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Research Article

Ameliorating Effect of *Apium graveolens* (Celery) Extracts on IL-6 Plasma Level and Expression of Caspase 3 on Liver in Animal Model of Lead Intoxication

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Abstract

Background and Objective: Lead poisoning (Pb) is a big problem because it is found in almost all objects in daily life such as vehicle fuel, water pipes, ceramics, cosmetics and others. Continuous lead exposure can increase ROS resulting in an increase in hepatic IL-6 and caspase 3 which replaces hepatic cell apoptosis. The objective of this study was to determine the effect of *Apium graveolens* (celery) extract on plasma IL-6 and hepatic caspase 3 levels. **Materials and Methods:** This study used a post-test control group design. The research subjects were 20 Wistar rats that met the inclusion criteria and were divided into 4 groups randomly, namely (a) Sham group that had no treatment, (b) Negative control group was induced with lead acetate 200 mg kg⁻¹ body weight/day without any treatment (c) Positive control group and (d) Treated group. On the 15th day, blood was taken to check IL-6 levels and tissue was taken for liver caspase 3 examination by immunohistochemical method. Data analysis used the one-way ANOVA test and continued with the *post hoc* LSD test. **Results:** The highest mean caspase 3 expression was in the control group 45.84 ± 4.39 pg mL⁻¹, while the mean of IL-6 plasma level was highest in the P1 641.33 ± 39.72 pg mL⁻¹ group. The Mann-Whitney test showed a significant difference in IL-6 levels between the study groups (p = 0.000). The Mann-Whitney test showed a significant difference in caspase 3 levels between the study groups (p = 0.000). **Conclusion:** Giving celery extract 300 mg kg⁻¹ body weight/day affects plasma IL-6 and hepatic caspase 3 levels in lead acetate-induced rats.

Key words: Celery extract, IL-6, caspase 3, lead intoxication, ROS (reactive oxygen species), antioxidant

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lead intoxication is a condition when human body were overexposure to lead that through the air, water, cosmetics and food sources¹. Lead interferes with a variety of body processes and toxic to many organs and tissues including the heart, bones, intestines, kidneys, reproductive and nervous systems². Lead that enters the body through the mouth will be absorbed by the small intestine and circulated to all organs including the liver³. The liver may experience injury due to an overabundance lead, which can elevate the production of reactive oxygen species (ROS) production and directly reduce antioxidant reserves⁴. This excess generation of oxygen-derived free radicals in the lead-affected organ also inevitably results in lipid peroxidation on the cell membrane and disrupts enzyme activity. This process will also trigger the production of proinflammatory cytokines including Interleukin 6 (IL-6). Prolonged inflammation causes apoptosis and necrosis of liver cells characterized by increased hepatic caspase 3 expression^{5,6}.

The intracellular mechanism of lead (Pb) effects can be intricate, as Pb acts as a competitive inhibitor, interfering with the absorption of essential bio-elements, replacing them in enzyme active sites, or binding to the SH groups of protein⁷. A prior investigation demonstrated that chronic administration of Pb-acetate to rats over a period of 2 week increased the occurrence of apoptotic bodies in the proximal tubular cells. Therefore, it plausible to hypothesize that Pb intoxication has an impact on the gene expression of apoptosis-related proteins^{8,9}.

The potent free radical scavenging capacity of natural antioxidants, sourced from medicinal plants, has garnered significant attention and thorough research due to its potential relevance to diverse diseases¹⁰. Within living systems, different antioxidant mechanisms may function by enhancing the levels of innate defenses by upregulating the expression of genes that encode enzymes and cytokines¹¹. *Apium graveolens* usually known as celery is a plant that is easily available and is commonly consumed as a vegetable. Celery contains several active antioxidant compounds such as apigenin, tannins, flavonoids, L-3-n-butylphthalide, sedanolide, linoleic acid, phenolics and essential oils found in almost all parts including roots, leaves and seeds^{12,13}.

Previous studies have proven that these antioxidants can improve the function and structure of the liver which is characterized by improvements in liver enzymes such as CYP2A5 and histopathological features of the liver¹⁴. In this study, *Apium graveolens* ethanol extract was used to explore

its antioxidant properties against the toxic effects of lead intoxication and compared it with vitamin E as a standardized treatment for toxication. Vitamin E as a standard source of antioxidants has functions as a hydrogen ion donor that can convert peroxy radicals (products of lipid peroxides) into less reactive tocopherol radicals, so they are unable to damage fatty acid chains¹⁵⁻¹⁸.

The present study aimed to analyze the phenolic compounds in the *Apium graveolens* ethanol extract and to explore its anti-inflammatory and antioxidant properties against the toxic effects of Pb lead intoxication by evaluating the gene expressions of caspase 3 in liver and IL-6 level in plasma to explore the molecular mechanisms responsible for the curative effects.

MATERIALS AND METHODS

Study area: The experiment was performed at SCCR Animal Model Research Center, Indonesia from May-September, 2022.

Animals and ethical clearance: The 24 male rats with strain Wistar weighing approximately 250-300 g were purchased from the Agricultural and Fishery Service of Salatiga, Indonesia. The animals were housed in optimal conditions: Temperature $22 \pm 2^\circ\text{C}$, humidity $55 \pm 10\%$ and artificial ventilation. Rats received a standard diet and drinking water *ad libitum*. All animals were kept under a rigorous cleaning and hygiene program and were monitored daily. The animals were housed in plastic cages with sawdust and each cage contained 5 animals. The minimum number of samples according to WHO provisions in 2019 is 5 individuals per treatment group multiplied by the number of treatment groups. To avoid loss of following, 1 experimental animal was added to each group so that the number of samples in this study was 24 Wistar rats. The experiments were done in approval by principles of the Bioethics Commission for Medical/Health Research, Faculty of Medicine, Sultan Agung Islamic University, Semarang No. 307/VIII/2022/Komisi Bioetik.

Preparation of ethanolic extraction of *Apium graveolens*:

The extract was conducted in the Integrated Laboratory, Diponegoro University. *Apium graveolens* ± 600 g dried at a temperature of $50-60^\circ\text{C}$ and blended into a dry powder. Then it was extracted by maceration method using 95% ethanol at 90°C for 17 hrs. The ethanol content is evaporated using IKA Rotary Evaporator RV10 (IKA, Baden-Württemberg, Germany) until the remaining thick extract remains. The thick

extract formed was *Apium graveolens* extract that was ready to use and then stored in a refrigerator at a temperature of 2-8°C until the treatment was given.

Determination of *Apium graveolens* phytochemical

content: Phytochemical screening of extracts was carried out to determine the content of secondary metabolites such as saponins and alkaloids using the Wagner method¹⁹, flavonoids using Willstatter test²⁰ and tannins, FeCl₃ 1% and triterpenoids using Lieberman Burchard²¹.

Animal models of lead-induced intoxicating:

Male white rats (*Rattus norvegicus*) of Wister, 3 months old, weigh 250 g, divided into 4 groups. The first group was control (normal, untreated), received distilled water without any treatments; the second group was negative control, lead-sulphate treated group that received freshly dissolved PbCOOH₂ in 2 mL distilled water at a dose of 200 mg kg⁻¹ body weight/day for 14 days peroral; third group was positive control, group that received freshly dissolved PbCOOH₂ in 2 mL distilled water at a dose 200 mg kg⁻¹ body weight/day for 14 days then treated with vitamin E at dose 50 IU kg⁻¹ body weight/day for 14 days peroral; fourth group was treated group, group that received freshly dissolved PbCOOH₂ in 2 mL distilled water at a dose 200 mg kg⁻¹ body weight/day for 14 days then treated with *Apium graveolens* at dose 300 mg kg⁻¹ body weight/day for 14 days peroral. The dose of celery extract was determined by previous studies that showed oral administration of celery extracts 300 mg/kg body weight/day in Wistar rats can protect liver function. Thus, the dose of celery extract used in this study was 300 mg/kg body weight/day²².

Sampling collection: After the last treatment administration of peroral PbCOOH₂, vitamin E and *Apium graveolens* extract then it was rapidly incised to expose the liver which was rapidly removed storage in neutral buffer formalin stored at room temperature to be processed into slide. Plasma was collected by retro orbital-puncture in Eppendorf tubes with anticoagulant for haematological then centrifuge to separate the pellet and supernatant.

Immunohistochemistry analysis of caspase 3 liver:

Immunohistochemistry for caspase 3 was performed on formalin-fixed, paraffin-embedded tissue on liver samples in all groups. Sections (5 µm thick) on positively charged slides were deparaffinized in xylene, hydrated in graded alcohol and pretreated for antigen retrieval in 10 mmol L⁻¹ citrate buffer, pH 6.0, in a decloaking chamber (Biogear, Netherland) at 95°C

for 15 min. Staining was performed using monoclonal rabbit anti-caspase 3 antibody (1:200 dilution, Invitrogen, San Diego, California, USA), Starr Trek Universal HRP Detection System (Biocare Medical, Pacheco, California, USA). Scoring expression of caspase 3 of the sample was analysed by semiquantitative visual evaluations by two observers using fractional area (ImageJ software). Overexpression was considered to be present when the intensity of tumour staining was at least twice the staining intensity of adjacent non-neoplastic liver.

Quantification of IL-6 plasma level by ELISA:

Frozen plasma at -80°C obtained from each group subject was thawed and assayed for IL-6 by use of a commercially available ELISA (Invitrogen IL-6 Rats, Thermofisher Scientific). The concentration was measured by iMark microplate absorbance reader (Bio-Rad, California, USA) capable of reading absorbance at 450 nm.

Statistical analysis:

Data analysis was performed using SPSS (version 20.0). Comparison analyses were performed using One-way Analysis of Variance (ANOVA), followed by *post hoc* tests to determine differences between groups. A p<0.05 was considered as significant.

RESULTS

Bioactive compounds of *Apium graveolens* extract:

This study evaluated the richness of *Apium graveolens* extract in antioxidants that might function as ROS scavengers. Secondary metabolites, the phytochemical compounds, then assessed by visualizing the colours produced by each compound. Phytochemical screening tests showed the presence of alkaloid, saponin, tannin, flavonoid and steroid while triterpenoid is absent.

Flavonoid compounds widely distributed in plants have many benefits on human health as free radical scavengers and are mainly responsible for the antioxidant capacity of plants. For this reason, further measuring of the flavonoid totals of the extract.

The results point out the presence of flavonoids. It might be the potential source of antioxidant activity of *Apium graveolens* extract. Quantitative flavonoid test by analysing the total flavonoid content in the ethyl acetate and ethanol extracts of *Apium graveolens*. Measurement of total flavonoid levels was carried out 3 times and the results obtained show the total flavonoid content (TFC) values of 14.8050, 13.55515 and 14.5543 corresponding to extract concentration of 1000 parts per million (PPM). The results of

the average TFC values in the *Apium graveolens* extract sample were 14.3036 ± 0.66 PPM.

Expression of caspase 3 in liver's lead-intoxication rats:

The effect of *Apium graveolens* extract on caspase 3 liver was analyzed by immunohistochemistry while the IL-6 plasma level was measured by ELISA. Both expressions of caspase 3 liver and IL-6 level plasma in the treatment group were

significantly decreased compared to those in the control negative group. However, expressions of caspase 3 liver and IL-6 plasma levels in the negative control group were higher compared to those in the treatment group.

In this study, the results of hepatic caspase 3 expression in a rat model were induced by lead acetate and therapy with celery extract. Image depicting caspase 3 Immunochemistry in the liver tissue of mice in sham group (Fig. 1a), negative

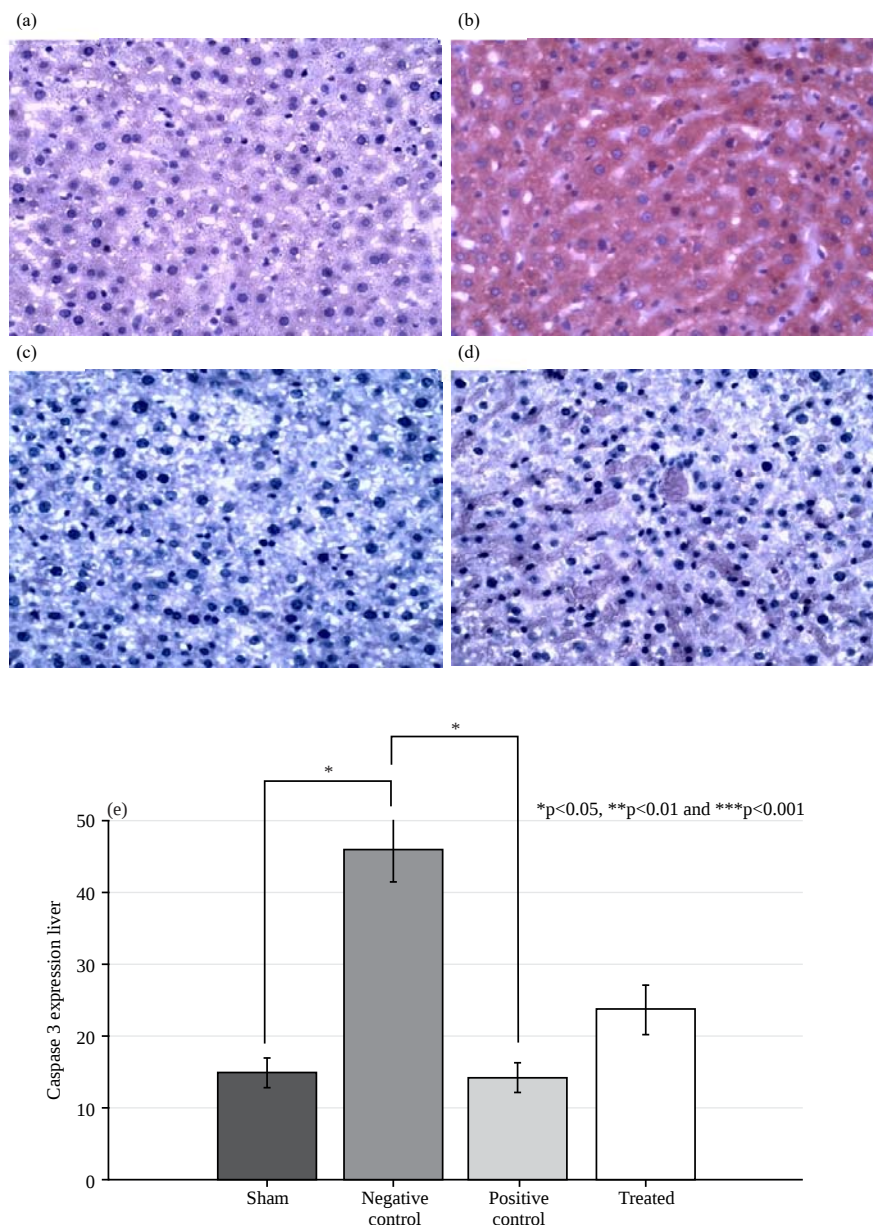


Fig. 1(a-e): Representative images of caspase 3 immunohistochemistry in the liver tissue, (a) Sham group, (b) Negative control group, (c) Positive control group, (d) Treated group, Original magnification: 40 $\times$ and (e) Percentage area of caspase 3 histological analysis for each group
Each bar shows the mean value (Standard deviation, n = 5) and * p<0.05

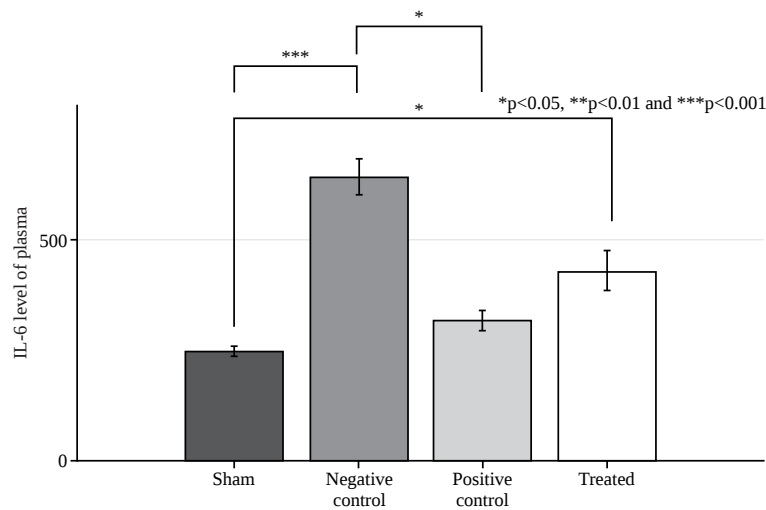


Fig. 2: Enzyme linked immunosorbent assay of IL-6 in plasma of the, sham group, negative control group, positive control group and treated group

*Indicates a significant difference from the control group ($p < 0.05$), **indicates a significant difference from the UVB group ($p < 0.05$, $n = 6$ per group) and the data represent Mean $\pm$ Standard deviation

control group (Fig. 1b), positive control group (Fig. 1c) and treated group (Fig. 1d). It shows that the highest increase in caspase 3 expression was found in the negative control group at $45.84 \pm 4.39\%$, while the lowest caspase 3 level was found in the positive control group at $14.15 \pm 2.01\%$ (Fig 1e). The results of the difference test between groups using the Mann-Whitney test obtained a value of $p = 0.020$ ($p < 0.05$) between the healthy control group and the negative control group. The results of the difference test between groups using the Mann-Whitney test got a p -value = 0.045 ($p < 0.001$) between the positive control group and the negative control group. Therefore, it can be interpreted that there is a significant difference in caspase 3 expressions between the groups (Fig. 1e).

Interleukin 6 level in plasma lead-intoxication rats: In this study, the results of plasma IL-6 levels were obtained in a rat model induced by lead acetate and therapy with celery extract. It is known that the highest increase in IL-6 levels was found in the negative control group of $641.33 \pm 39.72 \text{ pg mL}^{-1}$, while the lowest levels of IL-6 were found in the healthy control group of $245.334 \pm 11.15 \text{ pg mL}^{-1}$. The results of the difference test between groups using the Mann-Whitney test obtained a p -value = 0.044 ($p < 0.05$) between the healthy control group and the treatment and between the positive control and negative control. The results of the difference test between groups using the Mann-Whitney test got a p -value = 0.000 ($p < 0.001$) between the healthy control group

and the negative control group. Therefore, it can be interpreted that there is a significant difference in IL-6 levels between the groups (Fig. 2).

DISCUSSION

One of the organs that is often poisoned by lead exposure is the liver. Accumulation of lead excess in the liver can trigger excessive production of ROS, which leads to activation of the NF- κ B pathway, resulting in the production of inflammatory cytokines such as IL-6^{23,24}. In line with the statement, current findings showed that the IL-6 levels in positive control mice exposed to lead exhibited higher levels of IL-6 compared to the other groups (Fig. 2). These indicate that the positive control mice were in an inflammatory condition due to lead exposure²⁵.

Previous studies have revealed the role of anthocyanins and apigenins found in celery in increasing antioxidant enzymes such as SOD, GSH and GPx²⁶. It is known that antioxidant enzymes can reduce ROS levels. The decrease of ROS is due to the inhibition of the NF- κ B pathway leading to the reduction of pro-inflammatory cytokines generation, including IL-6²⁷. In current findings, the treated mice group showed a lower level of IL-6 (Fig. 2). This was in line with the previous study that reported a role for celery extract in lowering IL-6 levels to near normal in rats exposed to lead²⁸. In addition to increasing levels of antioxidant enzymes, celery extract could also reduce IL-6 through the activation of the

Nrf-2 pathway which can inhibit the activation of the NF- κ B pathway²⁹. An interesting finding shows that the level of IL-6 is not significantly different in both mice with lead and vitamin E treatment and mice with lead and celery extract treatment. This corresponds with previous findings, the ability of celery extract to reduce IL-6 levels was proven to be equivalent to vitamin E³⁰.

Decreased levels of IL-6 were also known correlate with the cessation of cell apoptotic processes^{31,32}. These reports were evidenced by the decrease in caspase 3 expression and in line with the decrease in IL-6 that occurred after the administration of celery extract (Fig. 1e). The IL-6 is known to trigger a series of activation of the apoptotic pathway through phosphorylation of STAT3 which then activates caspase 8 and then the activation of caspase 3³³. In addition, IL-6 also phosphorylates the Akt pathway which then directly activates caspase 3. The activated caspase 3 then triggers cell apoptosis, therefore, the decrease of IL-6 level correlates with a decrease in caspase 3³⁴. Celery extract in addition to the inhibitory pathway of IL-6 production also works through apigenin which inhibits STAT3 and Akt phosphorylation so that it can suppress caspase 3 activation which leads to the termination of the apoptotic process^{35,36}. Therefore, celery extract has the potential to avert toxicity induced by lead.

In this study, we did not analyze the oxidative stress level that can be characterized by the level of reactive oxygen species (ROS) and malondialdehyde (MDA). We also did not measure the antioxidant activity, which can represent the oxidative stress inhibition level in rats. The analysis of intracellular molecules controlling oxidative pathways, such as Nuclear Factor-Erythroid-2 related factor 2 (NRF-2) and Kelch-like ECH-Associated Protein 1 (KEAP-1) also needed to assess the ability of antioxidant enzymes contained in celery extract.

CONCLUSION

Apium graveolens extract had potentially been used as a peroral treatment of lead-intoxication by reducing caspase 3 in the liver and IL-6 level in plasma excessively on the lead-intoxication rats model. Celery ethanol extract at 300 mg kg⁻¹ body weight has the greatest effect in preventing oxidative stress in the lead intoxication rat model that showed the significance of plasma interleukin-6 levels and hepatic caspase 3 expression between the negative control group and the treatment group.

SIGNIFICANCE STATEMENT

Literature shows that antioxidants can improve liver function and structure. This study showed that *Apium graveolens* ethanol extract provides anti-inflammatory properties and prevents oxidative stress against lead intoxication-induced liver damage, compared with vitamin E as a standardized treatment. It showed a reduction in level of interleukin-6 and hepatic caspase 3, suggesting that celery extract has the potential to mitigate lead induced toxicity.

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