i> 600 mg/kg	61.28 ^c \pm 4.62	33.24 ^c \pm 3.27	44.63 ^c \pm 4.31

^{a-c}Different superscript within each column indicate significant difference between the means ($P < 0.05$)

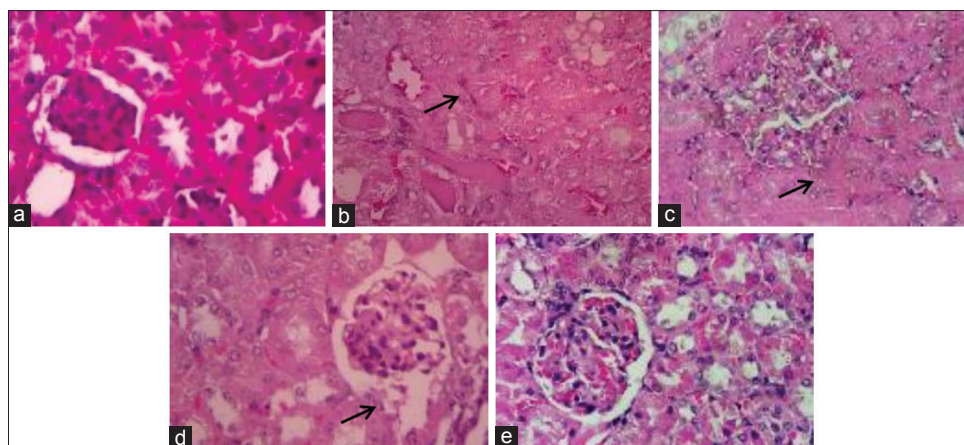


Figure 4: Histological study of pretreatment with chitosan-*Pinus merkusii* extract nanoparticle on lead acetate-induced nephrotoxicity. Normal morphology of liver sections in the negative control group (a). The lead acetate treated group showed necrosis (indicated by black arrows) (b). Rats treated with chitosan-*Pinus merkusii* extract nanoparticle 150 mg/kg BW and 300 mg/kg BW showed necrotic changes (c and d). Rats treated with chitosan-*Pinus merkusii* extract nanoparticle 600 mg/kg showed regeneration in hepatic cells damage (e). H and E $\times 400$

that chitosan-*P. merkusii* extract nanoparticle could attenuate oxidative stress by decreasing the lipid peroxidation (MDA level) in the lead-treated kidney.

The serum enzyme markers such as BUN and creatinine are recommended for the assessment of renal cell damage in preclinical studies as it is considered a more specific and sensitive indicator of kidney damage. Most increases BUN and creatinine levels are caused by kidney damage.^[9] The current work showed an increase in the levels of a BUN and creatinine in lead acetate-treated rat in comparison to the control and this may be due to the degeneration of renal cell by necrosis which causes leakage of these BUN and creatinine into blood circulation. The similar observation has reported that lead acetate treatment induced significant elevation of serum BUN and creatinine activities.^[4,5] Our results indicated that the chitosan-*P. merkusii* nanoparticle 600 mg/kg BW showed an improvement in the BUN and creatinine levels. This might be through its direct action on free radicals of lead acetate to prevent the renal cellular damage by maintaining its membrane integrity. Reduction of the BUN and creatinine levels near-normal levels suggested regeneration of renal cell with a healing of nephrotoxicity.

SOD and GPx are important antioxidant enzymes. They constitute a mutually supportive defense mechanism against ROS. Many studies have shown that lead has the high affinity for SH groups in several enzymes such as SOD and GPx, thus it can alter antioxidant activities by inhibiting functional SH groups in these enzymes.^[5,6] In the present study, the activity of SOD and GPx in rat kidney was decreased by lead acetate treatment. Lead acetate is known to cause free radical damage in tissues by increased ROS and by causing depletion of antioxidant reserves. This suggested that lead acetate exposure induced oxidative stress by inhibiting the activity of this antioxidant enzyme. Interestingly, the administration of chitosan-*P. merkusii*

extract nanoparticle increased the activities of SOD and GPx in the kidney of lead-treated rats, which might be due to the ability of chitosan-*P. merkusii* extract nanoparticle to reduce the accumulation of free radicals. Chitosan-*P. merkusii* extract nanoparticle acts as a scavenger for the oxygen-derived free radicals, thus protecting from kidney damage. The decrease in lipid peroxidation due to chitosan-*P. merkusii* extract nanoparticle has been attributed to alterations in the antioxidant defense system which includes enzymes such as SOD and GPx, which normally protects against free radical toxicity.

It has been reported that the lead toxicity condition can cause excessive production of ROS, there is an imbalance between the production of oxidants and the defense systems of antioxidant which may promote the induction of lipid peroxidation, proteins and DNA damage, leading to renal cell death via apoptosis or necrosis.^[7-9] Expression of the caspase-3 is a hallmark of apoptosis and can be used in cellular assays to quantify activators and inhibitors of the "death cascade."^[8] The results of this study showed that the expression of caspase 3 of kidney tissue was significantly increased on the lead acetate group. Dose-dependent manner of chitosan-*P. merkusii* extract nanoparticle decreased kidney tissue caspase 3 expression in lead acetate treatment. It has been also reported that tissue cell apoptosis induced by lead toxicity was associated with mitochondrial injury and changes in levels of apoptogenic proteins including Bcl-2, Bax, and caspase-3.^[7] In lead toxicity, the expression levels of caspase-3 and Bax significantly increased, while the levels of Bcl-2 significantly decreased. The renal apoptotic cell death is highly associated with lead loading and changes in caspase-3 expression may play an important role in this process.

The histopathological results demonstrating structural changes in kidney tissue of heavy metal toxicity such

as lead acetate were reported by some researchers. In the present study, histopathological view of kidney sections in the lead acetate group showed the renal cell damage (necrosis) as compared to the control group. The kidney damage (necrosis) was considered mild in the groups treated with chitosan-*P. merkusii* extract nanoparticle.

CONCLUSION

In conclusion, the present study indicates that the administration of chitosan-*P. merkusii* extract nanoparticle inhibits the effects of lead acetate-induced nephrotoxicity might be related with both antioxidant and anti-caspase 3. The administration of chitosan-*P. merkusii* extract nanoparticle lessened the effects of lead acetate-induced nephrotoxicity possibly by increasing antioxidant and inhibiting caspase 3 expression.

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Conflicts of interest

There are no conflicts of interest.

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