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ORIGINAL ARTICLE

Antioxidant and Anti-caspase-3 Activity of Chitosan-*Pinus merkusii* Extract Nanoparticle on Lead Acetate-induced Hepatotoxicity

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ABSTRACT

Background: Lead (Pb) is a hazardous environmental and industrial pollutant, which induces hepatotoxicity in both humans and animals. Lead acetate can cause the formation of an oxidative stress, resulting in the increase in the concentration of free radicals and decrease in antioxidant. Chitosan-*Pinus merkusii* extract nanoparticle has shown to possess powerful antioxidant properties. **Objective:** In the present study, we investigated the impact of Chitosan-*P. merkusii* extract nanoparticle against lead acetate-induced hepatotoxicity in rats. **Materials and Methods:** Chitosan-*P. merkusii* extract nanoparticle was characterized by dynamic light scattering (DLS) and scanning electron microscope (SEM). The fifty male rats were divided into control group (rats were given daily with distilled water), lead acetate group (rats were injected with lead acetate [15 mg/Kg BW i. p] for the 7 consecutive days), and the treatment group (rats were given the Chitosan-*P. merkusii* extract nanoparticle [100 mg, 200 mg, and 400 mg/Kg BW orally] once in a day for 11 days and on the 4th day, they were injected with lead acetate [15 mg/Kg BW i. p] for 7 days). On day 11, the rats' blood samples were taken by a cardiac puncture to measure the levels of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and alkaline phosphatase (ALP). Furthermore, rats were sacrificed, and liver tissues were collected to evaluate the malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). The liver tissues also were subjected to histological evaluations and immunohistochemical evaluations of the expressions of caspase-3. **Results:** The results showed that DLS showed the formation of Chitosan-*P. merkusii* extract nanoparticle with the size of 530.2 ± 30.27 nm. SEM images of the Chitosan-*P. merkusii* extract nanoparticles showed an irregular shape, and the morphology surface showed the rough surface. Injection of lead acetate (15 mg/Kg BW) for 7 days resulted in a significant ($P < 0.05$) increase in SGOT, SGPT, ALP, MDA level, and caspase-3 expression. Lead acetate also significantly ($P < 0.05$) decreased in SOD and GPx. Treatment with the Chitosan-*P. merkusii* extract nanoparticle (400 mg/Kg BW) significantly ($P < 0.05$) decreased the elevated SGPT, SGOT, ALP, MDA levels, and caspase-3 expressions as compared to lead acetate group. Treatment with Chitosan-*P. merkusii* extract nanoparticle (400 mg/Kg BW) also resulted in a significant ($P < 0.05$) increase in SOD and GPx, as compared to lead acetate group. Administration of lead acetate to rats can cause histopathological changes such as loss of the normal structure of hepatic cells, blood congestion, and necrosis whereas rats treated with Chitosan-*P. merkusii* extract nanoparticle showed an improvement in these changes, and the tissue appears with normal structures. **Conclusion:** This study indicates that Chitosan-*P. merkusii* nanoparticle could be a potent natural product that provides a promising hepatoprotective effect against lead acetate-induced hepatotoxicity in rats, through increasing antioxidant and inhibiting caspase-3 expression.

Keywords: Alkaline phosphatase, antioxidant, caspase-3 expression, Chitosan-*Pinus merkusii* nanoparticle, lead acetate, malondialdehyde, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase

SUMMARY

- The dynamic light scattering showed that the size of Chitosan-*Pinus merkusii* extract nanoparticle was 530.2 ± 30.27 nm
- Scanning electron microscope images of the Chitosan-*P. merkusii* extract nanoparticles showed an irregular shape, and the morphology surface showed the rough surface
- Administration of lead acetate (15 mg/Kg BW i. p) for 7 days resulted in a significant increase in serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP), malondialdehyde (MDA) level, caspase-3 expression, and hepatic cell necrosis. Lead acetate also resulted in a significant decrease in superoxide dismutase (SOD) and glutathione peroxidase (GPx)
- Treatment with the Chitosan-*P. merkusii* extract nanoparticle decreased the elevated SGPT, SGOT, ALP, MDA levels, and caspase-3 expression and inhibited hepatic cell necrosis. The Chitosan-*P. merkusii* extract nanoparticle also resulted in a significant increase in SOD and GPx
- This study concluded that Chitosan-*P. merkusii* extract nanoparticle could be a potent natural product that provides a promising hepatoprotective effect against lead acetate-induced hepatotoxicity in rats, through increasing antioxidant and inhibiting caspase-3 expression.

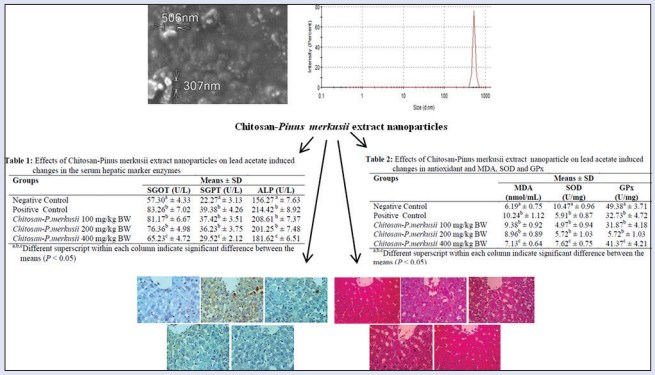

Table 1: Effects of Chitosan-Pinus merkusii extract nanoparticles on lead acetate induced changes in the serum hepatic marker enzymes

Groups	Means ± SD		
	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
Negative Control	$57.10^{a} \pm 4.33$	$22.25^{a} \pm 3.13$	$156.27^{a} \pm 7.63$
Positive Control	$83.26^{b} \pm 7.02$	$39.38^{b} \pm 4.26$	$214.42^{b} \pm 8.92$
Chitosom-P.merkusii 100 mg/kg BW	$81.17^{b} \pm 6.67$	$37.42^{b} \pm 3.51$	$208.61^{b} \pm 7.37$
Chitosom-P.merkusii 200 mg/kg BW	$76.36^{b} \pm 4.98$	$36.23^{b} \pm 3.75$	$201.25^{b} \pm 7.48$
Chitosom-P.merkusii 400 mg/kg BW	$65.23^{c} \pm 4.72$	$29.52^{c} \pm 2.12$	$181.62^{c} \pm 6.51$

[a,b,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 antioxidant and MDA, SOD and GPx

Groups	Means ± SD		
	MDA (nmol/mL)	SOD (U/mg)	GPx (U/mg)
Negative Control	$6.19^{a} \pm 0.75$	$10.47^{a} \pm 0.96$	$49.38^{a} \pm 3.71$
Positive Control	$10.24^{b} \pm 1.12$	$5.91^{b} \pm 0.87$	$32.75^{b} \pm 4.72$
Chitosom-P.merkusii 100 mg/kg BW	$9.38^{b} \pm 0.92$	$4.97^{b} \pm 0.94$	$31.85^{b} \pm 4.18$
Chitosom-P.merkusii 200 mg/kg BW	$8.96^{b} \pm 0.89$	$5.72^{b} \pm 1.03$	$5.72^{b} \pm 1.03$
Chitosom-P.merkusii 400 mg/kg BW	$7.13^{c} \pm 0.64$	$7.62^{c} \pm 0.75$	$41.37^{c} \pm 4.21$

[a,b,c]Different superscript within each column indicate significant difference between the means ($P < 0.05$)

Abbreviations used: DLS: Dynamic light scattering; SEM: Scanning electron microscope; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase; ALP: Alkaline phosphatase; MDA: Malondialdehyde; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; ROS: Reactive oxygen species; CAT: Catalase; BW: Body weight.

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INTRODUCTION

Lead is regarded as a potent occupational toxin, and its toxicological manifestations are well known in both humans and animals. The main sources of lead toxicity are leaded gasoline, industrial processes such as lead smelting and coal combustion, lead-based paints, lead-containing pipes, or lead-based solder in water supply systems, battery recycling, grids, and bearings.[1] There is no such level of lead that appears to be necessary or beneficial to the body, and no "safe" level of exposure to lead has been found. It has been reported that lead toxicity can interfere with the cardiovascular, central nervous, hematopoietic, renal, reproductive, and hepatic systems producing serious toxicity in human and animal.[2-5]

In living systems, the liver is considered to be highly sensitive to toxic agents. The study of lead acetate in enzymatic activities such as serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), and alkaline phosphatase (ALP) has been found to be of great value in experimental liver damage.[6-8] Various molecular, cellular, and intracellular mechanisms of lead hepatotoxicity have been proposed to explain the toxicological profile of lead that includes generation of oxidative stress, ionic mechanism, necrosis, and apoptosis.[2,9,10]

Excessive reactive oxygen species (ROS) formation and decreasing antioxidant can induce oxidative stress, leading to liver cell damage that can culminate in cell death. The generation of ROS such as superoxide ion (O_2^-), hydroxyl radical (OH^-), nitrogen oxide, hydroperoxides (HO_2), and hydrogen peroxide (H_2O_2) increased, and the production of antioxidant decreased.[2,6,11] Malondialdehyde (MDA) may be used as an indicator of cell membrane damage after exposure to ROS and free radicals. The increase in MDA levels in the liver suggests that antioxidant failure to inhibit ROS and formation of free radicals, which can enhance lipid peroxidation and causing hepatotoxicity. The MDA level is the direct evidence of liver tissue injury processes caused by free radicals.[6,10,11]

ROS is efficiently detoxified by antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) in normal healthy conditions. However, under lead toxicity condition which causes 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 hepatic cell death through apoptosis or necrosis.[1,2,12] It has been reported that hepatic apoptosis induced by lead toxicity was associated with mitochondrial injury and changes in levels of apoptogenic proteins including Bcl-2, Bax, and caspase-3.[12,13] In lead toxicity, the expression levels of caspase-3 and Bax significantly increased whereas the levels of Bcl-2 significantly decreased.[12,13]

It has been revealed that antioxidant activity or inhibition of generation of free radicals plays a crucial role in protection against lead acetate-induced hepatotoxicity.[2,10] Hence, it has been claimed that protective agents against free radicals, such as antioxidants, may be useful therapeutics for lead acetate toxicity in the liver. The natural product can be a good alternative as the antioxidant because of their low costs, availability, and lack of undesirable side effects.[14]

This study has been focused on natural products or herbal medicine having antioxidant properties namely Chitosan-*Pinus merkusii* for protect in lead acetate-induced liver cell damage. *Pinus* plants have many medicinal uses such as hemostatic, anthelmintic, liver tonic, diaphoretic, diuretic, antioxidant, flatulence, bronchitis, inflammations, and skin diseases.[15] It has been demonstrated that *Pinus* plant has phytochemicals including alkaloids, polyphenols, flavonoids, lignans, triterpenes, sterols, glycosides, triterpenoids, and saponins.[16] Recent research activities have shown that *Pinus* plant is an important source of Pycnogenol that contains proanthocyanidins (procyanidins).[17-19] Proanthocyanidins are potent as an antioxidant, antibacterial agents, anti-allergic, anti-inflammatory, cardioprotective, immune-stimulating, antiviral, and estrogenic activities.[19-21]

In recent years, the synthesis of natural product nanoparticles is an interesting issue of the nanoscience and nanobiotechnology.[22] Several research studies show that they are very important to the biosynthesis of the nanoparticles using a natural product, and they are suitable for large-scale biosynthesis of nanoparticles. In medicine, natural product like chitosan nanoparticles is used as drug carriers encapsulating a broad range of therapeutic agents to more efficient drug delivery into the target site. The nanoparticle delivery system offers advantages such as better bioavailability, solubility, efficacy, and encapsulation of the drug compared to conventional systems.[23] Nanochitosan is one of the natural products reported to exhibit adhesiveness, biodegradability, and biocompatibility and is widely used in biomedical and pharmaceutical applications.[24-26] Chitosan nanoparticles have drawn the attention of researchers for their controlled drug release properties and are used for both *in vitro* and *in vivo* applications.[27] They are also nontoxic and are known to possess many biological activities such as antibacterial, antioxidant, antihyperlipidemic, antidiabetic, anti-HIV, anti-inflammatory activities, drug delivery, and immunoenhancing, making it an ideal delivery agent for applications in medicine.[25,27-29] The objective of the present study was to investigate the antioxidant and anti-caspase-3 activity of the Chitosan-*P. merkusii* extract nanoparticle on lead acetate-induced hepatic cell damage in Wistar albino rats.

MATERIALS AND METHODS

Preparation of chitosan-*Pinus merkusii* extract nanoparticles

The chitosan-*P. merkusii* extract nanoparticle was prepared according to the procedure first reported based on the ionic gelation of chitosan-*P. merkusii* extract with sodium tripolyphosphate (TPP) anions.[30,31] 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 mins, and TPP solution was added drop wise under constant stirring. The ratio of chitosan: TPP solution was maintained at 2:1 throughout the experiment. The supernatant obtained was subjected to ultracentrifugation at 25,000 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).

Dynamic light scattering (DLS) was done using Malvern Instruments version 2.2 (Malvern Instruments Ltd, Enigma Business Park, Grovewood Road, Malvern, Worcestershire, WR14 1XZ, UK). The average particle size of the Chitosan-*P. merkusii* extract nanoparticle was determined.

Experimental animal

Male Wistar rats weighing approximately 200–250 g (2.5–3 months) were obtained from Gadjah Mada University, Yogyakarta, Indonesia, for experimental purpose. Rats were housed in plastic cages in an air-conditioned room with a temperature maintained at 26 ± 2°C and 12-h alternate light and dark cycles. The rats were given *ad libitum* with tap water and fed with standard commercial rat chow. This study was

reviewed by the Ethical Clearance Committee for preclinical research, Institute of Tropical Disease, Airlangga University and obtained ethical clearance under No. 178/ITD/1/2018.

Experimental design

The sample used 50 male rats were divided into 3 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 100 mg, 200 mg, and 400 mg/Kg BW orally once in a day for 11 days and on the 4th day, they 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 measure the levels of SGOT, SGPT, and ALP. Furthermore, rats were sacrificed, and hepatic tissues were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid. The supernatant was separated by centrifugation at 1000 g for 20 min at 4°C. The supernatant was used for the analyses of MDA and antioxidant enzymes (SOD and GPx). The liver was also fixed in a 10% neutral-buffered formalin solution for immunohistochemical evaluation of the expression of caspase-3 and histopathological evaluation of the liver damage.

Measurement of serum glutamic-pyruvic transaminase, serum glutamic-oxaloacetic transaminase, and alkaline phosphatase

Serum biochemical marker activities of SGPT, SGOT, and ALP were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits.[6]

Measurement of malondialdehyde

MDA was determined in the supernatant of homogenate liver tissue by the thiobarbituric acid (TBA) method which estimates the MDA formation. The concentration of MDA was measured at 532 nm and calculated by the absorbance coefficient of MDA–TBA complex.[6] MDA is expressed as nanomolecules MDA/mg tissue.[32]

Measurement of antioxidant enzymes

Tissue preparation for enzyme assay of rat liver was rapidly thawed from −70°C at room temperature for 5 min and manually homogenized in cold phosphate buffer (pH 7.4), and debris was removed by centrifugation at 3500 g for 10 min (Centrifuge 5415 R, Eppendorf AG, Hamburg, Germany). Supernatants were recovered and used for enzyme activity and protein assays.

The activity of SOD was measured with SOD detection kit according to the manufacturer's instructions. The SOD activity was then evaluated by the degree of inhibition of this reaction. The level of SOD was measured at 505 nm and through a standard curve and expressed as U/mg protein.[6,33]

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. GPx 1 unit was l mol of oxidized NADPH/min/mg of tissue. The GPx activity was expressed as U/mg protein.[6,33]

Histopathological examination of the liver damage

The tissue of the liver was fixed in a 10% neutral-buffered formalin solution, embedded in paraffin, and used for histopathological examination with hematoxylin and eosin stain.[6]

Immunohistochemical examination of the expression of caspase-3

The paraffin-embedded liver was cut into 4 µm sections and mounted on positively charged slides for the expression of caspase-3 immunohistochemistry. Immunocytochemical reactions were performed using the peroxidase/antiperoxidase (PAP) method.[13] Nonspecific peroxidase reactions were blocked with methanol containing 0.1% H_2O_2. The sections were also incubated with normal goat serum to avoid nonspecific reactions once the samples were incubated with specific antibodies against caspase-3 (dilution, 1:2000, Santa Cruz, CA, USA). Tissue sections were then washed with phosphate buffer and incubated with secondary antibodies (1:2000; Sigma, USA), before being washed in phosphate buffer again and finally incubated with the PAP complex (dilution, 1:200). The peroxidase reaction was carried out using a solution of 3,3'-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2 in Tris-HCl buffer (0.05 M, pH 7.6). After immunostaining, the liver sections were lightly counterstained with hematoxylin and observed under a light microscope.

Statistical analysis

Data were presented as means ± standard deviation. Statistical comparison of biochemical and antioxidant enzyme parameters was done using one-way analysis of variance, followed by the least significant difference (LSD) test using a statistical package program (SPSS version 17.0 (SPSS Inc, Chicago, USA)). In cases where ANOVA showed significant differences, LSD test was performed. $P < 0.05$ was considered to be statistically significant.

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 530.2 ± 30.27 nm as shown in Figure 2.

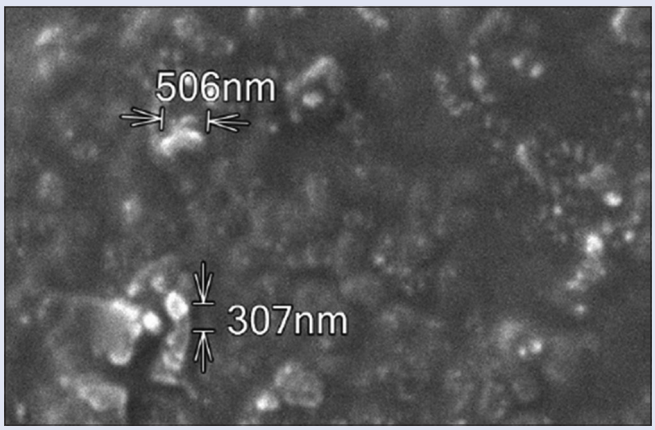

Figure 1: Scanning electron microscope images of Chitosan-*Pinus merkusii* extract nanoparticles

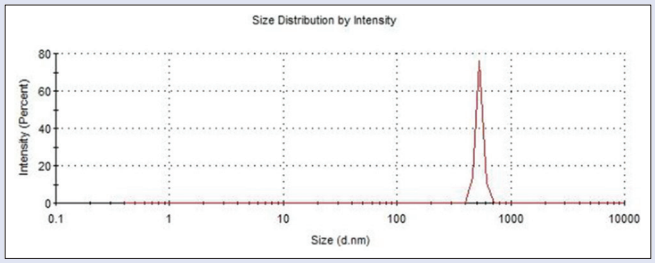

Figure 2: Size distribution of Chitosan-*Pinus merkusii* extract nanoparticles by Dynamic light scattering

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

An increase in the serum hepatic marker enzymes (SGOT, SGPT, and ALP) indicates liver damage. Analysis of these hepatic marker enzymes has been done to evaluate the hepatoprotective effect of Chitosan-*P. merkusii* extract nanoparticles in the lead acetate-treated rats. Lead acetate-treated groups showed a statistically significant ($P < 0.05$) increase in serum hepatic enzymes (SGOT, SGPT, and ALP) compared with the control group. In contrast, the groups pretreated with Chitosan-*P. merkusii* extract nanoparticles at a dose of 400 mg/Kg BW showed statistically significant ($P < 0.05$) decreased enzyme (SGOT, SGPT, and ALP) levels in a dose-dependent manner with respect to the lead acetate group toward normalization and close to the control group [Table 1].

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

Table 2 shows the results of the level of MDA, SOD, and GPx of liver tissue changes in all groups. In the lead acetate group, the level of MDA of liver tissue was significantly increased compared to the control group ($P < 0.05$). Treatment with Chitosan-*P. merkusii* extract nanoparticle at a dose of 400 mg/Kg BW markedly reduced liver tissue MDA in lead acetate treatment, which was significantly different from the lead acetate group ($P < 0.05$). Table 2 also shows the results of the lead acetate group; the level of SOD and GPx of liver tissue was significantly decreased compared to the control group ($P < 0.05$). Treatment with Chitosan-*P. merkusii* extract nanoparticle at a dose of 400 mg/Kg BW markedly enhanced liver 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

An increase in the expression of caspase-3 indicates liver cell apoptosis. Figure 3 shows the results of the expression of caspase-3 of liver cell apoptosis. In the lead acetate group, the expression of caspase-3 of liver tissue was significantly increased compared to the control group ($P < 0.05$). Treatment with Chitosan-*P. merkusii* extract nanoparticle at a dose of 400 mg/Kg BW markedly reduced liver tissue caspase-3 expression, which was significantly different from the lead acetate group ($P < 0.05$).

Table 1: Effects of Chitosan-*Pinus merkusii* extract nanoparticles on lead acetate-induced changes in the serum hepatic marker enzymes

Groups	Means±SD		
	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
Negative control	57.30^{a}±4.33	22.27^{a}±3.13	156.27^{a}±7.63
Positive control	83.26^{b}±7.02	39.38^{b}±4.26	214.42^{b}±8.92
Chitosan-P. merkusii 100 mg/kg BW	81.17^{b}±6.67	37.42^{b}±3.51	208.61^{b}±7.37
Chitosan-P. merkusii 200 mg/kg BW	76.36^{b}±4.98	36.23^{b}±3.75	201.25^{b}±7.48
Chitosan-P. merkusii 400 mg/kg BW	65.23^{c}±4.72	29.52^{c}±2.12	181.62^{c}±6.51

^{a,b,c}Different superscript within each column indicate a significant difference between the means ($P<0.05$). SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase; *P. merkusii: Pinus merkusii*; SD: Standard deviation; BW: Body weight

Table 2: Effects of chitosan-*Pinus merkusii* extract nanoparticle on lead acetate-induced changes in antioxidant and malondialdehyde, superoxide dismutase, and glutathione peroxidase

Groups	Means±SD		
	MDA (nmol/mg)	SOD (U/mg)	GPx (U/mg)
Negative control	6.19^{a}±0.75	10.47^{a}±0.96	49.38^{a}±3.71
Positive control	10.24^{b}±1.12	5.91^{b}±0.87	32.73^{b}±4.72
Chitosan-P. merkusii 100 mg/kg BW	9.38^{b}±0.92	4.97^{b}±0.94	31.87^{b}±4.18
Chitosan-P. merkusii 200 mg/kg BW	8.96^{b}±0.89	5.72^{b}±1.03	5.72^{b}±1.03
Chitosan-P. merkusii 400 mg/kg BW	7.13^{c}±0.64	7.62^{c}±0.75	41.37^{c}±4.21

^{a,b,c}Different superscript within each column indicate significant difference between the means ($P<0.05$). MDA: Malondialdehyde; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; BW: Body weight; SD: Standard deviation

Effects of Chitosan-*Pinus merkusii* extract nanoparticle on lead acetate-induced liver cell damage

Figures 4 shows the histological changes in the liver of control group that shows normal architecture with normal appearance of the hepatocytes and central vein. In the lead acetate group, showed hepatic cell damage (congestive and necrosis). In the rats, treated with Chitosan-*P. merkusii* extract nanoparticle, the number and morphological integrity of hepatic cells are being preserved. This indicates that the hepatotoxic effects of lead acetate were inhibited by Chitosan- *P. merkusii* extract nanoparticle [Figure 4].

DISCUSSION

In the field of medicine, the nanotechnology has developed with various aspects such as drug delivery and tissue engineering for the diagnosis of various diseases.[22,23] Due to new advances in nanotechnology, it is now possible to produce natural product nanoparticles that can be utilized in a variety of innovative ways. Nanoparticle-based natural product delivery systems offer enhanced drug stability, treatment efficacy, and penetration power compared to a pure natural product.[25]

A variety of polymers were used for herbal extract-loaded nanoparticles; however, chitosan has received great attention in both the medical and pharmaceutical fields.[23] Chitosan, a biodegradable and biocompatible polymer, is a modified natural carbohydrate and the second most abundant polysaccharide in nature.[25,27] We made *P. merkusii* extract which was loaded with chitosan nanoparticle with the use of sodium

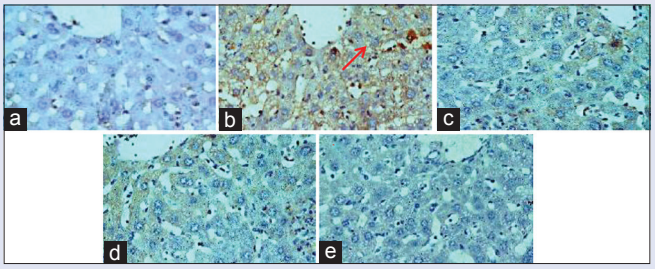

Figure 3: Immunohistochemical study of Chitosan-*P merkusii* extract nanoparticle on caspase-3 expression of lead acetate. The lead acetate-treated group showed caspase-3 expression (indicated by red arrows). Negative control group (a); Positive control group (b); Rats treated with Chitosan-*Pinus merkusii* extract nanoparticle 100 mg/Kg BW, 200 mg/Kg BW, and 400 mg/Kg (c-e)

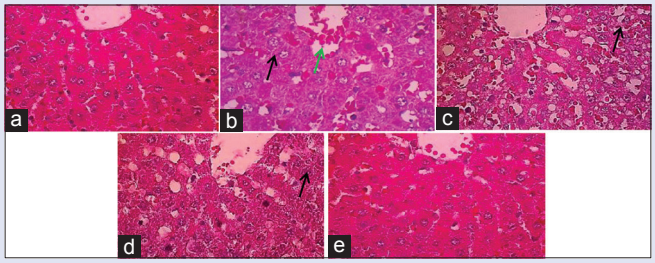

Figure 4: Histological study of pretreatment with Chitosan-*P merkusii* extract nanoparticle on lead acetate-induced hepatotoxicity. Normal morphology of liver sections in the negative control group (a). The lead acetate-treated group showed congestive (indicated by green arrows) and necrosis (indicated by black arrows) (b). Rats treated with Chitosan-*Pinus merkusii* extract nanoparticle 100 mg/Kg BW and 200 mg/Kg BW showed necrotic changes (c and d). Rats treated with Chitosan-*P merkusii* extract nanoparticle 400 mg/Kg showed regeneration in hepatic cells damage (e). H and E, ×400

TPP on ionotropic gelation method, which has more advantages over *P. merkusii* extract itself. Due to this, modification can improve biodistribution and increase specificity and sensitivity and reduce pharmacological toxicity.[26,27] The results of the DLS showed that the size of Chitosan-*P. merkusii* extract nanoparticle was 201.8 ± 14.6 nm. The SEM images of the Chitosan-*P. merkusii* extract nanoparticles showed an irregular shape, and the morphology surface showed the rough surface. In our study, administration of lead acetate showed a significant increase in MDA level, as a product of lipid peroxidation and showing oxidative damage to the liver. The various toxic effects induced by lead acetate in biological systems have been linked to increased MDA or lipid peroxidation.[1] The present investigation resulted in significantly increased MDA levels in the liver of lead acetate-treated rat in comparison to the control group. This means that it increased the oxidative stress in the lead acetate-treated rats. Therefore, the significantly lower levels of MDA in the liver tissues of Chitosan-*P. merkusii* extract nanoparticle-treated groups, as compared with the lead acetate group, indicate attenuation of lipid peroxidation. It is known that lead acetate-induced oxidative stress and tissue damage could be caused by two mechanisms including increased generation of ROS and by causing direct depletion of antioxidant reserves.[3] The lipid peroxidation can be increased by lead acetate administration, which may affect the cytoplasmic membranes and mitochondrial, causing more severe oxidative damage in the tissues and consequently releasing lipid HO_2 into circulation, which reflects the induction of oxidative stress.[1,2] In this study, 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' liver. Treatment of rats with Chitosan-*P. merkusii* extract nanoparticle at a dose of 400 mg/Kg BW prevented the levels of MDA to rise when the rats were challenged with lead acetate. This means that Chitosan-*P. merkusii* extract nanoparticle minimized the toxic effect of lead acetate through its antioxidant activity. The antioxidant protective mechanism decreases the oxidative stress and scavenges the free radical responsible for the liver damage and thus inhibits the lipid peroxidation as measured by MDA levels. The findings of this study suggest that Chitosan-*P. merkusii* extract nanoparticle could attenuate oxidative stress by decreasing the lipid peroxidation (MDA level) in the lead-treated liver.

Liver damage following lead acetate exposure is well characterized by elevated levels of plasma hepatic marker enzymes, which indicate cellular leakage and loss of functional integrity of hepatic membrane architecture.[8] The serum enzyme markers such as SGOT, SGPT, and ALP are recommended for the assessment of hepatocellular damage in preclinical studies as it is considered a more specific and sensitive indicator of liver damage. Low levels of SGOT, SGPT, and ALP are normally found in the blood, but when the liver is damaged or diseased, it releases SGOT, SGPT, and ALP into the bloodstream, which makes a rise in SGOT, SGPT, and ALP levels. Most increases in SGOT, SGPT, and ALP levels are caused by liver damage.[4,6] The current work showed an increase in the levels of SGOT, SGPT, and ALP in lead acetate-treated rat in comparison to the negative control, and this may be due to the degeneration of hepatocytes by necrosis which causes leakage of these enzymes into blood circulation. The similar observation has reported that lead acetate treatment induced significant elevation of serum SGOT, SGPT, and ALP activities.[5-7] Furthermore, Ibrahim *et al.* reported that the high SGOT, SGPT, and ALP activities are accompanied by high liver microsomal membrane fluidity, free radical generation, and alteration in the liver tissue. Our results indicated that Chitosan-*P. merkusii* nanoparticle has hepatoprotective activity against lead acetate-induced hepatotoxicity, where the pretreated groups with chitosan-*P. merkusii* nanoparticle (400 mg/Kg BW) showed an improvement in the SGOT, SGPT, and ALP levels. This might be through its direct action on free radicals of lead acetate to prevent the liver cellular damage by maintaining its membrane integrity. Reduction of serum transaminases near-normal levels suggested regeneration of hepatocytes with a healing of hepatic parenchyma.

The activities of SOD and GPx have been used to assess oxidative stress in cells. Many studies have shown that lead acetate has a 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.[1,2,6] In the present study, the activity of SOD and GPx in the rat liver was decreased by lead acetate treatment. This decreased SOD and GPx activities with lead acetate treatment is in agreement with previous studies. 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 liver 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 liver 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 protect against free radical toxicity. The primary mechanism of action of Chitosan-*P. merkusii* extract nanoparticle may involve the scavenging of free radicals, which can inhibit free radical formation.[16,26] It has been found a decrease in the MDA levels and an increase in the antioxidant enzyme parameters including SOD and

GPx in liver tissue of rats that were administered Chitosan-*P. merkusii* extract nanoparticle when compared with lead acetate group.

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 hepatic cell death through apoptosis or necrosis.[9,12] 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."[13] This study showed that in the lead acetate group, the expression of caspase-3 of liver tissue was significantly increased compared to the control group. Dose-dependent manner of Chitosan-*P. merkusii* extract nanoparticle decreased liver tissue caspase-3 expression in lead acetate treatment. It has been reported that hepatic apoptosis induced by lead toxicity was associated with mitochondrial injury and changes in levels of apoptogenic proteins including Bcl-2, Bax, and caspase-3.[13] In lead toxicity, the expression levels of caspase-3 and Bax significantly increased whereas the levels of Bcl-2 significantly decreased.

The histopathological results demonstrating structural changes in the liver tissue of heavy metal toxicity such as lead acetate were reported by some researchers. In the present study, histopathological view of liver sections in the lead acetate-treated group showed the hepatic cell damage (congestive and necrosis) as compared to the control group. The liver damage (congestive and necrosis) was considered mild in the groups treated with Chitosan-*P. merkusii* extract nanoparticle.

CONCLUSION

The results of the present study indicate that lead acetate-induced liver toxicity might be related with both oxidant and caspase-3. The administration of Chitosan-*P. merkusii* extract nanoparticle lessened the effects of lead acetate-induced hepatotoxicity possibly by increasing antioxidant and inhibiting caspase-3 expression. Further investigation of these promising protective effects of Chitosan-*P. merkusii* extract nanoparticle against lead acetate-induced hepatic cell damage may have a considerable impact on developing clinically feasible strategies to treat patients with lead acetate-induced liver toxicity.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Flora G, Gupta D, Tiwari A. Toxicity of lead: A review with recent updates. Interdiscip Toxicol 2012;5:47-58.
2. Hsu PC, Guo YL. Antioxidant nutrients and lead toxicity. Toxicology 2002;180:33-44.
3. Sudjarwo SA, Eraiko K, Sudjarwo GW, Koerniasari. Protective effects of piperine on lead acetate induced-nephrotoxicity in rats. Iran J Basic Med Sci 2017;20:1227-31.
4. Sudjarwo SA, Sudjarwo GW, Koerniasari. Protective effect of curcumin on lead acetate-induced testicular toxicity in Wistar rats. Res Pharm Sci 2017;12:381-90.
5. Ahmed MA, Hassanein KM. Cardio protective effects of *Nigella sativa* oil on lead induced cardio toxicity: Anti inflammatory and antioxidant mechanism. J Physiol Pathophysiol 2013;4:72-80.
6. Wardani G, Farida N, Andayani R, Kuntoro M, Sudjarwo SA. The potency of red seaweed (*Eucheuma cottonii*) extracts as hepatoprotector on lead acetate-induced hepatotoxicity in mice. Pharmacognosy Res 2017;9:282-6.
7. Attia AM, Ibrahim FA, Nabil GM, Aziz SW. Antioxidant effects of ginger (*Zingiber* officinale Roscoe) against lead acetateinduced hepatotoxicity in rats. Afr J Pharm Pharmacol 2013;7:1213-9.
8. Moussa SA, Bashandy SA. Biophysical and biochemical changes in the blood of rats exposed to lead toxicity. Rom J Biophys 2008;18:123-33.
9. Ibrahim NM, Eweis EA, El-Beltagi HS, Abdel-Mobdy YE. Effect of lead acetate toxicity on experimental male albino rat. Asian Pac J Trop Biomed 2012;2:41-6.
10. Kaushik A, Husain A, Awasthi H, Singh DP, Khan R, Mani D. Antioxidant and hepatoprotective potential of swaras and hima extracts of *Tinospora cordifolia* and *Boerhavia diffusa* in swiss albino mice. Pharmacogn Mag 2017;13:S658-62.
11. Jackie T, Haleagrahara N, Chakravarthi S. Antioxidant effects of *Etlingera elatior* flower extract against lead acetate-induced perturbations in free radical scavenging enzymes and lipid peroxidation in rats. BMC Res Notes 2011;4:67.
12. Xu J, Lian LJ, Wu C, Wang XF, Fu WY, Xu LH. Lead induces oxidative stress, DNA damage and alteration of p53, bax and bcl-2 expressions in mice. Food Chem Toxicol 2008;46:1488-94.
13. Yuan G, Dai S, Yin Z, Lu H, Jia R, Xu J. Sub-chronic lead and cadmium co-induce apoptosis protein expression in liver and kidney of rats. Int J Clin Exp Pathol 2014;7:2905-14.
14. Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. Biochim Biophys Acta 2013;1830:3670-95.
15. Kaushik D, Aggarwal A, Kaushik P, Mehra R, Rana AC. *Pinus roxburghii*-incredible gift in the lap of Himalayas. Int J Pharmacogn Phytochem Res 2010;2:29-35.
16. Li YY, Feng J, Zhang XL, Cui YY. Pine bark extracts: Nutraceutical, pharmacological, and toxicological evaluation. J Pharmacol Exp Ther 2015;353:9-16.
17. Kim NY, Jang MK, Lee DG, Yu KH, Jang H, Kim M, *et al.* Comparison of methods for proanthocyanidin extraction from pine (*Pinus densiflora*) needles and biological activities of the extracts. Nutr Res Pract 2010;4:16-22.
18. Ku CS, Mun SP. Characterization of proanthocyanidin in hot water extracts isolated from *Pinus radiata* bark. Wood Sci Technol 2007;41:235-47.
19. Ince I, Yesil-Celiktas O, Karabay-Yavasoglu NU, Elgin G. Effects of *Pinus brutia* bark extract and pycnogenol in a rat model of carrageenan induced inflammation. Phytomedicine 2009;16:1101-4.
20. Park IJ, Cha SY, Kang M, So YS, Go HG, Mun SP, *et al.* Effect of proanthocyanidin-rich extract from *Pinus radiata* bark on immune response of specific-pathogen-free white leghorn chickens. Poult Sci 2011;90:977-82.
21. Qadir M, Shah WA. GC-MS analysis, antibacterial, antioxidant and anticancer activity of essential oil of *Pinus roxburghii* from Kashmir India. Int J Res Pharm Chem 2014;4:228-32.
22. Saini R, Saini S, Sharma S. Nanotechnology: The future medicine. J Cutan Aesthet Surg 2010;3:32-3.
23. Kumari A, Kumar V. Nanotechnology: A tool to enhance therapeutic values of natural plant products. Trends Med Res 2012;7:34-42.
24. Ahmed TA, Aljaeid BM. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. Drug Des Devel Ther 2016;10:483-507.
25. Ghadi A, Mahjoub S, Tabandeh F, Talebnia F. Synthesis and optimization of chitosan nanoparticles: Potential applications in nanomedicine and biomedical engineering. Caspian J Intern Med 2014;5:156-61.
26. Khanmohammadi M, Elmizadeh H, Ghasemi K. Investigation of size and morphology of chitosan nanoparticles used in drug delivery system employing chemometric technique. Iran J Pharm Res 2015;14:665-75.
27. Shukla SK, Mishra AK, Arotiba OA, Mamba BB. Chitosan-based nanomaterials: A state-of-the-art review. Int J Biol Macromol 2013;59:46-58.
28. Wardani G, Mahmiah, Sudjarwo SA. *In vitro* antibacterial activity of chitosan nanoparticles against *Mycobacterium tuberculosis*. Pharmacog J 2018;10:162-6.
29. Friedman M, Juneja VK. Review of antimicrobial and antioxidative activities of chitosans in food. J Food Prot 2010;73:1737-61.
30. Vimal S, Abdul Majeed S, Taju G, Nambi KS, Sundar Raj N, Madan N, *et al.* Chitosan tripolyphosphate (CS/TPP) nanoparticles: Preparation, characterization and application for gene delivery in shrimp. Acta Trop 2013;128:486-93.
31. Sudjarwo SA, Wardani G, Eraiko K, Koerniasari. The Potency of nanoparticle of *Pinus merkusii* as immunostimulatory on male Wistar albino rat. Int J Nutr Pharmacol Neurol Dis 2018;8:10-5.
32. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
33. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc 2010;5:51-66.